Regulation of spindle assembly checkpoint (SAC) by phosphorylation and protein-protein interactions in *Drosophila melanogaster*

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Declaration

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other university.

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Abstract

Chromosome segregation is a complex, but subsequently error- prone process, who's accuracy is essential to prevent uneven DNA distribution between mother and daughter cells. Such unequal chromosome segregation can often result in aneuploidy, which is a prevalent phenotype of cancer cells, and so surveillance mechanisms must exist within the cell cycle to detect and correct the cause of such chromosome division errors, before allowing the cell to divide.

The Spindle Assembly Checkpoint (SAC) has evolved to monitor the interaction between microtubules, and the point at which they attach sister chromosomes, the kinetochore. By detecting attachment and resulting tension abnormalities, the SAC halts the metaphase to anaphase transition if chromosomes are not aligned correctly at the metaphase plate. By disallowing cell division to occur in the absence of proper chromosome alignment, the SAC minimises the frequency of uneven DNA distribution and the consequent problems this can incur. Silencing of the SAC, and normal cell progression is not promoted until correction mechanisms have achieved proper bioriented chromosome attachments.

The target of the SAC is widely accepted to be Cell Division Cycle 20 (Cdc20), which is the activator of the Anaphase Promoting Complex or Cyclosome (APC/C), the E3 ubiquitin ligase that drives cells into anaphase. By inhibiting Cdc20, the activity of the APC/C is halted, and cells are arrested at metaphase. A number of key proteins are believed to be involved in the sequestration of Cdc20, by incorporating it into an inhibitory Mitotic Checkpoint Complex (MCC). This MCC complex is believed to comprise of Cdc20, BubR1, Bub1 and Mad2, although there is speculation as to whether Mad2 is part of the complex, or merely promotes its formation.

The proteins involved in the MCC all localise to kinetochores with activation of the SAC, although it remains unclear as to whether the MCC forms at the kinetochore upon localisation of the various components, or can form in part or as a whole, moving to kinetochores upon SAC activation. Sub-complexes of the MCC have been detected outside of mitosis, which provide evidence in favour of a kinetochore-independent MCC formation. However, if this were the case, it could be assumed that modification (such as phosphorylation) to either MCC components or the APC/C itself would need to occur in mitosis or with SAC activation, allowing for APC/C inhibition only with SAC activation, and to prevent

non-specific inhibition of APC/C by the MCC elsewhere in the cell cycle. These issues still remain unclear.

In order to investigate further, the requirement of direct kinetochore localisation of MCC components in the formation of the complex, this thesis aims to provide evidence of the effect of disrupting such kinetochore localisation upon checkpoint function, as well as the impact of removal of Cdc20 modifications on MCC formation. In addition to this, the protein-protein interaction domains between Cdc20 and BubR1, proven essential for SAC function, are investigated within *Drosophila melanogaster.*

Collectively, the data in this thesis provides an insight into the regulation of SAC in *Drosophila*. The Cdk1/Cyclin B phosphorylation of Fizzy (the *Drosophila* homologue of Cdc20) is confirmed to have an effect on MCC formation, and can be mapped to three specific sites on the N-terminal of Fizzy, which are conserved across various species. In addition to the effect of Cdk1/Cyclin B phosphorylation on the interaction between Fizzy and other SAC proteins, the importance of the BubR1 KEN box motif on the Fizzy-BubR1-Mad2 interaction is confirmed, implicating another essential domain for MCC formation in *Drosophila.* With regard to kinetochore localisation of SAC components, a model is achieved in which a dramatic reduction of Mps1, previously shown to disturb kinetochore localisation of Mad1, Mad2 and BubR1 in *Xenopus*, confirms a role for Mad2 kinetochore localisation in SAC activation, even though Fizzy localisation is unperturbed. Overall, these findings may provide a useful insight into the complex relationships, kinetochore localisation requirements and inter-protein dependencies within the regulation of MCC formation and SAC signalling in *Drosophila melanogaster.*

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Chapter 1. Introduction

1.1. Normal Cell Cycle Progression

The primary function of the cell cycle is to accurately duplicate chromosomal DNA and segregate the copied DNA into two genetically identical daughter cells. These two processes define the major phases of the mammalian cell cycle; S phase (or synthesis phase), and M phase (Mitosis). Mitosis itself consists of nuclear division (true mitosis) brought about by the complex machinery of the mitotic spindle, and cytoplasmic division (cytokinesis). Within the cell cycle, two major checkpoints exist, to prevent cell progression in the presence of DNA damage and to prevent the onset of anaphase in the presence of misaligned chromosomes.

1.1.1. M Phase

M phase is comprised of 5 principle stages (Alberts et al., 2002) beginning with Prophase. Here, the replicated chromosomes condense and the mitotic spindle assembles outside of the nucleus, between the two centrosomes. Prometaphase then begins with nuclear envelope breakdown, allowing chromosomes to attach spindle microtubules via kinetochores. The chromosomes are then aligned at the spindle equator, between the spindle poles and sister chromatids and are attached to opposite poles of the spindle via kinetochore microtubules. This phase is referred to as metaphase. During the fourth phase of mitosis, anaphase, the sister chromatids are separated into the two sister chromatids, kinetochore microtubules shorten and spindle poles move apart to bring about chromosome segregation. The cell then moves into telophase, where the two sets of daughter chromosomes reach opposite poles of the cell and once there, condense. At this stage a new nuclear envelope forms around each set of segregated chromosomes, completing the formation of two daughter nuclei containing a diploid complement of chromosomes identical to that of the parent cell, marking the completion of mitosis. Following mitosis, the final stage of cell division is cytokinesis, or division of the cytoplasm, which "pinches" the cell into two independent cells each with their own identical nuclei. This series of events must always proceed in the stated

order, and mechanisms exist to ensure that the cell cycle cannot continue to the next stage without successful completion of the previous stage (figure 1.1).

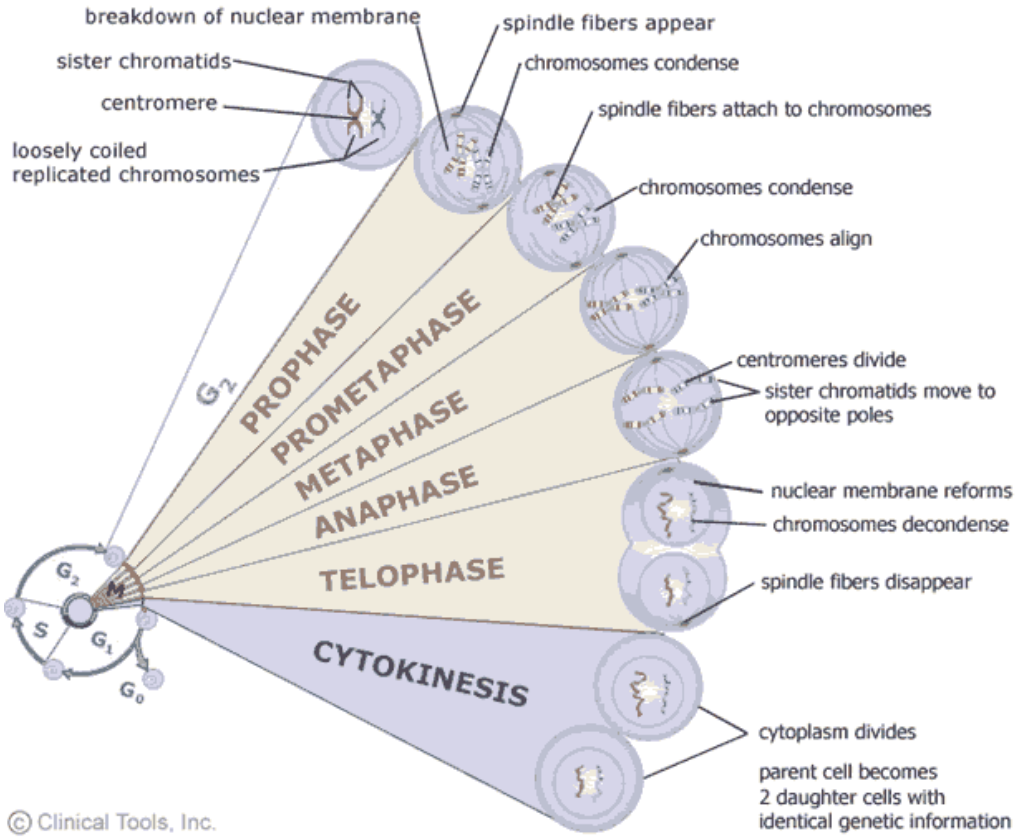


Figure 1.1. An overview of M phase and cytokinesis.

During early mitosis, the pairs of sister chromatids are attached to the mitotic spindle by microtubules. By mid-mitosis or metaphase, each of the pair of sister chromatids are attached to separate microtubules, originating from opposite poles of the spindle, with sister chromatid cohesion destroyed during anaphase, whereby microtubules pull the separated chromosomes to opposite ends of the cell. The two sets of chromosomes are packaged into two daughter nuclei, with constriction of the cytoplasm and deposition of a new membrane during cytokinesis marking the end of the cell cycle. Image taken from: http://www.le.ac.uk/ge/genie/vgec/he/cellcycle.html.

1.2. Introduction to the Mitotic Spindle Assembly Checkpoint

Referred to as the "guardian of the genome" (Iwanaga and Jeang, 2002) the spindle assembly checkpoint (SAC) ensures that all chromosomes are properly aligned at the metaphase plate before the cell cycle is allowed to progress to anaphase. This ensures that premature sister chromatid separation is avoided, and so daughter cells receive an equal and identical chromosome compliment. So why is this checkpoint so important? Chromosome instability and aneuploidy (abnormal chromosome number) has profound effects (Hassold and Hunt, 2001) and is linked to various cancers (Yuen et al., 2005). The SAC is critical in the preservation of euploidy (normal chromosome number), loss of which is a common characteristic of cancer cells, contributing to their malignant progression (Weaver and Cleveland, 2006; Weaver et al., 2006). Loss or downregulation of SAC components have been reported in a number of cancer cell lines. An example of such a component is the SAC protein Mad2. Mutation of SAC component Mad2 has been linked to diseases including gastric cancer (Kim et al., 2005) and has been found to be downregulated in cancer cell lines. With links between kinetochore dysfunction and cancer (Li and Benezra, 1996), the importance of the SAC in human disease is becoming ever more apparent. SAC components are commonly being researched as potential gene therapy targets where downregulated in cancer cell lines (Morozov et al., 2007) , as well as targets for anti-tumour therapies due to the lethality of cells with complete loss of checkpoint function (Kops et al., 2005).

1.2.1. Initial implications into the existence of a mitotic spindle assembly checkpoint by the identification of Mads and Bubs.

In 1991, two groups (lead by Andrew Murray and Andrew Hoyt) set out to isolate mutations in budding yeast, defective on any feedback mechanism preventing cells leaving mitosis until an intact spindle is present. The data they published identified two key groups of mutations, *mad* and *bub*, and resulted in the first implication of a mitotic checkpoint related to the dependency of microtubule function in mitotic exit.

(Li and Murray, 1991) isolated five mutants in *Saccharomyces cerevisiae*, mapped to three loci, named Mitotic Arrest Deficiency (*mad)* to reflect the progression of the mutants through mitosis whether or not a spindle is present

(and therefore, in the presence of unattached kinetochores). The *mad* mutants were shown to die at the end of nuclear division, presumably whilst attempting to segregate their chromosomes in the absence of a functional spindle. The technique they employed, utilised their understanding that a protein kinase, called Maturation Promoting Factor (MPF), regulates mitotic entry and induces mitotic spindle assembly. Benomyl (which inhibits microtubule polymerisation) was used to slow down microtubule assembly in *mad* mutants, allowing them more time to assemble their spindle. By observing activated MPF in *mad* cells as they progress through the cell cycle under this treatment, they showed that MPF levels in these mutants failed to stabilise. The consequence of this was decreased MPF levels pushing *mad* cells towards mitotic exit, regardless of their non-functional mitotic spindle. Premature mitotic exit in the presence of Benomyl resulted in cell death. The properties of these *mad* mutants, including the requirement of cells to pass through mitosis to be killed by Benomyl and the ability to override Benomyl-induced death using independent methods to delay mitotic exit, implicates that *mad* mutants are defective in a feedback control mechanism, now understood to be the SAC.

In addition to the work carried out by Li and Murray (1991), (Hoyt et al., 1991) used a similar approach (also in yeast), screening with Benzymidazole (a Benomyl-related compound) to isolate three mutants in which budding was uninhibited. The Budding Uninhibited by Benzymidazole mutants, named *bub*, proceeded to the next cell cycle stage to replicate their DNA and duplicate their spindle, despite unsuccessful completion of mitosis. By crossing *bub* mutants into an α-tubulin mutant, in order to replace chemical microtubule disruption with genetic disruption, they confirmed that the phenotype displayed by these mutants was microtubule-specific. They also demonstrated that this microtubule disruption alone was not sufficient to cause cell death. As with the *mad* mutants, cells were also required to progress through the cell cycle in order for lethality to incur. Also in accordance with results observed in *mad* mutants, the regulation of MPF activity was also affected in *bub* strains.

The identification of *mad* and *bub* mutants was the first time that the existence of a mitotic spindle assembly checkpoint, linked to MPF activity, was implied. At this time, many key SAC proteins had yet to be discovered, and so the understanding into the mechanisms by which Mad and Bub proteins may

regulate MPF was unclear. Subsequent studies into these proteins has lead to our current understanding of SAC components including Mad1, Mad2, Bub1, Bub3 and Bub-Related kinase 1 (BubR1), and provided a platform for the development of current models of spindle assembly checkpoint regulation of the metaphase to anaphase transition in mitosis.

Drosophila	*H. sapiens*	*X. leavis*	*S. cerevisiae*	*S. pombe*
Fizzy	Cdc20	Cdc20	Cdc20	Cdc20
BubR1	BubR1	BubR1	Mad3	Mad3
Mad2	Mad2	Mad2	Mad2	Mad2
Ald	Mps1	Mps1	Mps1	Mps1

Table 1.1. Key spindle checkpoint homologues.

Table showing the key *Drosophila* checkpoint protein names and their homologues in humans, *Xenopus*, and yeast.

1.3. The DNA Damage Checkpoint: A Brief Overview

The DNA damage response, or DNA damage checkpoint, acts at G1/S as well as within S-phase and G2/M (but not in mitosis) to block the cell cycle in the presence of any potentially lethal genetic errors which may result from chromosomal DNA damage. A highly conserved surveillance mechanism triggers a cascade of events which coordinate the repair of the detected lesion with cell cycle arrest. The checkpoint is only satisfied once the offending lesion has been successfully removed and repaired. Failure of the cell to detect DNA damage can have catastrophic effects, and has been linked to increased cancer susceptibility.

DNA damage can present itself in various forms. The nucleotides of DNA are continuously subject to modification by hydrolysis and oxidation reactions, these can lead to the introduction of genetic error of a number of types. External environmental factors can also contribute to genetic modification, such as exposure to ultraviolet (UV) radiation (Sancar and Reardon, 2004).

Alterations in DNA structure most commonly affect one DNA strand at a given site, resulting in a single strand break (ssb), however, it is possible for both strands of the DNA to be disrupted, resulting in a double strand break (dsb).

Because breaks in DNA are capable of fragmenting chromosomes and can often lead to chromosomal rearrangements if the DNA repair machinery accidently fuses broken DNA ends from different chromosomes, they are potentially very harmful and so must be repaired.

The DNA damage checkpoint does not respond to all genetic errors within chromosomal DNA, it only becomes active when the normal routes of DNA repair are unable rectify the problem, or the damage has caused replication forks to stall and produce abnormal DNA structures. In eukaryotes the DNA damage response is under the control of two members of the phosphoinositide three-kinase-related kinase family (PIKK) (Zhang et al., 2006), ATM and ATR (Lovejoy and Cortez, 2009; Cimprich and Cortez, 2008; Barr et al., 2003).

ATM is specialised to the repair of double-strand breaks (dsb), and its activation is required for checkpoint activation in response to these breaks (Pereg et al., 2006; Shiloh, 2003). ATR is also required for checkpoint activation in response to dsbs, but is also responsible for a number of other responses, such as those to nucleotide damage (ssbs) and stalled replication forks (Zhang et al., 2006). The specific recruitment of either ATM or ATR to sites of DNA damage is believed to be due to their localisation requiring different proteins or complexes. In the case of ATR, recruitment is believed to be via a protein named RPA, whereas ATM recruitment to dsbs is believed to be in response to its activation by binding of the MRN complex (Jazayeri et al., 2006) Once recruited, the ATM and ATR can initiate the DNA damage response, halting the cell cycle and initiating the relevant repair mechanisms.

Simple nucleotide alterations (single strand breaks) can be repaired by two systems; the first is base excision repair (BER) the second system is nucleotide excision repair. Double stranded breaks can be repaired by two mechanisms, non-homologous end joining (NHEJ) and homologous recombination. If repair mechanisms are unable to rectify DNA damage, the block on cell cycle progression may never be lifted, or more commonly, the cell will be subject to death by apoptosis.

1.4. The Eukaryotic cell cycle is highly ordered and tightly regulated

The cell cycle consists of a highly ordered sequence of biochemical events, triggered in a specific order and at specific time points. This control system is tightly regulated by a number of key components, mainly the cyclin-dependent kinases (Cdks). These biochemical switches control the transitions of the cell cycle through three main phases; cell cycle start, defining entry into the cycle at late G1 phase, and the two cell cycle checkpoints; the G2/M checkpoint (controlling entry into mitosis), and the spindle assembly checkpoint (SAC) which controls the metaphase to anaphase transition.

1.4.1. An Introduction to Cyclin-Dependent kinases

Cdks are small serine/threonine heterodimeric protein kinases that require association with a cyclin subunit for their activation. The kinase is inactive when it is not bound to its cyclin subunit, with the kinase activation site actively blocked in the absence of cyclin (De Bondt et al., 1993). Once bound, cyclin-Cdk must also undergo phosphorylation of a particular threonine residue of the kinase subunit activation loop (specific to each individual kinase) which is also required for its full activation (Zhang et al., 1998). Cyclin-Cdk dimers are relatively stable, with dissociation of those tested displaying half-lives of up to a number of h (Kobayashi et al., 1994). Each cyclin-Cdk complex promotes the activation of the next in sequence, maintaining the highly ordered fashion of the cycle. In most cases the concentration of kinase subunit is relatively constant throughout the cell cycle, whereas the concentration of the cyclin subunit oscillates, with this changing concentration of cyclins during the cell cycle believed to be one factor which leads to the specific timing of Cdk activation.

Members of the Cdk and cyclin families have binding surfaces which are largely interchangeable, thus allowing many different cyclin-Cdk combinations to form in cells. These contribute to a number of cellular processes, but with regard to those cyclins involved in regulation of the cell cycle, the classic model organises them according to which of the three main phases (G1, G2/M or Metaphase /anaphase) they control. In this model, cyclin E – Cdk2 activation is required to initiate S phase, cyclin A – Cdk1 is then responsible for its continuation, with B-

type cyclins and Cdk1 or Cdk2 responsible for initial entry into mitosis (Yamaguchi et al., 2000).

Active Cdks can also be switched off in a number of ways, examples of which include the degradation of the cyclin subunit by ubiquitin-mediated proteolysis (Jarviluoma et al., 2006), or being subject to association with inhibitor proteins or modification caused by inhibitory phosphorylations (Harvey and Kellogg, 2003).

1.4.2. Cell cycle regulators and their destruction by ubiquitin-dependent proteolysis

Ubiquitin dependent proteolysis of cell cycle regulators, such as cyclins, is one means by which the unidirectional nature of cell cycle transitions are achieved. The activation of Cdks, as well as proteolytic destruction of cell cycle regulators, is an irreversible mechanism which facilitates the highly ordered nature of cell cycle events (Tyers and Jorgensen, 2000).

Cyclins, Cdk inhibitors, as well as other regulatory proteins, are targeted for degradation by a process known as ubiquitination, whereby multiple copies of the 76-amino acid polypeptide ubiquitin are conjugated to proteins via isopeptide linkage. Ubiquitination is carried out in multiple steps; ubiquitin activation, conjugation, and protein ligation by a set of enzymes known as E1, E2 and E3 ubiquitin ligases (Pickart, 2001). Ubiquitinated proteins are then recognised and destroyed by a large ~2.5MDa multisbunit complex called the proteosome.

Ubiquitin ligases are categorised into four major classes, based on their structural motif; HECT-type, U-box-type, PHD-finger type and ring-finger-type (Ingham et al., 2004; Fang et al., 2003; Joazeiro and Weissman, 2000; Kamura et al., 1999; Ohta et al., 1999). Ring-finger ubiquitin ligases are further categorised into subfamilies, the largest of which is the cullin-based E3's, which contain a distinct globular C-terminal domain (or cullin homology domain) as well as a series of N-terminal five-helix-bundle repeats (cullin repeats). Two major classes of cullin-based ring-finger E3 ubiquitin ligases, the SKP1-CUL1-F-box-protein (SCF) complex, and the anaphase-promoting complex, or cyclosome (APC/C), have a central role in cell cycle transitions, regulating the

G1-S transition and the metaphase to anaphase transition respectively. As part of the same category of E3 ubiquitin ligases, the APC/C and SCF complex are therefore structurally similar to one another, yet their cellular functions are distinct, with APC/C active between anaphase to the end of G1, and SCF active from G1 to early M phase but is mainly functional at G1-S phase (Nakayama and Nakayama, 2006).

1.4.2.1. The SCF complex

The SCF is comprised of three core subunits, the RING protein Rbx1, the cullin Cul1, and Skp1. The SCF complex interacts with a number of interchangeable F-box bridging proteins via Skp1, which contain at least one F-box domain to mediate protein-protein interactions. This domain regulates the binding specificity of the SCF (Bai et al., 1996), allowing specific recruitment of target proteins to the SCF for ubiquitination, based upon the identity of the associated F-box protein (Feldman, 1997; Skowyra et al., 1997). The rate of substrate ubiquitination is also controlled by the changing affinity of the substrate to its corresponding F-box, rather than core enzyme activity, which remains largely unchanged throughout the cell cycle.

The recognition of target substrates by F-box proteins is usually dependent on modification, commonly in the form of substrate phosphorylation (von der Lehr et al., 2003; Yoshida et al., 2002; Nash et al., 2001; Feldman, 1997). The ability of the SCF to recognise phosphorylated residues on the substrate called phosphodegrons, allows substrate discrimination. A phosphodegron is one or a series of phosphorylated residues that directly interact with protein-protein interaction domain motifs (such as an F-box motif) in an E3 ubiquitin ligase, to link the substrate to the conjugation machinery (Winston et al., 1999). This means that until a specific cluster of residues within the target substrate are phosphorylated, they will not be recognised by their corresponding F-box, and cannot be targeted for degradation by the SCF. As these phosphorylations are often carried out by Cdks, this ubiquitin-mediated proteolysis can be linked to specific times in the cell cycle, based on levels of Cdk activity.

In yeast, the cell cycle can be divided into high and low Cdk activity states, with low activity lasting from telophase until late G1, and high activity from G1/S to

anaphase. The oscillating levels of high and low Cdk activity allow a distinct pattern of DNA replication and chromosome segregation (as low Cdk activity is required for the formation of prereplicatory complexes and high Cdk activity is required for initiation of DNA replication and subsequent drive into mitosis). During stages of low activity, SCF proteolysis is largely prevented, with ubiquitination by the APC/C possible, whereas SCF-dependent degradation is stimulated and APC/C activity inhibited in cell cycle stages where Cdk activity is high, allowing specific activation of SCF at G1/S and APC/C at mitosis (Nash et al., 2001).

1.4. The anaphase promoting complex / cyclosome (APC/C)

The ability of cells to regulate the metaphase-anaphase transition in the presence of misaligned chromosomes or unattached kinetochores is crucial in maintaining genome stability. The SAC prevents anaphase onset by targeting the activation of the anaphase-promoting complex or cyclosome (APC/C). The APC/C is a 1.5MDa E3 ubiquitin ligase complex, comprised of at least 12 subunits, which during normal mitosis, triggers the transition from metaphase to anaphase (reviewed in Morgan, 2007). The APC/C performs its function by assembling polyubiquitin chains on substrate proteins, which then targets these substrates for destruction by the 26S proteosome (a multi-subunit proteosome which targets multi-ubiquitinated proteins for degradation) in order to inactivate proteins who's action is no longer required (Thornton and Toczyski, 2006). The various targets of the APC/C contain amino acid sequence motifs which are believed to be required for their recognition and subsequent ubiquitination, the most frequently observed being destruction boxes (D-boxes) and KEN boxes (Peters, 2006).

The APC/C was Initially implicated in the destruction of mitotic cyclins during metaphase, (Clute and Pines, 1999; Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995) in a normal cell cycle, the APC/C is found in the interphase nucleus, spreading throughout the cytoplasm to associate with the spindle apparatus during mitosis. The activity of APC/C depends upon two cofactors, cell division cycle 20 (Cdc20) and Cdh1, which interact transiently with APC/C to activate it during early mitosis, and from late mitosis to the G1-S transition, respectively (Peters, 2006).

With important roles in cyclin proteolysis (Tang et al., 2001b; Irniger et al., 1995; King et al., 1995) APC/C ubiquitin ligase (E3) activity is required for cell proliferation in all eukaryotes, although the most well known function of the APC/C is its role in sister chromatid separation (Kudo et al., 2006). In order for chromosome segregation to occur, the cohesion between sister chromatids must be removed. This is initiated at metaphase by active APC/C^{Cdc20}-mediated ubiquitination of securin, a protein which functions as a co-chaperone and inhibitor of the protein separase. Once activated, separase cleaves a subunit of cohesin, the Scc1 complex, who's function is to hold sister chromatids together. This results in their separation and the subsequent onset of anaphase (Kudo et al., 2006). In the absence of APC/C, cells are unable to separate sister chromatids and progress through mitosis (Au et al., 2002; Peters, 2002).

During mitosis, phosphorylation of Cdh1 by CDK's (Zachariae et al., 1998), as well as binding of nucleoporins to Cdh1 (Jeganathan et al., 2006), prevents APC/C-Cdh1 binding. This ensures that APC/C^{Cdc20} is the major form of APC/C present. At the metaphase-to-anaphase transition, APC/C^{Cdc20} also initiates degradation of cyclin B, lowering Cdk1 activity. The previously described separase (free of securin due to APC/C degradation) is also capable of direct binding with cyclin B and inhibiting Cdk1 (Gorr et al., 2005). Inhibition of Cdk1 reduces the level of CDK activity rendering it insufficient to prevent Cdh1 from binding APC/C. Active APC/C^{Cdh1} also degrades Cdc20, leading to a complex switching mechanism from APC/C^{Cdc20} to APC/C^{Cdh1} as the cell undergoes mitotic exit (Peters, 2006).

1.4.1. The specific nature of APC/C activity

The action of APC/C requires one of two WD40 proteins as activators, Cdh1, or in the case of its mitotic role, Cdc20 (Burton and Solomon, 2001; Visintin et al., 1997). Although the precise function of these WD40 activators remains speculative, it has been suggested that they may interact dynamically with the APC/C to recruit substrates by their WD40 domains, or to enhance the specific activity of the APC/C (Yu, 2007; Peters, 2006). The substrate specificity of APC/C is critical, as its role in mitotic progression requires the ability to select different substrates for destruction at specific times. Understanding of how this specificity is achieved remains poor, but WD40 activator proteins Cdc20 and

Cdh1 appear to be key (Visintin et al., 1997). One model predicts that WD40 activator proteins act as substrate-recognition motifs to bind and “usher” substrates to the APC/C due to their ability to bind both substrate and the APC/C itself (Yamano et al., 2004), although, prior to this, a destruction box (D-box) motif was discovered on the N-terminus of cyclin B1 which was required for its ubiquitination by APC/C (Sancar et al., 2004).This is believed to be the most common motif recognised by the APC/C, which includes some version of the sequence RXXLXXXXXN, where R is arginine, X is any amino acid, L is leucine and N is asparagine. Loosely conserved D-boxes have been found to mediate the destruction of many APC/C substrates, functioning as recognition sequences for both APC/C^{Cdc20} and APC/C^{Cdh1}. Four different models have been proposed to explain the contribution of Cdc20 to D-box-dependent substrate recognition by APC/C (Yu, 2007).

The sequential model proposes that the D-box first binds to Cdc20, then to APC/C. In the composite binding pocket model, the D-box binding site is formed at the Cdc20-APC/C interface. In the multivalency model, both Cdc20 and APC/C contain binding sites for the D-box and other APC/C substrates. The final model, the allostery model, predicts a conformational change on APC/C triggered by Cdc20 binding which reveals a D-box binding pocket. Alternative destruction motifs also exist such as the KEN-Box motif found on Cdc20 (Pfleger and Kirschner, 2000). The KEN box motif includes versions of the amino acid sequence KENXXXN, where K is lysine and E is glutamate. It is understood that the KEN box is required for ubiquitination of substrates after anaphase and in G1 where only APC/C^{Cdh1} is active (Bashir and Pagano, 2004; Pfleger and Kirschner, 2000; Visintin et al., 1997). Although Cdc20 may be capable of direct binding with D-box motifs due to its WD40 domain (Kraft et al., 2005), it has been shown that the receptor for the D-box of Cyclin B is the protein-protein interaction domain or TPR domain of the APC3/Cdc27 subunit, not Cdc20 (Vodermaier, 2004; Vodermaier and Peters, 2004; Yamano et al., 2004). In addition, TPR-domain containing APC/C subunits may also interact with substrates independently of WD40 activators (Steen et al., 2008; Yamano et al., 2004). As well as the presence of a WD40 activator protein, the APC/C can only ubiquitinate substrates with the help of a further two co-factors, an ubiquitin-activating (E1) enzyme, and an ubiquitin-conjugating (E2) enzyme. E1

functions to create a thioester bond between its active-site cysteine and the C-terminal glycine residue of ubiquitin, resulting in ubiquitin activation (Hershko et al., 1981). Ubiquitin is then transferred to the active-site of the E2 molecule (Hershko et al., 1983), which with the joint activity of E3, transfers ubiquitin to the targeted substrate (Hershko and Ciechanover, 1998).

It is therefore believed that all APC/C interactions require ternary complex formation (between APC/C, the substrate, and one WD40 activator protein) in the presence of E1 and E2 for the ubiquitination reaction to occur (Passmore et al., 2005). One hypothesis (Baker et al., 2007) predicts that APC/C is loaded with the target substrate before the transfer of ubiquitin takes place through the association of an E2 enzyme. This model is described with regard to current understanding of the SAC and mitotic checkpoint complex (MCC), whereby the complex associates with APC/C to inhibit ligase activity until the SAC is satisfied. Once satisfied, and all chromosomes are properly aligned, the rapid destruction of cyclin B occurs. In prometaphase, cyclin B already associates with APC/C, yet it is not destroyed until the metaphase-anaphase transition (Jeganathan et al., 2006), in the presence of the WD40 activator Cdc20.

Closely related to the importance of the ability of the APC/C to target specific substrates is the mechanism by which it can ignore substrates until specific points in the cell cycle. The activity of APC/C oscillates throughout the cell cycle, with lower activity in S and G2 phase, and high during mitosis and G1. If the APC/C is active throughout, how does it select when to target a specific substrate and when to ignore it? One model proposes an explanation for SAC-independent destruction of APC/C substrates in that early mitotic APC/C substrates are very efficiently targeted to any residual checkpoint-independent APC/C^{Cdc20} (van Leuken et al., 2008). It also considers the possibility that APC/C^{Cdc20} may become increasingly active with its progressive phosphorylation immediately after nuclear envelope breakdown (NEB) (Lindqvist et al., 2007), whilst the checkpoint specifically prevents the ability of APC/C to recognise securin or cyclin B. At present it remains unclear as to whether specific features of APC/C substrates determine the timing of their destruction. Alternatively, since it has been observed that phosphorylated APC/C and Cdc20 are enriched at kinetochores (Lindqvist et al., 2007; Acquaviva et al., 2004), where cyclin B1 degradation is initiated upon

checkpoint release, it is proposed that different pools of APC/C, with regard to their intracellular localisation, may hold the answer (van Leuken et al., 2008).

1.5. The Kinetochore

Kinetochores themselves are large protein assemblies built upon the end or middle of centromeres. Their primary function is to create load-bearing attachments between chromosomes and microtubules in the dividing cell, upon which proper partitioning of sister chromatids to the daughter cell depends (Walczak and Heald, 2008; Wittmann et al., 2001).

Microtubule-kinetochore attachment itself is subject to various models. Initially a "search and capture" model was proposed, whereby microtubule dynamic instability was suggested to be a feature allowing them to explore space, selectively stabilising once they hit their targets (Waters et al., 1996). During mitosis, the target of these dynamic microtubules is the kinetochore, for which a crucial function is the stabilisation of kinetochore-bound microtubules (Shaw et al., 1998; Waters et al., 1996; Zhai et al., 1996). Evidence also exists that kinetochores can promote the growth of small microtubule stubs generated in their vicinity (Tulu et al., 2006; Telzer et al., 1975), as well as being capable of maintaining attachment to growing or disassembling microtubules (Shaw et al., 1998).

1.5.1. Kinetochore Feedback Mechanisms

Two proposed, and related, feedback mechanisms exist in which the kinetochore is fundamental, the first being the SAC. Here, cell cycle progression is synchronised with microtubule attachment, whereby sister chromatid cohesion and mitotic exit through degradation of Cyclin B is regulated by kinetochore-microtubule attachment or tension. The second feedback mechanism involves recognition of correct and incorrect kinetochore-microtubule attachments (Kelly and Funabiki, 2009; Pinsky and Biggins, 2005). Correct attachments, whereby sister kinetochores are attached to opposite spindle poles (bi-orientation or amphitelic attachment), are stabilised. This allows equal distribution of sister chromatids to daughter cells at anaphase (Li and Nicklas, 1995; Nicklas and Koch, 1969). Incorrect attachments such as merotelic (in which a sister is connected to both spindle poles) and syntelic

attachment (in which both sisters in a pair connect to the same spindle pole) fail to stabilise, allowing their subsequent correction (Lampson et al., 2004; Cimini et al., 2003; Nicklas and Koch, 1969).

The potential link between these two mechanisms lies with Aurora B (Santaguida and Musacchio, 2009). Aurora B has been proposed to be indirectly involved in the SAC, by destabilising incorrect or tension-less kinetochore-microtubule attachments. For this reason it has been suggested that Aurora B is required to activate the SAC in response to decreased tension, implicating its involvement in tension sensing across the centromere (Stern and Murray, 2001). A proposed mechanism for the action of Aurora B in destabilising incorrect attachment relates to its phosphorylation of members of the kinesin-13 family, which catalyse depolymerisation at the ends of microtubules (Moores et al., 2006) and contribute to correction of chromosome attachment errors (Kline-Smith and Walczak, 2004). The most well characterised member of this family is mitotic centromere associated kinases (MCAK). Aurora B phosphorylates multiple sites on MCAK to regulate its localisation to the inner centromere or kinetochore (Buster et al., 2007; Andrews et al., 2004). The destabilisation of tension-less kinetochore-microtubule attachments by Aurora B in turn leads to the recruitment of SAC proteins such as Mad2, BubR1 and Bub3 to the kinetochore to activate the checkpoint and arrest cell cycle progression (Pinsky et al., 2006; Pinsky and Biggins, 2005). Unattached kinetochores also fall into the category of tension-less. With nocodazole treatment (and resulting lack of attachment) Aurora B is active at kinetochores (Liu and Lampson, 2009; Liu et al., 2009), raising questions as to the direct implications of Aurora B activity in SAC control. However, the requirement of Aurora B for recruitment of SAC proteins to the kinetochore (which is a requirement of the SAC) in the presence of microtubule depolymerising drugs (Ditchfield et al., 2003; Hauf, 2003) strengthens the case for a direct involvement of Aurora B in SAC control and reinforces the link between kinetochore-microtubule attachment, error correction, and the SAC (Santaguida and Musacchio, 2009).

It is clear that the kinetochore is key in the SAC response, but what is the actual trigger that causes SAC to become active and prevent the metaphase to anaphase transition?

1.6.2. Kinetochore Stretch

SAC monitoring of kinetochore attachment as a crucial mechanism to ensure accurate chromosome segregation and genomic stability, is a widely accepted concept (Yuen et al., 2005). However, the less understood role of tension in SAC release remains the topic of much debate. Chromosome biorientation stretches chromatin between microtubules and attached kinetochores, and the potential affect of stretch within the kinetochore itself is often overlooked. In recent months, a new aspect of tension with relation to SAC has been brought to our attention. Two separate groups have focussed on the importance of tension in SAC release by attempting to distinguish between interkinetochore stretch (the stretch between sister kinetochores) produced by kinetochore-microtubule attachment, and intrakinetochore stretch (stretch within kinetochores).

1.6.2.1. Interkinetochore versus intrakinetochore stretch

The first (Uchida et al., 2009) used a HeLa cell line that stably expresses two components of the kinetochore, GFP- Centromere Protein A (CENP-A) to mark the inner kinetochore and mCherry-Mis12 to mark the outer kinetochore. Interkinetochore distances were measured by the distance between CENP-A markers, with intrakinetochore distance measured between CENP-A and Mis12 markers. Their findings showed that in metaphase cells, the Mis12 signal deviated from the pole, suggesting an increase of intrakinetochore length, whereas interkinetochore length changes were infrequent. These movements of CENP-A and Mis12 imply that kinetochores undergo repetitive extension and recoil or "kinetochore stretching". The second group, Maresca and Salmon, 2009, also used fluorescent markers with the generation of a *Drosophila* S2 cell line stably expressing mCherry centromere identifier (CID, a homologue of CENP-A) to mark the inner kinetochore and GFP-Ndc80 as the outer kinetochore marker (Cheeseman et al., 2006; Salmon et al., 2005).

The overall findings of both systems suggested a lesser importance of interkinetochore stretch in SAC release. Uchida et al took the approach of using low doses of microtubule poisons or depletion of condensin, a chromosomal protein complex known to confer integrity to centromeric chromatin (Gerlich et

al., 2006) to produce conditions in which kinetochore stretching is inhibited but interkinetochore stretch is retained. This would distinguish between the relevance of stretching kinetochores from stretching inner centromeres. It was observed that when treated with 7ng/ml nocodazole or depleted of condensin by RNAi, no obvious effect on interkinetochore stretch could be seen, yet intrakinetochore stretch was decreased and SAC inhibition was released. In agreement with this data, (Maresca and Salmon, 2009) showed that S2 cells treated with low concentrations of taxol reduced interkinetochore stretch whilst intrakinetochore stretch remained the same as in untreated control cells and the SAC was satisfied. Treatment with higher doses of taxol, both inter- and intrakinetochore stretch was reduced, and the SAC remained on.

Based upon the findings of both papers, it is intrakinetochore stretch and not interkinetochore stretch which has an effect on the SAC. Uchida et al propose a model by which biorientation induces stretching of centromeres (interkinetochore stretch). The kinetochores undergo intrakinetochore stretch sufficient to inactivate the checkpoint, and therefore promote SAC silencing. Silencing is not achieved when the intrakinetochore stretch is disturbed (by nocodazole or depletion of condensin), regardless of interkinetochore stretch, and the checkpoint remains active.

1.6.2.2. Models of kinetochore stretch

Two models are proposed to explain how kinetochores are stretched. The mechanical stretching model proposes that the stretching is caused by the mechanical forces between microtubules and kinetochores, with deformation based on an elastic property of kinetochores. The findings that centromere integrity is required for kinetochore stretching favour this hypothesis; however, intrakinetochore stretch and SAC release were observed in conditions too low to produce interkinetochore stretch. The second model proposes that stretching may be a result of conformational rearrangements of the components of the kinetochore itself. In this case, intrakinetochore stretching need not reflect the presence of tension. (Maresca and Salmon, 2009) agree with the second model proposed by Uchida et al. They also hypothesise that intrakinetochore stretch is generated by translocation of protein elements within the kinetochore structure. This may be in response to the attachment of dynamic microtubules, behaving

like a low-tension "mechanical switch" which controls SAC silencing by altering the localisation of regulatory factors within the kinetochore.

Although the mechanism by which intrakinetochore stretching occurs is unknown, the most likely answer would appear to lie with the conformational rearrangement of the kinetochore itself. Further studies into the exact rearrangements as well as how these translate into SAC release are essential in understanding the full picture with regard to SAC activation and silencing in response to tension.

1.6. Checkpoint Activation: Attachment and Tension

The primary signal that activates the SAC remains unclear. Although it is widely accepted that chromosomes which have failed to achieve bi-polar attachment to the mitotic spindle is the target of the checkpoint, a debate remains between models which propose lack of microtubule-kinetochore attachment as the checkpoint trigger and those which suggest a sensing mechanism which recognises lack of tension generated on the kinetochore. The fact that attachment and microtubule-kinetochore tension are interdependent also makes it difficult to determine which of these may play the larger role.

In the presence of any misaligned chromosome, unattached kinetochores recruit a number of evolutionarily conserved SAC proteins; Mps1, BubR1, Bub3, Mad1, Mad2 and Mad3 (May and Hardwick, 2006). In metazoans these are accompanied by additional proteins; Aurora B, CENP-E and the Rod-Zw10-Zwilch complex (May and Hardwick, 2006). When these SAC proteins are recruited close to the kinetochore it allows the formation of an inhibitory complex, the mitotic checkpoint complex (MCC). The MCC comprises of checkpoint proteins Mad2, Mad3/BubR1 and Bub3, as well as the APC/C activator Cdc20 (Sudakin et al., 2001). When part of this inhibitory complex, Cdc20 is unable to activate APC/C activity (Tang et al., 2001a; Fang et al., 1998a), therefore preventing exit from mitosis.

The SAC achieves inhibition of APC/C by preventing APC/C from binding its activator Cdc20, as well as promoting Cdc20 degradation to lower levels upon checkpoint activation (Chen, 2007). There are conflicting ideas about exactly how the APC/C is inhibited by the SAC, the most likely being a dynamic model

in which APC/C is regulated by Cdc20 binding by a complex comprised of SAC proteins, all of which have been shown to localise to, and concentrate at kinetochores during prometaphase (Maiato et al., 2004; Cleveland et al., 2003). The direct target of the SAC, therefore is Cdc20. Cdc20 is recruited to kinetochores independent of APC/C (Vigneron et al., 2004) from prometaphase to telophase (Raff et al., 2002; Kallio et al., 1998) Investigation in *Xenopus* (Vigneron et al., 2004) has shown that Mps1, Aurora B and CENP-E act upstream of other checkpoint proteins and are required to induce the correct localisation of Cdc20 during prometaphase (Kallio et al., 1998).

1.7. "Catalytic" model of checkpoint activation

There are currently two models aiming to explain how unattached kinetochores produce a diffuse signal to prevent APC/C activation in the cytoplasm. The first model suggests that Cdc20 is inactivated in the cytoplasm by association of Cdc20 or its inhibitors with the unattached kinetochores (Kallio et al., 1998) (Howell et al., 2000). In support of this "catalytic model" it has been reported, as is the case for other SAC proteins such as Mad2, Cdc20 associates with mitotic kinetochores transiently. However, the dynamic exchange of Cdc20 with kinetochores is thought to be four times faster than those published for Mad2 (Kallio et al., 2002). This distinct difference in Cdc20 trafficking may be related to the mechanism by which the checkpoint signal is broadcast from unattached kinetochores and diffused throughout the cell. A current model (Kallio et al., 2002) suggests that rapid circulation of Cdc20 at all unattached kinetochores, already containing high concentrations of SAC proteins such as Mad2, BubR1 and Bub3, involves binding either directly to kinetochores or indirectly through another SAC protein (Eg. Bub3) (Fraschini et al., 2001). This binding is predicted to be via WD-40 domains of Cdc20, leaving binding domains for inhibitory proteins Mad2 and BubR1 free to then interact with Cdc20 and prevent it from activating APC/C. The rapid dynamic exchange of Cdc20 at the kinetochore may therefore be a mechanism allowing Cdc20 to continuously sample kinetochores and maintain cell-wide APC/C inhibition (Kallio et al., 2002).

1.8. "Sensitisation" model of checkpoint activation

A second model the "APC/C sensitisation model" proposes that checkpoint-active kinetochores "sensitise" the APC/C to inhibition by the inhibitory mitotic checkpoint complex (Sudakin et al., 2001). This model could be achieved by rapid exchange of APC/C with kinetochores, however existing evidence of APC/C kinetochore localisation conflicts this "exchange" theory (Topper et al., 2002). Another SAC component, the kinase Mps1, is a proposed diffusible signal from the unattached kinetochore, as it binds APC/C and may therefore sensitise it to inhibition by the MCC (Liu et al., 2003). It is however yet to be proven as to whether Mps1 exchanges rapidly between kinetochores as would be required in this model. To understand and determine the most likely mechanism for Cdc20 inhibition it is important therefore that we understand the localisation of other SAC components.

The hypothesis that unattached kinetochores are the activator of the SAC was first proposed by a study in mitotic rat PtK cells, in which electron microscopy and live-cell imaging revealed that a single unattached kinetochore was capable of arresting cells at metaphase. This coupled with the finding that laser ablation of the centromere and associated sister kinetochores (to destroy the last monooriented chromosome) relieved the SAC, allowing cells to pass into anaphase, provided further support for the attachment hypothesis. The study went on to destroy one kinetochore on a bi-orientated chromosome in the presence of an attached monooriented chromosome, revealing that under this condition anaphase continued. This suggested that the SAC signal is not generated by the attached kinetochore of a monooriented chromosome. Finally with ablation of the remaining monoorientated chromosome, the SAC was rendered inactive. Overall the study concluded that molecules in or near the unattached kinetochore of a monoorientated chromosome is the likely trigger for the SAC (Rieder et al., 1995). Studies have been carried out in yeast in which mutants defective for the four evolutionarily conserved proteins of the Ndc80 kinetochore complex were used. The Ndc80 complex is a conserved outer kinetochore component required for microtubule binding (reviewed by (Cheeseman and Desai, 2008; Ciferri et al., 2005). Ndc80 mutant cells are unable to attach chromosomes to the mitotic spindle, and, in all cases were confirmed to be SAC defective (McCleland et al., 2003; He et al., 2001;

Schramm et al., 2001; Wigge and Kilmartin, 2001). In addition to this, another attachment hypothesis which links kinetochore-microtubule binding to the SAC with regard to the Ndc80 complex introduces the KMN "super complex" comprised of KNL-1/AF15Q14/SPC105/Blinkin, Mis12 complex and Ndc80 complex, shown to be a critical microtubule-binding interface on kinetochores and required for attachment (Emanuele et al., 2007). Two of the proteins which form the KMN complex, Ndc80 (Hec1 in human cells) and KNL-1 (homologous to human Blinkin) are microtubule interacting proteins (Buffin et al., 2007; Cheeseman et al., 2006), both of which have been implicated in the SAC by direct interaction with SAC proteins. If this is the case, competition between signalling and microtubule binding may exist (Burke and Stukenberg, 2008).

The SAC protein Mps1 kinase, required for SAC and in some organisms for the duplication of microtubule organising centres (Winey and Huneycutt, 2002), has also been implicated in regulating microtubule attachment (Jones et al., 2005). This link between the SAC and the microtubule-kinetochore binding interface further supports the attachment model of SAC activation. In recent months the attachment model with respect to SAC linkage has been expanded upon with regard to unattached kinetochores catalyzing the production of an inhibitory complex that requires Mad2 as a template for Cdc20-BubR1 binding (Kulukian et al., 2009), (Li and Benezra, 1996). This study used an in vitro assay for Cdc20-stimulated ubiquitination of APC/C to demonstrate that unattached kinetochores on purified chromosomes (equating to quantities of around ten unattached chromosomes) generate a diffusible Cdc20 inhibitor, or possibly inhibit Cdc20 already bound to APC/C.

Using molecular markers for the lack of attachment at kinetochores is another approach taken to support the tension hypothesis. Mad2 localisation studies have shown that it binds unattached kinetochores in prometaphase and is lost from the kinetochore in metaphase once microtubule-kinetochore attachments are made (Chen et al., 1996; Li and Benezra, 1996). To distinguish between the localisation of Mad2 to unattached kinetochores and those with reduced tension, micromanipulated cells and cells treated with taxol, were examined for Mad2 localisation. Taxol is a microtubule-stabilising treatment which results in sister kinetochores moving closer together (an indication of loss of tension). It was shown that Mad2 does not accumulate on the kinetochore under these

conditions, and so it has therefore been proposed that Mad2 specifically marks unattached kinetochores (Waters et al., 1998). There are, however, limits to the use of SAC proteins such as Mad2 as markers for unattached kinetochores. It has been argued that cells treated with taxol always show at least one kinetochore capable of staining with Mad2, and so the possibility exists that taxol alters microtubule occupancy. It has also been shown that in cells treated with monastrol, which blocks spindle-pole separation and results in syntelic attachments which are attached but lack tension, Mad2 accumulates on the majority of kinetochores (Kapoor et al., 2000). This indicated that Mad2 kinetochore localisation may not be specific to unattached kinetochores. It may, however be due to the attachments generated by monastrol treatment not being at their full microtubule occupancy. It has been argued that the reduced microtubule occupancy may result in decreased stability of attachments that are not under proper tension, and therefore taxol may activate the checkpoint by creating unoccupied microtubule-binding sites, not lack of tension (Pinsky and Biggins, 2005).

1.9. The Mitotic Checkpoint Complex (MCC)

Both of the two presented models to account for the inhibition of Cdc20 activity by the SAC, the "catalytic" and "APC/C sensitisation" models, share a common component, the presence of an inhibitory complex, the MCC. The MCC is comprised of SAC proteins Mad2-BubR1-Bub3 and Cdc20 (Sudakin et al., 2001). Other smaller complexes have also been reported in yeast, *Xenopus* and mammalian cells upon checkpoint activation including Mad2-Cdc20 and Cdc20-BubR1-Bub3 (Chen, 2002; Millband and Hardwick, 2002). The MCC binds the APC/C, eliminating its ubiquitin-ligase activity on securin and cyclin B (Morrow et al., 2005; Poddar et al., 2005; Sudakin et al., 2001; Fang et al., 1998a). Although the MCC may be a key inhibitor of APC/C and therefore potentially the most important component of the SAC, it relies on many other SAC components upstream. These include Mad1, essential for the interaction between Mad2 and Cdc20 (De Antoni et al., 2005) and Mps1, phosphorylation of which is required for the localisation of checkpoint proteins including Bub1, Bub3, Mad1 and Mad2 (Zhao and Chen, 2006).

Regarded as the primary SAC effector, the MCC accumulates in mitosis and appears to associate with APC/C (Morrow et al., 2005; Hardwick et al., 2000). Within the complex both Mad2 and Mad3 are able to directly bind Cdc20, the interaction between Mad3 and Cdc20 requires all SAC proteins, whereas the only essential proteins required for the Mad2-Cdc20 interaction are Mps1 and Mad1 (Hardwick et al., 2000; Hwang et al., 1998). Partially purified MCC from mitotic HeLa cells has indicated that Mad2, Bub3, BubR1 and Cdc20 exist in apparently equal stochiometries (Sudakin et al., 2001) yet it is presently unclear as to how and when the components of the complex interact to form the MCC. Theories as to the location of MCC formation take two standpoints: at the kinetochore and in the cytosol, independent of kinetochore interaction. In order to fully analyse which is the more likely explanation, the localisation and binding capabilities of the components of the MCC must be considered.

The least characterised MCC component is the kinase Bub3, the binding partner of BubR1, required for its localisation (as well as that of Bub1) (Taylor et al., 1998) Although Bub3 has never been shown to bind APC/C or Cdc20 directly, it is clearly an essential SAC component, as without it cells are unable to arrest in the presence of spindle damage (Lopes et al., 2005).

1.10. Cdc20: the proposed key target for inhibition by incorporation into the MCC

Cell division cycle 20 (Cdc20), the activator and substrate adaptor of APC/C, is a key molecule for the metaphase to anaphase transition, and is believed to be the primary target of the SAC in its route to APC/C inhibition (Fang et al., 1998a; Kramer et al., 1998). Expression of Cdc20 is regulated at several levels throughout the normal cell cycle. Both Cdc20 protein and RNA levels reach a peak during mitosis (Haugwitz et al., 2004; Morris et al., 2003; Kramer et al., 2000; Prinz et al., 1998), with the generation of functional Cdc20 (by proper protein folding) aided by the CCT chaperonin in order to promote activation of the APC/C (Camasses et al., 2003).

1.10.1. Normal Cdc20 activity

In a normal cell, Cdc20 localises to the centrosomes during interphase, with slight exclusion from the nuclei. It then rapidly accumulates in the nuclear

region, localising at kinetochores and the metaphase plate, later displaying microtubule and centrosomal association during mitosis (Raff et al., 2002). Distinct domains of Cdc20 are required for its association with kinetochores (WD-40 repeats) and centromeres (the NH_2 terminal region of Cdc20, containing the Mad2 binding domain) (Kallio et al., 2002). Cdc20 associates with mitotic kinetochores transiently, as is the case for Mad2, but has a reported turnover rate four times faster than Mad2 exchange (Kallio et al., 2002). Kinetochore associated Cdc20 is reported to exchange with a cytoplasmic pool, independent of microtubules. This supports a concept whereby transient association of Cdc20 with unattached kinetochores maintains APC/C inhibition towards targets whose ubiquitination or degradation are required for anaphase onset (Kallio et al., 1998). This lead to the theory that Cdc20 circulates rapidly at all kinetochores, binding via WD-40 repeats (potentially via another WD-40 repeat containing SAC protein such as Bub3). The WD-40 repeat binding at kinetochores leaves free the NH_2 terminal domain for Mad2 binding and or possible interaction with another SAC protein BubR1, while at the kinetochore Cdc20 accepts inhibitory proteins which prevent it from binding APC/C (Kallio et al., 2002). Cdc20 also directly binds BubR1, through a Mad3 homology domain of BubR1. This interaction is dependent upon SAC protein Mad2 (Davenport et al., 2006). This has not yet been proven to be the case in *Drosophila*.

1.11. Cdc20 phosphorylation

Cdc20 in mammalian cells has been shown to be phosphorylated by MAPK, Cdc2 protein kinase Cdk1 and the SAC protein Bub1 (Tang et al., 2004a), the kinase activity of which is stimulated upon SAC activation. Failure of Bub1 to phosphorylate Cdc20 results in a defective SAC, implicating this event as essential in the SAC function of Cdc20 (Tang et al., 2004a). However, some controversy exists as to the role of phosphorylation of Cdc20 by Bub1 in the SAC as it has been shown that in *Xenopus* egg extracts depleted for endogenous Bub1, SAC function can be restored with the addition of a kinase-inactive Bub1 mutant (Chen, 2002). Whether these discrepancies are the result of differences in SAC mechanisms between organisms or due to experimental limitations remains unproven.

A number of highly conserved sites within the N-terminal of human Cdc20 have been implicated as potential phosphorylation sites and can be observed over various species including *Xenopus* and *Drosophila*. In *Xenopus laevis,* four of these; Ser 50, Thr 64, Thr 68 and Thr 79, are believed to be required for SAC function. Mutation of these sites substantially decreases Cdc20 phosphorylation by Cdk1 in mitotic egg extracts (Kramer et al., 2000), and with their introduction in order to generate phosphorylation-deficient Cdc20 mutants, the SAC is rendered inactive (Chung and Chen, 2003). The role of these four phosphorylation sites in SAC function has also been investigated with regard to the interaction of Cdc20 with other checkpoint proteins Mad2, BubR1 and Cdc27. With a single mutation to any of the four implicated sites, reduction in the interaction between Cdc20 and Mad2 in response to unattached kinetochores is observed, with loss of all four sites displaying a marked decrease (Visconti et al., 2010; Chung and Chen, 2003). The phosphorylation of Cdc20 at these positions is believed to be mainly a result of Cdc2 (Cdk1) activity, as with inhibition of Cdk1 by high concentration p21/Cip1/Waf1, Cdc20 phosphorylation in wild type extracts can no longer be observed (Visconti et al., 2010). Cdk1 activity has been shown to have an effect on the interaction between Cdc20 and APC/C as well as Cdc20-Mad2 and Cdc20-Cdc27 in HeLa cells. With the inhibition of Cdk1 activity, Cdc20-Mad2 binding is greatly reduced, with no apparent effect on Cdc20-Cdc27 (Visconti et al., 2010). It should however be considered that a noticeable decrease in Cdc20 phosphorylation is also observed with inhibition of MAPK by U0126, suggesting that, although MAPK is not essential, Cdk1 and MAPK may together play a role in Cdc20 phosphorylation (Chung and Chen, 2003).

Overall Visconti et al (Hagting et al., 2002), suggest a hypothesis whereby Cdk1 may phosphorylate APC/C, a requirement of its activity, but at the same time phosphorylate Cdc20 to stabilise Cdc20-Mad2 interactions. This in turn lessens Cdc20-APC/C binding to provide a precondition for active checkpoint proteins such as Mad2 to interact with Cdc20 in response to unattached kinetochores. In this case, once correct spindle assembly is satisfied, and checkpoint proteins are no longer activated, APC/C-Cdc20 can begin to form. Alternatively, Cdk1 activity may be regulated by the checkpoint mechanism so that upon completion

of the mitotic spindle assembly, a drop in activity dissociates Cdc20 from other active checkpoint proteins allowing reactivation of APC/C-Cdc20.

Cdc20 regulation involves changes in its abundance during the cell cycle, with levels negligible during G1, rising to their peak in mitosis before rapidly declining (Fang et al., 1998b; Weinstein, 1997). But with findings which implicated cell-cycle dependent Cdc20 phosphorylation, it is now believed that this also has a role in the regulation of Cdc20 and its ability to activate the APC/C. Levels of Cdc20 phosphorylation in mammalian cells have been reported to be maximal during G2, dropping during the G2-M phase transition, whilst expression of Cdc20 is actually still rising (Weinstein, 1997). This finding implied that Cdc20 phosphorylation actually precedes its increase in abundance. Subsequent investigation into conditions in which Cdc20 is phosphorylated suggested an association between increased phosphorylation and mitotic arrest, both by the microtubule depolymerising agent nocodazole (Kramer et al., 1998) and in *Xenopus* eggs naturally arrested in metaphase II by cytostatic factor (CSF) (Lorca et al., 1998). This lead to questions as to whether the phosphorylation of Cdc20 may negatively regulate the action of the APC/C, as the observed increase of Cdc20 phosphorylation during mitotic arrest corresponds with the inhibition of APC/C activity observed during the spindle checkpoint response.

There are conflicting reports regarding the role of Cdc20 phosphorylation in the regulation of APC/C activity. At least three kinases (Cdk1, MAPK and Bub1) have been implicated in Cdc20 phosphorylation, with various potential phosphorylation sites observed across species. It is therefore plausible that phosphorylation of Cdc20 at different sites, by a number of different kinases, may have different roles in the checkpoint response.

1.11.1. Cdc20 phosphorylation by Cdk1

An early paper into Cdc20 phosphorylation by Cdk1/cyclin B, using bacterially expressed renatured Cdc20, converted it into a form capable of activating APC/C (Kotani et al., 1999), although subsequent attempts to replicate this were unsuccessful (Kramer et al., 2000). As these initial attempts to uncover the role of Cdc20 phosphorylation were deemed unreliable, an *in vitro* system

was set up in order to study the differential effects of Cdc20 and Cdh1 phosphorylation on the activity of APC/C in HeLa cells (Kramer et al., 2000). Here, synchronised HeLa cells were used to purify cyclosomes from different cell cycle stages using gel filtration chromatography. The effect of Cdc20 and Cdh1 on the cyclin-ubiquitin ligase activity of the cyclosomes was then examined. Cdh1 was shown to stimulate cyclosome activity in all cell cycle stages, whereas Cdc20 only stimulated its activity in samples subject to nocodazole arrest (M phase) or after release from nocodazole arrest (M-phase exit). No effect was observed in G1 or S-phase. This confirmed the previous correlation between Cdc20 phosphorylation and conditions in which the APC/C is inactive.

This system was then used to investigate the effect of Cdh1 and Cdc20 phosphorylation using a two-step incubation in which S-labelled proteins were incubated in the presence or absence of Cdk1/cyclin B in a reaction mixture containing MgATP. Protein kinase activity was then terminated with staurosporine before the addition of cyclosomes from M-phase cells and the cyclin-ubiquitin ligation reaction was carried out as done previously. This confirmed that Cdk1/cyclin B (as well as Cdh1) phosphorylation of Cdc20, inhibited its ability to stimulate cyclosome activity. In order to eliminate the possibility that Cdk1/cyclin B phosphorylation observed was not due to the presence of other proteins, which could potentially phosphorylate and inhibit the action of Cdc20, a mutant of Cdc20 in which seven out of eight potential Cdk1/cyclin B phosphorylation sites were converted to alanine (serine 41, 408 and 452; threonine 55, 59, 70 and 157) was tested. It was observed that the phosphorylation-defective mutant Cdc20 protein was capable of stimulating cyclosome activity, confirming that the effect of Cdc20 phosphorylation upon cyclosome activation was specific. The effect of phosphorylation on binding to the cyclosome was then determined by co-immunoprecipitation with anti-Cdc27 antibody, which showed an abolished Cdc20-cyclosome interaction upon Cdk1/cyclin B phosphorylation and a proper interaction in between the cyclosome and the mutant protein. Overall, this paper provided a link between Cdc20 phosphorylation and checkpoint control via the inhibition of APC/C control, yet the connection with a checkpoint-dependent control mechanism remained unclear.

Following these findings, the requirement of cyclin-dependent kinases in the spindle checkpoint was investigated further (D'Angiolella et al., 2003). This study illustrates the stabilisation of Cdk1 (as well as Cdk2) activity under checkpoint conditions in HeLa cells, and goes on to investigate the role of Cdks in the interactions between Cdc20, Mad2 and the APC/C in order to identify a mechanism by which Cdk phosphorylation affects the activation of APC/C. On the basis that when cells are released from nocodazole arrest, activation of the APC/C ^{Cdc20} requires the dissociation of Mad2 from Cdc20 (Fang et al., 1998b), experiments were carried out to attempt to establish whether the Cdc20 phosphorylation by Cdk has an effect on its ability to dissociate from Mad2. For this they used the Cdk inhibitor Roscovitine in checkpoint arrested cells. Cdk inhibition was found to override checkpoint-dependent arrest in HeLa cells, with immunoprecipitation experiments on cells at time points after Roscovitine treatment showing a drastic reduction in Cdc20-Mad2 interaction, but a maintained Cdc20-Cdc27 (APC/C) interaction. This was consistent with previous data in that without Cdk1 phosphorylation, Cdc20 binds (and activates) APC/C (Kramer et al., 2000) but also lead to the hypothesis that Cdk activity restrains APC/C^{Cdc20} activation during checkpoint arrest by stabilising the Cdc2—Mad2 interaction, thus preventing Cdc20 from activating the APC/C. In support of this hypothesis, HeLa proteins were separated on longer SDS-PAGE runs to visualise mobility changes of Cdc20 from checkpoint arrested cells at various time points after Cdk inhibition. Cdc20 mobility increased after treatment with Roscovitine, suggesting that it becomes dephosphorylated, with the timing of this dephosphorylation compatible with the timing of the increase in Cdc20-APC/C binding and decrease in Cdc20-Mad2 binding observed in the previous experiment.

Because Roscovitine inhibits both Cdk1 and Cdk2, further analysis was carried out to distinguish which cyclin-dependent kinase was likely to be responsible for this. *Xenopus* egg extracts, arrested in M-phase by CSF, in which Cdk1 activity is sustained by redundant mechanisms so that Cdk2 can be inhibited without affecting Cdk1 activity (Tunquist et al., 2002) were incubated with S-labelled Cdc20 or mutant Cdc20 (as described in (Kramer et al., 2000) which had been pre-treated with active Cdk1. These were treated with a low concentration of p21/Cip1/Waf1, which selectively inhibits Cdk2. SDS-PAGE analysis displayed

a normal Cdc20 gel mobility shift. In contrast with high concentrations capable of also inhibiting Cdk1, the mobility shift was prevented. This implied that Cdk1 and not Cdk2 was responsible for the phosphorylation of Cdc20.

To test whether Cdk1-dependent phosphorylation affected Cdc20 interactions with Mad2 and Cdc27, S-labelled wild type Cdc20 was analysed, with the non-phosphorylatable mutant Cdc20 (Kramer et al., 2000) used as a control. Higher binding of Cdc27 was observed with the mutant Cdc20 than with the wild-type protein, with levels of Cdc20-Mad2 interaction higher in the wild type. This confirms previous findings that Cdk1 inhibition induces dissociation of Cdc20 from Mad2, and a transient increase of Cdc20-APC/C. To investigate the significance of the observed decrease in Mad2-Cdc20 interaction with Cdc20 phosphorylation in Mad2-dependent checkpoint arrest, pre-phosphorylated wild-type and mutant Cdc20 proteins were incubated with a CFS-arrested extract, before CSF was inactivated and cyclin degradation was measured. Cyclins remained stable in the extract containing wild-type Cdc20, but degradation of cyclins was restored in the extract containing the non-phosphorylatable mutant. This suggested that Mad2-dependent checkpoint arrest was maintained in the presence of Cdk1-phosphorylated wild type Cdc20, but not in mutant protein.

Although Cdk1 activity appears to regulate the SAC by stabilising the Cdc20-Mad2 interaction, regulation of the mechanism by which Cdk1 phosphorylation of Cdc20 inhibits its ability to activate the APC/C remains largely speculative. It was proposed that during mitosis, Cdk1 may perform positive phosphorylations of APC/C (Hagting et al., 2002) but at the same time lessening the interaction between APC/C and Cdc20 by Cdc20 phosphorylation, and so providing a precondition for checkpoint proteins to interact with Cdc20 upon activation of the SAC. Upon satisfaction of the SAC, checkpoint proteins are no longer recruited and Cdc20 can go on to interact with and activate APC/C. It is, however, unclear at present whether the phosphorylation which has been implicated to maintain the interaction between Cdc20 and Mad2 is also required for the interaction to occur in the first place. Alternatively, the pathways that control Cdk1 activity (D'Angiolella et al., 2003) may be sustained by the checkpoint mechanism, with completion of mitotic spindle assembly leading to a proteolysis-dependent drop in Cdk1 activity. This would allow the interaction between Cdc20 and APC/C, amplified by the dissociation of other checkpoint

proteins from Cdc20 (D'Angiolella et al., 2003). At this point, further investigation into the effect of other checkpoint proteins and their Cdk1-influenced interaction with Cdc20 would need to be investigated in order to implicate either hypothesis.

The potential phosphorylation sites which were substituted for alanine in the mutant Cdc20 protein are conserved in human and *Xenopus* and all reside in the N-terminal of Cdc20, a region already identified as a Mad2 binding domain (Zhang and Lees, 2001). The previously described studies implicate the phosphorylation of Cdc20 by Cdk1 to be important in maintaining the interaction of Cdc20 with Mad2, but whether it is needed for the interaction to take place, and what effect it has on the binding of Cdc20 to other components of the MCC has yet to be investigated. Also, although these findings strongly implicate Cdk1 in this phosphorylation, no measures to rule out or implicate any other kinases were carried out.

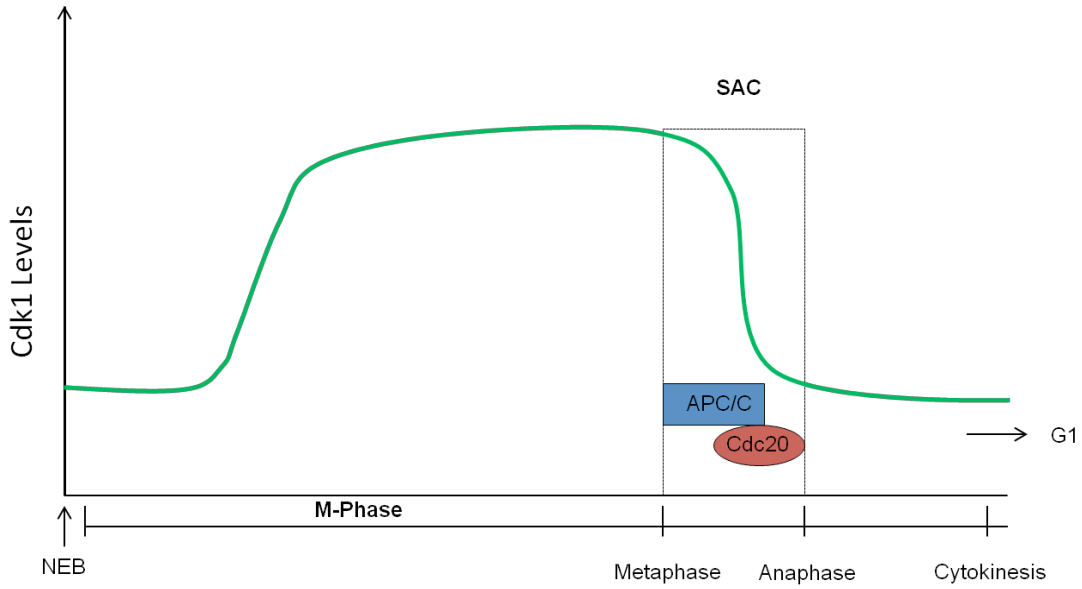


Figure 1.2. Graph to illustrate Cdk1 levels during M-phase.

After nuclear envelope breakdown (NEB), Cdk1 levels rise and remain stable throughout M-phase until after the metaphase to anaphase transition. Here, in the presence of the SAC, Cdk1 levels are high and APC/C^{Cdc20} is the major form of APC/C. At the later stages of mitosis, after the SAC, Cdk1 levels drop rapidly and remain low as cells move into G1.

1.12.2. Cdc20 phosphorylation by MAP Kinase

Cdc20 also contains several Ser/Thr-Pro sequences that are potential targets for MAPK, a kinase already implicated in checkpoint signalling (Wang et al., 1997; Minshull et al., 1994). Chen et al 2003 used a MAPK inhibitor (U0126) to block MAPK activity, and detected a reduced gel mobility shift in *Xenopus* extracts treated with this inhibitor. This implied that Cdc20 phosphorylation is, at least in part, carried out by MAPK. A mutant was then generated which made substitutions at the five conserved sites within the Mad2 binding domain, with Ser50 and Ser114 residues replaced with Ala and Thr64, Thr68 and Thr79 replaced with Val. Mitotic egg extracts for this mutant as well as wild-type Cdc20 were labelled with ^{32}P, with failure of the mutant Cdc20 to incorporate ^{32}P implying that these mutations prevented mitotic phosphorylation. By later creating single substitution mutants for the five conserved sites and repeating the procedure, it was shown that all single mutants apart from Ser114 displayed a significant decrease in ^{32}P. It was concluded that the four sites likely to be involved in mitotic phosphorylation of Cdc20 were Ser50, Thr64, Thr68 and Thr79.

The role of MAPK in these phosphorylations was pinpointed further with the use of two-dimensional tryptic phosphopeptide analysis of ^{32}P-labelled wild-type Cdc20.Three major peptides were revealed, one of which disappeared and was replaced with a fourth peptide in U0126-treated extracts. Peptide mapping of the five single-mutant Cdc20 showed that this missing peptide correlated with undetectable phosphorylation at the Thr64 and Thr68 mutants. This suggested that MAPK phosphorylates either Thr64 or Thr68 of *Xenopus* Cdc20.

In order to determine whether this phosphorylation was required for regulation of the SAC, it was observed that after incubation with sperm nuclei and nocodazole, phosphorylation-deficient mutants for each individual conserved site or mutants in which all four sites had been substituted for Ala or Val, all display de-condensed chromosomes indicative of failure to maintain mitotic arrest in response to improper or unattached kinetochores. This implies that in order for Cdc20 to be inhibited in response to the SAC signal, all four conserved sites must be phosphorylated. But what role does this phosphorylation have in

Cdc20 inhibition and are these sites required for Cdc20 to be inhibited by the MCC complex?

To determine whether the phosphorylation-deficient Cdc20 mutants were capable of interacting with other checkpoint proteins, they then immunoprecipitated Cdc20 from metaphase extracts treated with nocodazole and performed immunoblot analysis to reveal that when all four phosphorylation sites are disturbed, levels of associated Mad2 and BubR1 are greatly reduced. This suggests that the phosphorylation of *Xenopus* Cdc20 at Ser50, Thr64, Thr68 and Thr79 is important for its association with BubR1 and Mad2 in response to unattached kinetochores. This phosphorylation can be partially attributed to MAPK, which is enriched at kinetochores during mitosis (Mandell et al., 1998; Shapiro et al., 1998), inhibition of which by U0126 reduced the mobility shift of Cdc20 in mitotic extracts (Chung and Chen, 2003), although the importance of other mitotic kinases such as Bub1 and Cdc2 and Cdk1 cannot be ruled out.

The phosphorylation status of these conserved sites within the N-terminal region of *Xenopus* Cdc20, all of which overlap with the previously proposed Mad2 binding domain of Cdc20 (Zhang and Lees, 2001), appear to be unquestionably linked to the ability of Cdc20 to interact with Mad2. This would suggest that the formation of the MCC to activate the SAC in response to unattached kinetochores requires phosphorylation of Cdc20 at these sites. The phosphorylation of Cdc20 in order to bind the MCC and eliminate activation of the APC/C is a plausible hypothesis. However, the exact role of this phosphorylation, as well as confirmation of the kinases involved specifically in regulation of other MCC components and their interactions within MCC formation must be understood in order to combine all current proposed models into a more detailed understanding of the SAC mechanism.

1.12.3. Cdc20 phosphorylation by Bub1

The least characterised kinase believed to be involved in Cdc20 phosphorylation is Bub1. In a 2004 study (Tang et al., 2004b) Bub1 was depleted in HeLa cells by RNAi in the presence of spindle damaging agent nocodazole. While almost all control cells arrested in mitosis, a significant

proportion of Bub1 depleted cells failed to undergo mitotic arrest, and exited from mitosis without completing cytokinesis. This implied that Bub1 was required for mitotic arrest of HeLa cells in response to spindle damage, as expected due to the essential nature of Bub1 as a SAC component. Because Bub1 contains a kinase domain, they went on to test whether it phosphorylated Cdc20. A wild-type Bub1 and kinase-inactive Bub1 ($Bub1^{k82A}$) were coexpressed with Bub3, shown to always associate with Bub1 in HeLa cells, and purified the resulting Bub1 complexes that would potentially associate with Cdc20. This showed that in the case of the wild-type, at a molar ratio of Bub1:Cdc20 of 1:10, and assuming each Cdc20 molecule was phosphorylated at one site on average, 74% of Cdc20 was phosphorylated. In contrast, the kinase-deficient mutant failed to phosphorylate Cdc20. In this study, Plk1, Mps1, Aurora A and B, as well as BubR1 were tested and all failed to phosphorylate Cdc20. As did two members of the MAPK family, p38 and extracellular signal-related kinase 1 (ERK1). This is inconsistent with previous findings by Chen *et al*. (2003) in *Xenopus* extracts. This could be due to differences between human and *Xenopus* Cdc20, or by differences in the way in which MAPK regulates Cdc20 phosphorylation between species, but the inconsistency between these studies should be noted.

In support of the role of Bub1 in mitotic arrest, Bub1 was then shown to inhibit APC/C^{Cdc20} *in vitro* using a reconstituted APC/C ubiquitination assay with a fragment of Cyclin B1 as a substrate. Again, wild-type Bub1 (plus Bub3) was shown to inhibit the activity of APC/C, with the kinase-deficient mutant failing to do the same. In an attempt to determine which sites on Cdc20 were required for the Bub1-dependent phosphorylation which lead to inhibition of APC/C, mass spectrometry was used. Endogenous Cdc20 from nocodazole-treated HeLa cells and protein treated with Bub1-Bub3 (already shown to phosphorylate Cdc20 *in vitro*) both identified the same six Ser and Thr residues, located in the N-terminal of Cdc20 (Ser41, Ser72, Ser92, Ser153, Thr157 and Ser161). These sites are different from the two potential phosphorylation sites which were identified in a 2003 study from the same species (Kraft et al., 2003). Here, two potential Thr residues were identified (Thr70 and Thr 146), although it was not determined as to which kinase was implicated in phosphorylation at these sites. Another factor which may have had an influence on this discrepancy is the

isolation method used to purify the endogenous protein, which differed between studies. (Kraft et al., 2003) isolated only APC/C-bound Cdc20 via the use of an anti-Cdc27 antibody, whereas (Tang et al., 2004a) isolated all Cdc20 within the cellular population via an anti-Cdc20 antibody. Why these two pools of Cdc20 might display different sites available for phosphorylation remains unclear.

Tang *et al*. then followed the route of mutating the potential Cdc20 phosphorylation sites to alanine to test the ability of this mutant Cdc20 to be phosphorylated. It was observed that the recombinant mutant Cdc20 was only weakly phosphorylated by Bub1-Bub3 *in vitro*, indicating that the six sites identified were involved in Bub1-mediated phosphorylation of Cdc20. Interestingly, this recombinant Cdc20 was as efficient as the wild-type protein in promoting the activity of the APC/C, with Bub1-Bub3 failing to inhibit the activity of the APC/C-mutant Cdc20 complex. Although it cannot be ruled out that Bub1 itself could be phosphorylating APC/C directly for its inhibition, these results may suggest that it is the phosphorylation of Cdc20, not other core APC/C subunits, that is required for inhibition of APC/C^{Cdc20} by Bub1 (Tang *et al*., 2004).

In contrast to studies into the importance of Cdc20 phosphorylation by MAPK and Cdk1 on the spindle checkpoint, investigation into the effect of mutation to potential Bub1 phosphorylation sites had less convincing effects. Although cells expressing the mutant Cdc20 were observed to progress through mitosis without cytokinesis in the presence of nocodazole or taxol, in cells where the endogenous Cdc20 was depleted by siRNA, only 50% of cells expressing the phosphorylation mutant Cdc20 protein escaped mitotic arrest. A reduction of the levels of securin were also observed, indicative of cells progressing through mitosis. This does imply that Bub1 phosphorylation of Cdc20 has a role in the functional checkpoint response, although it appears that the checkpoint is only partially defective with a reduction in Bub1 phosphorylation.

In support of the idea that the checkpoint is only partially defective with reduction in Bub1 phosphorylation, data into the interaction of Cdc20 with other checkpoint proteins in the absence of Bub1 phosphorylation was tested. It was observed that BubR1 and Mad2 were still capable of binding Cdc20 when the potential Bub1 phosphorylation sites were mutated. It was also observed that

mutant Cdc20 still localised to kinetochores, arguably in support of the idea of a kinetochore-dependent MCC formation in the checkpoint response. A repeat APC/C ubiquitination assay also identified that the mutant Cdc20 was efficiently inhibited by Mad2 and BubR1. This implies that the phosphorylation of Cdc20 by Bub1 is not involved in the formation of the MCC complex. It is more likely, based on the findings of Tang *et al*., 2004. that Bub1 inhibits APC/C^{Cdc20} catalytically and its involvement in Cdc20 phosphorylation may be related to the diffusible signals of the checkpoint response, rather than the formation of the APC/C inhibitory complex.

1.12. The role of Cdc20 phosphorylation in the SAC remains unclear

The connection between phosphorylation of Cdc20 and the SAC is apparent, with loss of Cdk1 and MAPK phosphorylation resulting in a defective checkpoint and reduced association with checkpoint proteins Mad2 and BubR1 (Chung and Chen, 2003; D'Angiolella et al., 2003; Kramer et al., 2000), and partially defective checkpoint observed with the loss of Bub1 kinase activity (Tang *et al.,* 2004). However, with regard to the exact role of Cdc20 phosphorylation in the SAC response pathway, many issues remain unclear. It is most likely that modification by each kinase is related to a specific Cdc20 function, may take place at different phosphorylation sites depending on the kinase involved, and these phosphorylations are likely to be specific to cell cycle stage and timed in accordance with their function. The contribution of each kinase in the overall phosphorylation of Cdc20 has not yet been established, the localisation of checkpoint proteins when kinase activity toward Cdc20 is disrupted, and the various interactions which are believed to occur in order for formation of the SAC inhibitory mitotic checkpoint complex have not been fully explored.

With regard to the role of phosphorylation in the inhibition of Cdc20 by the MCC, it has already been observed that Mad2 and BubR1 association with Cdc20 is disrupted when MAPK and Cdk1 phosphorylation is abolished (Chung and Chen, 2003; Kramer et al., 2000), but the reason behind this failure of MCC components to interact is not understood. Where failure of these proteins to interact has been observed, their localisation has not been studied and so opens the debate as to whether a failure of non-phosphorylatable Cdc20 to

localise to unattached kinetochores may contribute to disrupted MCC formation, resulting in an abolished checkpoint.

Although with the loss of Bub1 phosphorylation, Cdc20 was shown to localise to kinetochores (Agarwal et al., 2003), in this system, checkpoint protein interaction remained intact and the checkpoint itself was only partially defective. It cannot be ruled out that the reason the checkpoint was not abolished completely in this case was that Bub1 phosphorylation of Cdc20 is not required for its localisation to unattached kinetochores, therefore allowing normal Cdc20 localisation when Bub1 kinase activity is lost, and its subsequent interaction with other kinetochore localising checkpoint proteins. It would be interesting to see, with the loss of Cdk1 or MAPK phosphorylation of Cdc20 and subsequent failure of Cdc20-Mad2 and Cdc20-BubR1 interactions, whether Cdc20 is still observed at kinetochores.

1.14. Cdc20 binds directly to Bub-Related Kinase1

Cdc20 also has binding sites on Bub-Related kinase 1 (BubR1), a central component of the MCC, with important roles in processes directly linked to, and within the SAC. As well as the role of BubR1 in SAC, it is also directly implicated in microtubule-kinetochore attachments (in *Drosophila* S2 cells) (Maia et al., 2007), where monooriented and unattached chromosomes are displayed in BubR1-depleted cells, and BubR1 is reported to dissociate from kinetochores upon biorientation (Skoufias et al., 2001; Taylor et al., 2001). Along with this observation, reports into simultaneous BubR1 and CENP-E depletion reveals a role for CENP-E in the rescue of the partially unattached kinetochore phenotype in BubR1 depleted extracts. This implies that BubR1 and CENP-E may act antagonistically to correct inappropriate kinetochore attachment (Maia et al., 2007), ultimately resulting in SAC silencing.

The essential nature of BubR1 in the SAC, and its role in the interaction of SAC proteins Mad2-Cdc20-Bub3 with each other and the kinetochore, has been shown by impaired complex formation and kinetochore localisation in BubR1 immmunodepleted cells (Chen, 2002). BubR1 itself is present throughout the cell cycle (Li and Benezra, 1996) but has been shown to localise to unattached kinetochores, as illustrated by its outer kinetochore plate localisation in HeLa

cells (Johnson et al., 2004; Taylor et al., 2001), where it is reported to be phosphorylated (Wu et al., 2000; Chan et al., 1999; Fang et al., 1998a; Li and Benezra, 1996) (Wu et al., 2000). Hyperphosphorylation of BubR1 at unattached kinetochores has been shown to be a process directly enhanced by the presence of Mad1(Chen, 2002).

The localisation of BubR1 to kinetochores requires the action of Bub1, a structurally related protein that binds kinetochores but dissociates upon microtubule attachment (as is the case for SAC components CENP-E and CENP-F), although repression of Bub1 is yet to have any proven negative effect on the function of the SAC. This implies that the localisation of BubR1 must be under the control of a further mechanism which overrides the loss of Bub1 and allows kinetochore localisation of BubR1 upon SAC activation in its absence (Johnson et al., 2004). This control mechanism for BubR1 localisation has a proposed model involving another SAC component aurora B. Repression of aurora kinase activity in Bub1-repressed cells, shows a dependency for Aurora kinase activity in the localisation of BubR1 in the absence of Bub1 (Morrow et al., 2005). This result could suggest two possible explanations, the first being that Aurora B and Bub1 may play redundant roles in a timer mechanism. The other, and favoured, mechanism is that the lethality observed by depletion of both Bub1 and Aurora B is due to the checkpoint being composed of two arms, one arm dependent on Bub1 and the other on Aurora B, with the common denominators of the two arms being BubR1 and Mad2. This provides a plausible explanation as to why BubR1 and Mad2 are essential to SAC function, yet Bub1 and Aurora B alone are not (Morrow et al., 2005).

In checkpoint arrested cells BubR1 has been shown to have up to twelve times higher binding affinity with Cdc20 than Mad2, and is therefore reported to be a highly potent Cdc20 inhibitor (Fang, 2002). This promotes the suggestion that although increased levels of either Mad2 or BubR1 alone can themselves inhibit APC/C, they are likely to mutually promote each other's Cdc20 binding, together displaying synergistic function to inhibit Cdc20-APC/C (Fang, 2002). Although BubR1 is a kinase capable of phosphorylating Cdc20 directly (Wu et al., 2000), no evidence exists that this phosphorylation has any effect in the control of Cdc20 activity (Fang, 2002).

1.15. BubR1-Mad2 binding enables BubR1-Cdc20 interaction

Two Cdc20 binding sites exist on BubR1, one appearing to have no effect on the SAC, the other being highly specific to Mad2-bound Cdc20. Mutation of this specific domain weakens the BubR1-Cdc20 interaction and reduces SAC function (Davenport et al., 2006). The fact that mutation of this binding domain merely weakened Cdc20-BubR1 binding rather than causing the interaction to be abolished lead to the suggestion that more than one checkpoint complex containing the MCC components Cdc20, BubR1, Mad2 and Bub3, may exist, and that this may play a role in a two-step mechanism of Cdc20 inhibition (Davenport et al., 2006). Checkpoint inhibition in this case would require prior binding of Mad2 to Cdc20 in order to sensitise Cdc20 to BubR1 binding and inhibition. Davenport's model of the two-step inhibition of Cdc20 proposes that the N-terminal of Cdc20 physically blocks binding of BubR1 until it is bound by Mad2, at which point a conformational change of Cdc20 exposes a previously unavailable BubR1 binding site. This binding between the N-terminal of Cdc20 and BubR1 is shown in this research to be the region of interaction linked to SAC. In this model, since evidence existed to imply that the second BubR1 binding site of Cdc20 is not relevant to SAC function, this domain is ignored. Furthermore, the suggestion that the BubR1 involved in this reaction is already Bub3-bound is made, although this is yet to be proven. No suggestion as to whether the formation of the MCC complex in the two-step inhibition of Cdc20 model requires the presence of the kinetochore is provided. In order for the two-step inhibition model to be widely accepted, the relationship of other SAC proteins with those in the model, and the requirement of the kinetochore, whether direct or indirect via a protein such as Mad1, should be further investigated.

1.16. SAC proteins dynamically interact with kinetochores

Evidence does exist that Cdc20 associates transiently with mitotic kinetochores, along with SAC protein Mad2. The reported turnover rate for Cdc20 is four times faster than Mad2, implying that Cdc20 cycles rapidly on and off the kinetochore (Kallio et al., 2002). Cdc20 kinetochore localisation is believed to be via the WD-40 repeats, of which Cdc20 has seven (Kallio et al., 2002). A rapid exchange between kinetochores and a cytoplasmic pool of Cdc20 independent

of microtubules supports a previously proposed concept whereby transient association of Cdc20 and unattached kinetochores serves to maintain APC/C inhibition towards targets whose ubiquitination or degradation are required for anaphase onset (Kallio et al., 1998). The addition of Cdc20 kinetics at kinetochores lead to a modified theory in which Cdc20 circulates rapidly at all kinetochores (binding via WD-40 repeats). This binding may potentially be in conjunction with other WD-40 repeat-containing SAC proteins, a prime candidate for which is Bub3. Upon binding, an N-terminal domain of Cdc20, believed to contain a Mad2 binding site, remains available for the Cdc20-Mad2 interaction. This may also be responsible for association of other SAC proteins BubR1 and Bub3 to form the MCC complex responsible for preventing the activation of APC/C by Cdc20 (Kallio et al., 2002).

1.17. Potential mechanisms for Mitotic Checkpoint Complex (MCC) formation

The emerging picture of the potential mechanisms of MCC formation are in consensus with regard to the importance of one common interaction, Cdc20-Mad2. Mad2 is clearly one of the key SAC proteins, conserved in all eukaryotes (Musacchio and Salmon, 2007; Taylor et al., 2004). Unlike SAC component Cdc20, Mad2 localises inside of the nucleus throughout the entire cell cycle, however, reason for this nuclear localisation and its role in SAC function is unknown. In the nucleus Mad2 is recruited to the kinetochore during mitosis (Taylor et al., 2004; Cleveland et al., 2003) interacting transiently, but at a slower rate to that of Cdc20 (Kallio et al., 2002). Mad2 has been shown to associate directly with the APC/C in HeLa cells upon activation of the SAC by nocodazole treatment (Li and Benezra, 1996). Further investigation in *Xenopus* into this association illustrates the ability of Mad2 binding to APC/C to inhibit its activity (Li et al., 1997), dissociating from the cyclosome with inactivation of the SAC. However, the biochemical mechanism promoting the Mad2-APC/C interaction is unknown. The most widely accepted major role for Mad2 in the SAC is in the formation of the MCC, with the most commonly accepted sub-complex of MCC comprising of Mad2-Cdc20 (Taylor, Scott et al 2004). Cdc20 has two distinct Mad2 binding domains (Mondal et al., 2006). The N-terminal domain of Cdc20 is the most commonly known functional Mad2 binding domain (Zhang and Lees, 2001). It has since been reported that the C-terminal domain

of Cdc20 contains at least two functional domains in the SAC, one of which is directly involved in Mad2 binding (Xiong et al., 2008). The contribution of a second Mad2 binding domain in the Mad2-Cdc20 interaction has shown to reflect upon interaction through the first binding site. Blocking of the C-terminal Mad2 binding site of full-length Cdc20 destabilises its interaction with Mad2 via the N-terminal binding site. This suggests that binding of Mad2-Cdc20 is optimal through interactions of multiple domains (Xiong et al., 2008).

1.17.1. Mad2 adopts two distinct states

Mad2 itself displays three unusual properties. The first of these is that it adopts two distinct natively folded states (Mapelli et al., 2007; Luo et al., 2004), referred to as (O) open-Mad2 and (C) closed-Mad2. Secondly, these distinct states engage in a conformational dimer, essential for SAC function (Mapelli et al., 2007). Finally, closed-Mad2 has been reported to form topological links with Mad1 and Cdc20. It has been reported that the recruitment of Mad2 to kinetochores requires another SAC protein Mad1, and that this recruitment is essential for the efficient formation of a Mad2-Cdc20 complex (Hardwick, 2005)

1.17.2. Mad2 models: Template versus exchange

Two models exist to explain the role of Mad1 in the formation of Mad2-Cdc20 interaction, the "Mad2 exchange" and "Mad2 Template" models (Mapelli et al., 2006; Hardwick, 2005; Nasmyth, 2005) (figure 1.2). The Mad2 exchange model proposes that Mad1 recruits open-Mad2 at the kinetochore, changing its conformation to closed-Mad2, which then dissociates from the kinetochore and is capable of binding Cdc20. Closed-Mad2 is also formed when open-Mad2 binds its checkpoint target Cdc20 (Nezi et al., 2006). The exchange model, therefore proposes that Mad1 is the catalyst in the conformational change from open to closed-Mad2, which is in turn required for Cdc20 binding (Luo et al., 2004). The exchange model has been criticised by structural Mad2 studies which claim that Mad1 and Cdc20 bind the same Mad2 domain. If this were the case, Mad1 and Cdc20 would be in direct competition for Mad2 binding, eliminating the possibility of Mad1 activation of Mad2-Cdc20 binding (De Antoni et al., 2005). Studies into Mad2 kinetics also failed to show switching kinetics of Mad2 which are suggested by this model (Ibrahim et al., 2008).

The second model, the Mad2 template model overcomes the problem of Mad1 and Cdc20 competing for the same Mad2 binding domain. This model introduces the ability of Mad2 conformers (open and closed-Mad2) to bind one another in a conformational dimer (De Antoni et al., 2005; Luo et al., 2004). The presence of an open and closed-Mad2 conformational dimer was further tested and illustrated in *S. cerevisiae* (Nezi et al., 2006). The main proposals put forward by this model are that there is a Mad1-bound closed-Mad2 pool at the kinetochore and a non-exchanging pool of open-Mad2 in the cytosol. The open-Mad2 kinetochore receptor is a tight complex between Mad1 and closed-Mad2 (Sironi et al 2002). In this model a stable kinetochore-bound form of closed-Mad2 is formed, and acts as a receptor for open-Mad2. The model therefore does not imply that Mad1 and Cdc20 compete for Mad2 binding and also provides an explanation for the existence of two distinct Mad2 pools previously identified by FRAP analysis (Shah et al., 2004).

The implications of the Mad2 template model are that Mad1-closed-Mad2 at kinetochores is activated in order to trigger the conformational rearrangement of open-Mad2 needed for Cdc20 binding (Nasmyth, 2005). This Closed-Mad2 - Cdc20 would then presumably dissociate from Mad1-Mad2. In this case there must be a cytosolic pool of Mad1- closed-Mad2 and that this pool is unable to catalyse the structural rearrangement of Mad2, which should only occur in the presence of unattached kinetochores. It also implies that cells contain some

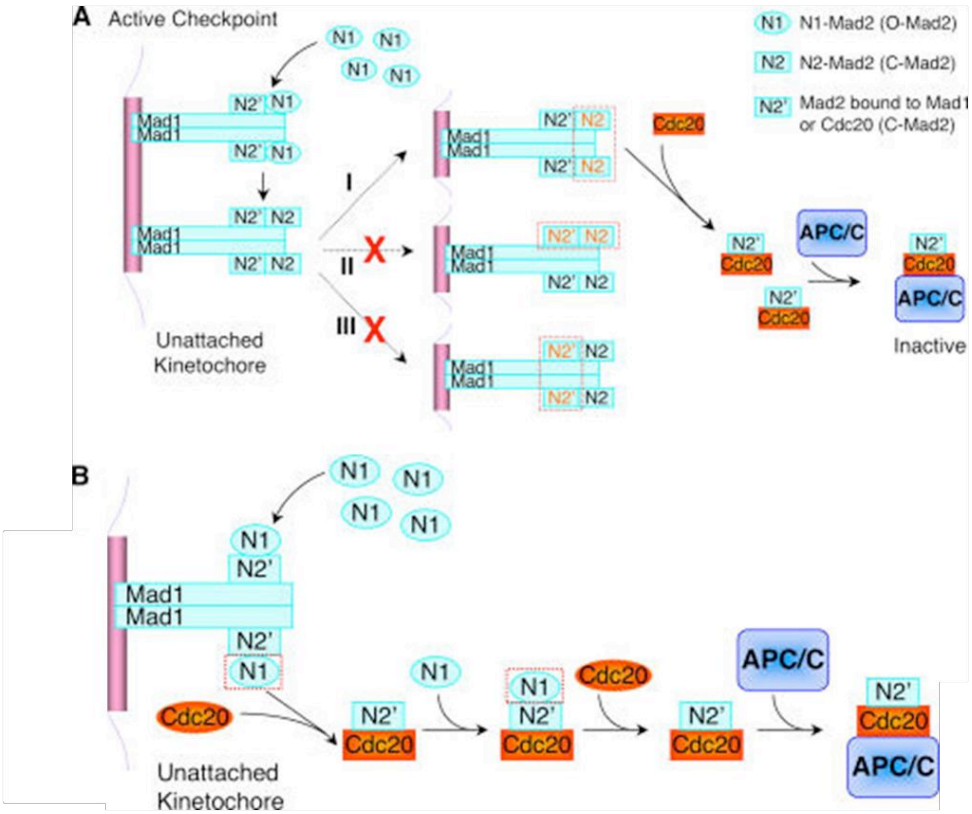


Figure 1.3. Two-state versus template model of structural Mad2 activation within the SAC.

(A) The two-state Mad2 model. Mad2 binds to Mad1 and adopts the N2' conformation. Upon checkpoint activation, the Mad1–Mad2 complex is recruited to the unattached kinetochores. Another copy of N1–Mad2 is recruited to Mad1, mainly through its binding to N2'–Mad2, and is then converted to N2–Mad2. N2–Mad2 is either directly passed on to Cdc20 from Mad1 or dissociates from Mad1 to form a transient dimeric intermediate that then binds to Cdc20. Because Mad1 exists as a homodimer, three pathways can be envisioned for the transfer of N2–Mad2 from Mad1 to Cdc20: (I) the two loosely bound N2–Mad2 molecules dissociate from Mad1 as a unit; (II) one tightly bound N2'–Mad2 and one loosely bound N2–Mad2 dissociate as a unit; (III) the two tightly bound N2'–Mad2 molecules dissociate as a unit. Pathways I and III require the dimerization of Mad1. Pathway III is equivalent to the so-called “exchange” model (De Antoni et al., 2005). Only pathway I is consistent with FRAP studies in Ptk cells. (B) The Mad2 template model. In this model, N2'–Mad2 bound to Mad1 recruits N1–Mad2, which is passed on to Cdc20. The Cdc20-bound N2'–Mad2 recruits another N1–Mad2. In this way, the N2'–Mad2–Cdc20 can self-propagate away from the kinetochores. Taken from (Yu, 2006)

kind of mechanism for preventing the positive feedback loop based on Cdc20-Mad2 in the absence of kinetochore signals. For this reason, if the template model is to hold, regulation of the catalytic function of closed-Mad2 must be tightly controlled by kinetochores (Musacchio and Salmon, 2007). Recent *in silico* investigation into the inhibition of Cdc20 by Mad2 also provides criticism, this time mathematical, of the template model as it stands. Although it displays robust switching behaviour, the application of experimentally derived parameters to the template model does not predict Mad2 levels which can be confirmed as plausible by experimental research. This leads to the suggestion that Mad2 is not capable of completely sequestering Cdc20, but may do so alongside additional binding partners. A potential candidate for this is BubR1 (Ibrahim et al., 2008).

1.18. A role for Mps1 in SAC signalling

Mps1 was first identified as a dual-specificity kinase, whose levels are increased in various tumour cells (Lindberg et al., 1993; Mills et al., 1992). It is one of the many protein kinases implicated in the SAC, with depletion of this protein in yeast to human cells resulting in the checkpoint being overridden (Fisk et al., 2004). Across these species, Mps1 has been shown to regulate normal mitotic progression, chromosome congression, and cytokinesis (Jelluma et al., 2008; Fisk et al., 2004). It has also been suggested that, alongside its role in the SAC, Mps1 is also required to correct chromosome attachment errors, phosphorylating Borealin (the chromosomal passenger complex partner of Aurora B). In Mps1- depleted human cells, chromosome alignment defects have been shown to be rescued by the introduction of a form of Borealin where phosphorylation sites are mutated, with partial restoration of Aurora B activity (Jelluma et al., 2008). This form of Borealin did not, however, restore the SAC. This implies that although Borealin is the Mps1 target essential to allow its phosphorylation and activation of Aurora B, and contribute to chromosome attachment correction, other targets must be important for its function in the SAC.

1.18.1. Mps1 regulation during the cell cycle

During the cell cycle Mps1 is tightly regulated. During G1, Mps1 is diffusely distributed throughout the cell, relocating to the nucleus and centrosomes during the G2/M transition. Mps1 localises to kinetochores in prophase and prometaphase, or with activation of the SAC (Stucke et al., 2004; Liu et al., 2003; Stucke et al., 2002; Friedman et al., 2001). At these times, Mps1 activity is dramatically increased (Kang et al., 2007; Mattison et al., 2007; Stucke et al., 2004; Liu et al., 2003; Stucke et al., 2002). Mps1 dissociates from kinetochores at metaphase, or upon satisfaction of the SAC. Mps1 is believed to adopt a unique inactive state, with kinase activation upon Mps1 autophosphorylation (Xu et al., 2009). This autophosphorylation of key sites within the N-terminal domain of Mps1 is believed to be important, along with phosphorylation of the Mps1 C-terminal, for kinetochore targeting as well as SAC signalling (Xu et al., 2009). Mps1 in human cells has been found to be required for the kinetochore localisation of other checkpoint proteins, Mad1 and Mad2 ((Zhao and Chen, 2006; Vigneron et al., 2004; Abrieu et al., 2001), with Mps1 knockdown in SW480 cells shown to inhibit Mad2 kinetochore targeting (Liu *et al*. 2009). Data from *Xenopus* egg extracts suggests that Mps1 also recruits Bub1, BubR1 and Cenp-E (Wong and Fang, 2006; Zhao and Chen, 2006; Abrieu et al., 2001). In human cells, Mps1 depletion by RNAi triggers premature anaphase, often displaying misaligned chromosomes. A wild-type Mps1 transgene was capable of rescuing this phenotype, but a catalytically inactive Mps1 mutant was not (Tighe et al., 2008). This catalytically inactive Mps1 can restore kinetochore localisation of Mad1, but not Mad2. This implies that in human cells, Mps1 catalytic activity is required for SAC function and Mad2 recruitment. With the essential role of Mps1 in the kinetochore localisation of various checkpoint proteins, Mps1 knock-down experiments could prove useful when investigating the formation of the MCC with regard to the requirement of direct kinetochore interaction.

1.19. A role for chemical inhibition of Mps1

A number of groups have approached the investigation into the effect of Mps1 on SAC function using chemical inhibition of the kinase, rather than its deletion. Various compounds have been identified as potent Mps1 inhibitors, and have been tested for their specificity to this SAC component, as well as their effect on other SAC proteins. This approach is becoming increasingly popular, as with identification of Mps1 as an essential SAC component, the loss of which leads to aneuploidy and cell death (Janssen et al., 2009), Mps1 has become a potential target for anticancer drugs.

The earliest report of the chemical inhibition of Mps1 comes from a 2005 paper (Schmidt et al., 2005). Here, the previously reported c-jun amino-terminal kinase (JNK) inhibitor SP600125 was shown to abolish SAC function in a JNK-independent fashion in human U20S cells. Sequence analysis revealed a degree of similarity between the ATP-binding pocket of JNK and the human Mps1 binding domain, which lead to investigation into the ability of SP600125 to inhibit human Mps1 kinase. This revealed that, in fact, human Mps1 kinase was inhibited to a greater degree than JNK. Rescue experiments using SP600125-resistant mutants of Mps1 confirmed that this compound acts upon Mps1 to inhibit the SAC. Previous studies into the effect of SP600125 have implicated a role in the inhibition of a number of other kinases, although the only reported case which is believed to be involved in mitotic progression is cyclin B/Cdk2 (Bain et al., 2003). Schmidt et al., 2005 then confirmed the relative specificity of SP600125-mediated Mps1 inhibition by displaying the failure of SP600125 to significantly inhibit Cyclin B/Cdc2, BubR1 and Aurora B, although the mild inhibition of these kinases could not be ignored in the disruption to SAC signalling in response to this compound.

With regard to the effect on other SAC components in the presence of the proposed Mps1 inhibitor SP600125, the Mps1-dependent kinetochore localisation of BubR1 was consistent with previous reports (Liu et al., 2003; Taylor et al., 2001) in that BubR1 kinetochore recruitment in response to nocodazole and taxol treatment of cells was significantly reduced in the presence of the Mps1 inhibitor. This, however, was not the case for SAC protein Mad1, previously shown to require Mps1 for its kinetochore localisation (Abrieu

et al., 2001). With inhibition of Mps1 by SP600125, the localisation of Mad1 to kinetochores appeared largely unaffected (Schmidt et al., 2005). This finding lead to the proposal of a model in which the presence, but not the kinase activity of Mps1, is required for Mad1 kinetochore localisation. This seems reasonable in that all studies which deplete Mps1 (Abrieu et al., 2001) show an effect on Mad1 kinetochore recruitment, yet those which merely inhibit the activity of Mps1, for example using anti-Mps1 antibody (Liu et al., 2003) or chemical inhibition by SP600125 (Schmidt et al., 2005) do not.

Although SP600125 appears to inhibit Mps1, the fact that it displays mild inhibition of other kinases means that more specific compounds are required for further investigation into Mps1 kinase activity and SAC function. A recent review (Lan and Cleveland, 2010) compares the more recent attempts at identifying potent Mps1 inhibitors, with varying inhibitory concentrations, specificities and sometimes conflicting results with regard to their effect on SAC components. Discrepancies exist between Mps1 inhibitors as to which of the SAC proteins require Mps1 activity for their localisation to kinetochores, potentially due to the nature and timing of their action.

Three novel inhibitors of endogenous human Mps1 have been described in the last twelve months. Firstly, a pair of inhibitors, Mps1-IN-1 and Mps1-IN-2 were identified (Jelluma et al., 2010) which use a stable shRNA approach in human U2OS cells. Although Mps1-IN-2 remains largely uncharacterised, Mps1-IN-1 inhibition of Mps1 was shown to inhibit the kinetochore recruitment of Mad1 and Mad2, as well as producing a chromosome misalignment phenotype. It does, however, have an effect on Aurora B kinase activity, and so the observations reported cannot be completely attributed to Mps1 inhibition by this compound.

Another compound, AZ3146, described to be a more potent and specific inhibitor of Mps1 than Mps1-IN-1 and Mps1-IN-2, has been shown have no significant effect on the activity of BubR1, Cdk1 or Aurora B (Hewitt et al., 2010). Of a screen of 50 other kinases, only four kinases were mildly inhibited by AZ3146, namely JNK1, JNK2, FAK and KIT. In this study, AZ3146 was shown to override SAC-mediated mitotic arrest in HeLa cells blocked with microtubule inhibitors, thus confirming the role of Mps1 in SAC arrest, and is particularly interesting as it attempts to use this inhibitor to investigate the role

of Mps1 activity in the formation of the MCC complex at kinetochores. The level of Mad2 recruitment to kinetochores was decreased by approximately 85% with AZ3146 inhibition, with only a partial effect on the recruitment of Mad1. This data is consistent with previous observations associated with loss of Mps1 activity (Tighe et al., 2008), and so provide a basis for the relevance of AZ3146 as a useful tool for probing Mps1 function, although questions existed as to why Mad1 localisation was only partially effected by AZ3146 when other Mps1 chemical inhibitors such as Reversine show dramatic effects on Mad1 localisation (Hewitt et al., 2010).

It was possible that the difference in the effects of Reversine in comparison to AZ3146, with relation to their effect on Mad1 localisation, could be associated with the cell cycle stage at which the chemical inhibitor acts (Hewitt et al, 2010). By devising a set of experiments involving the use of MG132 and nocodazole, regimens were set up to test the effect of the Mps1 inhibitor when cells were synchronised in mitosis before AZ3146 treatment, in comparison to cells which enter mitosis after the addition of AZ3146. Under conditions whereby all detected mitotic cells had entered mitosis before AZ3146 exposure, Mad2 kinetochore localisation was disrupted, yet Mad1 kinetochore recruitment was largely unaffected. However, when cells were first treated with MG132 before the addition of nocodazole (so that all cells should be in mitosis with aligned chromosomes stripped of Mad1 and Mad2, with the addition of nocodazole triggering a SAC response to recruit Mad1 and Mad2 to kinetochores) the addition of AZ3146 showed that neither Mad1 nor Mad2 localised to kinetochores. When these experiments were repeated with Reversine instead of AZ3146, identical results were obtained. This lead to a hypothesis that it is not the timing of the inhibitor which lead to initial discrepancies between potential Mps1 inhibitors, but that Mps1 may be involved in two distinct steps within mitosis, possibly to recruit Mad1 to kinetochores upon mitotic entry, then to promote continuous Mad2 localisation (Hewitt et al., 2010). However, various data exists to suggest that the Mad1-Mad2 complex is very stable (Kwiatkowski et al., 2010; Howell et al., 2004) which conflicts with this idea. A more plausible explanation for the data observed by Hewitt et al (2010), attempts to uncover a role for Mps1 in the Mad2 template model of MCC formation, based on the observation that Mps1 inhibition by AZ3146 appeared to abolish all Mad2

kinetochore localisation, when it should have only had an effect upon ~50% of kinetochore-bound Mad2 levels.

1.20. A possible role for Mps1 in the Mad2 template model of MCC formation

Because a key step in SAC activation in the Mad2 template model of MCC formation is the recruitment of O-Mad2 to kinetochore-bound C-Mad2, Hewitt et al (2010) hypothesised that perhaps Mps1 activity is required for the localisation of O-Mad2 to Mad1-C-Mad2 complexes at the kinetochore. It was speculated that the antibody used to detect Mad2 may have shown it to be absent at kinetochores with chemical inhibition of Mps1, when actually C-Mad2 should still have been present. Upon testing the specificity of the anti-Mad2 antibody used (SM2.2 antibody) it was speculated that C-Mad2 was not being detected.

This was further confirmed by the use of Mad2 mutants unable to adopt the closed Mad2 conformation in comparison to Mad2 mutants which can bind Mad1 but not dimerise. It was shown that in the presence of AZ3146, mutant Mad2 rendered unable to dimerise was unaffected by Mps1 inhibition, whereas the Mad2 mutant unable to adopt the closed conformation was absent from kinetochores. This lead to the idea that Mps1 is required during mitosis to recruit O-Mad2 to kinetochore-bound Mad1-C-Mad2 complexes. This inclusion of Mps1 in triggering the Mad2 template model of MCC formation builds upon previous models, but the mechanism by which Mps1 may achieve this is still uncertain. It is not likely to be a direct affect, as the Mad2 template mechanism has been investigated *in vitro* without the inclusion of Mps1 in the past (Vink et al., 2006). A more likely explanation is that Mps1 may act upon a cellular inhibitor in order to exert its function on this system.

1.21. Requirement of Kinetochores in Mitotic Checkpoint Complex Formation

Directly related to the localisation and binding patterns of the key SAC proteins, there are two basic observations derived from experimental systems in various species, from humans to yeast, for the role of the kinetochore in the SAC (Kim and Burke, 2008). The primary observation is that SAC proteins such as Mad2, Mps1 and BubR1 localise to unattached kinetochores (Howell et al., 2004). The

second observation is that a complex of SAC proteins referred to as the mitotic checkpoint complex (MCC) has been shown to be a potent inhibitor of APC/C. In this model the checkpoint proteins dynamically interact with unattached kinetochores, are released as the MCC which then diffuses from the kinetochore to inhibit APC/C (Musacchio and Salmon, 2007; Cleveland et al., 2003). However, the role of the kinetochore in the formation and action of the MCC remains incompletely described, with studies in yeast providing evidence that the presence of the kinetochore may not be required for the formation of the MCC (Nezi et al., 2006). It has also been shown that an interphase pool of MCC exists, indicating that the complex forms independently of unattached kinetochores during a normal cell cycle (Sudakin et al., 2001).

A possible explanation of the mechanism of MCC formation is that the formation of a primary Mad2-Cdc20 sub-complex functions to promote the further recruitment of the other MCC components into the final complex (Musacchio and Salmon, 2007). This argument is strengthened by research in human cells which shows that binding of the N-terminal region of BubR1 to Cdc20 requires previous binding of Mad2 with Cdc20 (Davenport et al., 2006), an indication of a two-step Cdc20 inhibition process requiring Cdc20 to be sensitised to Mad2 by prior binding with BubR1. The subunit comprised of Mad2-Cdc20 itself is insufficient for complete sequestering of Cdc20 (Ibrahim et al., 2008). In all cases BubR1 is also required for full checkpoint activation (Sudakin et al., 2001). There is also evidence to suggest that in order for Cdc20 to bind Mad2 or BubR1, it must first be phosphorylated. Kinases implicated as important in this phosphorylation include Bub1, MAPK and Cdk1 (Tang et al., 2004a; Chung and Chen, 2003; D'Angiolella et al., 2003; Yudkovsky et al., 2000). As well as the mechanism of complex formation, the location of MCC formation is also poorly understood. Two arguments exist, that of MCC formation at the kinetochore, and that of kinetochore-independent cytosolic MCC formation.

1.21.1. Kinetochore based MCC formation

The kinetochore-based theory of MCC formation is immediately obvious due to the localisation of all SAC components to unattached kinetochores (Shah and Cleveland, 2000). A strong argument for the requirement of the kinetochore in MCC formation reverts back to the previously explained Mad2 template model

of Cdc20 binding (Nezi et al., 2006), where Mad1, which is stably kinetochore bound in prometaphase (De Antoni et al., 2005; Howell et al., 2004; Chung and Chen, 2002) binds open-Mad2 to allow its conformational change to closed-Mad2 (Luo et al., 2004; Sironi et al., 2002). This Mad1-closed-Mad2 interaction does not appear to dissociate or move from the kinetochore during checkpoint activation (De Antoni et al., 2005; Howell et al., 2004) and accounts for the stable pool of Mad2 observed (Shah et al., 2004). The strong requirement for the presence of the kinetochore in the formation of the Mad2-Cdc20 subunit, referred to as the "seed" For MCC formation (Musacchio and Salmon, 2007) implies that the requirement of the kinetochore in MCC formation is probable.

Another perspective may be derived from investigation into the composition of the MCC complex, in order to attempt to clarify whether the MCC observed in interphase cells (Sudakin et al., 2001) is the same complex, the components of which have also been reported to preferentially bind APC/C in the SAC (Morrow et al., 2005; Hardwick et al., 2000; Fang et al., 1998a). Recent experiments subjecting salt eluate of anti-Cdc27 immunoprecipitate from checkpoint-arrested extracts to immunodepletion by anti-Cdc20 confirm that Cdc20, BubR1 and Mad2 are in a complex in mitotic cells (Braunstein et al., 2007). However it also indicated that MCC may only be partly responsible for APC/C inhibition, as after the removal of Cdc20 and accompanying complex components, inhibition of APC/C remained. This suggests that there are potentially other inhibitory factors which may be associated with the APC/C when the SAC is active, either enhancing or prolonging the action of the MCC. Furthermore, it was also observed that much less MCC components were observed to be associated with APC/C in cells which had exited SAC arrest (activated extracts) than in SAC-arrested extracts. Data suggests that the reason for these lower levels is due, not to the dissociation of MCC from the APC/C, but to the dissociation of a large part of the MCC itself (Braunstein et al., 2007).

Although other studies have suggested MCC dissociation (Sudakin et al., 2001) the timing of this was not investigated and therefore somewhat cast aside. It may be argued that this finding implies that MCC is formed upon activation of the SAC and then dissembles upon SAC exit, and therefore conflict with the hypothesis that MCC exists outside of mitosis and that the same complex is involved in the SAC. It also coincides with the kinetochore-dependent formation

of MCC, as the MCC in this case is forming upon localisation of SAC proteins to the unattached kinetochore. A potential theory for this is that the requirement of Mad2 and BubR1 to bind Cdc20 is also needed for inhibition of APC/C ^{Cdc20} rather than simply to sequester Cdc20 to inhibit APC/C indirectly but until the mechanisms involved in causing MCC formation upon SAC activation, and the potential disassembly of this complex upon SAC exit are understood, this remains unclear.

1.21.2. Kinetochore independent MCC formation

The argument for MCC formation independent of kinetochore interaction is at first glance controversial, with such strong links between SAC proteins and recruitment to kinetochore presented. However, there are also arguments against requirement for kinetochore interaction. The first being the reported interphase presence of MCC. With mature kinetochores only existing in mitosis and MCC potentially existing throughout the cell cycle (Sudakin et al., 2001) the possibility of kinetochore-independent MCC formation becomes relevant. MCC has been detected in *S. cerevisiae* cells with defective SAC, in which a key protein for the assembly of the kinetochore, Ndc10, has been mutated (Poddar et al., 2005). This was also the case for normal metaphase-arrested cells. MCC has also been detected in HeLa cells, independent of unattached kinetochores, in active interphase cells. It was also shown that in this case the MCC detected in non-mitotic cells did not have an effect on the APC/C. This lead to the prediction that mitotic APC/C potentially undergoes modification allowing rapid interaction with a pool of MCC (Sudakin et al., 2001). This potential existence of MCC throughout the cell cycle, only exerting its effect in mitosis, puts forward the possibility that the role of the kinetochore is merely to sensitise the APC/C to inhibition by MCC rather than having a direct involvement in MCC formation (Musacchio and Salmon, 2007). This model proposes that other SAC proteins (uninvolved in MCC) may be important in the recruitment as well as the sensitisation of APC/C at kinetochores. This may be plausible as some evidence exists which suggest APC/C may localise to kinetochores during SAC activation (Acquaviva and Pines, 2006; Vigneron et al., 2004). One potential problem with the sensitisation model arises with studies unable to support the finding of interphase MCC (Morrow et al., 2005; Chung and Chen, 2002; Fang, 2002; Chan et al., 1999). But if in fact MCC is present in interphase cells, is this

MCC composed of the same subunits as mitotic MCC? Could it actually be that, although reportedly containing the same components, they actually differ enough in composition for this to be the reason behind the lack of APC/C inhibition by interphase MCC extracts? There is a possibility that differences in composition between mitotic and interphase MCC exist, with only mitotic MCC capable of APC/C inhibition. It is yet to be tested as to whether the APC/C actually does undergo a conformation change which could explain this occurrence, or whether mitotic MCC extracts can inhibit APC/C from non-mitotic extracts.

1.22. The use of Model Organisms

A model organism is a non-human species used to investigate biological processes, whereby the common descent of all living organisms and subsequent conservation of many biological phenomenon, genetic materials and regulatory and developmental pathways, allows findings to provide insight into the workings of other organisms. They are widely used to explore causes and treatment of human disease, in applications deemed unethical or unfeasible for human experimentation, and tend to be small in size, with a short generation time. Other common traits which are considered when choosing a model organism include accessibility, manipulation, genetics and potential economic benefit. It must, however, also be recognised that caution must be aired when making generalisations about human processes based on observations within model systems alone.

Particular areas of research tend to favour specific model organisms, based on their unique characteristics. For example, the use of the bacterium *Escherichia coli* has been used in research into molecular genetics, due to its rapid growth rate, simple nutritional requirements, well established genetics and completed genomic sequence. A second example is the use of yeast, such as *Saccharomyces cerevisiae,* in studies of the cell cycle due to the many similarities between human and yeast cell cycles, as well as regulation by homologous proteins. Another organism widely used in the study of cell cycle regulation and genetics is *Drosophila melanogaster* (Greek for dark-bellied dew lover – also known as the vinegar fly or fruit fly).

1.23. An introduction to *Drosophila melanogaster*

Drosophila melanogaster has been studied as a model organism for over 100 years, advantageous in its small size, rapid development period (Figure 1.3) and short (2 week) life cycle, and ability to produce large numbers of progeny (~50 offspring). A great deal is known about the developmental genetics of the fruit fly, laboratory techniques for the use of this model are well established, information is freely available with the establishment of websites such as “FlyBase” and “Interactive Fly” and the genome of *Drosophila* was sequenced in 2001.

Drosophila have four pairs of chromosomes; X-chromosome, and chromosomes two, three, and four. The right (R) and left (L) arms of chromosomes two and three, as well as the X-chromosome, are roughly comparable in size. Chromosome four is approximately five times smaller. An important feature of *Drosophila* genetics is the total absence of recombination (the process in which DNA molecules are broken and the fragments rejoined in new combinations) in males. Recombination in females is still achieved, the control of which can be achieved by another advantageous tool in *Drosophila* genetics, balancer chromosomes.

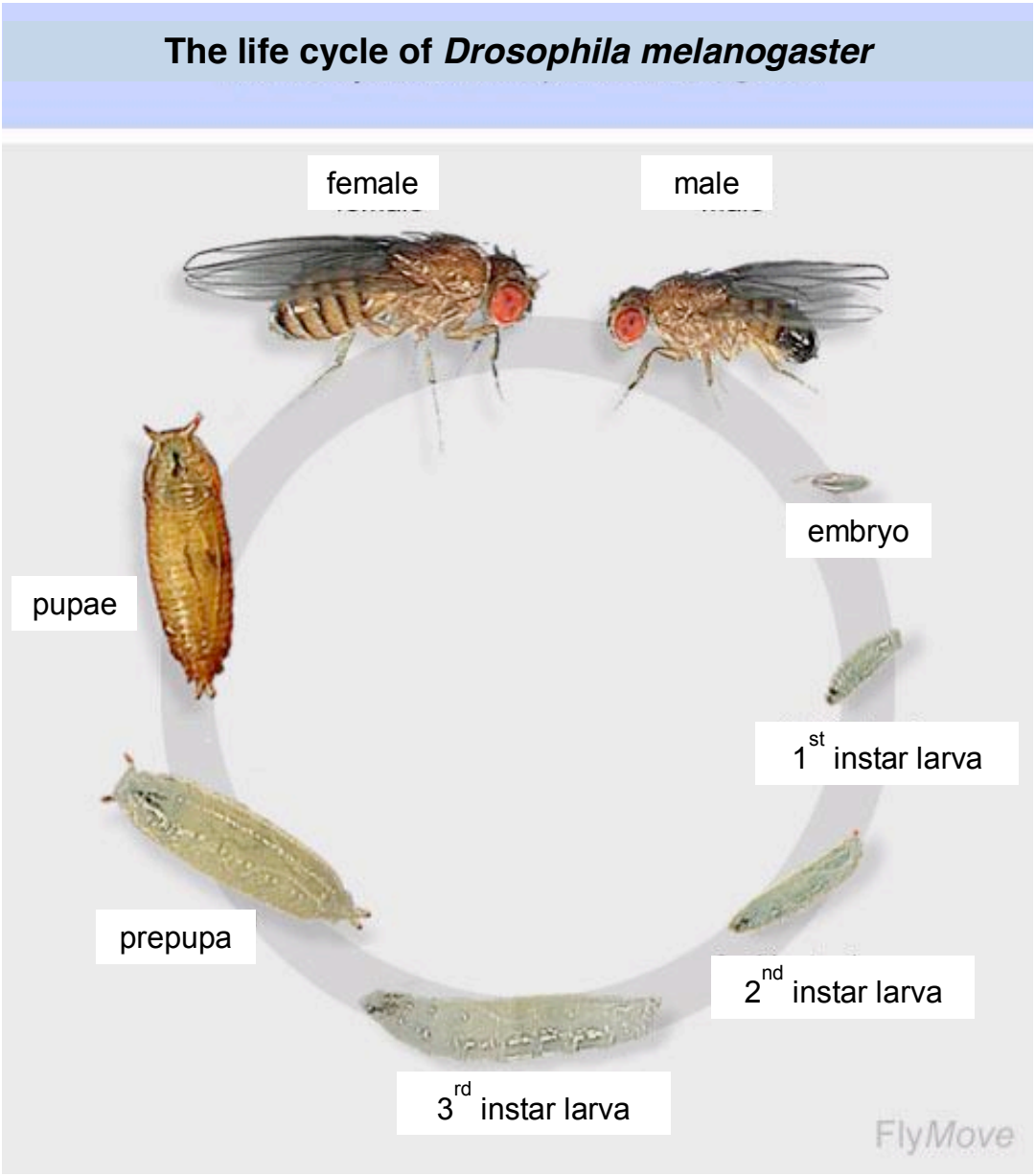


Figure 1.4. The *Drosophila* life cycle.

At 25ºC, the development period from embryo to adult fly is approximately 8.5 days. Embryos hatch approximately 12-15 h after laying, and grow for around four days, within which they moult at 24 h and 48 h into 2nd and 3rd instar larvae respectively. Larvae then encapsulate in the puparium to undergo a four-day metamorphosis period, after which the adult flies emerge. Image taken from: http://flymove.uni-muenster.de/.

1.23.1. The use of genetic tools in Drosophila research

1.23.1.1 Balancer chromosomes

The use of balancer chromosomes in the *Drosophila* model is one of the features which set it apart from that of many other organisms. Balancer chromosomes exploit the finding that multiply inverted chromosomes are unlikely to undergo exchange with their normal homologues. These chromosomes, which as a result of multiple breaks and rejoinings (usually by radiation), have scrambled gross sequences which consequently disrupt the synopsis between homologous chromosomes during meiotic prophase. When these multiply inverted chromosomes are also linked to a marker mutation, they become able to allow populations of flies carrying defined homozygous lethal mutations to be maintained as a heterozygous or "balanced lethal" stock, as well as allowing segregation analysis as transmission of the homologue from parent to progeny can be unambiguously tracked. By suppressing the reproductive fitness when balancer chromosomes are carried homozygously it ensures that the population it is carried in does not become fixed for the balancer chromosome. Since the X chromosome must exist in a hemizygous state in males, X-chromosome balancers tend not to contain recessive lethals, but instead, recessive female sterility mutations. Balancer chromosomes can therefore be defined as possessing three main properties: they suppress recombination with their homologs, carry dominant markers and negatively affect reproductive fitness when carried homozygously.

Many balancers exist for the X, second, and third chromosomes but are not required for chromosome four, as there is no exchange on this chromosome. The naming of balancer chromosomes tends to begin with the letter representing their chromosome (F for first – which is the X chromosome, S for second, and T for third). This is followed with an M for multiply inverted, a number, and often the genetic symbol for the principle marker or mutation carried by that balancer. An example of these are the second chromosome marker *SM6,Cy* (real name *In(2LR)SM6, al^{2}, Cy dp^{lv1} cn^{2} sp^{2}*) which carries the dominant marker Curly and produces a phenotype in adult flies in which wings

are curled upward instead of lying flat to the body, and *TM6BTb* (real name In(3LR) TM6, Hu e) which carries a dominant allele of Antennapedia (Hu) and the additional dominant Tubby (Tb), which causes a shorter and fatter body shape, and is therefore a good marker for larval and pupal stages as well as adult flies. The tubby balancer chromosome is especially useful in the identification of homozygous mutant flies, in lines which are not viable to adult stages. In these lines, rather than investigating the introduced mutation or modification of interest using the most common tissue (the embryo) it is often the third instar larvae neuroblast cells, selected from larvae absent of the balancer characteristic, which are used for imaging or preparation of samples for biochemical analysis.

1.23.1.2. P-elements

Another powerful genetic tool present in *Drosophila* are DNA transposable elements. Transposable elements are genetic units that can insert into a chromosome, exit, and relocate, therefore altering their position within the genome. An example of DNA transposable elements that exist naturally in *Drosophila,* and are subsequently exploited as a tool for genetic research, are P-elements. P-elements were discovered as a result of the study of asymmetrical hybrid dysgeneisis, whereby females of laboratory fly strains were mated with males from the natural population. Mating of these different strains resulted in abnormal phenotypes manifested in the germline, such as increased frequency of chromosome dysjunction, higher rates of mutation, and sterility, phenotypes which did not occur when laboratory strain males were mated with natural females. This phenomenon was later investigated with flies divided into P strains (paternal contributing) and M strains (maternal contributing), whereby any P strain male can induce dysgenesis in a cross with an M strain female but not vice versa. This is believed to be due to the ability of wild type females to produce a transposase inhibitor. Construction of recombinant chromosomes showed several regions within the P chromosome were able to cause dysgenesis. The nature of these P-specific sequences were first identified by mapping the DNA of *w* mutant flies, which carry mutations as a result of DNA insertions at the w locus which inactivate the gene and result in a white-eye phenotype, found amongst the dysgenic hybrids, and the inserted sequence was named the P-element.

P-element insertions form a classic transposable system, whereby individual elements vary in length but are homologous in sequence. P-elements contain a gene, composed of four exons and three introns, that encodes transposase (the enzyme that binds to the ends of a transposon and catalyzes it's relocation to another part of the genome). Each contains a 31bp inverted repeat at each terminus, and generate direct repeats of target DNA of 8bp upon transposition (Muster et al., 1983). Transposition requires ~15bp of terminal DNA. The transposase binds to 10bp sequences adjacent to the 31bp inverted repeats and inserts the P-element by a non-replicative "cut and paste" mechanism. Insertion of the P-element may cause mutation to the new site, and have a deletion effect at the donor site. These P-elements are transcribed in both germline and somatic tissues, but only activated in the germline. This tissue specificity is believed to be conferred by a change in splicing pattern, in which within somatic cells the first two introns are excised and a protein which represses transposase activity is transcribed, or in the germline where an additional splicing event occurs to remove the third intron and connect all reading frames to translate transposase.

The mobility of these elements can be exploited in *Drosophila* genetics to mark the position of genes and to facilitate gene cloning. As P-elements inserted into genes *in vivo* disrupt genes randomly, they create mutants with different phenotypes, marking the presence of the mutation. These can subsequently be selected for cloning of the mutant gene by way of transposon tagging, in which the mutant gene is cloned with the use of P-element segments as a probe. P-elements can also be used to insert genes, which may be generated to carry a specific point mutation or be fused to a marker or tag such as green fluorescent protein (GFP). This would then allow for the inserted fusion protein to be visually tracked within cells, to study localisation of that protein as well as any phenotype it may cause.

1.23.1.3. The use of P-elements to insert genes

This use of P-elements to insert donor genes into the germline was first demonstrated by a procedure designed by Rubin and Spralding in 1983 (Rubin and Spradling, 1983). The recipient genotype is homozygous for a given mutation, for example rosy (*ry*-) which confers a characteristic eye colour

phenotype. Embryos from this line are collected at around cycle nine when it still exists as a single multinucleated cell. At this stage, the nuclei destined to form germ cells are clustered at one end of the embryo. As P-elements mobilise only in germ cells, embryos at this stage are injected with two types of DNA, the first being a bacterial plasmid carrying a defective P-element into which the wild-type gene (for example *ry+*) has been inserted. This deleted element is not able to encode transposase, and is therefore unable to transpose without the help of a second injected DNA, or helper plasmid, containing a full element. The resulting flies are phenotypically still rosy mutants, but their offspring will include large numbers of *ry+* flies. The presence of stable Mendelian inheritance of the *ry+* gene suggests that it is located on a chromosome.

The gene of interest can be generated to contain point mutations, with the use of PCR site-directed mutagenesis in the construction of the P-element, and as mentioned previously, can also be designed to express the protein of interest tagged to a second protein, such as a fluorescent marker.

1.24. The relevance of *Drosophila* as a model organism

With all of this considered, use of *Drosophila* genetics is often an elegant means by which to study a wide array of cellular processes. Even though the human genome is almost four-fold larger than that of the fly, they carry comparable numbers of gene families and there is a remarkable conservation between human and *Drosophila* genes. Many genes involved in human disease can be directly linked to orthologues in flies, enabling the use of genetic techniques in *Drosophila* to establish their function in humans, and thus develop treatments. This is particularly true of genetic and developmental diseases, due to the wide understanding of these processes in flies.

Many genes involved in cell cycle regulation, including those which make up the mitotic spindle assembly checkpoint, are also orthologous between human and *Drosophila*. With the use of described genetic techniques, fly lines can be generated to study the effects of modification to these genes including deletion, overexpression and functional domain mutation, and the effect this has on cell progression and checkpoint functionality. Localisation of tagged proteins can be easily visualised throughout the cell cycle in embryo and neuroblast cells, with

the rapid life cycle of flies allowing easy sample collection for this as well as biochemical analysis.

1.25. Aims and objectives

The series of events leading to inhibition of Cdc20 in the SAC response has been widely studied, and a general consensus exists as to the importance of the formation and incorporation of Cdc20 into a mitotic checkpoint complex in order to inhibit its function as an APC/C activator. The conditions required in order for this MCC to form is the subject of much debate, with the requirement of direct kinetochore interaction, and therefore the kinetochore localisation of SAC proteins, one of the key questions remaining.

A number of conserved domains across various SAC proteins have been identified and deemed important in the interaction between MCC components, but many of these have never been confirmed in *Drosophila*, and the role of these domains in the kinetochore localisation of SAC proteins is often largely overlooked. To be able to visualise the localisation patterns of Cdc20 and other SAC components in *Drosophila* models, whereby potential functional motifs and domains have been removed, or upstream players in the SAC response omitted, could provide a useful tool in building our understanding of factors essential for SAC protein interactions, and the resulting effect on their kinetochore localisation and subsequent ability to be incorporated in the MCC.

The main aims of this project were to generate working systems with which we could investigate the role of specific conserved residues or upstream SAC proteins in kinetochore-based MCC formation within *Drosophila*. Specifically:

- To confirm the role of specific residues within Cdc20 which are potentially involved in its phosphorylation using transgenic approaches, and to investigate a potential requirement of this phosphorylation in Cdc20 kinetochore localisation and MCC formation.

- To develop bacterial and yeast three hybrid systems which will allow us to confirm a role for the BubR1 KEN box motif on its ability to bind Cdc20 in the presence and absence of Mad2.

- To use transgenic approaches to develop a model whereby the requirement of Mps1 in the localisation of other SAC proteins can be

investigated, and the effect on the ability of cells to elicit a SAC response under these conditions.

Chapter 2. Materials and Methods

2.1 Reagents and Buffers

2.1.1 Fly Food (1L)

100g Polenta maize meal

50g Brown sugar

25g Yeast

12.5g Agar

0.4g Sorbic acid

2g Benzoic acid

0.9g Nipagen

H_2O up to 1L

The mixture was heated to dissolve components, aliquoted into vials and allowed to set.

2.1.2 Apple Juice Agar

30% Apple Juice

70% H_2O

3% Agar

2.1.3 50x TAE (Tris-Acetate-EDTA) Buffer

2M Tris

1M Acetic acid

50mM EDTA

pH adjusted to 8.5 with NaOH and autoclaved to sterilise.

2.1.4 6x DNA Loading Dye

10mM Tris-HCl (pH 7.6)

0.03% Bromophenol Blue

0.03% Xylene cyanol FF

60% Glycerol

60mM EDTA

2.1.5 10x PBS

Dulbecco's Phosphate Buffered Saline Buffer from Sigma.

2.1.6 1x PBS-T

1x PBS (Sigma)

0.1% Tween-20

2.1.7 1x Protein Sample Loading Buffer

2% w/v SDS

5% v/v 2-mercaptoethanol

60mM Tris (pH 6.8)

Bromophenol Blue

10% Glycerol

2.1.8 20 x Protein Gel Running Buffer

NuPAGE® MOPS SDS Running Buffer from Invitrogen.

2.1.9 Transfer Buffer for Western Blotting

25mM Tris

192mM Glycine

20% Methanol

2.1.10 Blocking Buffer for Western Blotting

Odyssey Blocking Buffer from Li-Cor.

2.1.11 2x YT Medium

10g Tryptone

10g Yeast extract

5g NaCl

De-ionised H_2O up to 1L

pH adjusted to 7.4 with NaOH and autoclaved to sterilise.

2.1.12 LB Agar medium

10g NaCl

10g Tryptone

5g Yeast extract

20g Agar

De-ionised H_2O up to 1L

pH adjusted to 7.0 with NaOH and autoclaved to sterilise.

2.2. DNA Purification Buffers

2.2.1. Wizard® Plus SV Minipreps DNA Purification System

Cell Lysis Solution:

0.2M NaOH

1% w/v SDS

Cell Resuspension Solution:

50mM Tris-HCl (PH 7.5)

10mM EDTA

100µg/ml RNase A

Neutralisation Solution:

4.09M Guanidine hydrochloride

0.759M Potassium acetate

2.12M Glacial acetic acid

Final pH ~ 4.2

Column Wash Solution:

162.8mM Potassium acetate

22.6mM Tris-HCl (pH 7.5)

0.109mM EDTA (pH 8.0)

Plus 95% ethanol as directed.

2.3.DNA Gel Extraction Buffers

QIAquick® Gel Extraction Kit from Qiagen.

200mM Tris (pH 7.4)

200mM NaCl

1mM EDTA (pH 7.4)

10mM 2-mercaptoethanol

2.4. Inhibitors

1mM phenylmethylsulfonyl fluoride (PMSF)

2µg/ml Leupeptin

2µg/ml Aprotinin

2µg/ml Pepstatin

100µM Benzamidine

2.5.Kinase Assay Buffers

2.5.1. Basic Homogenisation Buffer

Both Homogenisation buffers A+B have the same basic components:

5ml basic mix:

20mM Tris (pH 7.5)	- 100µl of 1M stock
2mM EGTA	- 50µl of 200mM stock
2mM DTT	- 10µl of 1M stock
100mM NaCl	- 125µl of 4M stock

A) ***Contains protease inhibitors only*****:**

10µg/ml Aprotonin

10µg/ml Leupeptin

100µM PMSF

1mM Benzamidine

B) Contains both protease and phosphatase inhibitors:

10µg/ml Aprotonin

10µg/ml Leupeptin

100µM PMSF

1mM Benzamidine

50mM NaF

0.1mM $NaVO_4$

10mM β Glycophosphate

2.5.2. Kinase Wash Buffer

50mM Tris (pH 7.5)
10mM MgCl
1mM EGTA

2.5.3. Kinase Reaction Mix (70*µ*l – sufficient for 7 reactions)

Made up in kinase reaction buffer:
50mM Tris (pH 7.5)
10mM $MgCl^2$
1mM EGTA
1mM DTT
Plus:
100µM cold ATP
245ng CDK1/CycB (35ng per reaction)
Finally:
0.5µCui/µl ATP^{32P} (35µCui total activity)

2.6. *S. cerevisiae* media

2.6.1. YPD media

1% (w/v) Bacto-yeast extract

2% (w/v) Bacto-peptone

2% (w/v) glucose

20mg/L adenine hemisulphate

10mg/L L-histidine

20mg/L L-leucine

10mg/L L-tryptophan

8mg/L uracil

10mg/L methionine

To make solid YPD, 2% (w/v) Bacto-agar was added.

2.6.2. Minimal SD media

1% (w/v) Bacto-yeast nitrogen base

2% (w/v) glucose

To make solid SD, 2% (w/v) Bacto-agar was added.

2.7. Fly Methods

2.7.1. *Drosophila* Stock Maintenance

Unless otherwise stated, stocks can be maintained by periodic mass transfer of adults onto approximately 5ml fresh fly food in a 20ml vial. A small amount of baker's yeast is sprinkled over the surface of the fly food within the vial before use. To transfer, vials are tapped to bring flies away from the plug before it is removed. The vial is then rapidly inverted over one containing fresh food and tapped to bring flies to the base of the new vial before plugging. General stocks are maintained at 18ºC at which temperature generation time from egg to adult is approximately 19 days. These stocks are transferred every three weeks. The temperature for normal experimental conditions is 25ºC. At this temperature the generation time is around 21 days, and stocks must be transferred every two

weeks for maintenance, or every day if flies are required for embryo harvesting, up to five days prior to use.

2.7.2. Embryo Harvesting

Flies are transferred into a fresh vial containing either general fly food as before, or to encourage laying, into vials containing 30% apple juice agar. In either case a tiny amount of baker's yeast is sprinkled onto the surface of the food/agar, and the vial inverted and tapped to remove excess before transferring flies as before. Flies are kept at 25ºC for the appropriate amount of time for laying. This will depend upon the application for which embryos will be used. After this time, flies are transferred to a fresh vial, 1xPBS-T (0.1%) is added to the vial containing the embryos, and using a soft paintbrush, embryos are carefully dislodged from the surface of the food/agar. Embryos are then collected and washed three times with 1xPBS-T (0.1%) using a vacuum manifold to remove food and debris.

2.7.3. Drosophila Viability Studies

Well fed flies were transferred to fresh vials containing 30% apple juice agar and left for up to 3h. Embryos were harvested as described in section 2.7.2. and transferred onto 3% agar plates supplemented with a spot of baker's yeast paste as food once hatched. Plate lids were modified to incorporate a ventilation hole, plugged with sponge. One hundred embryos per plate, in ten groups of ten embryos were transferred, all of which were collected from the same embryo harvest. Plates were sealed with parafilm to avoid escape, and left at 25ºC for development. This was replicated five times per line. Once hatched, first instar larvae were counted and monitored to third instar stages where they were counted again and transferred into general food vials for development into pupae. The number of third instar larvae which developed into pupae stages was recorded, and vials were left for further development and hatching into adult flies.

2.7.4. Embryo Dechorionation – Manual Method

For applications such as live imaging of embryos and microinjection, embryos must be dechorionated manually. To manually dechorionate embryos, normal harvesting is carried out and embryos transferred onto a microscope slide prepared with double-sided tape. This adhesive surface holds the embryos in position to allow the chorion to be removed manually. Fine forceps are then used to gently massage the chorion to separate it from the embryo. Once separated, forceps can be used to remove the top part of the chorion, and the embryo can then be lifted away, leaving the remaining chorion adhered to the slide.

2.7.5. Embryo Dechorionation – Bleach Method

For applications such as heptane/methanol fixation and immunostaining, embryos were dechorionated using the bleach method. Embryos were left in the vacuum manifold after harvesting and 60% thin bleach was added for two min with regular agitation. The bleach was then drawn away and embryos washed three times in 1xPBS-T (0.1% tween) followed by once in dH_2O.

2.7.6. Preparation of Living Syncytial Embryos for imaging

Well fed adult flies were transferred into a fresh vial (fly food or apple agar) and left for 45-60 min. Embryos were harvested (as in 2.7.2) and manually dechorionated as described in section 2.7.4. A thin strip of glue was placed onto a coverslip and left for 3 min before the dechorionated embryos were placed onto and adhered to the glue strip, and immediately immersed in Voltalef 10S oil (a 5:1 mixture of Holocarbon 700 oil and 27 oil, Sigma) to prevent dehydration. The living embryos were then visualised using confocal microscopy.

2.7.7. Heptane/Methanol Fixation of Embryos

Embryos were harvested as normal and dechorionated using the bleach method. Embryos were then transferred to a 1.5ml eppendorf tube and 500µl heptane was added to permeabilise the vitteline membrane. An equal volume of cold methanol was added immediately and the tube shaken vigorously for one min. Embryos were left to settle to the bottom of the tube and for the heptane

and methanol to separate. The heptane and methanol were carefully removed. Any old, and therefore unsuitable, embryos will float between the heptane and methanol layers and were also removed. Embryos were then washed three times in cold methanol. At this point they can be stored at 4ºC, or the methanol can be removed before use.

2.7.8. Formaldehyde Fixation of Embryos

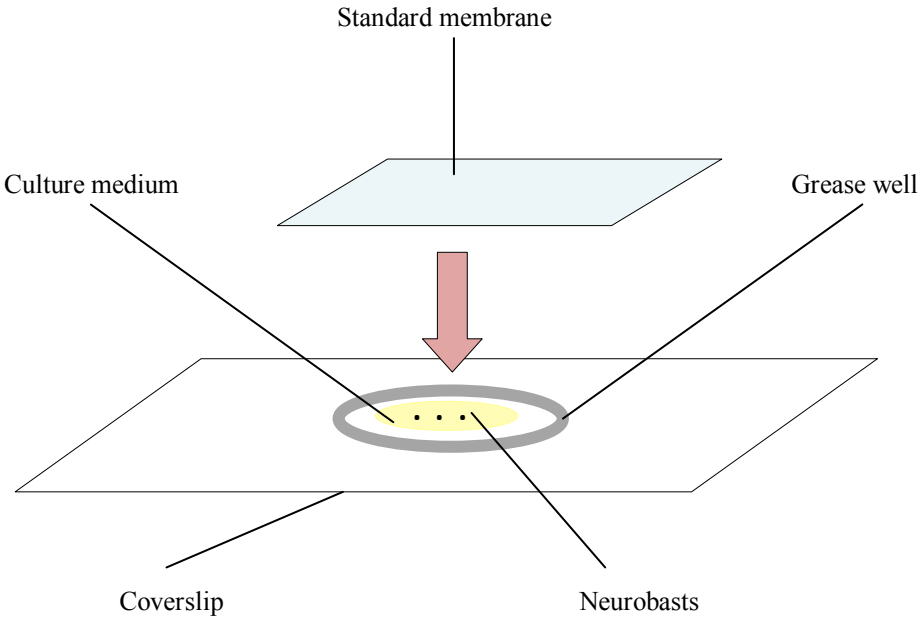
In cases whereby embryos express a transgenic fluorescent protein signal, and the retention of this signal is desired, heptane/methanol fixation is unsuitable. In these cases formaldehyde fixation was carried out. Embryos were harvested and dechorionated using 60% bleach as before, transferred into a 1.5ml eppendorf tube and incubated with 750µl 3.7% formaldehyde in 1xPBS for 30 min at room temperature with gentle rotation. With proteins now preserved, the formaldehyde was carefully removed and replaced with 500µl cold methanol and incubated at -20ºC for 15 min for further fixation. At this point embryos can be stored at -20ºC or the methanol removed before use.

2.7.9. Preparation and Imaging of Third Instar Larval Neuroblasts

Drosophila late third instar larvae were removed from vials and washed in 1x PBS to remove food debris and placed in a drop of 1xPBS on a clean microscope slide for dissection. Brains were isolated by holding the larval body and mouth parts with fine forceps and pulling in opposite directions. The adjacent tissue was carefully removed and the isolated brain placed in a fresh drop of 1x PBS on a clean coverslip. Once 2-5 brains were isolated in this way, they were covered and lightly flattened with a second coverslip, each brain into a disc of approximately 2mm in diameter. Excess liquid was removed using filter paper and the flattened brains were observed under a dissecting microscope to ensure adequate spread of the brain tissue. The coverslip was sealed around the periphery, leaving a small unsealed area for ventilation, using 10S oil. When prepared this way, brains were flattened to provide clear protein localisation images, although cells were non-viable under these conditions. Images were recorded using a spinning disc confocal microscope.

2.7.10. Preparation and Imaging of Live Third Instar Larval Neuroblast

Drosophila late third instar larvae neuroblasts were dissected as described in 2.7.9. A coverslip was prepared, using a fine paintbrush to create a well in the centre of the coverslip using grease. The fresh brain was mounted into a drop of *Drosophila* S2 culture medium containing penicillin and streptomycin, plus any appropriate drugs/inhibitors within the well. A piece of breathable standard membrane was cut to size and lowered onto the sample, using a soft paintbrush to flatten the sample and seal the membrane onto the grease well. Neuroblasts were gently flattened using a soft paintbrush, allowing excess culture medium to escape from the grease well to leave a minimal layer of media around the partially flattened neuroblasts. The slide was then visualised using a spinning disk confocal microscope. Using the appropriate settings, cells in these conditions were viable for up to three h.



2.7.11. Imaging TF4;B4-2 Neuroblast cells

Imaging settings were optimised for the TF4 and TF4;B4-2 fly lines to allow the highest quality image capture whilst still allowing cells to be viable. The settings used in all cases were as follows:

Bit-scale enabled: Yes

Laser: 22%

Exposure: 150ms

2.7.12. Imaging TM2-413;B4-2 Neuroblast cells

Imaging settings were optimised for the TM2-413 and TM2-413;B4-2 fly lines to allow the highest quality image capture whilst still allowing cells to be viable. The settings used in all cases were as follows:

Bit-scale enabled: Yes

Laser: 17%

Exposure: 150ms

2.8. Molecular Biology

2.8.1. Polymerase Chain Reaction (PCR)

The DNA insert fragments used to generate all constructs, including those used in antibody production and purification, were amplified from cDNA plasmids or genomic DNA using PCR. In these instances, HotStarTaq® DNA Polymerase (Qiagen) was used. A typical reaction mixture using the HotStarTaq®kit had a total volume of 100µl per reaction, assembled as follows:

Component	Volume	Final Concentration
10x PCR Buffer	10µl	1x
dNTP Mix (10mM of each)	2µl	200µM of each dNTP
Sense primer (50µM)	1µl	0.1-0.5µM
Antisense primer (50µM)	1µl	0.1-0.5µM
Template DNA	Variable	1µg maximum
HotstarTaq® polymerase	0.5µl	2.5 units
Distilled water		up to 100µl -

For amplification of DNA templates used in reverse transcription reactions to generate DNA, GoTaq® HotStart Polymerase (Promega) was used. A typical 100µl reaction mix was assembled as follows:

Component	Volume	Final Concentration
GoTaq® Flexi Buffer	20µl	1x
$MgCl_2$ Solution (25mM)	4µl	2.0mM
PCR nucleotide mix (10mM)	1µl	0.2mM each dNTP
Sense primer (50µM)	1µl	0.1-0.5µM
Antisense primer (50µM)	1µl	0.1-0.5µM
Template DNA	Variable	1µg maximum
GoTaq®HotStart Polymerase	0.5µl	2.5 units
Nuclease-free water	up to 100µl	-

Variations in reaction mixture depended on the length DNA being amplified and the primers used. In all cases the PCR reaction was carried out on a Hybaid PCR Sprint Thermal Cycler (Thermo Scientific, SPRT001). A typical program is as follows:

Initial activation step:	5 min	95ºC
3-step cycling (25 Cycles):		
- Denaturation	1 min	94ºC
- Annealing	1 min	55ºC (T_m)
- Extension	1 min	72ºC
Final extension:	5 min	72ºC
Hold:	-	4ºC

The annealing temperature was calculated according to primer sequence length using the following formula: Tm = 2AT + 4GC. The extension time was varied according to the length of the target fragment, for both HotstarTaq® polymerase and GoTaq®HotStart Polymerase 1 min per 1Kb of DNA to be amplified was allowed. All oligonucleotides were purchased from Sigma-Genosys (details of primers can be found in table 2.1). PCR products were analysed by agarose gel electrophoresis (see 2.11.3), and bands of expected size were excised and purified using QIAquick® Spin Gel Extraction Kit (Qiagen).

2.8.2 Restriction Digest

All restriction enzymes (Promega) were used with their optimal buffer and incubated at their optimal temperature, usually 37ºC (in accordance with the

Promega Life Sciences catalogue 2008) for a minimum of 1.5 h. A typical digest was be performed in a volume of 20µl on 0.2-1.5µg of substrate DNA, assembled in the following order:

Component	**Volume**
Sterile water	Variable
10x Buffer	2µl
Acetylated BSA	0.2µl
DNA	Variable
Mix by pipetting, then add:	
Restriction enzyme	0.5µl

For applications such as sub-cloning, whereby DNA fragments generated were to be purified or vectors cut and ligated, this was scaled up to a 100µl final reaction volume in order to allow higher yields of DNA fragments to be isolated for use. All digests were analysed by agarose gel electrophoresis (see section 2.8.3).

Table 2.1. Primers used within this thesis.

Primer name		Details	Sequence	Enzyme site
BACTH				
JY424	5’	BubR1 N-terminal WT fragment	GTCGACTATGGACTTTGACAATG CGAAAGAGAACATTCAGCG	*Sal*I
JY425	5’	BubR1 N-terminal KEN-AAA mutant fragment	GTCGACTATGGACTTTGACAATG CGGCAGCGGCCATTCAGCCG	*Sal*I
JY426	3’	BubR1 N-terminal fragment	GAGCTCTGAGGCCTGTTCTTGGCC ACAAAGTTTGTCGGAAAT	*Stu*I
JY427	5’	BubR1 Kinase domain fragment	CATATGATTGCCTCGTTT	*Nde*I
JY428	3’	BubR1 Kinase domain fragment	GAGCTCGGTTTCTGCAATATCGTG TTA	*Sac*I
JY435	5’	pUT18 adaptor oligo	CTGCAGGCTCGAGTGGTACCAGG CCTCATATGGAATTCGAGCTC	*Sph*I
JY436	3’	pUT18 adaptor oligo	AATTGAGCTCGAATTCCATATGA GGCCTGGTACCACTCGAGCCTGC AGCATG	*Eco*RI
JY453	5’	P25N adaptor oligo (phosphorylated)	CGATATGAGCGGATAACAATTTC ACACAGGAAACAGCTGCGGCCGC ACTAGTGCATGCAT	*Cla*I
JY454	3’	P25N adaptor oligo (phosphorylated)	CGATGCATGCACTAGTGCGGCCG CAGCTGTTTCCTGTGTGAAATTGT TATCCGCTCATAT	*Cla*I
JY457	5’	Mad2 fragment for BTH	GCG GCGGCC GCATGTCAA CTGCCCAGGCGA C	*Not*I
JY458	3’	Mad2 fragment for BTH	GCG GCA TGC TTA AGT GCT CAT CTT GTA GTT GAC	*Sph*I
YTH				
JY472	5’	BubR1 N-terminal WT	AA TAT AAC CCG GGG ATG GAC	*Sma*I

		fragment	TTT GAC AAT GCG	
JY473	5’	BubR1 N-terminal KEN-AAA mutant fragment	A TAT AAC CCG GGG ATG GAC TTT GAC AAT GCG GCA GCG GCC ATT CAG CCG	*Sma*I
JY474	3’	BubR1 N-terminal fragment	AT AAT TGC TAG CCG TCG CTC GTT AAG TTC C	*Nhe*I
JY475	5’	BubR1 Kinase domain fragment for Y2H	TTA TAT CAT ATG ATT GCC TCG TTT ATG AAA G	*Nhe*I
JY476	3’	BubR1 Kinase domain fragment	TTA TAT GTC GAC CTA TTT CTG CAA TAT CGT G	*Sal*I
JY477	5’	Fizzy fragment	AA TAT AAC CCG GGG ATG TCG CAG TTC AAT TTT GTG	*Sma*I
JY478	3’	Fizzy fragment	TTA TAT GTC GAC CTA ACG GAT GCT CTG TCG GAA C	*Sal*I
JY481	5’	Adaptor oligo for use in pBridge MCSII	C ATG GCG CGG CCG ATG CAC GGA	*Nco*I
JY482	3’	Adaptor oligo for use in pBridge MCSII	G ATC TCC GTG CAT CGG CCG CGC	*Bgl*II
JY489		Mad2 fragment for MCSII of pBridge	TTAAATGCGGCCGCATGTCAACT GCCCAGGCG	*Not*I
JY480	3’	Mad2 fragment for MCSII of pBridge	AT ATACCATGGTTAAGTGCTCATCTT GTAGTTG	*Not*I
JY490		pBridge MCSII adaptor oligo	GGCCAATTTTACGACCATGGGGA TTAA	*Not*I
JY491		pBridge MCSII adaptor oligo	CGTTAAAATGCTGGTACCCCTAAT TCTAG	*Bgl*II
Antibody				
JY437	5’	BubR1-MBP fusion	GAATTCATGGACTTTGACAATGC G	*Eco*RI
JY438	3’	BubR1-MBP fusion	GTCGACAATCGGAATATTTCCCTC A	*Sal*I

JY439	5’	Fizzy-MBP fusion	GAATTCTCCGGCGATAAGTCCGAT	*EcoR*I
JY440	3’	Fizzy-MBP fusion	GTCGACCCCCTCCTGGATCCACGA	*Sal*I
JY441	5’	Mps1-MBP fusion	GAATTCGATAGCATAAGCTTCTCC	*EcoR*I
JY442	3’	Mps1-MBP fusion	GTCGACAGGAAGGTTGGTGGTGTA	*Sal*I
dsRNAi				
JY467	5’	Fizzy 3'UTR + T7 Promoter	GCGTAATACGACTCACTATAGtatgctcagacctttagaactgtt	-
JY468	3’	Fizzy 3’UTR + T7 Promoter	GCG TAATACGACTCACTATAGtcagaatgtcaagcattttattta	-

2.8.3. DNA Gel Electrophoresis

Typically 0.8% agarose gels in 1x TAE running buffer were used for analysis, unless otherwise stated. After dissolving with heat, the gel was cooled and ethidium bromide (CLP®) was added to a final concentration of 0.5µg/ml before pouring. DNA samples were mixed with 1x DNA loading dye before gels were run in a DNA running tank (Minnie the gel cicle, Hoefer). In each case a 1Kb DNA ladder (New England Biolabs) was run alongside DNA samples in order to determine the size of DNA fragments. Gels were run at a constant voltage of 100V for 20-40 min in 1x TAE running buffer. Visualisation of bands by integrated ethidium bromide (under UV light) was photographed by BioGene BV2040 gel documentation system using Grabber 3.00D software.

2.8.4. DNA Extraction from Agarose Gels

For extraction of DNA fragments 70bp-10Kb, bands of interest were excised from agarose gels following electrophoresis and DNA was extracted using the QIAquick® Spin Gel Extraction Kit (Qiagen), according to standard protocol (including optional steps). In cases where concentrated DNA was required, like samples were pooled and run through a single spin column to ensure maximum binding, with elution in 30µl buffer EB, applied directly to the centre of the column membrane and incubated for 1-2 min before spinning.

2.8.5. Ligation of DNA fragments into Vectors

Ligation was performed in a sterile 0.5ml eppendorf tube in a final reaction volume of 10µl. Reactions were set up with a Vector Plasmid concentration to insert DNA concentration of 1:9, together with 1U T4 DNA Polymerase in 1x ligase buffer (Roche). The ligation was carried out on a Hybaid PCR Sprint Thermal Cycler (Thermo Scientific, SPRT1001) for 16-20 h at 16ºC.

2.8.6. Transformation of DNA constructs into Bacteria

Unless otherwise stated, *Escherichia coli* NovaBlue strains (Novagen and lab) were used for amplification of all plasmids. *Escherichia coli* BL21 strains (Novagen) were used for the expression and purification of proteins. 5µl of the ligation reaction (see section 2.8.5) were added to 50µl of competent cells in a 1.5ml eppendorf tube and incubated on ice for 30 min, briefly vortexing after 15 min. Cells were then heat shocked for 45 sec at 42ºC before being returned to ice for a further 2 min. 500µl of S.O.C (Super Optimal broth with Catabolite repression) medium (Novagen) or LB was added to the cells and incubated with shaking at 37ºC for 1 hour. Transformed cells were plated onto 2% LB agar containing the appropriate antibiotic. For constructs allowing blue/white selection, X-gal (final concentration 40µg/ml) and IPTG (Isopropyl β-D-Thiogalactopyranoside) (final concentration 120µg/ml) were added. Plates were inverted and incubated overnight at 37ºC.

2.8.7. Plasmid DNA Preparation

Single colonies were picked from plates using sterile tips and grown in 7-9ml LB medium, containing the appropriate antibiotic, in a 50ml falcon tube overnight at 37ºC with 200rpm shaking in an orbital incubator (Gallenkamp, Sanyo). Overnight cultures were spun at 10000x*g* for 5 min, supernatant was poured off and inverted tubes blotted on a clean paper towel to remove excess media. The plasmid DNA was then extracted using the Wizard® *Plus* SV Minipreps DNA Purification System, according to standard centrifugation protocol (see Promega technical bulletin for Wizard® *Plus* SV Miniprep DNA Purification System). The final plasmid DNA was eluted from the spin column in 60µl nuclease free water, applying the water to the column membrane and incubating for 2-5 min before elution.

2.8.8. DNA Sequencing

Routine sequencing was performed to verify the correct insert sequence within each construct generated. Miniprep samples, prepared using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega), were diluted so that 15µl contained approximately 200ng/µl DNA and were subsequently sent to The Sequencing Service, College of Life Sciences, University of Dundee. Sequencing primers were dependent upon the vector used. Common primers include T3/T7 and SP6 (for vectors such as pGEM-T and pCAL-n).

2.9. Biochemical Methods

2.9.1. SDS-PAGE Gel and Western Blotting

Protein samples were prepared in 1x protein sample loading buffer, at a concentration which allowed optimal loading of 10-20µl per sample. Samples were boiled for 10 min at 95ºC and spun at 1300rpm for 30 sec to remove debris before loading onto a NuPAGE® 10% or 12% Bis-Tris pre-cast gel (Invitrogen). To separate proteins, the gel was run at 200V for around 60 min (variable depending on the size of the protein of interest) in NuPAGE® MOPS SDS Running Buffer (Invitrogen), within a Novex Mini-Cell gel tank (Invitrogen). For gels run longer than 90 min, gel tanks were packed with ice to avoid overheating. Proteins were then transferred onto a 0.2µm reinforced Cellulose Nitrate Membrane (Schleicher & Schuell) in transfer buffer at 70V using a Trans-Blot Cell (BioRad). Membranes were blocked at room temperature for 1 hour with shaking in Odyssey Blocking Buffer (Li-Cor). Primary antibodies were prepared in 1:1 Odyssey Blocking Buffer (Li-Cor): PBS-T (0.1%) at a dilution of 1:500, and incubated overnight at 4ºC with shaking. Following primary antibody incubation, membranes were washed three times for 10 min in PBS-T (0.1%) with shaking. Infrared-labelled secondary antibodies, Alea Flour 680 goat anti-rabbit IgG (Molecular Probes) and IRDye 800 donkey anti-mouse IgG (Rockland), were added at a dilution of 1:5000 in 1:1 Odyssey Blocking Buffer (Li-Cor): PBS-T (0.1%) and incubated away from light for 1 hour at room

temperature with shaking. The membrane was washed three times in PBS-T as before, then a final time for 5 min in PBS to remove excess Tween-20. Membranes were then scanned using the Odyssey® Infrared Imaging System (Li-Cor). The fluorescence intensity of bands of interest was quantified using TINA (version 2.09) software.

2.9.2 Antibody Preparation and Purification

2.9.2.1. Generation of pMalc2x constructs, and protein expression

BubR1, Mad2 and Mps1 full length fragments were generated by PCR (see primer table 2.1) and subcloned into *EcoRI –SalI* of pMalc2x expression vector (see 2.8.5). Constructs were transformed into BL21 cells (see 2.8.6.) and the protein expression induced with IPTG. Inductions for pMalc2x and pCal BubR1, Fizzy and Mps1 constructs were set up by inoculating a single transformed colony into 4ml of 2XYT broth with appropriate antibiotic and grown overnight at 37ºC with shaking (200rpm). 100µl of each culture were diluted into 1L 2XYT media supplemented with 2g glucose, and grown overnight at 16ºC. The following day, once the OD of the cultures had reached approximately 0.9nm, cultures were induced with 0.2mM IPTG and grown at 20ºC at 200rpm for five h. Cultures were spun at 4000*g* for 20 min and pellets resuspended in 50ml column buffer + inhibitors, ready for column purification.

2.9.2.2. Affinity Column Antibody Purification

Affinity columns were prepared as described by the AminoLink Plus Coupling Gel protocol. Approximately 30 mg of antigen were coupled per 2.5 ml of beads (5.0 ml 50% slurry). Sera were defrosted at room temperature before being transferred to 50 ml Falcon tubes and placed on ice. Storage buffer was drained from the affinity column before washing with 2.5 ml PBS. The sera was drawn through the column, with flow through collected in a 50 ml Falcon tube on ice. This was repeated several times to ensure all antibody was removed. After the final sera step, the column was drained before adding 2.5 ml PBS and allowing

this to drain into the collection tube. The column was then washed with 50 ml (20 column volumes) of 0.5M KCl (in PBS), then drained and washed with 25 ml (10 column volumes) of PBS. After the last traces of PBS were drained from the column, 10 x 1 ml of 0.1M Glycine were sequentially added to the column to elute the antibody in 1 ml aliquots into 1.5 ml eppendorf tubes containing 100 µl of 1.0M Tris-HCl (pH 8.8). As the fractions were eluted, the tubes were placed on ice. 10 µl of each fraction was added to 200 µl of Bradford reagent to assess which fractions contained the eluted protein. The peak fractions (typically fractions 3-5) were combined in another tube, with final antibody concentration determined by measuring the OD at 280 nm.

2.9.3. Cdc20 in vitro Kinase Assay

2.9.3.1. Drosophila Embryo Collection and Storage

Flies were kept in large collection vials on 30% fruit juice agar, supplemented with liquid and dry yeast, for 2 days to achieve optimal laying.1-4h embryos were harvested. Using a vacuum manifold, embryos were washed 3x in (1X) PBS-T 0.1%, 2x in (1X) PBS-T, then 1x in dH_2O. Embryos were de-chorionated in 60% thin bleach for 2min with agitation, then washed again 3x in (1X) PBS-T 0.1%, 2x in (1X) PBS-T, then 1x in dH_20. Embryos were transferred to a clean 1.5ml tube before being snap frozen in liquid nitrogen before being stored at -80ºC until use. From this point, embryos were not allowed to thaw.

2.9.3.2. Cross-linking GFP Antibody to DYNAL Dynabeads

The protocol described is for 100µl of Dynabead slurry= 50µl pure beads. (This is sufficient for 5x kinase samples/reactions @ 20µl per sample). 100µl of resuspended slurry was transferred to a clean 1.5ml tube before being placed on a magnetic separator for 1-2min, and the supernatant removed. Beads were washed by fully re-suspending in 500µl of 0.1M Na-Phosphate buffer (pH 8.1), before being returned to the magnet for 1-2min and the supernatant removed. This wash step was repeated 3x further. After the final wash, beads were returned to their original volume (in this case 100µl) of Na-Phosphate buffer (pH 8.1) including 10µl of the GFP-serum (Abcam Rabbit polyclonal to GFP –ab290)

(90μl Na-Phosphate buffer, 10μl serum). Beads were incubated at room temperature, with rotation, for 50min.The beads were then washed in 500µl Na-Phosphate buffer (pH8.1) x4 as described previously. After the final wash, beads were re-suspended in the original volume (100µl) of Na-Phosphate buffer. Beads were now ready for use, or could be stored at 4ºC (with 0.1% azide) until use.

2.9.3.3. Drosophila Embryo Extract Preparation

One kinase assay sample (one lane of final input gel) = 0.07g of embryos (prepared as above). A pestle and mortar was stood in a small tray filled with liquid nitrogen and 0.07g of frozen embryos were ground into a powder using the frozen pestle and mortar. The powder was then transferred to a clean 1.5ml tube (also standing in liquid nitrogen to prevent thawing of sample). 100µl of ice cold homogenisation buffer A was added to the powder and the powder allowed to dissolve on ice with gentle agitation. The sample was left on ice for a further 10min to allow lysis to occur (flicking tube to agitate after the first 5min, then returning to ice for the final 5min). The sample was centrifuged at max speed for 30min at 4ºC, and the supernatant transferred to a clean tube (x2), before being spun at max speed for 15min at 4ºC and the supernatant removed and transferred to a clean tube as before (x2). The final cleared lysates were transferred to a clean 1.5ml tube, ready to be used in the immunoprecipitation experiment (IP).

2.9.3.4. Immunoprecipitation

GFP-Dynabeads were equilibrated in homogenisation buffer A by placing beads on a magnetic separator for 1-2min, removing supernatant, and re-suspending in 500µl of homogenisation buffer A (x3). Beads were resuspended in homogenisation buffer A to their original volume (in this case 100µl). For each sample, 20µl of equilibrated GFP-Dynabeads (equivalent to 10µl pure beads) were added to the cleared lysate/embryo extract (equivalent to 0.07g of *Drosophila* embryos). The samples were incubated overnight with rotation at 4ºC.

The following day, the beads/samples were placed on a magnetic separator for 1-2min and the supernatant removed (but not discarded – used in post-IP blot).The beads were then washed (using the magnetic separator as before) x4 in 500µl homogenisation buffer B (containing both phosphatase and protease inhibitors). The beads were now ready for use in the kinase reaction.

2.9.3.5. The Kinase Reaction:

The beads were washed (using the magnetic separator as before) in 500µl kinase wash solution (x5). After the final wash, beads were re-suspended in 100µl kinase wash buffer and stored on ice until use. At this point, 3µl of beads from each sample were taken, added to 27µl of 2x protein sample buffer (SB) and boiled 5-10min to be later run on a western blot as the loading control for the kinase gel. The kinase reaction mix (without ATP^{32P}) was prepared, sufficient for all samples. ATP^{32P} was added to the appropriate amount of prepared kinase reaction mix (in accordance to its current activity) to a final activity of 50µCui/100µl. Beads were isolated from supernatant using a magnetic separator. 10µl of the complete *kinase reaction mix* were added to each bead sample, before samples were placed at 30ºC for 30min to allow the kinase reaction to occur. 10µl of 2xSB was added to each bead sample and boiled at 95ºC for 5min. Samples were placed briefly on ice before the beads were again separated from the supernatant using the magnetic separator. The supernatant was transferred to a clean tube for loading onto the kinase gel (a pre-cast 4%-15% gradient gel). Full samples were loaded in each case, and the gel run at 200V until the smallest protein of interest (in this case histone) and the largest protein (in this case GFP-Fizzy) were at appropriate positions on the gel according to the size marker. After running, the gel was washed in 5% TCA, 1% Na Pyrophosphate for 1 h with shaking. The wash was changed and left overnight with shaking. The following day, the 5% TCA, 1% Na Pyrophosphate wash was changed and the gel incubated for a further hour. The gel was placed in Safe Stain for 2h, then washed and dried between gel-drying films in 1% glycerol. After drying (1-2days), the gel was exposed to phospho-image plate for around 5-6 h and analysed.

2.10. S. cerevisiae techniques

2.10.1. Y190 strain and growth conditions

The *S. cerevisiae* strain Y190 (genotype: *MAT***a** *gal4-542 gal80-538 his3 trp1-901 ade2-101 ura3-52 leu2-3,112* URA3::GAL1-LacZ Lys2::GAL1-HIS3cyh^r) as used in the yeast three hybrid experiments in this thesis. Cultures were grown at 30ºC in either rich YPD or minimal SD media. SD media was supplemented (as appropriate) with adenine hemisulphate (20mg/L), L-histidine (10mg/L), L-leucine (20mg/L), L-methionine (10mg/L), L-tryptophan (10mg/L) and uracil (8mg/L) (All supplied by Sigma Aldrich). When making solid media, 2% (w/v) Bacto-Agar was added.

2.10.2. Lithium acetate transformation of S. cerevisiae cells

To account for all 14 samples, 300ml mid-log-phase-growing ($OD_{660\ nm}$ 0.4; $5x10^6$ cells/ml) Y190 cells were split into 3 x 100ml aliquots, each of which were pelleted by centrifugation, washed with 1ml LiAc/TE (0.1M LiAc pH 7.5, 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0) and resuspended in 1ml LiAc/TE. 200µl of cells were mixed with 20µg denatured salmon sperm DNA (Stratagene), 1-10ng of plasmid DNA and 1.2ml PEG/LiAc/TE (0.1M LiAc pH 7.5, 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 40% (w/v) PEG 4000), and incubated with shaking for 30min at 30ºC. Cell suspensions were heat shocked at 42ºC for 15min, then pelleted at 6,000 rpm for 30 sec. Cells were washed with 1ml dH_2O and plated onto selective SD agar plates which were incubated at 30ºC for 3-4 days.

2.10.3. β-galactosidase filter assay

Colonies were transferred onto Whatman #3 filter paper by placing the filter across the surface of the plate, and peeling away. The filters were then placed in liquid nitrogen for 30sec and then allowed to thaw at room temperature on a flat surface. 3 ml Z-buffer (60 nM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1mM $MgSO_4.7H_2O$), 8.1 µl β-mercaptoethanol and 40 µl X-gal (20mg/ml in dimethylformamide) was added to the lid of a petri dish and Whatman #50 filter paper was placed into the lid and allowed to absorb the buffer. The thawed #3 filter was then carefully placed on top of the #50 filter,

colony side up. Plates were incubated at 37ºC until the development of blue colour.

Chapter 3: Investigating the role of Cdc20 phosphorylation by Cdk1 in Cdc20 localisation and its incorporation into the MCC upon SAC activation

3.1. Introduction

Cdc20 regulation involves changes in its abundance during the cell cycle, with levels negligible during G1, rising to their peak in mitosis, before rapidly declining (Weinstein, 1997). But with findings which implicated cell-cycle dependent Cdc20 phosphorylation, it is now believed that this also has a role in the regulation of Cdc20 and its ability to activate the APC/C. Levels of Cdc20 phosphorylation in mammalian cells have been reported to be maximal during G2, dropping during the G2-M phase transition, whilst expression of Cdc20 is actually still rising (Weinstein, 1997). This finding implied that Cdc20 phosphorylation actually precedes its increase in abundance. Subsequent investigation into conditions in which Cdc20 is phosphorylated suggested an association between increased phosphorylation and mitotic arrest, both by the microtubule depolymerising agent nocodazole (Kramer et al., 1998) and in *Xenopus* eggs naturally arrested in metaphase II by cytostatic factor (CSF) (Lorca et al., 1998). This lead to questions as to whether the phosphorylation of Cdc20 may negatively regulate the action of the APC/C, as the observed increase of Cdc20 phosphorylation during mitotic arrest, corresponds with the inhibition of APC/C activity observed during the spindle checkpoint response.

There are conflicting reports regarding the role of Cdc20 phosphorylation in the regulation of APC/C activity. At least three kinases (Cdk1, MAPK and Bub1) have been implicated in Cdc20 phosphorylation, with various potential phosphorylation sites observed across species. It is therefore plausible that phosphorylation of Cdc20 at different sites, by a number of different kinases, may have different roles in the checkpoint response. Whether the MCC forms at unattached kinetochores with recruitment of individual components, or already exists as subunits which come together upon checkpoint activation to form the complex as a whole, is still a topic for debate. If MCC subunits such as Cdc20-Mad2 exist outside of the checkpoint response and in non-mitotic cells,

potentially only forming the complex as a whole upon checkpoint activation, would these too be affected by the inability of Cdc20 to be phosphorylated? Could the phosphorylation of Cdc20 by kinases who's activity peaks at mitosis or upon activation of the checkpoint, be involved in the difference between checkpoint protein complexes which are formed at kinetochores during the SAC response and those which already exist?

The experiments in this chapter were designed to establish a *Drosophila* working model, in which the potential effect of disturbing the potential phosphorylation of Cdc20 by Cdk1 could provide a system for studying MCC formation in which Cdc20 kinetochore localisation is perturbed. If this were to be successful, the requirement of direct kinetochore interaction with Cdc20 in the formation of the MCC could be established, providing a new and insightful method for investigating the formation of inhibitory complexes upon SAC activation in *Drosophila.*

3.2. Results:

3.2.1 *Drosophila* Fizzy carries three potential Cdk1 consensus phosphorylation sites on Thr63, Thr78 and Thr82 within the N-terminal of the protein.

The *Drosophila* homologue of Cdc20 (Fizzy) contains an N-terminal Mad2-binding domain, as observed in humans and *Xenopus* (Fig 1A). To identify whether the N-terminal of *Drosophila* Cdc20 also contains potential sites for Cdk1 phosphorylation, the protein sequence of Fizzy was analysed using a bioinformatic motif scan website (http://scansite.mit.edu/motifscan_seq.phtml), which performs proteome-wide prediction of cell signalling interactions using short sequence motifs (Kraft et al., 2005).

Scansite analysis highlighted three potential consensus sequences for Cdk1 phosphorylation, all of which reside within the N-terminal region, the predicted Mad2 binding region of Fizzy. The residues implicated for phosphorylation were Thr63, Thr78 and Thr82. These residues are consistent with the conserved phosphorylation sites observed in human and *Xenopus*.

3.2.2 Generation of transgenic fly lines for the study of Fizzy Thr63, Thr78 and Thr82 as potential phosphorylation sites.

In order to study the relationship between the phosphorylation on these three potential Cdk1 sites (Thr63, Thr78 and Thr82) and their relevance on the SAC function of Fizzy, previous lab members point-mutated these residues to alanine singly, doubly or all three together by site-directed mutagenesis PCR. These mutated plasmid DNA constructs were then fused with a GFP-tag at each of their 5'end, at the beginning of the open reading frame (ORF). These fusion DNA constructs were then subcloned into a P-element-mediated transgenic expression vector (pWRubq vector, from Nick Brown, Cambridge University) as described in materials and methods. Transgenic fly lines were generated by standard P-element-mediated transgenic procedure (Salmon and Ahluwalia, 2009) to express either a wild-type GFP-tagged Fizzy protein (TF4 line in stock) or a potential phosphorylation defective mutant protein (TF41) in which Thr63, Thr78 and Thr82 were all substituted for Ala using PCR-site-directed

mutagenesis (Figure 3.1B) (performed by G. Morley, microinjection performed by M. Pal). In order to study the effect of the potential phosphorylation site mutations on the localisation of Fizzy on the mitotic apparatus (particularly the kinetochores) as well as the interaction with other SAC components, a *Drosophila* line null for endogenous Fizzy protein was needed. In this case, once the GFP-Fizzy fusion protein is introduced, the only Fizzy protein present would be that of the GFP-fusion proteins. This would allow comparison between ectopically expressed GFP-tagged wild-type and mutant Fizzy, in the absence of endogenous protein. The absence of endogenous protein avoids the potential of masking the effects of the mutation on the GFP-fusion proteins.

Three *fizzy* mutant alleles were found to be available from Flybase, within the Bloomington stock centre. They are fzy^{1}, fzy^{3} and fzy^{EP1028}. *fzy1* and fzy3 alleles are embryonic lethal but the phenotypes could not be rescued by insertion of the wild-type GFP-*fzy* transgene, and are suspected to contain additional point mutations (Laine et al., 2009). These lines were deemed unsuitable for use in this particular study. The fzy^{EP1028} is a weak allele as it still expresses around 20% endogenous protein, with homozygotes viable (although sick). Therefore, this line was chosen for use in this study, and the transgenes were introduced into wild-type flies as a control (in the presence of endogenous Fizzy) and also in the fzy^{EP1028} mutant background. This would allow for analysis of the mutant GFP-fusion protein to be performed in the relative absence of the endogenous protein.

After introduction of *tf4* and *tf41* transgenes into the fzy^{EP1028} background, although the wild-type and mutant GFP-fusion proteins were successfully expressed, unfortunately, levels of endogenous Fizzy were unexpectedly detected at normal levels (data not shown). The reason for this is unknown, with the potential of mistakes during genetic crosses unlikely (although not impossible) due to these genetic recombinations being repeated twice. We therefore failed to express the GFP-fusion in a *fizzy* mutant background, in that only the TF4 and TF41 flies which express mutant Fizzy in the presence of wild-type levels of endogenous Fizzy were successful (data not shown).

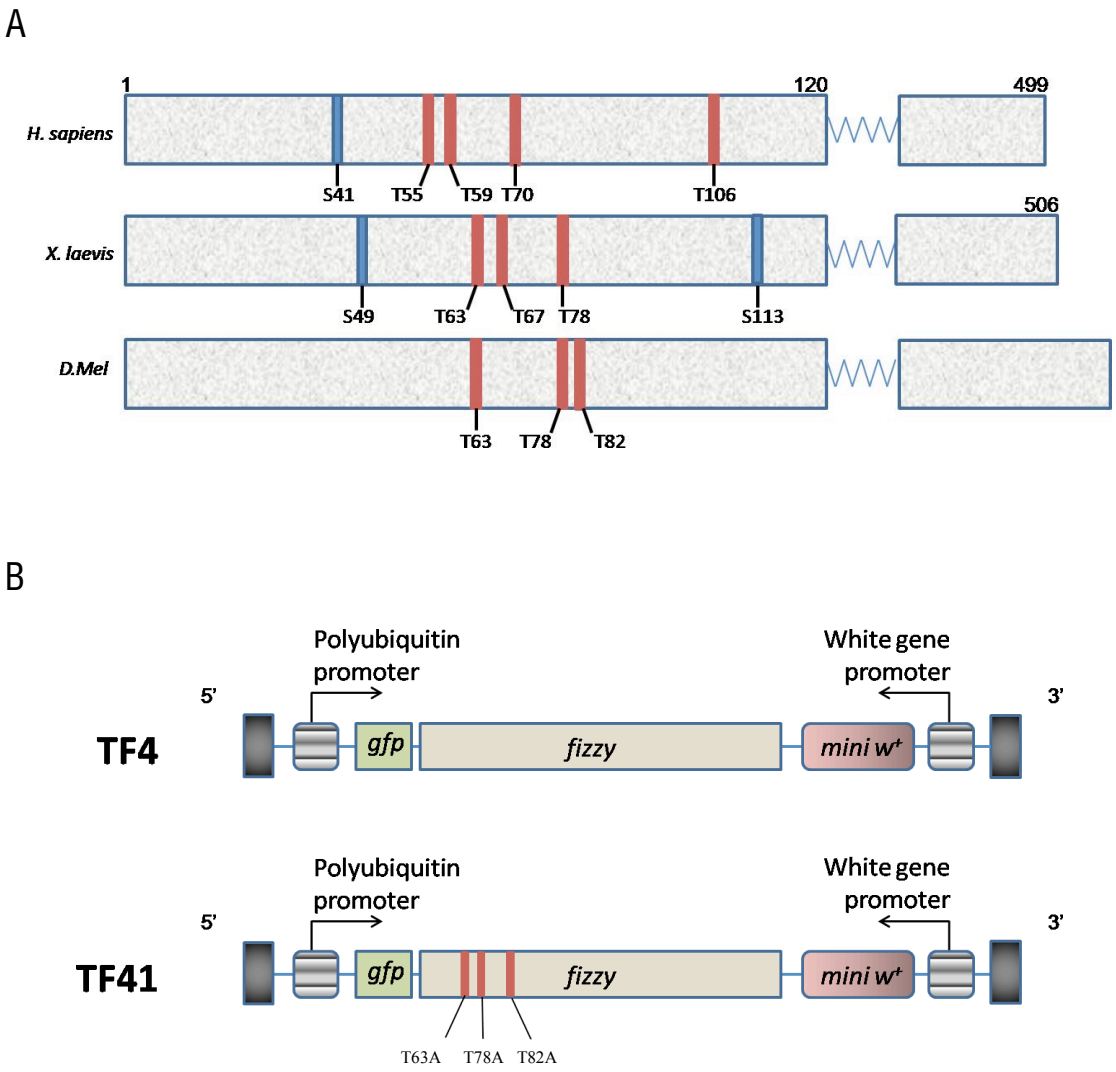


Figure 3.1. Potential regions of interest within *Drosophila fzy*, and subsequent constructs, for use in generating potential phosphorylation-site mutant fly lines.

A) Diagram to highlight the conserved potential Cdk1 phosphorylation sites in human and *Xenopus* Cdc20 in comparison to *Drosophila fizzy*. Threonine at positions 63, 78 and 82 of the *Drosophila* sequence are consistent with evolutionarily conserved sites within the human and *Xenopus* sequences. B) Diagram to represent the P-element constructs used to generate the TF4 GFP-Fizzy control and TF41 GFP-Fizzy phosphorylation site mutant transgenic lines. Constructs were generated using PCR for the wild-type TF4 and by PCR site-directed mutagenesis to substitute threonine 63, 78, and 82 of Fizzy for alanine in the mutant TF41 line. P-element constructs were then used to insert the wild-type GFP-tagged Fizzy protein or the mutant GFP-Fizzy protein into a background potentially null of endogenous Fizzy.

Single mutants for these lines were also generated, with either Thr63, Thr78 or Thr82 substituted for Ala in the presence of endogenous Fizzy. If these sites are targets for phosphorylation, some sites may be specific to phosphorylation by a specific kinase, be it a single site or all three sites combined. For this reason, it was decided to focus upon the TF41 mutant line, carrying all three mutated sites.

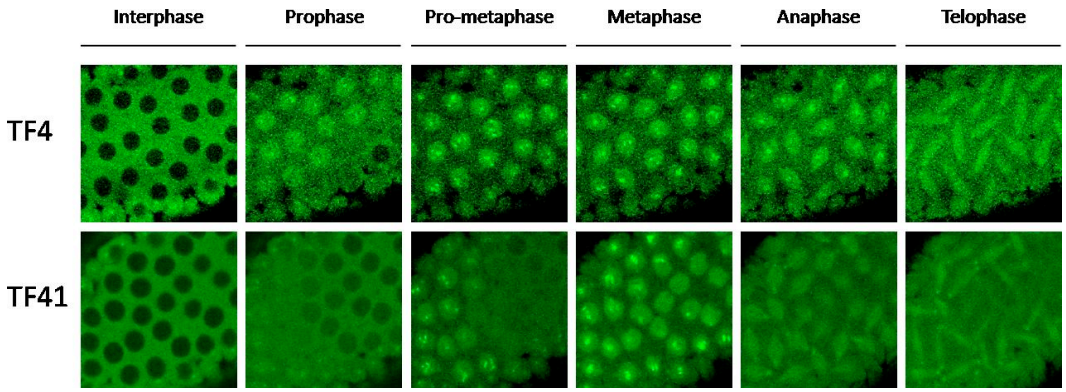


Figure 3.2. In the presence of endogenous Fizzy, the TF41 mutant GFP-fusion protein localises to kinetochores.

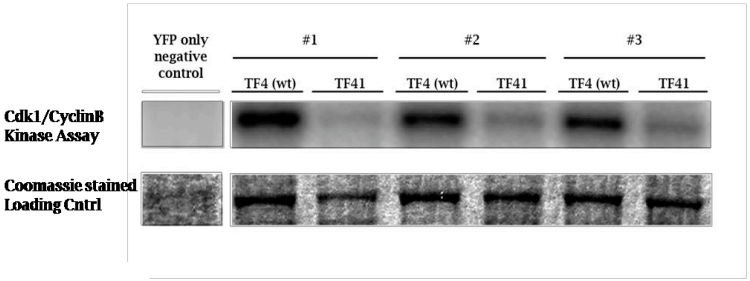
The TF41 GFP-fusion protein displays normal localisation patterns in the presence of endogenous Fizzy (bottom panel), as shown by time-lapse imaging in embryos. This is comparable with that observed in the TF4 line (top panel) throughout mitosis.

3.2.3 In the presence of endogenous Fizzy, the triple mutant Fizzy GFP-fusion protein localises normally throughout the cell cycle.

To determine whether the mutant Fizzy protein was capable of localising to the kinetochore in the TF41 fly line, GFP was visualised in syncytial embryos using a Leica confocal microscope. It was also visualised in the wild-type TF4 control line for comparison. Fizzy was clearly localised to the kinetochore during metaphase. Wild-type localisation patterns were observed throughout the cell cycle, with Fizzy absent from the interphase nucleus, moving from cytosol to nucleus during prophase. Fizzy was then observed to remain associated with the mitotic spindle throughout anaphase and telophase (Fig. 2). These data

suggest that in the presence of endogenous Fizzy, Thr63, Thr78 and Thr82 are not required for proper localisation of the GFP-fusion protein throughout the cell cycle in syncytial embryos.

A



B

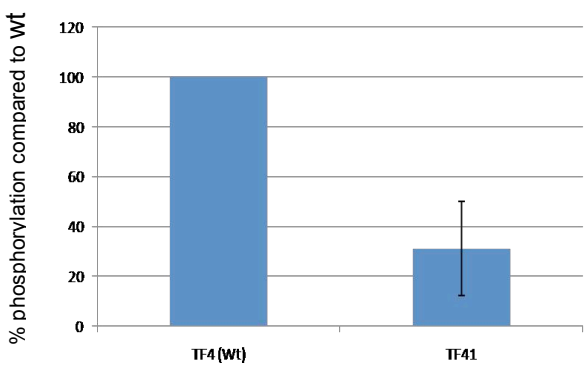


Figure 3.3. *In vitro* kinase assay results show a role for T63, T78 and T82 in Cdk1 phosphorylation of *Drosophila* Fizzy.

A) Cdk1 *in vitro* kinase assays were carried out to determine the phosphorylation status of the mutant and wild-type GFP-fusion proteins. Embryos from a YFP-only line were used as a negative control. Immunoprecipitation to Dynal Dynabeads cross-linked to anti-GFP antibody (Abcam) was carried out using 0-4hour embryo extracts, to isolate the fusion proteins and YFP control. These were then subject to an *in vitro* kinase assay and incorporated P^{32} isotope activity was visualised using a phospho-image plate. Experiments were carried out in triplicate. It was observed that the TF41 mutant line has a significant decrease in incorporated P^{32} (top panel), implying that the phosphorylation of this fusion protein is decreased in comparison to wild-type fusion protein. B) Quantification using TINA software revealed a decrease in Cdk1 phosphorylation in the TF41 mutant GFP-fusion protein of 69% compared to that of the TF4 wild-type fusion protein. Error bars represent standard deviation.

3.2.4 Thr63, Thr78 and Thr82 of *Drosophila* Fizzy are phosphorylated by Cdk1 *in vitro*.

Cdk1 was found to phosphorylate *Xenopus* and human Cdc20 (refer to introduction 1.12.) at residues conserved across both species as well as *Drosophila*. Because Scansite analysis of *Drosophila* Fizzy revealed that Thr63, Thr78 and Thr82 are predicted phosphorylation sites with Cdk1 recognition motifs, it was decided that analysis of the TF41 mutant GFP-fusion protein should be analysed with regard to the effect of these mutations on phosphorylation by Cdk1.

An *in vitro* kinase assay was performed on the triple mutant GFP-Fizzy fusion protein, isolated from TF41 embryos, as well as on the wild-type GFP-Fizzy fusion protein isolated from TF4 embryos as a control. 0-4 hour embryos were collected and homogenised over liquid nitrogen, with cleared lysate subjected to co-immunoprecipitation with anti-GFP antibody to isolate the GFP-fusion proteins. A Cdk1/Cyclin B kinase assay was performed on the isolated fusion proteins in the presence of ATP^{32P}, with levels of incorporated ATP detected using a phospho-image plate.

It was observed that in the absence of intact Thr63, Thr78 and Thr82 residues, phosphorylation of *Drosophila* Fizzy by Cdk1/Cyclin B was reduced by 69% (Figure 3.3). This implies that these residues are involved in the phosphorylation of Fizzy, and that a proportion of this phosphorylation can be attributed to Cdk1/Cyclin B.

3.2.5 Mutation of Thr63, Thr78 and Thr82 of *Drosophila* Fizzy does not affect its interaction with the APC/C.

After establishing that Thr63, Thr78 and Thr82 are phosphorylated by Cdk1/Cyclin B *in vitro*, the next step was to identify whether loss of phosphorylation at these sites had any effect on the ability of Fizzy to associate with the APC/C. Samples from the anti-GFP immunoprecipitation used to isolate the wild-type (TF4) and triple mutant (TF41) GFP-fusion proteins for the previous kinase assay, were probed by western blot for Cdc27, a subunit of APC/C. The levels of Cdc27 found to associate with the triple phosphorylation mutant Fizzy protein were comparable to the association of Cdc27 with wild-

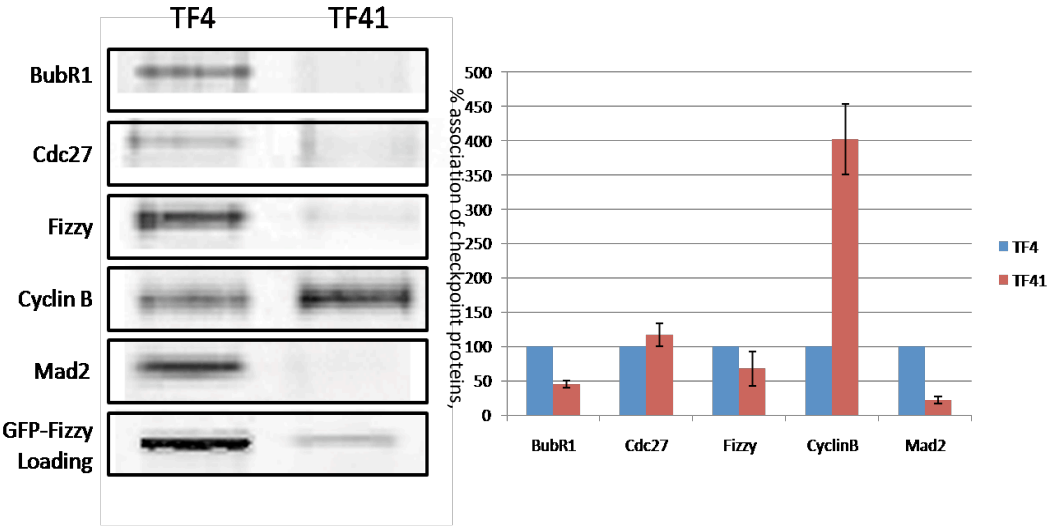


Figure 3.4. Western blot analysis to show levels of associated checkpoint proteins with wild-type TF4 and mutant TF41 fusion proteins in non-synchronised 0-4hour embryo extracts.

(Representative of extracts used for the *in vitro* kinase assay in fig. 3.3).

Immunoprecipitation with anti-GFP antibody (Abcam) shows that Mad2 and BubR1 association with GFP-Fizzy is reduced with the mutation of Cdk1 phosphorylation sites (as shown in the TF41 samples). Quantification of associated proteins when standardised as a percentage of that observed in wild-type GFP-Fizzy TF4 samples (as displayed in the bar chart to the right) shows that binding of wild-type and mutant GFP-Fizzy fusion proteins to endogenous Fizzy is consistent with one another, as is binding to the APC/C subunit Cdc27. Cyclin B affinity is increased four-fold in the TF41 fusion protein compared to wild-type. Experiments were carried out in triplicate, imaged using the Li-Cor Odyssey® infrared imaging system, with protein levels quantified using TINA software and samples from TF4 and TF41standardised against one another using GFP-Fizzy loading as reference. Error bars represent standard deviation.

Wild type GFP-Fizzy (Fig.3.4). This suggests that within this IP, mutation at these sites may cause a 69% decrease in Cdk1/Cyclin B phosphorylation, but does not affect the ability of Fizzy to associate with the APC/C.

3.2.6. Mutation of Thr63, Thr78 and Thr82 of *Drosophila* Fizzy has an effect on its ability to interact with other checkpoint proteins, implicating a role for Cdk1 phosphorylation in MCC formation.

To address the effect of the potential role of Cdk1 phosphorylation of *Drosophila* Fizzy on the interaction between mutant GFP-Fizzy and other SAC proteins, the anti-GFP immunoprecipitation samples were probed with anti-BubR1, anti-Mad2, anti-Cyclin B antibodies (Fig. 3.4). It was observed that 80% less Mad2 protein associated with the triple mutant GFP-fusion protein than with the wild-type GFP-Fizzy fusion protein. The ability of the mutant protein to interact with BubR1 was also decreased, with less than 50% BubR1 protein observed in the TF41 blot in comparison to the TF4 wild-type sample. This suggests that Cdk1/Cyclin B phosphorylation of *Drosophila* Fizzy at Thr63, Thr78 and Thr82 is likely to be required for efficient formation of the MCC.

With regard to the association of Fizzy with mitotic the cyclin, cyclin B, probing the immunoprecipitation samples with anti-Cyclin B antibody revealed a dramatically increased association of cyclin B with the triple mutant fusion protein (approximately four-fold), in comparison to that associated with the wild-type fusion protein (Fig. 3.4). This implies that Cdk1 phosphorylation of Fizzy is involved in its interaction with cyclin B, although how a decrease in Cdk1 phosphorylation would result in this increased association is not yet understood.

In order to assess the potential contribution of endogenous Fizzy in the results observed, the previously described immunoprecipitation samples were also probed with anti-Fizzy antibody (Fig. 3.4). Although the association of endogenous Fizzy with the triple mutant fusion protein appeared to be reduced in comparison to wild-type, this reduction was not significant. This implies that endogenous Fizzy is capable of associating with the mutant protein, and that phosphorylation of Fizzy at Thr63, Thr78 and Thr82, is likely to have no involvement in any interaction between Fizzy molecules. This should be taken into consideration when subsequently discussing these data.

3.2.7. GFP-Fizzy fusion proteins extracted from mitotically synchronised embryos display similar, but not identical, patterns of association with APC/C and checkpoint proteins.

The previous immunoprecipitation results were carried out on non-synchronised 0-4 hour syncytial embryos. Within a non-synchronised cell population, it has been previously determined within the lab, the mitotic index is approximately 4%. MG132 treatment of *Drosophila* embryos has been shown previously within the lab to result in approximately 80% of cells arresting in mitosis. In order to see whether the association between Fizzy and other checkpoint proteins observed in the previous experiments, and therefore representative of the

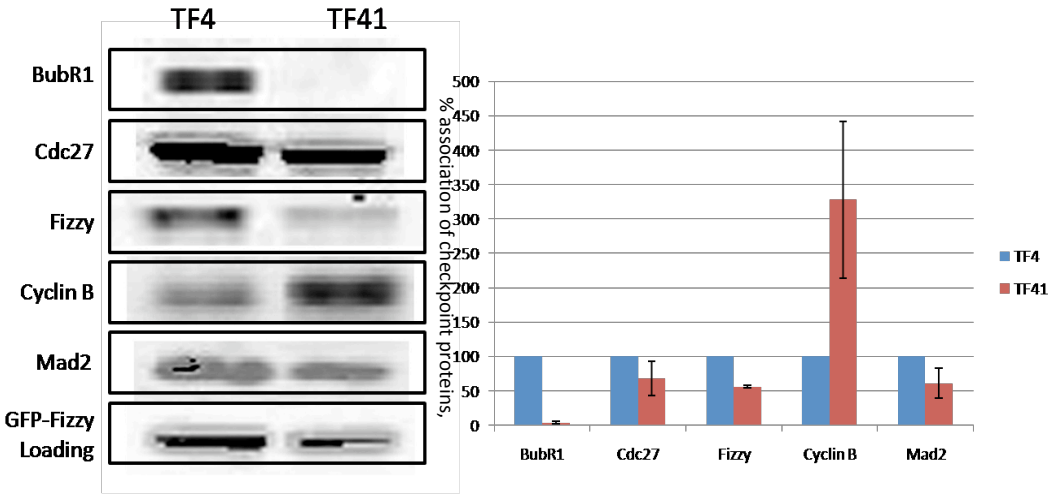


Figure 3.5. Western blot analysis to show levels of associated checkpoint proteins with Wild-type (TF4) and reduced phosphorylation mutant (TF41) in mitotically synchronised (MG132-treated) 0-4hour embryo extracts.

Immunoprecipitation with anti-GFP antibody (Abcam) shows that BubR1 binding is again reduced in the mutant extracts, as is the association of GFP-Fizzy with Mad2, although to a lesser extent. Binding of wild-type and mutant GFP-Fizzy fusion proteins to endogenous Fizzy appears less than in non-synchronised extracts, with association of the mutant GFP-fusion protein and endogenous Fizzy 50% less than that of wild-type GFP-Fizzy. Cdc27 is again consistent between samples, with Cyclin B affinity dramatically increased in the TF41 fusion protein compared to TF4 wild-type. Experiments were carried out in triplicate, imaged using the Li-Cor Odyssey® infrared imaging system, with protein levels quantified using TINA software and TF4 and TF41 samples standardised against one another by GFP-Fizzy loading controls. Error bars represent standard deviation.

general cell cycle, were consistent with embryos synchronised in mitosis, 0-4hour TF4 and TF41 embryos were treated with MG132 before repeating the anti-GFP immunoprecipitation experiment as before.

The general pattern of association between checkpoint proteins, Cyclin B and APC/C subunit Cdc27 with the triple phosphorylation site mutant and wild-type GFP-Fizzy fusion proteins, remains consistent with those observed in non-synchronised samples (Fig. 3.5). The association between Fizzy and APC/C displayed no significant difference between wild-type and mutant protein, whilst the association between Fizzy and Cyclin B significantly increased with the loss of Thr63, Thr78 and Thr82 in the triple mutant protein. Consistent with the non-synchronised extracts, the interaction between Fizzy and Mad2, as well as that between Fizzy and BubR1, are reduced in the TF41 mutant sample. This supports the finding that phosphorylation by Cdk1 at Thr63, Thr78 and Thr82 appear to be required for efficient association of checkpoint components.

Interestingly, although Mad2 and BubR1 interaction with Fizzy show the same decrease in interaction, it appears that the extent of this decrease differs in the mitotic samples in comparison to the fusion proteins extracted from non-synchronised embryos (Figs. 3.4 & 3.5). Whereas the levels of BubR1 detected in the anti-GFP immunoprecipitation from non-synchronised embryos was reduced by around 55% in the mutant line compared to wild-type, this decrease was far more dramatic in the western blot analysis of fusion proteins from mitotically synchronised embryos. Here, levels of BubR1 were reduced to less than 10% of the levels observed in wild-type. In contrast, the decrease in Fizzy-Mad2 interaction in the phosphorylation mutant GFP-fusion protein samples compared to that of wild-type, appears less in the samples from mitotically synchronised embryos. It is unclear as to whether these differences are significant.

With regard to the interaction between GFP-Fizzy fusion proteins and endogenous Fizzy, it appears that although on average the triple mutant GFP-fusion protein from mitotic samples showed decreased levels of associated endogenous Fizzy in comparison to wild-type, whether this slight difference has

any significance on the data is unlikely, but is yet to be investigated further (Figs. 3.4 & 3.5).

3.3. Discussion

The contribution of Cdk1 to the overall phosphorylation of *Drosophila* Cdc20 (Fizzy) has never been specified. With potential conserved Cdk1 sites identified within Fizzy, there is a possibility that Cdk1 may phosphorylate Fizzy in order to promote its function within the SAC response. With questions into the role of kinetochore localisation of SAC proteins in MCC formation, and studies yet to establish a system whereby Cdc20 or *Drosophila* Fizzy localisation can be perturbed, we set out to test whether these phosphorylation sites may affect the ability of Fizzy to localise to the kinetochore upon SAC activation. If this were to prove to be the case, interactions between Fizzy and other SAC components could be investigated in the absence of Fizzy kinetochore localisation, thus providing a *Drosophila* working model which will allow more of an insight into the requirements for MCC formation.

With the use of generated transgenic fly lines, it has been possible to isolate GFP-tagged wild-type and potential phosphorylation-deficient mutant Fizzy fusion proteins from syncytial embryos, in order to analyse their phosphorylation by Cdk1. These data suggest that Thr63, Thr78 and Thr82 of *Drosophila* Fizzy are phosphorylated *in vitro*, and that approximately 70% of this phosphorylation could be attributed to the activity of the mitotic cyclin dependent kinase Cdk1 (and cyclin B). The three residues tested all reside within the N-terminal of Fizzy, consistent with conserved phosphorylation sites observed in *Xenopus* and human Cdc20.

Previous studies of Cdk1 phosphorylation of Cdc20 linked this to the role of Cdc20 in the spindle checkpoint, with inhibition of Cdk1 activity resulting in a defective checkpoint response, believed to be attributed to the finding that non-phosphorylatable Cdc20 is capable of binding APC/C in the presence of SAC triggers. Although the effect of the triple phosphorylation-site mutant *Drosophila* Fizzy in the presence of checkpoint-arresting agents was not tested directly in syncytial embryos, it was observed that removal of Thr63, Thr78 and Thr82 of Fizzy, had no effect on its binding to APC/C subunit Cdc27 in both mitotic and

non-synchronised embryo extracts (Figs. 3.4 & 3.5). This could potentially imply that *Drosophila* Fizzy is likely to behave in the same fashion, although at present it is not possible to reliably test this directly.

Originally, we anticipated examining the functions of the GFP-tagged Fizzy fusion proteins in a *fizzy* weak allele genetic background in which the endogenous Fizzy protein levels were significantly reduced. Unfortunately, the efforts of introducing the TF4 and TF41 transgenes into the *fizzy* weak allele (fzy^{EP1028}) genetic background failed. The resulting fly lines expressed the wild-type GFP-fusion protein or the triple mutant GFP-fusion protein in the presence of wild-type levels of endogenous Fizzy. For this reason, they are not suitable for use in testing the behaviours of the ectopically-expressed GFP-tagged wild-type or triple phosphorylation-deficient Fizzy under conditions which should trigger the SAC, as the endogenous protein present would likely satisfy the checkpoint response, and arrest cells regardless of whether phosphorylation of the fusion protein occurred.

If we were to assume that phosphorylation of Fizzy by Cdk1 is required to block its association with APC/C in the checkpoint response, by allowing it to localise and subsequently associate with other checkpoint components as part of the MCC, we might expect that the triple mutant protein would display reduced interaction with MCC components such as Mad2. Previous studies in humans and *Xenopus* have shown that with mutation of potential Cdk1 phosphorylation sites, there is a decreased association with checkpoint proteins Mad2 and BubR1. Consistent with this, immunoprecipitation experiments to isolate the complexes associated with the GFP-tagged phosphorylation-deficient mutant and wild-type Fizzy fusion proteins revealed that this is also the case in *Drosophila* Fizzy (Fig. 3.4). Fizzy-associated Mad2 levels were decreased by over 80% in the phosphorylation-deficient mutant sample, compared to that of wild-type, Similarly, a reduction of over 50% BubR1 associated with Fizzy was detected in the phosphorylation mutant sample. These results were obtained using extracts made from non-synchronised embryos. In a non-synchronised population, only approximately 4% of *Drosophila* syncytial embryos are in mitosis (unpublished results, carried out by another member), and past studies from other systems have implied that Cdc20 can form sub-complexes of the

MCC (such as Cdc20-Mad2) outside of mitosis. Hence, it was not known whether our data were representative of the interactions of Fizzy specifically in mitosis. In the non-synchronised population, TF41 Fizzy has reduced ability to associate with Cdc27, BubR1 and Mad2 but increased ability to bind with cyclin B.

To test whether these results would also be observed in mitotic extracts, similar co-immunoprecipitation experiments were performed to isolate the complexes that associated with GFP-tagged Fizzy fusion proteins from MG132-treated mitotically-synchronised embryos. These experiments revealed a similar pattern of GFP-Fizzy-Mad2 and GFP-Fizzy-BubR1 interaction in that, with the mutation of Thr63, Thr78 and Thr82, the association of these checkpoint proteins is decreased. This implies that the patterns observed are representative of the mitotic behaviour of Fizzy, and so it is not unreasonable to link the decreased association with defective formation of the MCC complex. In order to test this further, it would be interesting to probe for the presence of associated Bub1 and Bub3 (other predicted MCC components), to verify whether the phosphorylation of Fizzy is required for specific interactions, or formation of the MCC as a whole. Unfortunately no Bub1 or Bub3 antibodies were available during the course of this study. Interestingly, a coomassie stain of a gel on analysing immunoprecipitation samples for the TF4 and TF41 proteins isolated for the kinase assay displayed numerous differences in visible bands, representing associated proteins, between the wild-type and mutant samples (Fig. 3.5). The predicted positions of Mad2 and BubR1 can account for two bands observed in the wild-type samples which appear to a far lesser extent in the mutant samples, but the other proteins which display differences between the TF4 and TF41 isolated fusion protein immunoprecipitation samples are yet to be identified.

Although the significance of the differences in the extent of the decreased association between Fizzy-Mad2 and Fizzy-BubR1 in non-synchronised and mitotic extracts is questionable, it cannot be ruled out that it could have some potential significance. Levels of associated BubR1 were seen to decrease more dramatically in mitotically-derived fusion protein samples, whereas Mad2 levels decreased to a lesser extent in protein extracted from mitotically-synchronised

embryos than with protein extracted from non-synchronised embryos (figs 3.4 & 3.5). It could be speculated that the interaction of Fizzy with Mad2 when forming sub-complexes outside of mitosis, may be affected more by lack of Cdk1 phosphorylation than the Fizzy-Mad2 interaction which occurs in the formation of the MCC. Further research into the differences between the interactions of Fizzy-Mad2 outside of mitosis would need to be carried out. However, with past studies questioning the presence of Mad2 in the MCC itself (Nilsson et al., 2008), and a recent paper from our own laboratory implicating that BubR1, but not Mad2, is required for recruitment of Fizzy to the kinetochore in *Drosophila* (Cheeseman, 2009), this notion cannot be completely dismissed at present.

A recent publication by our lab also highlights the greater significance of BubR1 in the MCC than that of Mad2 (Li et al., 2010). By inserting a GFP-Fizzy transgene into the Mad2 EY21687 fly line, characterised as null for endogenous Mad2 by western blot analysis and proven to be unable to induce a SAC response, it was shown that in the absence of Mad2, Fizzy is still capable of normal kinetochore localisation. In contrast, with insertion of the GFP-Fizzy transgene into a BubR1-null line (obtained from the Bloomington stock centre), it was observed that in the absence of BubR1, Fizzy was unable to localise to kinetochores in mitosis, even when the SAC is potentially induced by treatment with colchicine. Alongside this data, the fact that the EY21687 Mad2-null line is viable, whereas the BubR1-null line is homozygous lethal at the larval/pupal stage, indicates a more prominent role for BubR1 than for Mad2 in the sequestration of Fizzy within the SAC response. This also suggests that in *Drosophila* Mad2 and BubR1 are likely to form complexes with Fizzy independently of one another. This data is supportive of that of Pines et al (2008), who claimed that Mad2 may not be a component of the MCC complex, but rather, has a role in retaining the localisation of Cdc20/Fizzy once the SAC is activated.

In addition to analysis of associated APC/C and checkpoint proteins, immunoprecipitation samples were probed for the association of Cyclin B. In both non-synchronised and mitotically-derived samples, the association of Cyclin B with the mutant GFP-fusion protein was significantly increased in comparison to wild-type. It is known that Cdc20 targets Cyclin B, and that for

normal mitotic progression this associated Cyclin B must dissociate. At present it is not known why the reduction in Cdk1 phosphorylation of Fizzy would result in a greater association with Cyclin B. However, two possible scenarios which could be investigated in the future are whether this is a result of the phosphorylation mutant protein being rendered constitutively active, or whether Cdk1 phosphorylation of Fizzy is required to allow cyclin B dissociation, thus resulting in the observed excess. At present the effect of Cdk1 phosphorylation of Cdc20 on its association with mitotic cyclins in other organisms has not been investigated.

One aspect of these data which must be taken into consideration, is the fact that the fly lines generated for analysis contained wild-type levels of endogenous Fizzy. The checkpoint is likely to be effective in these cells as a result of this, and so *in* vivo analysis of the mutant GFP-fusion protein is not reliable due to the masking effect of endogenous Fizzy. It is possible that, because the mutant GFP-fusion protein is capable of interacting with the endogenous protein (as shown in Figs. 3.4 & 3.5), the normal localisation of this protein throughout the cell cycle (Fig 3.2) could be attributed to this association. For this reason, the localisation of Fizzy when Thr63, Thr78 and Thr82 are mutated, as well as whether this mutant Fizzy is capable of a checkpoint response, can only be investigated reliably in the absence of the endogenous protein.

Unfortunately, the re-generation of transgenic lines in a Fizzy-null background was too time consuming to consider as part of this study, as was the generation of constructs allowing for transfection into a stable cell culture line, such as *Drosophila* S2 cells, allowing for subsequent dsRNAi depletion of endogenous protein. In an attempt to create an environment whereby the wild-type and triple mutant Fizzy GFP-fusion proteins exist in an environment free of endogenous Fizzy protein, dsRNA to the 3'UTR of Fizzy was generated for microinjection into TF4 and TF41 syncytial embryos. This would target endogenous Fizzy, but not the transgenic protein, thus allowing localisation of the triple mutant GFP-Fizzy protein to be analysed in the absence of endogenous Fizzy. It could also help verify the effect of disruption of the three potential Cdk1 phosphorylation

sites on SAC arrest and kinetochore localisation of GFP-Fizzy in the absence of endogenous Fizzy.

Previous studies microinjected dsRNA into syncytial embryos to successfully deplete mitotic Cyclin B (McCleland et al., 2009), possibly due to the fact that

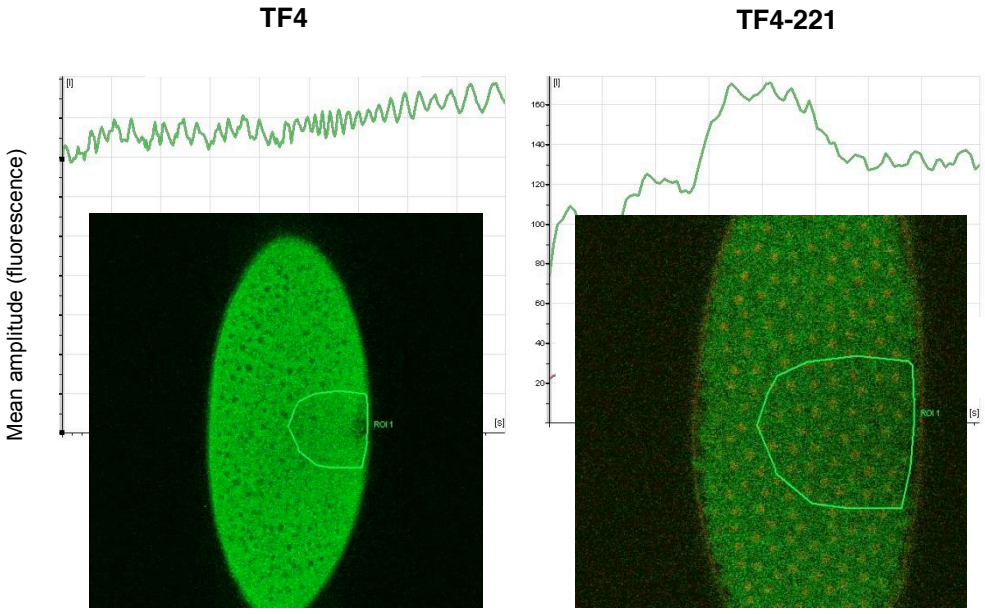


Figure 3.6. GFP dsRNAi injection into TF4 and TF4-221 embryos is unable to provide a phenotype to suggest GFP levels are depleted at the site of injection.

No visible reduction in GFP signal was observed over time at the site of GFP-dsRNA injection (highlighted in green) in either the original TF4 line or a derivative line which carries TF4 plus RFP histone (TF4-221). The pattern of the mean fluorescence amplitude only fluctuates with changes in cell cycle stage, presumably as Cdc20 localisation patterns change, but the overall presence of the GFP signal was unaffected. No obvious decrease in GFP signal was observed. This implies that the dsRNAi injection was unable to inhibit GFP turnover in embryos. Injections were carried out on more than five TF4 and TF4-221 embryos.

Cyclin B is a rapid turnover protein. Fizzy/Cdc20 is also a rapid turnover protein targeted by APC/C-mediated ubiquitination, supported by the presence of a destruction box and KEN box motif, although in *Drosophila* embryos it has not been confirmed that Fizzy is completely degraded every cell cycle. Therefore, there is a good possibility that *Drosophila* may be suitable for dsRNAi in embryos too. If this is the case, it would provide an alternative means to

conditionally knockout the endogenous Fizzy from TF4 or TF41 embryos so the experiments discussed above could be performed.

As a control to assess whether dsRNAi injection of embryos has a global or localised effect, dsRNA for GFP was produced and injected into TF4 nuclear division cycle 5 stage embryos and TF4-221 embryos, which carry the TF4 transgene as well as RFP-tagged histone as a DNA marker. If successful, this would cause a drop in GFP fluorescent intensity signals monitored by confocal microscope in areas where the dsRNA had been microinjected and effective. If this effect sustained long enough, injected embryos could be collected for western blot analysis to verify the knockout. If only a local effect of the dsRNAi depletion of the fluorescent signals was observed, any potential effects on the mitotic progression caused by depleting the endogenous Fizzy would need to observe the presence of any altered cell phenotypes locally around the injection site. Unfortunately, multiple attempts failed to produce any visible drop in GFP fluorescent intensity signals with the microinjection of dsRNA against the GFP mRNA. We have also microinjected dsRNA against the 3'UTR region of the *fizzy* gene nonetheless, with the intention of attempting to deplete the endogenous Fizzy from TF41 syncytial embryos. This also failed to yield any visible or interpretable results from multiple microinjection attempts (Fig. 3.6). For this reason, the dsRNAi approach in embryos was deemed too unreliable for use.

Chapter 4.The *Drosophila* BubR1 KEN-box motif is required for its interaction with Cdc20 (Fizzy).

4.1. Introduction

BubR1 has been shown in various systems to bind to Cdc20, alone or as part of the MCC, in order to sequester the activity of Cdc20 and prevent it from activating the APC/C. Two BubR1 Cdc20 binding sites were identified within BubR1, one in the N-terminal of BubR1 and the other in the C-terminal (Davenport et al., 2006). A previous report from the same group, illustrated that the N-terminal half of BubR1 was sufficient for SAC function (Harris et al., 2005). This leads to speculation that the N-terminal Cdc20 binding site of BubR1 was likely to be relevant in SAC signalling.

Whether any other domains within the N-terminal region of BubR1 carry importance in its ability to bind Cdc20 in the SAC response remained unclear until research into conserved KEN box motifs, which are conserved from yeast to humans. In other systems, two KEN box motifs exist, whereas only the N-terminal KEN box is present within *Drosophila* BubR1. Previous research into their importance revealed that in yeast, the N-terminal KEN box is required for SAC signalling ((Sczaniecka et al., 2008; Pfleger and Kirschner, 2000) (Zich and Hardwick, 2010), whereas the second KEN box motif is not. This is consistent with reports into the ability of the BubR1 N-terminal region to be sufficient for SAC signalling (Malureanu et al., 2009).

With regard to *Drosophila*, it had previously never been tested as to whether the single N-terminal KEN box behaves in a similar fashion to that observed in yeast Mad3. The single KEN box of *Drosophila* BubR1 may have a conserved function, or may possibly carry out additional roles due to the lack of a second KEN box. If *Drosophila* was to be used as a model for the study of the BubR1 KEN box in SAC function, it would first need to be established as to whether mutation of the BubR1 KEN box disrupted the interaction between BubR1 and Cdc20. We speculated that, as in other systems, the BubR1 KEN box will be essential for the BubR1-Cdc20 interaction, but whether Mad2 will be required for this interaction is questionable. In order to test this, a bacterial three-hybrid

experiment was designed to confirm the roles of both Mad2 and the BubR1 KEN box in the BubR1-Cdc20 interaction.

Whether the single KEN box of *Drosophila* BubR1 behaves in the same way to human and yeast N-terminal KEN boxes remains unknown. In these systems, although the second KEN box is not required for MCC formation, it is required for the checkpoint to remain active. Since *Drosophila* BubR1 lacks this second KEN box motif, it would be interesting to determine whether the single N-terminal KEN box has additional roles in checkpoint signalling to those observed in other systems, potentially compensating for the lack of a second KEN box motif. Understanding the role of the *Drosophila* BubR1 KEN box in SAC signalling in comparison to other systems may help to uncover the role of the second KEN box observed in humans and yeast. It may also provide a greater understanding of the formation of the MCC complex in this system, should the interaction between *Drosophila* BubR1 and Cdc20 be reliant upon this motif, and whether or not this interaction is dependent upon Mad2 as observed in other models.

Within this chapter we establish bacterial and yeast three hybrid systems, whereby the KEN-box-dependent manner of *Drosophila* BubR1 and Cdc20 can be investigated, in the presence and absence of Mad2. It is observed that the single N-terminal KEN box of *Drosophila* BubR1 has a similar SAC role to that conserved across other species, in that is required for the BubR1-Cdc20 interaction. This implicates the potential importance of the *Drosophila* BubR1 KEN box in the formation of the MCC.

4.1.1 Principle of the Bacterial Two Hybrid System

The bacterial three hybrid system used in this thesis is modified from the reconstituted signal transduction pathway based two hybrid protocol, supplied by Euromedex (Karimova et al., 1998). Here, a bacterial two hybrid system was established which allowed *in vivo* screening of functional protein-protein interactions, based on reconstitution of a signal transduction pathway which exploits the positive control exerted by cAMP.

Previous studies had shown that *B. pertussis* produces a calmodulin-dependent adenylate cyclase toxin, encoded by the *cyaA* gene, the catalytic domain of which exhibits high activity in the presence of calmodulin, and very low activity in its absence (Karimova et al., 1998). It was observed that this catalytic domain could be cleaved into two complimentary fragments (T25 and T18) which remain associated in a fully active ternary complex in the presence of calmodulin (Munier et al., 1991). In the absence of calmodulin the two fragments did not exhibit detectable activity, due to physical separation. Karimova et al. (1998) reasoned that if these complimentary fragments were fused to putative interactive proteins and expressed in an *E. coli* strain deficient for calmodulin or calmodulin-related proteins, the interacting proteins would be brought into close proximity and lead to re-association of complimentary fragments, resulting in cAMP synthesis. As cAMP is responsible for regulation of various genes, including those involved in catabolism of carbohydrates such as maltose(A., 1983), the restoration of the catalytic domain and its expression under the control of *lacZ,* would provide sufficient calmodulin-independent residual activity to restore the ability of the strain to ferment maltose. This could then be scored by plating onto maltose or X-gal plates.

The plasmids used by (Karimova et al., 1998) were constructed by modifying pACYC184 and pBluescript II KS to express either the T25 or T18 fragment of CyaA respectively. A multicloning site was fused to the C-terminal of T25 to facilitate construction of fusions with foreign protein, similarly the T18 fragment was fused in frame with α*lacZ* of pBluescript II KS, downstream of its multiple cloning site. To test the reliability of the system, first they co-transformed the two plasmids (pT25 and pT18) alone (empty of any interacting proteins) as well as a control plasmid which contained the full catalytic domain into a calmodulin

lacking bacterial strain. When plated onto agar supplemented with maltose, cells transformed with independently expressed fragments remained white, whereas those transformed with the full catalytic domain were red. This proved that without interacting proteins, the two fragments were incapable of contact and subsequent formation of a full catalytic domain.

Next, positive control plasmids were constructed in which sequences encoding interacting proteins were inserted into the multiple cloning sites of the pT25 and pT18 vectors. These control plasmids, pT25-zip and pT18-zip, contained DNA sequence that encodes a leucine zipper derived from yeast transcriptional activator GCN4. When pT25-zip and pT18-zip were co-transformed as before, the resulting colonies were red. This showed that functional complementation of T25 and T18 was as a result of interaction between the inserted leucine zipper motifs. As a negative control, pT25-zip was co-transformed with pT18, and pT25 with pT18-zip. In support of previous results, colonies were white, and so no functional complementation of the T25 and T18 fragments was observed.

The designed bacterial two hybrid system was proven capable of providing an effective means by which to detect protein-protein interactions in a bacterial model. The proteins tested were derived from yeast, indicative of its ability to analyse eukaryotic proteins. Because this system involves the generation of a regulatory molecule (in this case cAMP) it is possible to spatially separate the physical association of the interacting proteins from the transcriptional activation events which are dependent upon cAMP synthesis. This allows analysis of interactions which do not take place in the vicinity of the transcriptional machinery and so could be particularly useful in the study of proteins in the cytosol, or for co-localisation of two given proteins. This is not the case in yeast-based two hybrid systems. Along with this, the bacterial transformation protocol is quick and does not require any specialist knowledge, as it follows standard transformation protocol as used in the preceding molecular cloning steps.

4.2. Results

4.2.1 Creating a bacterial based three hybrid system, derived from pT25 and pT18 vectors, to test the interaction between Cdc20 and BubR1 in the presence of Mad2.

Due to the extensive restriction sites found within *Drosophila bubr1*, standard pT25 and pT18 vectors were unsuitable for use without prior modification of the MCS. As well as this, these vectors are only designed for a traditional two-hybrid approach. In order to accommodate the third protein (Mad2), a second MCS under the control of its own promoter was inserted into pT25. Replacement and additional multiple cloning sites were synthesised as adaptor oligos (Sigma Aldrich) which, after excision of the original MCS sequence, were inserted into the expression vectors to create the modified plasmids (named p25NJY and pUT18JY respectively) (Figs. 4.1 & 4.3). The various constructs generated for analysis in the bacterial three hybrid assay are listed in table 4.1. BACTH vectors were a kind gift from Richard Daniel (University of Newcastle).

4.2.2 Bacterial Three Hybrid Analysis was unable to detect interaction between BubR1 and Cdc20, even with the inclusion of Mad2.

With the use of bacterial strain BTH1001 as a host, none of the constructs generated, including wild-type full length BubR1 combined with both Cdc20 and Mad2, displayed the reporter phenotype of blue colonies in the presence of IPTG and X-gal. All colonies were white, showing that the T18 and T25 fragments had not been brought together as a result of interaction between hybrid proteins. This was also, as expected, observed in all negative control transformations. Co-transfection of the positive control constructs, pT18-zip and pT25-zip, displayed blue colonies indicative of an interaction between inserted proteins. This confirmed that the protocol and experimental conditions were suitable for reporting the interaction between hybrid proteins (Fig. 4.5).

4.2.3 The bacterial Three Hybrid system appears unsuitable for investigating the BubR1-Cdc20 interaction.

In order to verify whether the co-transfected constructs of interest had actually been expressed in the BTH1001 host strain during experimental conditions, colonies were inoculated in liquid LB from double transformants containing all three proteins of interest, full-length BubR1, Cdc20 and Mad2, and induced in the presence of IPTG at 37ºC for 1 h. Protein samples were collected from these cells and subject to western blot analysis. A sample prepared from *Drosophila* S2 cells was also run, to act as a comparison for indication of the band sizes of the three proteins. It was observed that bands were detectable which corresponded to the Cdc20 band, but bands in the expected positions of full-length BubR1 and Mad2 was not detected. This implies that the bacterial cells were not expressing the full length BubR1 protein (Fig. 4.6). The western blot itself was subject to excess background noise, likely to be due to the fact that the protein generated to raise the antibody was purified from a bacterial host too, this however, did not alter the fact that not all of the proteins of interest could be detected. It can be assumed that the co-transformation of the two constructs was successful, as they confer different antibiotic resistance and so without both constructs, cells could not grow on selective plates containing both antibiotics.

Because it could not be confirmed that the host strain could express all of the proteins of interest, it was decided that the system was unsuitable for use in the investigation of the interaction between BubR1 and Cdc20. Although previous studies implied that this system was suitable for the expression of eukaryotic protein, only fairly short proteins were tested. BubR1 is significantly larger, and it cannot be ruled out that this caused the failure of its expression in full. In order to determine whether the BubR1 KEN box is required for its interaction with Cdc20 in the absence or presence of Mad2, a yeast three hybrid system was employed.

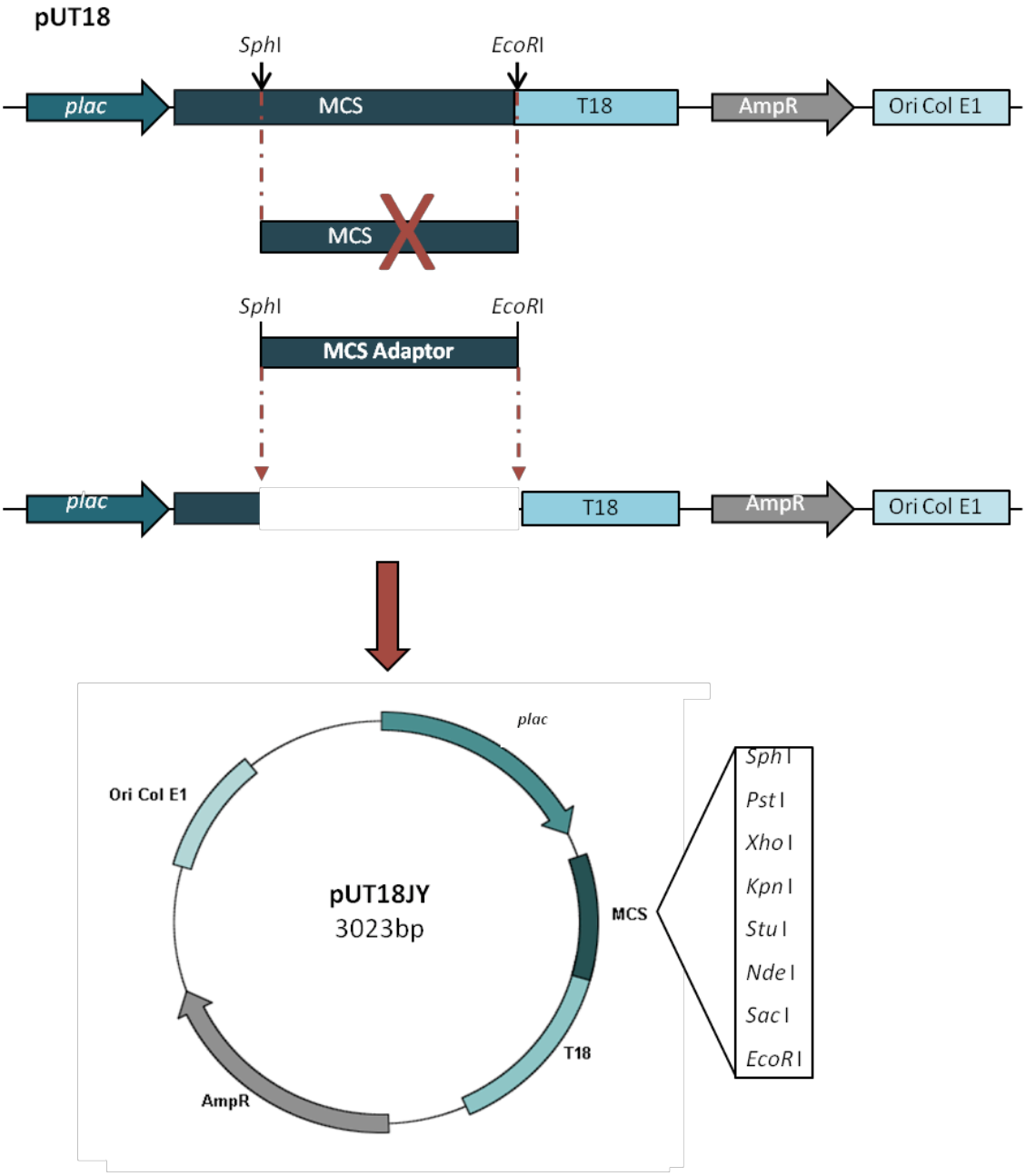


Figure 4.1. Schematic diagram to show the modification of pUT18 vector to pUT18JY.

The *Sph*I-*Eco*RI region of the pUT18 MCS was excised and replaced with a custom adaptor to include the required restriction sites for generation of the final constructs. This adaptor was produced by annealing two complimentary oligonucleotides, designed to ligate into the *Sph*I-*Eco*RI region. The resulting vector, pUT18JY, with adaptor MCS restriction sites is illustrated.

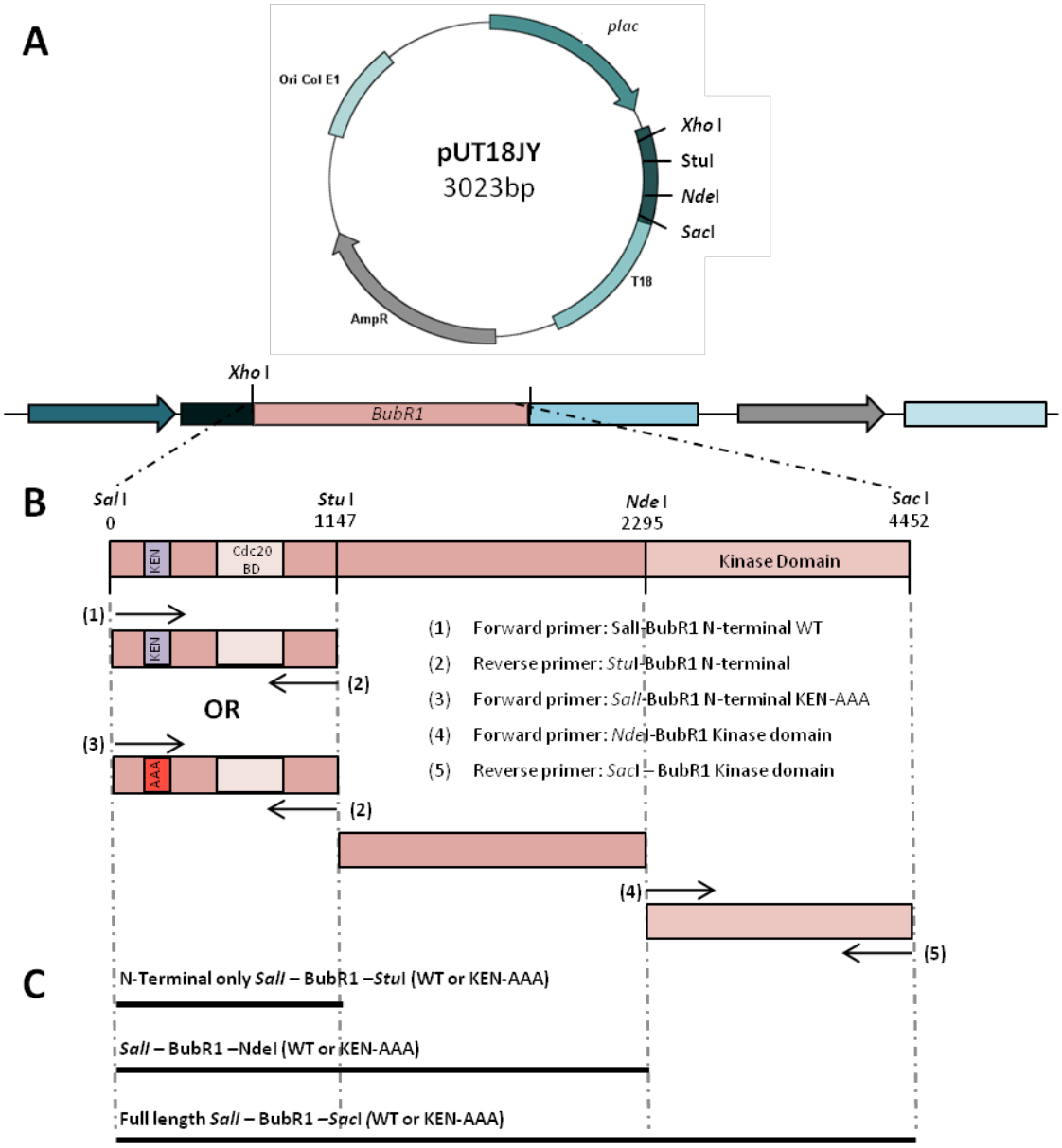


Figure 4.2. Diagram to show the sub-regions of BubR1 used to generate pUT18JY-BubR1 constructs to be analysed using the bacterial three hybrid system.

pUT18JY vector contains restriction sites suitable for insertion of three BubR1 fragments. N-terminal and kinase domain regions were modified by PCR to incorporate the mutations to the KEN box and the desired restriction sites. The mid-region was excised directly from BubR1 cDNA clone LD23835 between *Stu*I and *Nde*I (B). Constructs containing full length *Sal*I-*Sac*I, kinase deletion *Sal*I-*Nde*I, or N-terminal *Sal*I-*Stu*I BubR1 were generated, containing either an intact or mutated KEN box motif (C).

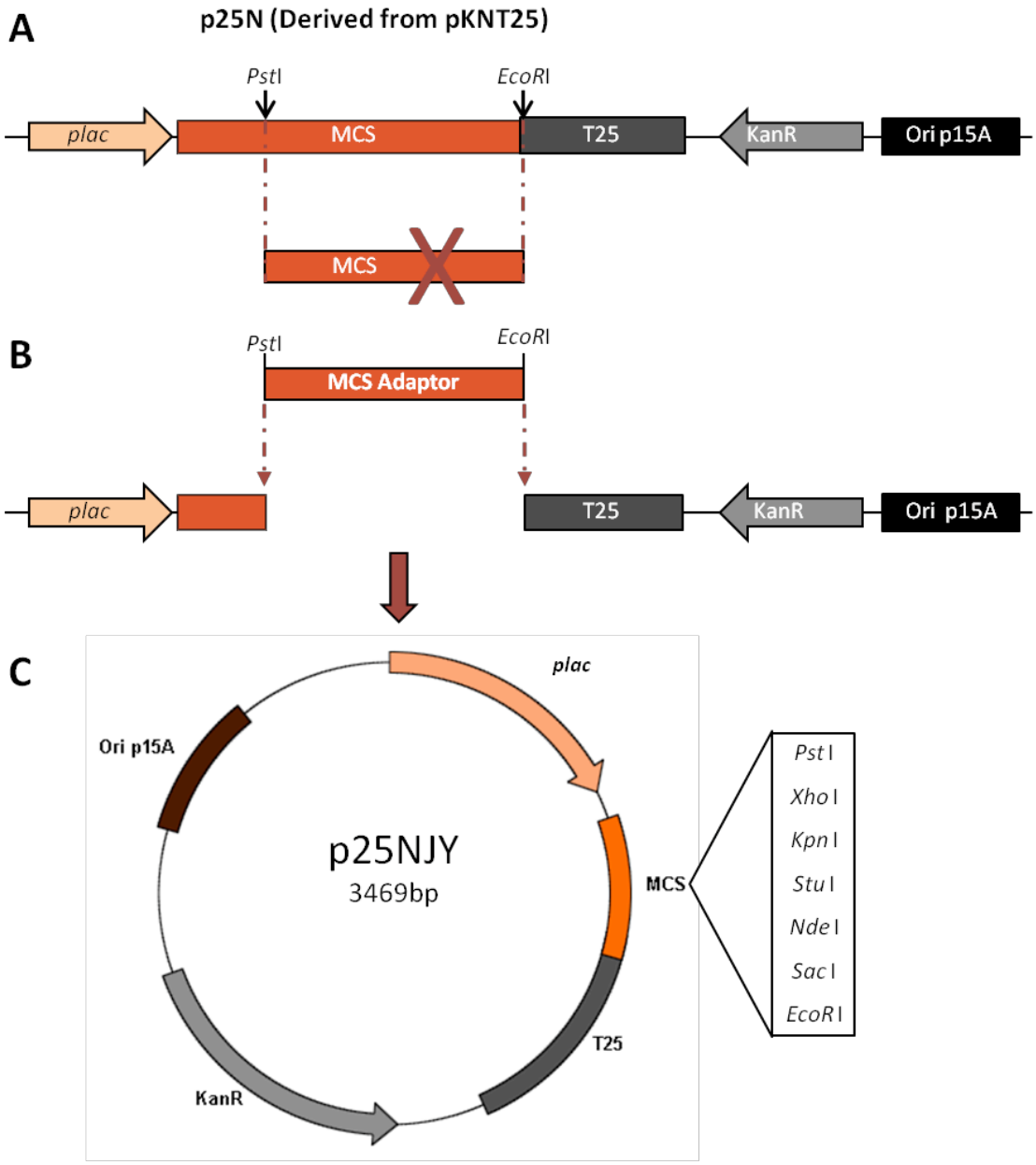
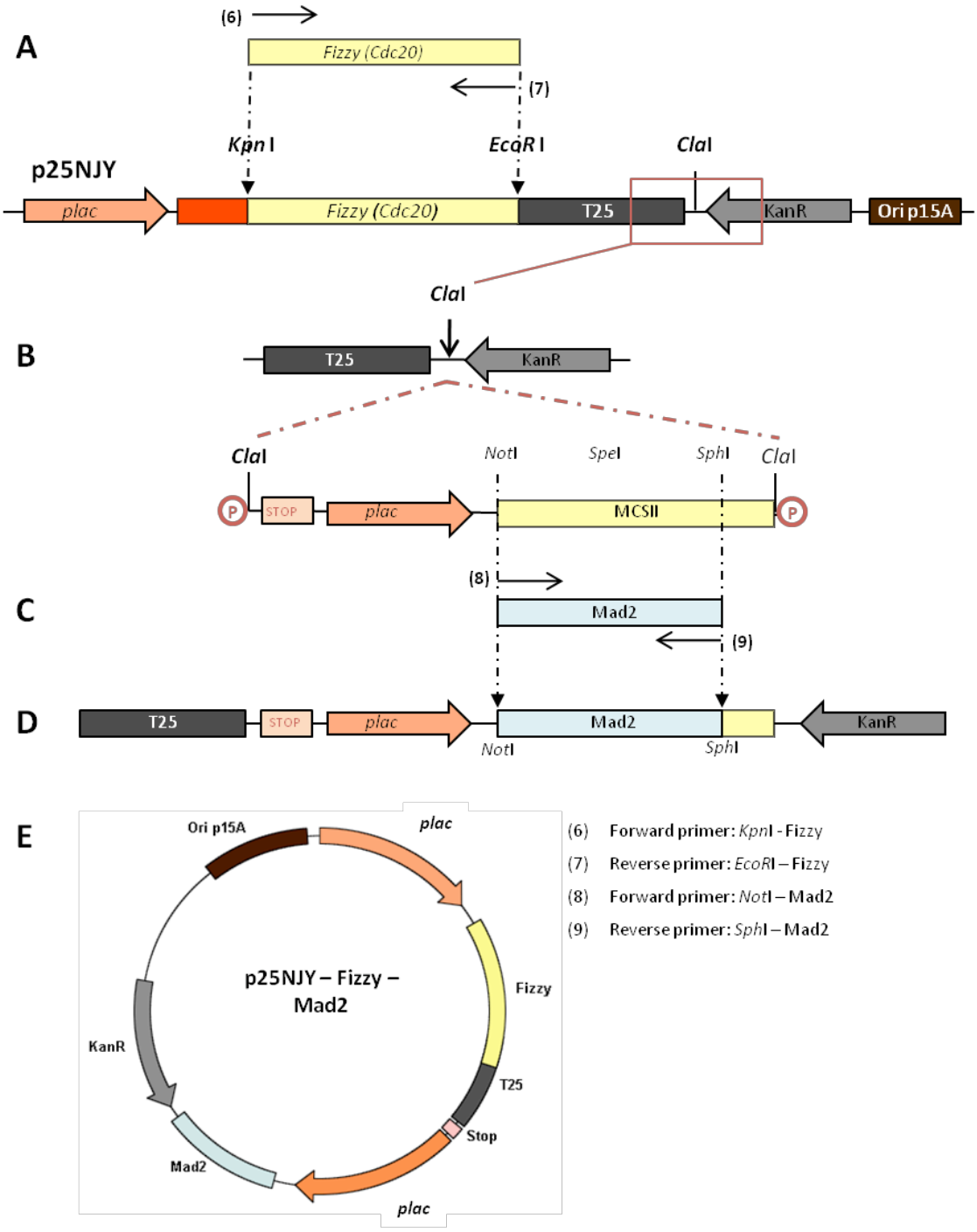


Figure 4.3. Schematic diagram to show the modification of p25N vector to p25NJY.

The *Pst*I-*Eco*RI region of the p25N MCS was excised and replaced with a custom adaptor to include the required restriction sites for generation of the final constructs. This adaptor was produced by annealing two complimentary oligonucleotides, designed to ligate into the *Pst*I-*Eco*RI region. The resulting vector, p25NJY, with adaptor MCS restriction sites is illustrated.



(6) Forward primer: *Kpn*I - Fizzy

(7) Reverse primer: *EcoR*I – Fizzy

(8) Forward primer: *Not*I – Mad2

(9) Reverse primer: *Sph*I – Mad2

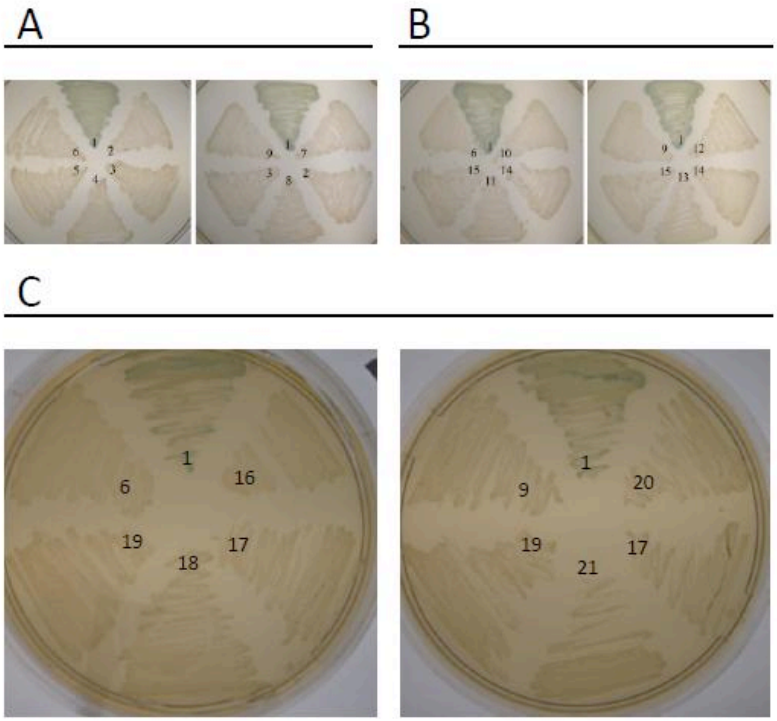
Figure 4.4. Schematic diagram to show the insertion of *fizzy* into p25NJY and the subsequent modification to include a second MCS under the control of its own promoter, for the insertion of *mad2*.

(A) Full length *fizzy* was modified by PCR to incorporate the required restriction sites and inserted into *Kpn*I-*Eco*RI of p25NJY. This is the protein which will be expressed as a T25-fusion. (B) In order to incorporate a third protein into the assay, a second multiple cloning site was created within the *Cla*I site which exists after the T25 motif. An adaptor was created by annealing two complimentary oligonucleotides which encode a stop codon to end Fizzy-T25 translation, and a *plac* promoter sequence to drive a second MCS into which *mad2* will be inserted. This adaptor was phosphorylated at both 5' and 3' ends so that the construct could be treated with alkaline phosphatase to prevent religation in the absence of the adaptor. (C) Full length *mad2* was modified by PCR with required restriction sites at either ends. This *mad2* fragment was designed to also carry its own stop codon. (D) *mad2* was inserted into *Not*I-*Sph*I sites of the newly generated MCSII. (E) The resulting construct contained full length *fizzy* in MCSI for the expression of a Fizzy-T25 fusion protein, and full length *mad2* in MCSII of p25NJY for the expression of Mad2 as a third and separate hybrid protein under the control of its own promoter.

Table 4.1. Bacterial Three Hybrid constructs

Modified Vector Name	**Original Vector**	**Modification information**
pUT18JY	pUT18 (Euromedex)	MCS of pUT18 (*Sph*I-*Eco*RI) replaced with adaptor oligo for the insertion of a replacement MCS.
p25NJY	P25N (Euromedex)	MCS of p25N (*Pst*I-*Eco*RI) replaced with adaptor oligo for insertion of replacement MCS.

Construct Name	**Insert Information**	**Restriction Sites**
pUT18JY N-terminal BubR1-*Stu*I WT	N-terminal WT BubR1 (Pstn 1-1147) in pUT18JY	*Sal*I-*Stu*I
pUT18JY N-terminal BubR1-*Stu*I KEN-AAA	N-terminal KEN-AAA BubR1 (Pstn 1-1147) in pUT18JY	*Sal*I-*Stu*I
pUT18JY BubR1-*Nde*I WT	WT BubR1 (Pstn 1-2295) in pUT18JY	*Sal*I-*Nde*I
pUT18JY BubR1-*Nde*I KEN-AAA	KEN-AAA BubR1 (Pstn 1-2295) in pUT18JY	*Sal*I-*Nde*I
pUT18JY Full Length BubR1 to *Sac*I WT	Full length WT BubR1 (Pstn 1- 4452) in pUT18JY	*Sal*I-*Sac*I
pUT18JY Full Length BubR1 to *Sac*I KEN-AAA	Full length KEN-AAA BubR1 (Pstn 1- 4452) in pUT18JY	*Sal*I-*Sac*I
P25NJY-Fizzy	Full length Fizzy (Pstn 1-1578) in p25NJY	*Kpn*I-*Eco*RI
P25NJY-Fizzy-Mad2	Full length Fizzy (Pstn 1-1578) in p25NJY. Construct then modified to introduce MCSII into *Cla*I site using adaptor oligos. Once modified, full length Mad2 (Pstn1-624) was inserted into MCSII.	*Kpn*I-*Eco*RI *Not*I-*Sph*I



BubR1

CONSTRUCT COMBINATION KEY :

1.	pKT25-Zip	+ pUT18C-Zip
2.	**pUT18JY N-terminal BubR1 to *Stu*I WT**	**+ p25NJY-Fizzy**
3.	pUT18JY N-terminal BubR1 to *Stu*I WT	+ pKT25-Zip
4.	**pUT18JY N-terminal BubR1 to *Stu*I KEN-AAA**	**+ p25N-Fizzy**
5.	pUT18JY N-terminal BubR1 to *Stu*I KEN-AAA	+ pKT25-Zip
6.	p25NJY-Fizzy	+ pUT18C-Zip
7.	**pUT18JY N-terminal BubR1 to *Stu*I WT**	**+ p25NJY-Fizzy-Mad2**
8.	**pUT18JY N-terminal BubR1 to *Stu*I KEN-AAA**	**+ p25NJY-Fizzy-Mad2**
9.	p25NJY-Fizzy-Mad2	+ pUT18C-Zip
10.	**pUT18JY BubR1 to *Nde*I WT**	**+ p25NJY-Fizzy**
11.	pUT18JY BubR1 to *Nde*I WT	+ pKT25-Zip
12.	**pUT18JY BubR1 to *Nde*I KEN-AAA**	**+ p25NJY-Fizzy**
13.	pUT18JY BubR1 to *Nde*I KEN-AAA	+ pKT25-Zip
14.	**pUT18JY BubR1 to *Nde*I WT**	**+ p25NJY-Fizzy-Mad2**
15.	**pUT18JY BubR1 to *Nde*I KEN-AAA**	**+ p25NJY-Fizzy-Mad2**
16.	**pUT18JY Full length BubR1 to *Sac*I WT**	**+ p25NJY-Fizzy**
17.	pUT18JY Full length BubR1 to *Sac*I WT	+ pKT25-Zip
18.	**pUT18JY Full length BubR1 to *Sac*I KEN-AAA**	**+p25NJY-Fizzy**
19.	pUT18JY Full length BubR1 to *Sac*I KEN-AAA	+ pKT25-Zip
20.	**pUT18JY Full length BubR1 to *Sac*I WT**	**+ p25NJY-Fizzy-Mad2**
21.	**pUT18JY Full length BubR1 to *Sac*I KEN-AAA**	**+ p25NJY-Fizzy-Mad2**

Figure 4.5. Bacterial three hybrid analysis failed to provide information on the requirement of the BubR1 KEN box in the BubR1-Fizzy interaction, either in the presence or absence of Mad2.

(A) The interaction between the BubR1 N-terminal to *Stu*I fragment, (B) as well as that of BubR1 to *Nde*I kinase deletion (C) and full length BubR1 to *Sac*I (C) with Fizzy were co-transfected in host strain BTH1001 in the absence (left panels) and presence (right panels) of Mad2. The only combination of constructs which produced a positive interaction, indicated by blue colonies in the presence of X-gal, is the positive control pKT25-zip plus pUT18C-zip transfection. As even full-length wild type BubR1 does not produce a positive interaction, no information can be obtained from these data. The combinations used in the assay are numbered, and listed in the key. Bold print indicates those experiments which were designed to test the effect of mutation of the KEN box domain in the interaction between BubR1 with Fizzy in the presence and absence of Mad2. Control experiments are listed in normal font.

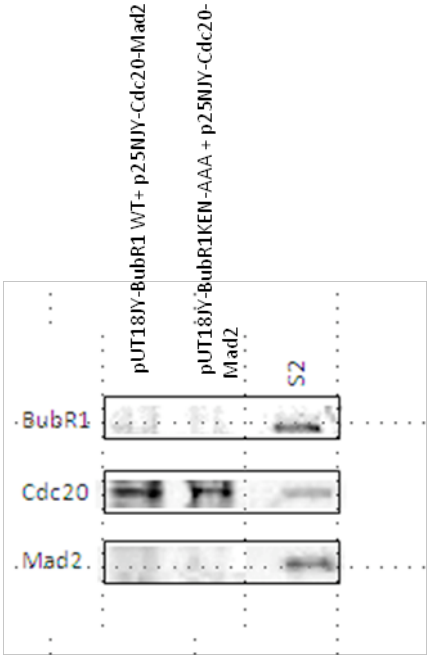


Figure 4.6. Western blot analysis of transfected BTH1001 cells reveal that only Cdc20 can be detected.

The right hand lane shows bands detected in an S2 cell extract when probing with anti-BubR1, anti-Fizzy (Cdc20) and anti-Mad2 antibodies. The extracts probed from bacteria co-transfected with pUT18JY containing full length WT or KEN-AAA BubR1 and p25NJY containing Cdc20 and Mad2 respectively. The only protein which shows a clear band at the correct position is Cdc20. It appears that full length BubR1 (WT or mutant) and Mad2 may not be expressed by host cells, or may be very unstable.

4.2.4 Principle of the Yeast Hybrid System

Yeast two and three-hybrid analysis systems are widely used to investigate protein-protein interactions, and are available commercially. The yeast three hybrid experiments carried out in this thesis uses an activation domain containing vector, pGBKT7, from Clontech's Matchmaker™ Gold Two-Hybrid system, alongside a modified version of the Clontech binding domain containing three-hybrid vector, pBridge™. When used together, these vectors allow investigation of ternary protein complexes, whereby the interaction of two proteins of interest may require the presence of a third protein to "bridge" the interaction, be it physically or by modification to one or both proteins of interest.

The Matchmaker™ system is a GAL4-based three hybrid assay, in which a bait protein is expressed as a fusion to the GAL4 binding domain of pBridge™ and the prey protein is expressed as a fusion to the GAL4 activation domain (Fields and Song, 1989) (Chien et al., 1991) of pGBKT7. The third or "bridge" protein is expressed from a second multiple cloning site of pBridge™. pBridge™ is a shuttle vector which replicates autonomously in *S.cerevisiae* and carries the *TRP1* nutritional marker. The bait protein within MCSI of pBridge™ is expressed by yeast host cells from the constitutive *ADH1* promoter, with termination of this transcription by an *ADH1* termination signal. This hybrid protein is targeted to the yeast nucleus by nuclear localisation signals which are an intrinsic part of the GAL4 DNA-binding domain(Osborne et al., 1995). The additional gene of interest is cloned into MCSII, located downstream of another nuclear localisation sequence and an HA epitope. This "bridge" protein is conditionally expressed from the *MET25* promoter in response to methionine levels within the growth medium. This enables the expression of the bridge protein to be switched on and off by replica plating of the host colonies. pGBKT7 expresses the bait protein fused to the DNA-binding domain of GAL4. Fusion proteins are highly expressed from the constitutive *ADH1* promoter.

When bait and prey proteins interact, they bring together the activation and binding domains in order to activate the transcription of four reporter genes (*AUR1-C, HIS3, ADE2 and MEL1*). These allow detection of protein-protein interactions in different ways, and can be used in combination to reduce

background activity. *AUR1-C* encodes a drug reporter, *HIS3* can be used to screen for

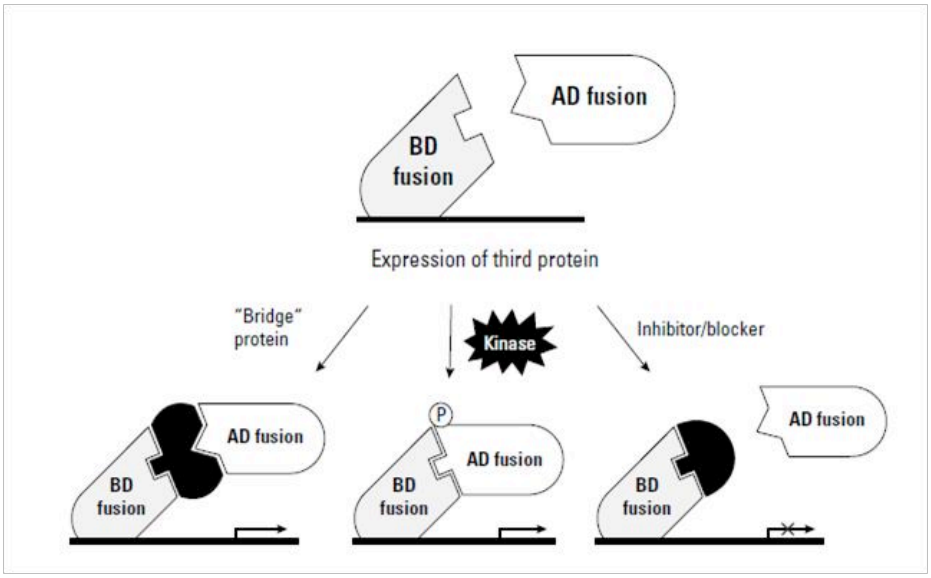


Figure 4.7. The mechanisms of the yeast three-hybrid system.

pBridge expresses both the DNA-BD fusion and the third, or bridging protein. The activation domain fusion is expressed from a separate vector (in the case of this thesis, pGBKT7). The conditionally expressed bridging protein can play either a structural role (left), a modifying role (middle) or an inhibitory role (right) in the interaction between the two hybrid proteins of interest, which restores reporter gene expression. (Taken from Clontech pBridge™ vector information sheet, protocol No: PT3212-5).

interactions in yeast strains unable to synthesise, and therefore grow, in the absence of histidine, as when bait and prey proteins interact, Gal4-responsive *His3* expression and allows the host strain to grow on histidine-deficient medium. *ADE2* works in the same fashion, but enables strains unable to synthesise adenine to grow on medium lacking adenine in the presence of an interaction. Finally, *MEL1* encodes α-galactosidase, which in the presence of a bait-prey interaction, is expressed and secreted by the yeast cells, causing them to turn blue in the presence of X-α-Gal. These reporter genes are under the control of three independent Gal4 responsive promoter elements (G1, G2 or M1). The protein binding sites within these promoters are different but each is related to the 17-mer consensus sequence in the UAS region which is

recognised by Gal4 (Giniger and Ptashne, 1988; Giniger et al., 1985). This ensures that any prey proteins which interact with unrelated sequences flanking, or within the UAS (false positives) are automatically screened out. (All information regarding Matchmaker™ and pBridge™ is taken from the Matchmaker™ Gold Yeast Two-Hybrid System User manual 2010, and pBridge™ vector information sheet, respectively).

4.2.5 Generation of pGBKT7 and modified pBridge constructs for three hybrid analysis of the BubR1-Cdc20 interaction in the presence and absence of Mad2.

Before *bubr1* or *fizzy* were subcloned into pBridge, it was first modified for the insertion of the *mad2* fragment. MCSII of pBridge was removed and replaced with an adaptor oligo containing an *Nco*I site between *Not*I and *Bgl*II. The Mad2 fragment was then inserted into *Nco*I-*Not*I of the modified vector, pBridgeJY. BubR1 was again cloned in three separate fragments, with the N-terminal fragment either coding for wild-type protein or with the KEN box substituted for AAA. This was inserted into *Sal*I-*Sma*I sites of pBridgeJY and *Xho*I-*Sma*I sites of pGBKT7, a kind gift from Rachel Davies (University of St Andrews). *fizzy* was inserted as a single fragment into the same *Sal*I-*Sma*I or *Xho*I-*Sma*I sites of JYpBridge-Mad2 or pGBKT7 respectively (figure 4.8).

Final plasmid DNA constructs were subjected to restriction digest and gel electrophoresis for confirmation of appropriate band pattern, and DNA sequence analysis was carried out (University of Dundee Sequencing and Services) to verify the presence of the PCR site-directed point mutation of the KEN box motif to AAA (figure 4.10).

4.2.6 The BubR1 KEN box is required for its interaction with Cdc20, and BubR1- Cdc20 appear to interact in the presence and absence of Mad2 in Y190.

Wild-type BubR1 interacts with Cdc20 (Fizzy) in the presence of Mad2, when expression from MCSII of pBridgeJY is switched on. This was displayed by positive blue colonies in the three hybrid X-Gal assay. In contrast, with mutation

of the BubR1 KEN box to AAA, negative white colonies were observed. This suggests that the *Drosophila* BubR1 KEN box is required for its interaction with Fizzy, in the presence of Mad2. In order to determine whether Mad2 was required in this interaction, the transfected Y190 strains, a kind gift from Jeremy Brown (University of Newcastle), were re-streaked onto plates containing methionine in order to switch off the expression of Mad2 from MCSII of pBridgeJY. Under these conditions, wild-type BubR1 still seems to interact with Fizzy, again indicated by the positive blue colonies of the three hybrid X-Gal assay.

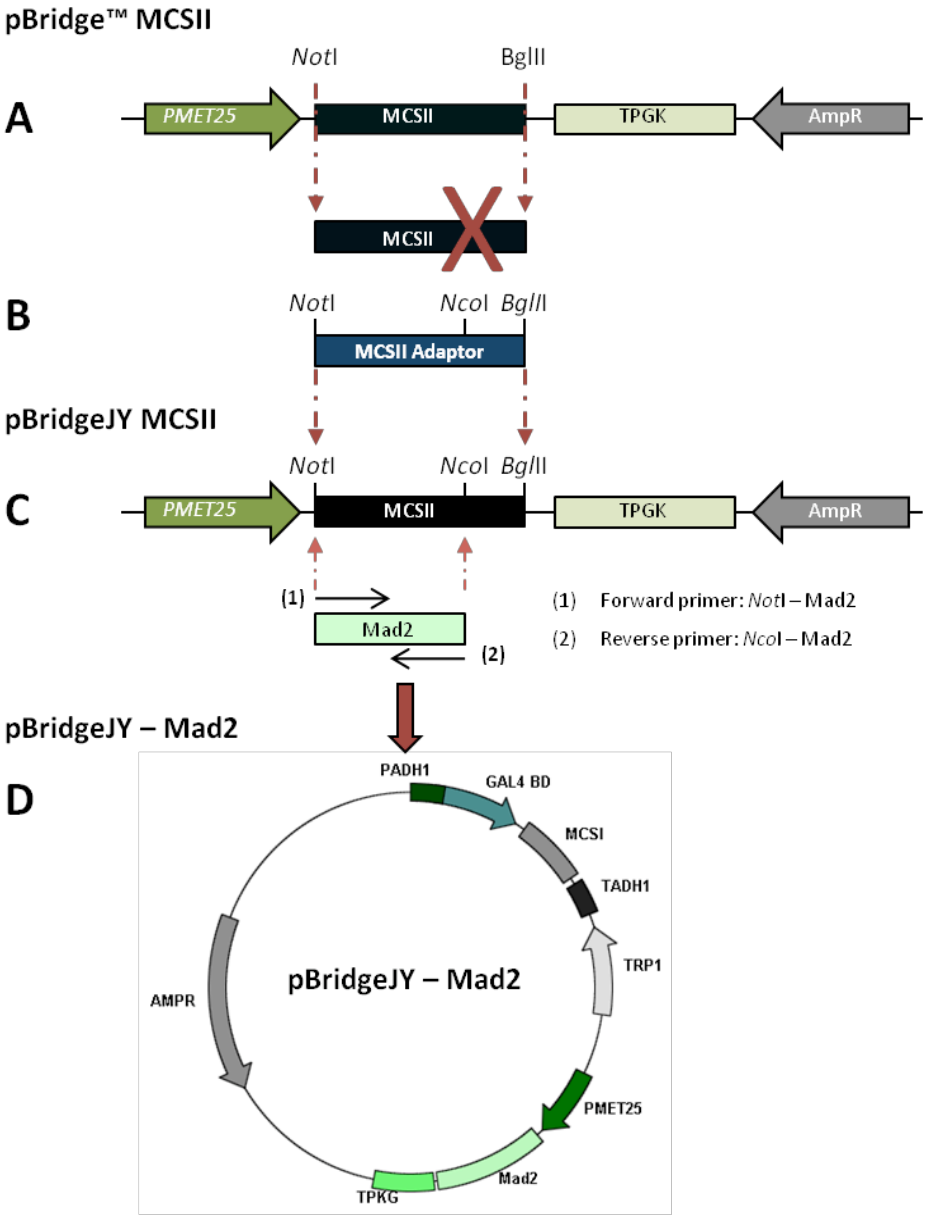


Figure 4.8. Schematic diagram to show the modification of pBridge™ vector to pBridgeJY.

(A) The *Not*I-*Bgl*II region of pBridge MCSII was excised and replaced (B) with a custom adaptor to include an *Nco*I site. This adaptor was produced by annealing two complimentary oligonucleotides, designed to ligate into the *Not*I-*Bgl*II region. (C) Full length *mad2* was modified by PCR with required restriction sites at either ends and ligated into *Not*I-*Nco*I of pBridgeJY MCSII. (D) This generated the pBridgeJY-Mad2 construct into which either *bubr1* or *fizzy* will be inserted later.

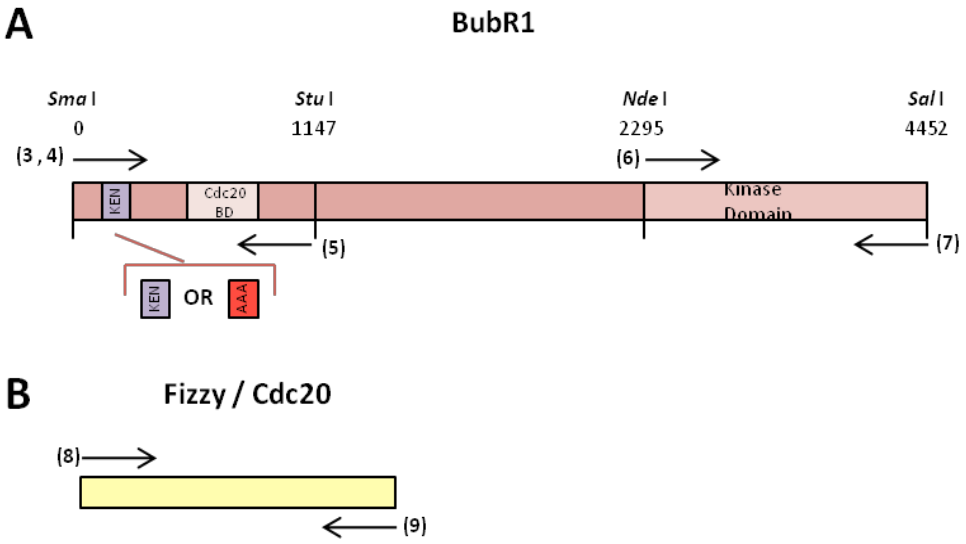


Figure 4.9. Diagram to show how full length *bubr1* and *fizzy* were constructed for insertion into yeast three hybrid vectors.

(A) Full length wild-type and KEN box mutant *bubr1* were constructed in three fragments within pGEM-T cloning vector, and subsequently cut and ligated into either pGBKT7 or pBridgeJY-Mad2 vectors. (B) Full length *fizzy* was modified by PCR. Constructs were inserted into pGBKT7 or pBridgeJY-Mad2 using *Sma*I – *Xho*I or *Sma*I – *Sal*I sites respectively. Oligos used for generation of the various fragments can be found in table 2.1. 3 and 4 represent 5' primers for wt *bubr1* and KEN mutant *bubr1*, with 5 representing the 3' reverse primer for this fragment. Oligos 6 and 7 are the forward and reverse primers for the *bubr1* kinase domain fragment. Oligos 8 and 9 represent the forward and reverse primers used to generate the *fizzy* fragment.

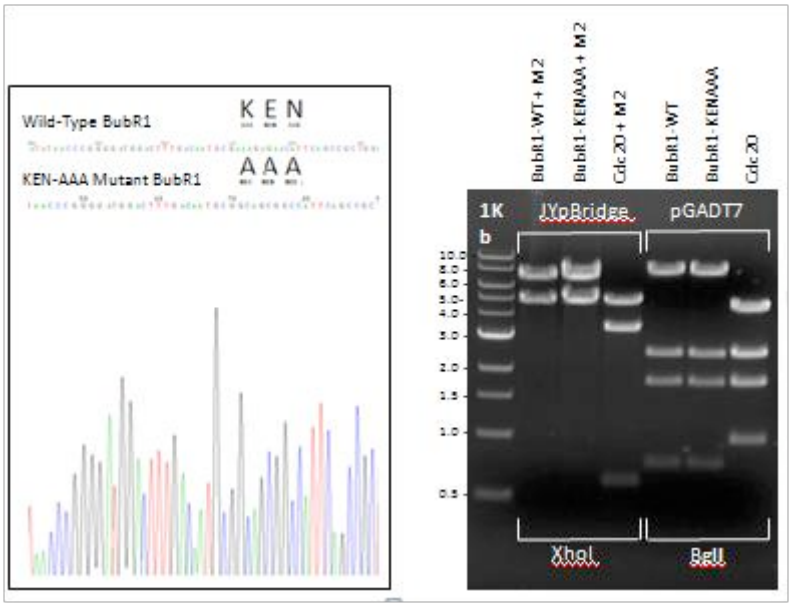


Figure 4.10. pBridge vector based yeast three hybrid plasmid DNA constructs were verified using both DNA sequence analysis and restriction digest.

pBridge-BubR1 was sequenced in the wild-type and KEN-AAA mutant constructs to ensure that the N-terminal KEN box was intact in the wild-type, and substituted for AAA in the mutant. The resulting chromatograms verify that the region containing the KEN box motif is correct in each case (left). Following sequence analysis of constructs, they were subject to restriction digest and gel electrophoresis to confirm that they display the expected band patterns (right). In each case, the band patterns corresponded with those expected (which are described in table 4.2).

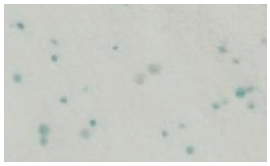
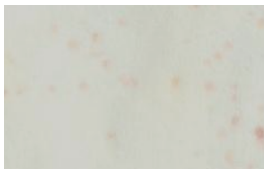
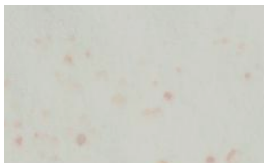
Table 4.2. Yeast Three Hybrid Constructs

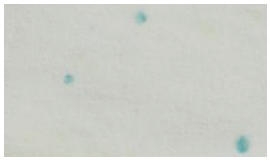
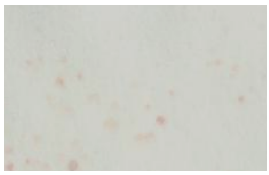
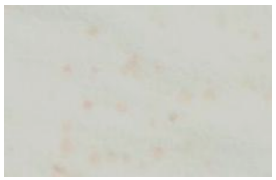
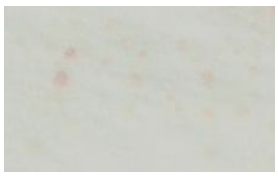
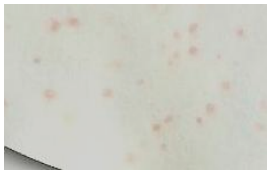
Modified Vector Name	Original Vector	Modification information
pBridgeJY1 (Amp^r)	pBridge (Clontech) (Amp^r)	MCSII of pBridge (*Not*I-*Bgl*II) replaced with adaptor oligo for the insertion of MCSII containing *Nco*I (*Not*I-*Nco*I-*Bgl*II).

Construct Name	**Size**	**Insert Information**	**RE Sites used**	**Test Digest:**	**Ref**
pBridgeJY1-Mad2 (Amp^r)	7.1Kb	Full length Mad2(Pstn1-624) in pBridgeJY1 (MCSII)	*Not*I- *Nco*I	Data not shown	
pBridgeJY1-BubR1(WT) -Mad2 (Amp^r)	11.5Kb	Full length Wild-type BubR1(Pstn1-4392) in pBridgeJY1 (MCSI) plus Mad2 (Pstn1-624 (MCSII))	*Sma*I-*Sal*I *Not*I-*Nco*I	Cut *Xho*I: Expect: 6.7Kb, 4.8Kb	Fig 4.10 Lane2
pBridgeJY1-BubR1(KENAAA) -Mad2 (Amp^r)	11.5Kb	Full length KEN-AAA mutant BubR1(Pstn1-4392) in pBridgeJY1 (MCSI) plus Mad2(Pstn1-624 (MCSII))	*Sma*I-*Sal*I *Not*I-*Nco*I	Cut *Xho*I: Expect: 6.7Kb, 4.8Kb	Fig 4.10 Lane 3

pBridgeJY1-Cdc20-Mad2 (Amp^r)	8.7Kb	Full length Cdc20 (Pstn11-1578) in pBridgeJY1(MCSI) plus Mad2 (Pstn1-624 (MCSII))	*SmaI*-*SalI* *NotI*-*NcoI*	Cut *Xho*I: Expect: 4.79Kb, 3.32Kb, 0.59Kb	Fig 4.10 Lane 4
pGADT7-BubR1(WT) (Amp^r)	12.38Kb	Full length Wild-Type BubR1(Pstn1-4392) in pGADT7	*Sma*I-*Sal*I (*Xho*I)	Cut *Bgl*II: Expect: 7.6Kb, 2.3Kb, 1.7Kb, 0.7Kb	Fig 4.10 Lane 5
pGADT7-BubR1(KENAAA) (Amp^r)	12.38Kb	Full length KEN-AAA mutant BubR1 (Pstn1-2392) in pGADT7	*Sma*I-*Sal*I (*Xho*I)	Cut *Bgl*II: Expect:7.6Kb, 2.3Kb, 1.7Kb, 0.7Kb	Fig 4.10 Lane 6
pGADT7-Cdc20 (Amp^r)	9.56Kb	Full length Cdc20 (Pstn1-1578) in pGADT7	*Sma*I-*Sal*I (*Xho*I)	Cut *Bgl*II: Expect: 4.4Kb, 2.44Kb, 1.7Kb, 0.9Kb	Fig 4.10 Lane 7

Table 4.3. Yeast Three Hybrid Results

Co-transfected constructs		Expressed proteins	% positive colonies	Int	Image of membrane
A pBridgeJY1-Cdc20-Mad2	pGADT7-BubR1(WT)	BubR1 (WT) Mad2 Cdc20	96	+	
B pBridgeJY1-Cdc20-Mad2	pGADT7-BubR1(KENAAA)	BubR1KENAAA Mad2 Cdc20	1	-	
C pBridgeJY1-Cdc20-Mad2	pGADT7 (empty)	Mad2 Cdc20	0	-	
D pBridgeJY1-Mad2	pGADT7 (empty)	Mad2	0	-	
E pGADT7-BubR1(WT)	pBridgeJY1-Mad2	BubR1 (WT) Mad2	1	-	
F pGADT7-BubR1(KENAAA)	pBridgeJY1-Mad2	BubR1KENAAA Mad2	0	-	
pGADT7	-	-	No growth		
pBridgeJY1-Mad2	-	Mad2	No growth		

Co-transfected constructs		Expressed proteins	% positive colonies	Int	Image of membrane
G pBridgeJY1-BubR1(WT) -Mad2	pGADT7-Cdc20	BubR1 (WT) Mad2 Cdc20	85	+	
H pBridgeJY1-BubR1(KENAAA) -Mad2	pGADT7-Cdc20	BubR1KENAAA Mad2 Cdc20	3	-	
I pBridgeJY1-BubR1(WT) -Mad2	pGADT7 (empty)	BubR1 (WT) Mad2	1	-	
J pBridgeJY1-BubR1(KENAAA) -Mad2	pGADT7 (empty)	BubR1KENAAA Mad2	0	-	
K pBridgeJY1-Mad2	pGADT7 (empty)	Mad2	0	-	
L pBridgeJY1-Mad2-Cdc20	pGADT7 (empty)	Mad2 Cdc20	0	-	
pGADT7 (empty)	-	-	No growth		
pBridgeJY1-Mad2	-	Mad2	No growth		

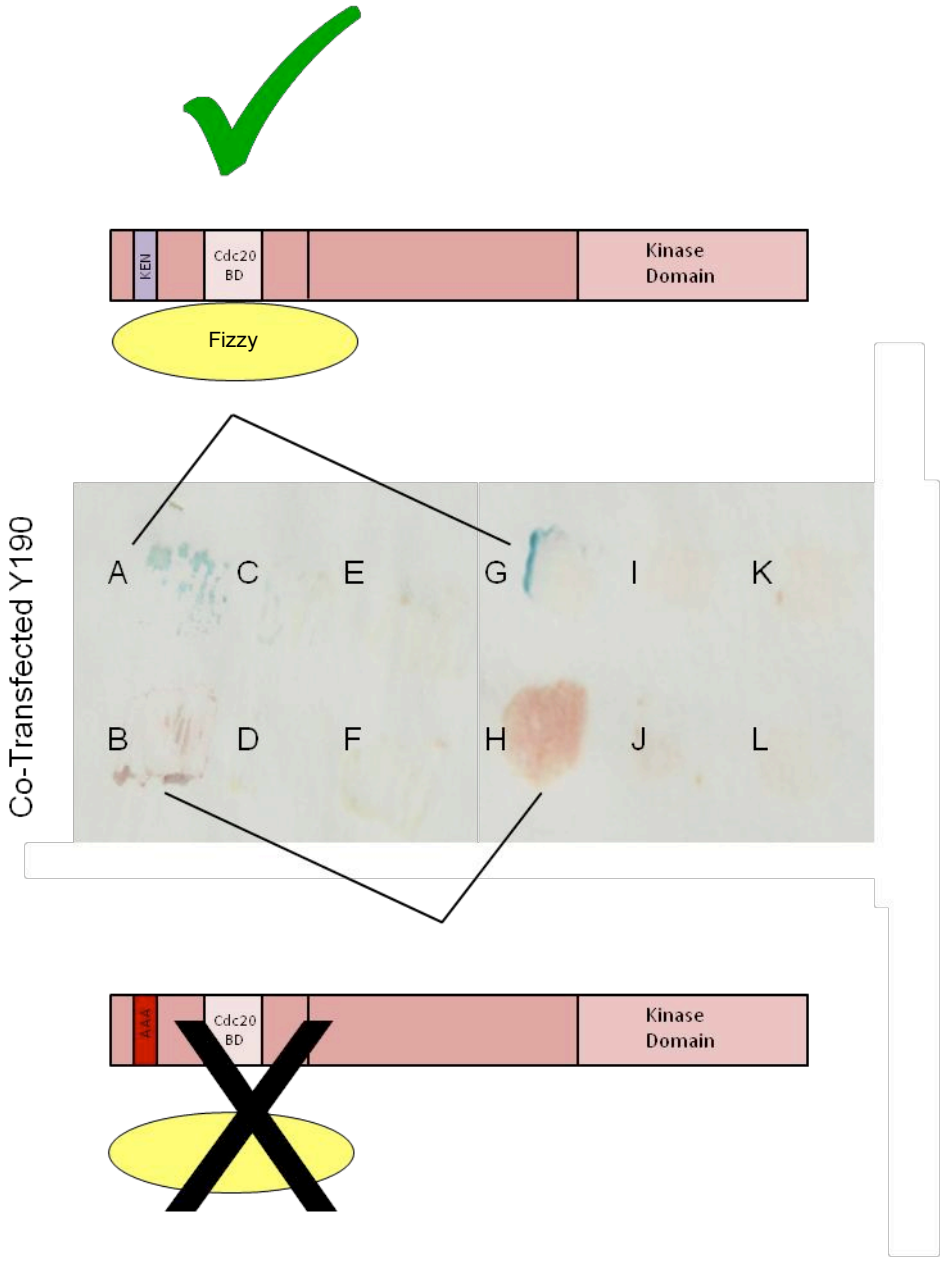


Figure 4.11. Without expression of Mad2, Wild-type BubR1 but not KEN-AAA mutant BubR1, is capable of interacting with Cdc20.

When the transfected Y190 colonies (see table 4.3. for transfection combinations) were streaked, then patched onto plates which contained methionine in order to silence expression of Mad2, wild-type BubR1 still appears to interact with Cdc20, as indicated by the blue colonies in the three hybrid X-gal assay. The interaction between BubR1 and Cdc20 is destroyed with mutation of the KEN box domain, as indicated by white colonies.

4.3 Discussion

Although it is unclear as to why the bacterial three hybrid system was unable to efficiently express all of the proteins of interest, this results in its use in this particular study of the interaction between *Drosophila* BubR1 and Cdc20 unreliable. Whether the proteins of interest failed to express at all, or were not folded correctly when expressed in a bacterial system, is not certain. However, preliminary western blot analysis suggested that bands to represent the full-length BubR1 protein of interest appeared absent (Figure 4.6).

However, using the yeast three hybrid system, these data provide evidence that, as in other systems, the BubR1 KEN box is required for the interaction between BubR1 and Fizzy in *Drosophila*. Without an intact BubR1 KEN box, BubR1 and Cdc20 appear unable to interact. It could be suggested that the BubR1 KEN box has its primary function in the interaction of BubR1 with Cdc20 within the MCC complex, rather than as a mitotic degron, although further investigation would be required in order to fully confirm this.

A previous study by Davenport *et al, (*2006) studied the interaction of human BubR1-Cdc20 in the absence and presence of Mad2 using the Clontech yeast three hybrid system. They showed that the human BubR1-Cdc20 interaction required the N-terminal of BubR1, and that Mad2 was required for this interaction to take place. Although this paper used pBridge vector to incorporate Mad2 as a bridging protein, when analysing the BubR1-Cdc20 interaction in the absence of Mad2 pBridge was not employed. Instead pGADT7 was used in combination with pGBKT7 vector in the traditional two hybrid system, with only BubR1 and Cdc20 fragments present, rather than utilising pBridge throughout by exploiting the *MET25* promoter which controls expression of the bridging protein and switching Mad2 expression on and off with replica plating onto methionine- and methionine+ plates, respectively. Why this study chose not to be consistent in their use of vectors is unclear. Perhaps the inclusion of Mad2 as a three hybrid system was introduced after the traditional two hybrid vector combination with only BubR1 and Cdc20 failed to produce a positive result? This should be taken into consideration when analysing the *Drosophila* three hybrid data, as it cannot be ruled out, without the use of western blot, that even in the presence of methionine, Mad2 may still be expressed.

Although the yeast three hybrid assay appears to have provided data consistent with other systems, the limitations of this system must still be taken into consideration. As is the case for the bacterial three hybrid assay, it is unclear as to whether all eukaryotic proteins expressed by yeast will be correctly folded, thus allowing interaction of true binding partners. It seems unlikely that the negative results observed in these data are due to false negatives incurred by such issues, as although BubR1 is expressed as either wild-type or to contain point mutations to the KEN box motif, it has not been implicated that this minor modification would lead to differences in protein folding in comparison to wild-type. Two hybrid experiments in mammalian cells have been established to attempt to overcome problems with eukaryotic protein folding, however at present, no commercial vector kits are available for mammalian three-hybrid analysis, and the effect of point mutations in protein folding will remain an issue as long as any hybrid technique is employed. In addition to this, it may be argued that the effect of the fusion of proteins of interest with transcription factors may affect their ability to interact with true binding partners. This should not be problematic within the yeast three hybrid assay used to obtain these data, again, due to the fact that wild-type BubR1 is shown to interact with Cdc20 as would be expected.

Because the proteins of interest (BubR1 and Cdc20) have been shown previously to interact by co-immunoprecipitation experiments (including those observed in chapter 3), and these data relate to the ability of these proteins to interact with introduction of a point mutation to the BubR1 KEN box motif, other limitations of the yeast three hybrid system such as binding of proteins which would not exist in the same cellular compartment and would never therefore interact in their natural environment, are not relevant. This is supported by the fact that BubR1 and Cdc20 are known to co-localise to kinetochores during mitosis. It can therefore be assumed that the positive interaction observed between wild-type BubR1 and Cdc20 in the yeast three-hybrid assay is a true interaction, relevant to events which occur within *Drosophila* cells.

By employing a three hybrid technique rather than the traditional two hybrid assay, problems incurred by the absence of additional proteins required for modification of interacting proteins, or to act as a bridge between interacting proteins, is minimised. By introducing Mad2 to the assay, which was previously

shown by Davenport, (2006) to be required for the BubR1-Cdc20 interaction, this issue is overcome. Most positive clones will activate all reporters, however it is possible that a particular prey fusion-protein may be inaccessible to a specific UAS, causing the failure of that reporter to activate even in the presence of a positive interaction. Again, a Clontech yeast strain is available which can be used for any one of the reporters, or if combined with total dropout media, all four at once. Unfortunately this strain was not available for use in this study, hence the use of Y190.

Taking system limitations into consideration, it is not unreasonable to consider the data obtained a reliable indicator that the BubR1 KEN box is required for the BubR1-Cdc20 interaction within *Drosophila*. Now that this has been verified, the effect of this mutation to the spindle checkpoint as a whole should be investigated within the *Drosophila* model. It would be interesting in the future to examine exactly how the lack of intact BubR1 KEN box would affect the formation of the MCC as a whole, and whether the kinetochore localisation of BubR1 or Cdc20 would be disturbed as a result. In order to study this further, other group members have attempted to create transgenic fly lines to express a fluorescently-tagged mutant BubR1 protein in a BubR1-null background. If successful, this would allow the study of BubR1 localisation as well as the study of the ability of these lines to display mitotic arrest in the presence of checkpoint-inducing agents such as colchicine when an intact KEN box motif is absent. If a different fluorescent tag such as RFP (Red Fluorescent Protein) was fused to Cdc20, and this was able to be incorporated into this line, study of Cdc20 localisation, as well as co-immunoprecipitation experiments to determine which MCC components were capable of interacting with Cdc20 or BubR1 could also be investigated. Another approach would be to create stably transfected *Drosophila* cell culture lines (such as S2) to express the mutant BubR1 protein, removing endogenous protein by dsRNAi. With the use of cell or embryonic fixation and immunostaining techniques, the microtubule structure and chromosome morphology could be investigated in relation to checkpoint defects caused by the removal of the BubR1 KEN box, with the use of α-tubulin and histone H3 antibodies, respectively.

In addition to this, these lines could be potentially useful in the investigation of BubR1 turnover. It would be interesting to see exactly how much of an effect the

loss of an intact KEN box would have of BubR1 proteolysis, to provide evidence of whether the BubR1 KEN box is involved in protein degradation as well as interaction, and which function of this motif is likely to be the most prominent. These would both be time consuming projects, but could provide some important insights into the role of BubR1 in the MCC and checkpoint signalling as a whole.

Chapter 5: *Drosophila* Mps1 is required for the SAC, and is involved in Mad2, but not Cdc20 localisation.

5.1. Introduction

Mps1 protein kinase has been implicated in various mitotic processes, and plays an essential role in the SAC response (Howell et al., 2004). Its role in the SAC is believed to be related to recruitment of key SAC proteins BubR1 and Mad2 as well as Mad1 and Bub1, as shown in human and *Xenopus* (Zhao and Chen, 2006; Howell et al., 2004; Vigneron et al., 2004; Liu et al., 2003; Martin-Lluesma et al., 2002; Abrieu et al., 2001). The effect of Mps1 depletion on the localisation of Cdc20 has not been reported in these systems, and no studies exist to confirm the SAC role of Mps1 in *Drosophila.*

As Mps1 is implicated in the kinetochore recruitment of MCC components, and questions still exist into the requirement of the kinetochore localisation of SAC proteins in the formation of the MCC, a model whereby some or all checkpoint proteins are not capable of localising to unattached kinetochores, could provide a useful tool in the future investigation of the SAC response.

A study by the Gilliland lab which carried out a germline clone screen for meiotic mutants in *Drosophila*, identified a lethal *mps1* allele with rare escapers, which could be potentially useful in the study of the role of Mps1 in the SAC (Page et al., 2007). This allele, ald^{B4} (referred to as B4-2 from hereon in) was recovered on chromosome 3R, failed to compliment the meiotic phenotype of the wild-type ald^{1} allele, and could be rescued by the presence of the P{ald^{+}} construct, which contains a functional copy of ald^{+}. Sequencing analysis revealed that the B4-2 contains an early T to C nonsense mutation, which changes glutamine at position 48 to a stop codon. For these reasons, it was speculated that B4-2 line was null for Mps1, although characterisation of this line was never carried out.

In this chapter we characterise the Mps1 B4-2 *Drosophila* line, and confirm that it has dramatically reduced levels of Mps1 in comparison to wild-type levels. It was therefore deemed suitable for use in establishing a *Drosophila* system for the study of GFP-Cdc20 and GFP-Mad2 localisation in a background

significantly depleted for Mps1, in order to verify its role in SAC response and the kinetochore localisation of these SAC components in living neuroblast cells. It is observed that a significant decrease in Mps1 expression renders *Drosophila* lines non-viable, and unable to elicit a checkpoint response. Whilst Cdc20 localisation appears unaffected by the decrease in Mps1 expression, Mad2 kinetochore recruitment is dramatically affected.

5.2. Results

5.2.1. Generation of *Drosophila* lines incorporating GFP-Fizzy or YFP-Mad2 into the Mps1 B4-2 weak allele background.

Fly lines already available in the lab, which express GFP-Fizzy (TF4) or YFP-Mad2 (TM2-20) in a normal wild-type background, were crossed into the B4-2 background, on the third chromosome (J. Huang). This line was previously shown to have only 4% viability and to be rescued by wild-type Mps1, therefore likely to be null for Mps1 (Gilliland lab), although Mps1 levels within this fly line had never before been confirmed by western blot. The resulting fly lines, referred to as TF4;B4-2 and TM2-20;B4-2, were maintained as a heterozygous stock using the TM6-Tubby balancer chromosome.

5.2.2. Introduction of GFP-Fizzy or GFP-Mad2 into the *mps1* weak allele background further reduces viability of B4-2 flies.

Viability studies analysing the progression of development of homozygotes of the original B4-2 fly line was consistent with previous reports by Gilliland et al, in that although approximately 16% of flies hatched, only around 4% survive to viable adult flies (Fig. 5.1). The remaining 12% died upon hatching, with wings still folded. With the incorporation of either GFP-Fizzy or YFP-Mad2 into the B4-2 weak allele background, the viability of B4-2 homozygotes was reduced, with the resulting flies being non-viable. The TF4;B4-2 and TM2-20;B4-2 lines can survive no later than pupae stages (Fig. 5.1 & 5.2).

5.2.3. The TM2-20;B4-2 fly line was deemed unsuitable for use in identifying Mad2 kinetochore localisation in the B4-2 background, whereas in the TF4-B4-2 line kinetochore localisation of Cdc20 was clearly observed.

Using spinning disk confocal microscopy to image TF4 and TM2-20 third instar larvae neuroblasts, in the wild-type background, YFP- Fizzy can be observed at what can be assumed to be kinetochore – like localisation during mitosis. The protein is seen congregating at the centre of the cell at metaphase, becoming tightly organised before the cell moves into anaphase. This localisation is also observed in the TF4;B4-2 line. In the TM2-20 control line, as well as the TM2-

20;B4-2 line, the high expression of the YFP-Mad2 fusion protein, and its nuclear localisation appears to mask any kinetochore localisation in metaphase. Other Mad2-fusion protein lines have been shown to display kinetochore localisation of Mad2 in a wild-type background (Kindly provided by Roger Karess). It is for this reason that the TM2-20 YFP-Mad2 fusion is unsuitable for such studies, as this distinct kinetochore localisation of YFP-Mad2 cannot be visualised. For this reason, another fly line had to be generated, in which the Mad2-fusion protein was expressed from its endogenous promoter was generated.

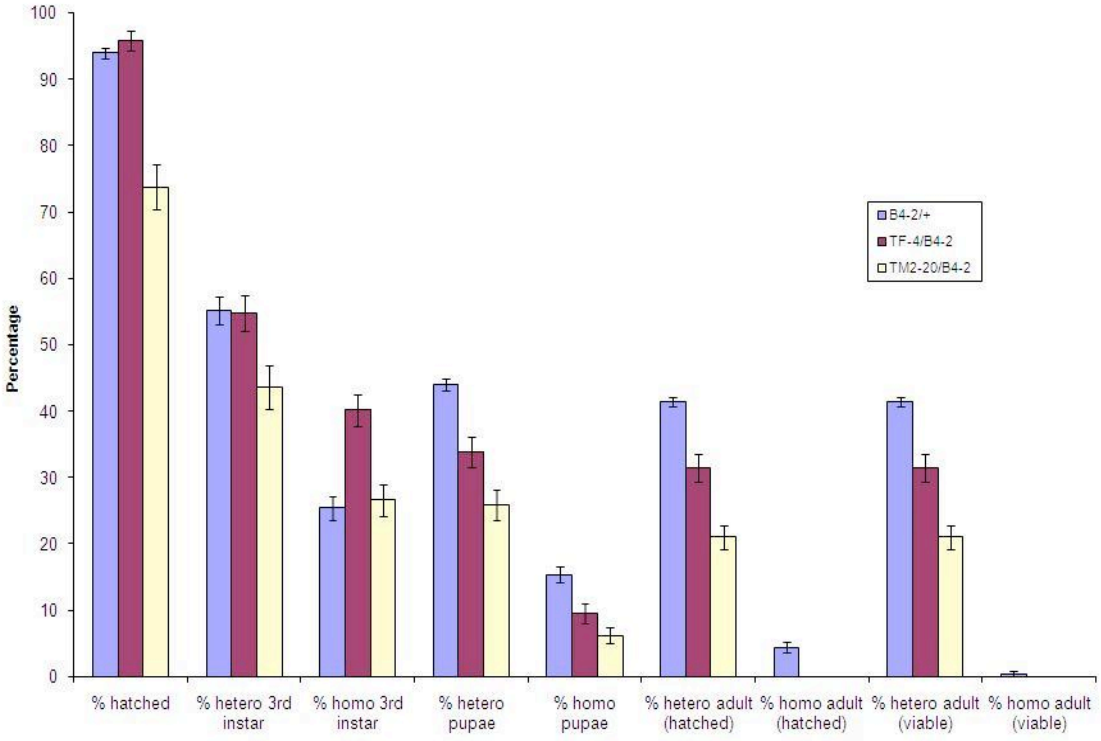


Figure 5.1. Full viability data for TF4;B4-2 and TM2-20;B4-2 Mps1-Null *Drosophila* lines.

The original B4-2 Mps1-null line has 1.6% viability, in-keeping with initial characterisation (of 2% viability) by the Gilliand lab. With the addition of the *fizzy*-GFP transgene (TF4;B4-2) into the Mps1-null background, this phenotype is enhanced and flies are non-viable. This is also the case with the addition of the *mad2*-YFP transgene (TM2-20;B4-2). Both lines have improved viability as compared to the B4-2/- line up to 3rd instar larvae, particularly with overexpression of Mad2, but are unable to develop past this point. Viability was recorded in groups of 20 embryos-flies, repeated ten times to count 200 embryos-flies in total. Error bars represent standard deviation.

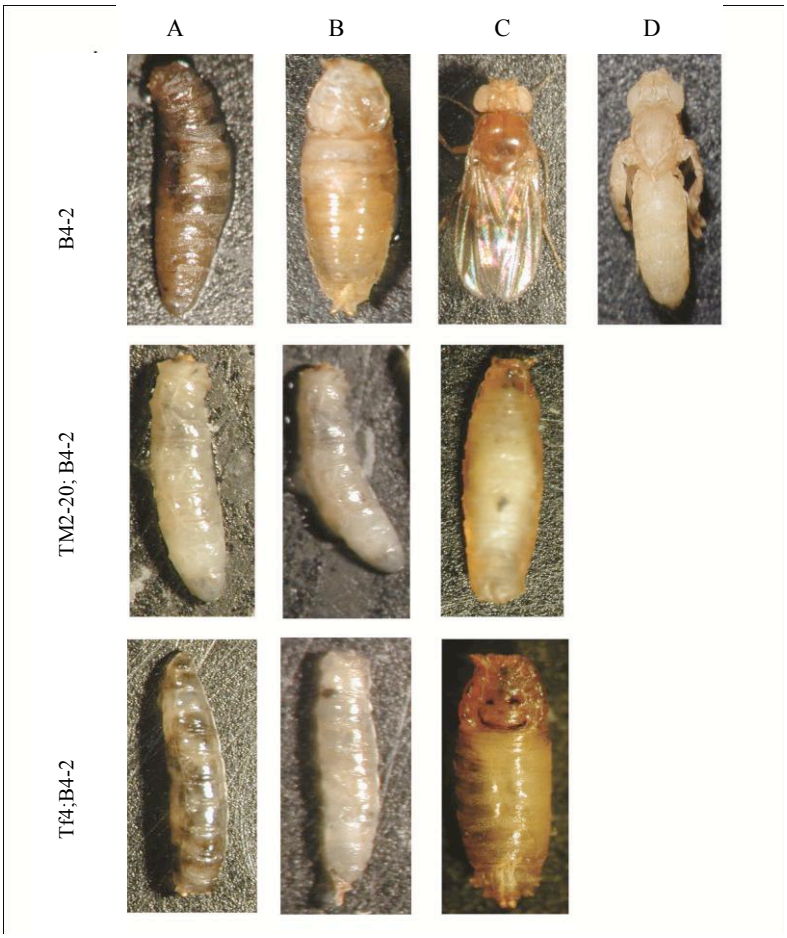


Figure 5.2. Mps1-reduced *Drosophila* Line Phenotypes.

The B4-2/- Mps1-reduced line suffers a large proportion of embryonic death with viability at 3rd instar stages lower than with overexpression of Mad2-YFP or Fizzy-GFP (see Fig. 5.1). Many 3rd instar larvae show signs of tissue abnormalities throughout the larvae, resulting in lethality (top panel A). With overexpression of Mad2, less severe and more localised tissue abnormalities which appear as dark patches can be observed (middle panel A and B). This is similar to the phenotype observed with Fizzy-GFP overexpression (bottom panel B). Over half of the TM2-20;B4-2 and TF4;B4-2 3rd instar larvae die in the late stages (bottom panel A), with surviving larvae capable of reaching pupae with relatively normal phenotypes (compare top panel B with C in middle and bottom panels). The original B4-2 line has 4.4% hatching, 2.8% of which are extremely sick and die within hours (top panel D) leaving1.6% hatch as healthy viable adults (top panel C).

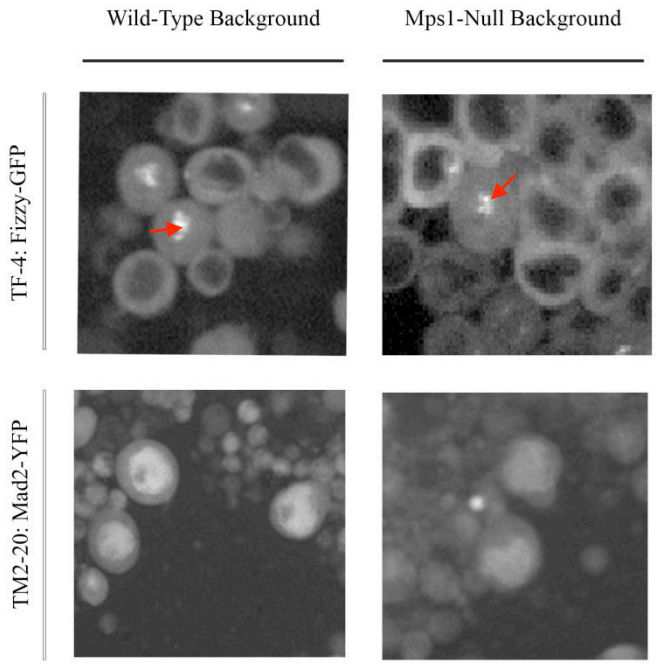


Figure 5.3. Preliminary data suggests that in the Mps1 B4-2 background Fizzy-GFP is still able to localise to the kinetochore in the TM2-20;B4-2 Mad2YFP line, the nuclear localisation of Mad2 appears normal. However, the kinetochore localisation of Mad2 is undetectable in both wild-type control and mutant lines.

In the wild-type background Fizzy-GFP localises to the kinetochore during metaphase (indicated by red arrows, top left panel) in third instar larvae neuroblast cells. The same pattern of localisation can be observed when Fizzy-GFP exists in the Mps1-reduced background (red arrow, top right panel). Under wild-type conditions, Mad2 is localised to the nucleus (bottom left panel). In the Mps1-reduced background Mad2-YFP cannot be observed on the kinetochore in either control (bottom left panel) or mutant background (bottom right panel). Nuclear localisation appears lessened (bottom right panel), although this has not been fully quantified. As the kinetochore localisation of Mad2 is not visible in either line, they are unsuitable for use in the investigation of the effect of Mps1 knockdown on the kinetochore localisation of Mad2-YFP. A minimum of 10 neuroblasts per line were analysed in order to draw these conclusions.

5.2.4. Generation of GFP-Mad2 fly lines, expressing the Mad2-fusion protein from its endogenous promoter, is suitable the insertion into the B4-2 background and allow subsequent analysis of Mad2 localisation.

The TM2-413 GFP-Mad2 line, with the transgenic insertion on the third chromosome, under the control of its native promoter was obtained from R. Karess, and was previously shown to be able to display Mad2 kinetochore localisation. TM2-4-13 expresses a GFP-Mad2 fusion protein from the endogenous Mad2 promoter. Unfortunately, B4-2 is also on the third chromosome and is homozygous lethal. This makes the TM2-413 line unsuitable for recombination with the B4-2 line, for use in the above study. In order to overcome this problem, a new transgenic line, derived from the TM2-413 line, was generated (J. Huang) using a (Δ2-3) G6B (α immobilisable transposase C transgene construct) based genetic hopping technique. This inserted the transgene on the X-chromosome. In this line, kinetochore localisation of GFP-Mad2 in neuroblast cells can be observed using spinning disk confocal microscopy. This localisation was not as striking as that observed in the TF4 or TF4;B4-2 line, but is very apparent when the TM2-413 control line is treated with colchicine and cells are arrested at metaphase (data not shown). Genetics techniques were used to introduce this line into the B4-2 background, resulting in the TM2-413;B4-2 line (carried out by J. Huang and T. Leung) in which the GFP-Mad2 fusion protein, deemed suitable for identification of potential kinetochore localisation, exists in the B4-2 background.

5.2.5. Generation of an anti-Mps1 antibody for use in confirming the reduced levels of endogenous Mps1 in the B4-2 and B4-2-derived fly lines.

An anti-Mps1 antibody was raised in rabbit against full-length *Drosophila* Mps1. The Mps1 sequence of interest was generated by PCR and subcloned into pMal-c2x expression vector, for IPTG induction in BL21 cells (see materials and methods). The protein was purified and separated by SDS-PAGE before being stained using SafeStain and excised from the gel. The protein was sent for immunisation by Cambridge Bioscience, with serum purified by affinity column. The resulting antibody was tested for band-specificity in *Drosophila* S2 cell

samples, and displayed a specific band at 71KD, the expected position of endogenous Mps1 (Figure 5.4).

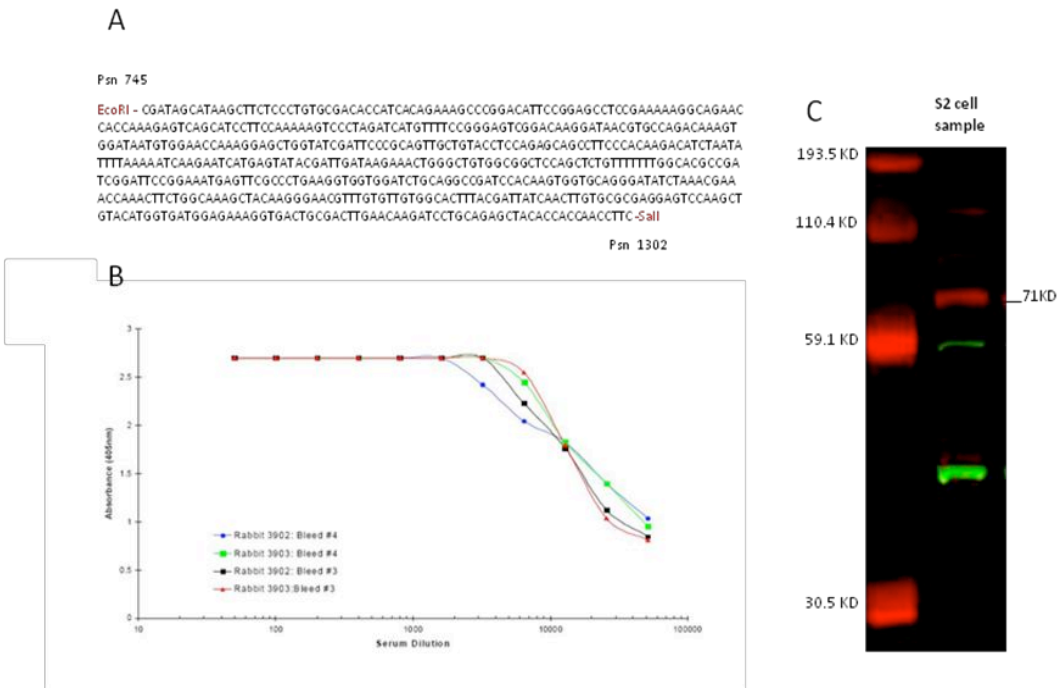


Figure 5.4. Generation and characterisation of a polyclonal antibody to *Drosophila* Mps1 (ald).

(A) Position 745 – 1302 of the *Drosophila* Mps1 (ald) nucleotide sequence was subcloned into pMal-C2x MBP-fusion protein plasmid using *Eco*RI –*Sal*I, for the generation of Mps1-MBP fusion protein used for immunisation. (B) Elisa tests (pre-final bleed) against Mps1 protein (without MBP tag) show that all immunised rabbits have generated anti -Mps1 antibody in response to the fusion protein. All rabbits display detectable antibody at an absorbance of 1.5 (405nm) at a serum dilution of between 1:10000 (observed in rabbit 3902) and 1:50000. Antibody from the serum of rabbit 3902 was affinity-purified for use. (C) Western blot analysis of S2 cell samples show a clear detectable band of 71KD, the size of the *Drosophila* Mps1 (ald) protein, implying that this antibody is specific.

5.2.6. Western blot analysis to characterise the B4-2 and B4-2-derived fly lines displays significantly reduced levels of endogenous Mps1 as well as incorporation of GFP-fusion proteins of interest.

Western blot analysis carried out on samples prepared from dissected third instar larvae neuroblasts confirmed that the B4-2 fly line carried dramatically reduced Mps1 expression, of approximately 16%. This reduction in Mps1 expression is retained when the B4-2 line was used to generate TF4;B4-2 and TM2-20;B4-2 lines. The TM2-413;B4-2 line also displays the reduction of endogenous Mps1. Western blot analysis also confirmed that the TF4-B4-2 line carries the GFP-Fizzy fusion protein, and that the TM2413-B4-2 line carries the GFP-Mad2 fusion protein. This confirms that the localisation patterns observed when analysing these lines represents GFP-Fizzy in the TF4;B4-2 line and GFP-Mad2 in the TM2-413;B4-2 line (Fig. 5.5).

5.2.7. With an 84% reduction in endogenous Mps1 expression, Fizzy kinetochore localisation is not perturbed, but Mad2 kinetochore recruitment appears significantly reduced in third instar larvae neuroblasts.

Quantification of kinetochore-like localisation patterns of GFP-Fizzy in TF4;B4-2 neuroblast cells, compared to that of the TF4 control line, confirmed that with an 84% reduction in Mps1 expression, there is no affect on the amount of Fizzy recruited to kinetochores (Fig. 5.6). This is not the case for GFP-Mad2, which shows very weak kinetochore-like localisation at metaphase (Fig. 5.7) (also previously observed by T. Leung). When neuroblast cells were treated with 10μM colchicine, the GFP-Mad2 control line showed an active SAC, with cells arresting at metaphase, displaying strong and persistent GFP-Mad2 kinetochore localisation. In the B4-2 background, GFP-Mad2 localisation under the same conditions was reduced by approximately 80% (Figure 5.7). Although some Mad2 was recruited to kinetochores, the SAC was not activated in the presence of colchicine, and cells progressed into anaphase.

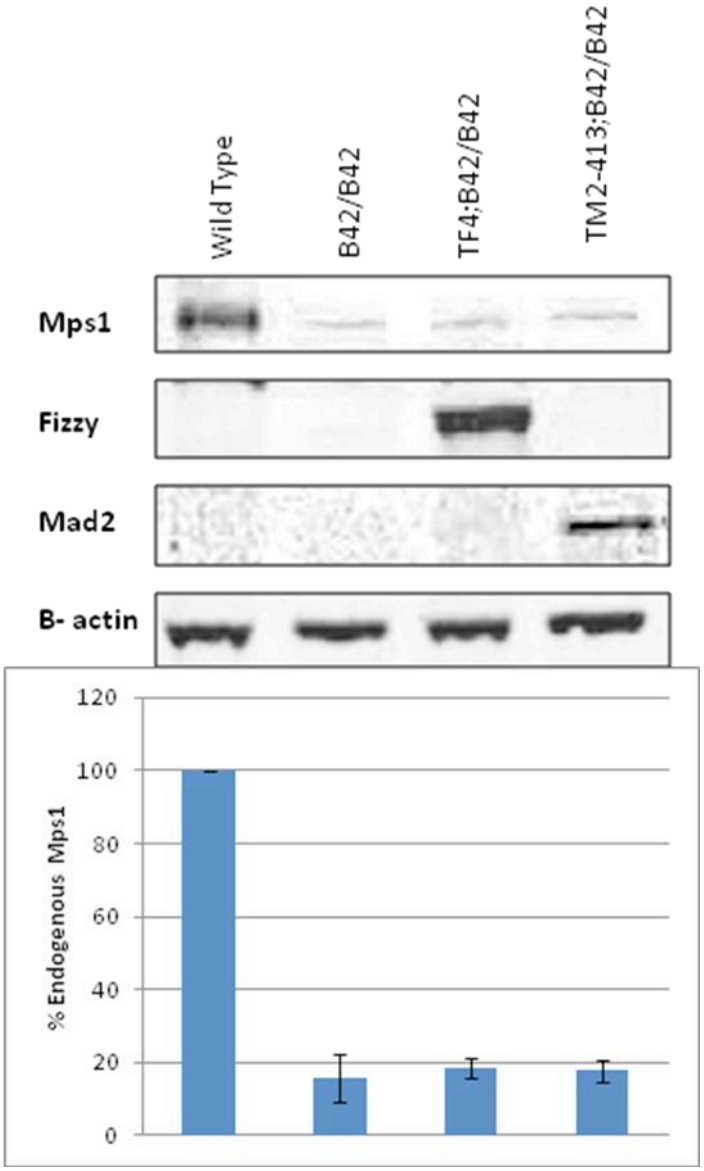


Figure 5.5. Western blot analysis indicates a dramatic decrease in endogenous Mps1 levels in the B4-2 background, as well as the success of incorporated Mad2 and Fizzy GFP-fusion proteins.

In comparison to wild-type neuroblasts, the TF4;B4-2 fly lines have had GFP-Fizzy successfully inserted into the B4-2 background, showing a clear band detected by anti-Fizzy antibody at the position of GFP-Fizzy and only displaying approximately 17.1% endogenous Mps1. A similar level of Mps1 remaining in the TM2-413;B4-2 line (approximately 16.5%), with this line clearly producing a band representing GFP-Mad2, as detected by anti-Mad2 antibody. The original B4-2 line displayed an average of 15.5% endogenous Mps1. Beta actin was used as a loading control. Western blot analysis was carried out in triplicate,

with band intensities quantified using TINA software. Error bars represent standard deviation.

5.2.8. Although GFP-Fizzy is recruited to kinetochores at normal levels, and GFP-Mad2 can be weakly detected in the absence of 84% endogenous Mps1, the spindle assembly checkpoint is defective.

With colchicine treatment of third instar neuroblast from both TF4 GFP-Fizzy and TM2-413 GFP-Mad2 control lines, cells arrest at metaphase with strong kinetochore localisation of fusion proteins, indicative of a functional spindle assembly checkpoint with their expression in a wild-type background. When the fusion proteins are expressed in the B4-2 background, as TF4-B4-2 and TM2413-B4-2, colchicine treatment of third instar neuroblasts failed to arrest cells at metaphase (Fig. 5.8). Instead, cells progress through metaphase but fail to divide properly. The striking GFP-Mad2 kinetochore localisation observed with colchicine treatment of the TM2-413 control line is not observed when the fusion protein is expressed in the B4-2 background. Failure of cells to arrest at metaphase in the presence of colchicine indicates that with an 84% reduction of endogenous Mps1, even though Fizzy localises to kinetochores properly, and some GFP-Mad2 localisation can be detected, the spindle checkpoint fails to produce an anaphase-wait signal and so can be considered defective.

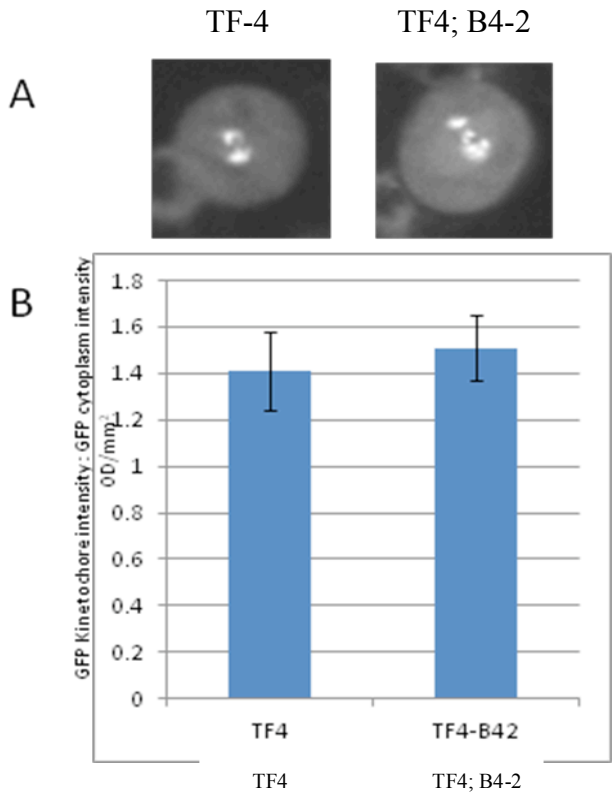


Figure 5.6. The level of kinetochore localisation of Fizzy during metaphase is not affected by reduction in Mps1 (ald) expression.

(A) During metaphase, Fizzy in Mps1-mutant third instar larvae neuroblasts is localised to kinetochores. (B) The intensity of Fizzy at kinetochores in Mps1 mutant TF4; B4-2 neuroblasts (in comparison to cytoplasmic levels) during metaphase, is comparable to that of wild-type TF4. Imaging was carried out using a Visitech spinning disk confocal microscope, with laser intensity and exposure settings standardised for the collection of comparable images. Twenty metaphase cells were quantified in each case. Kinetochore intensity was quantified using MetaMorph software. Error bars represent standard deviation.

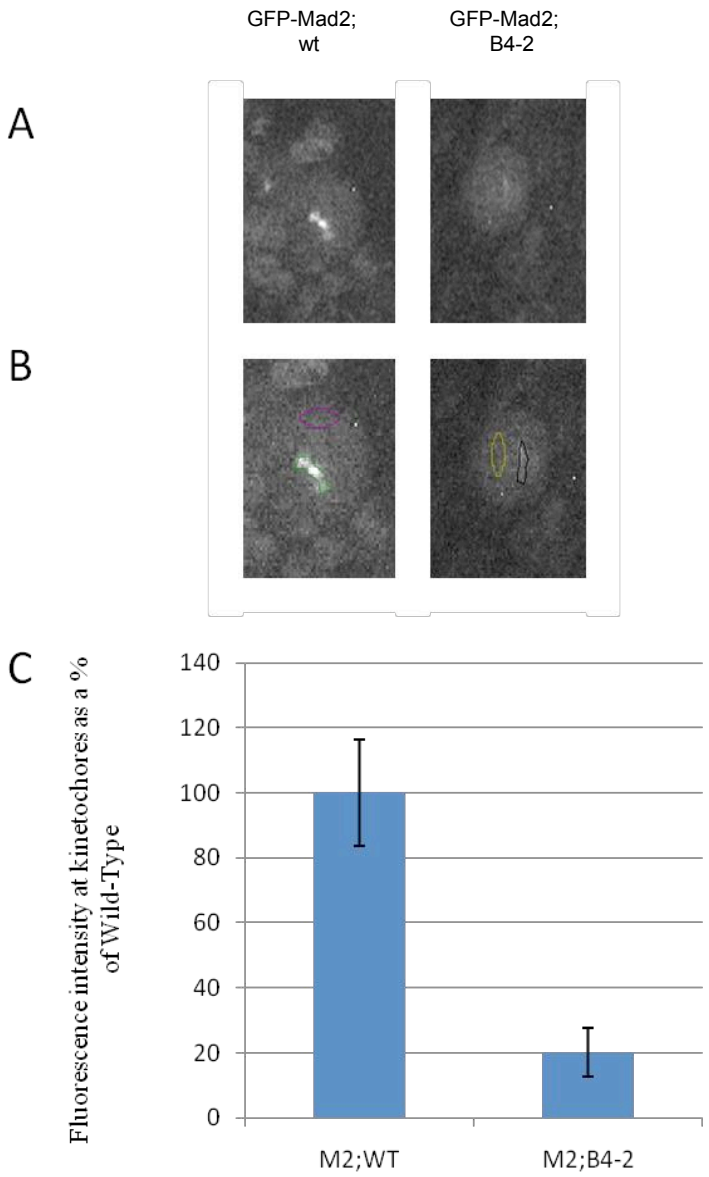


Figure 5.7. Kinetochore recruitment of GFP-Mad2 is decreased by almost 80% in the B4-2 background.

(A) Images of neuroblast cells in metaphase. GFP-Mad2 in the wild-type background (left panel) is strongly recruited to kinetochores with colchicine treatment, whereas in the B4-2 background (right panel) GFP-Mad2 kinetochore localisation is dramatically reduced. (B) An example of the regions used to quantify kinetochore localisation using MetaMorph software. (C) Graph to show quantification results for GFP-Mad2 fluorescence intensity at kinetochores in the wild-type and mutant B4-2 backgrounds, in colchicine-treated neuroblast cells. In the B4-2 background, only 20.5% of wild-type levels of GFP-Mad2 is recruited to kinetochores. Error bars represent standard deviation.

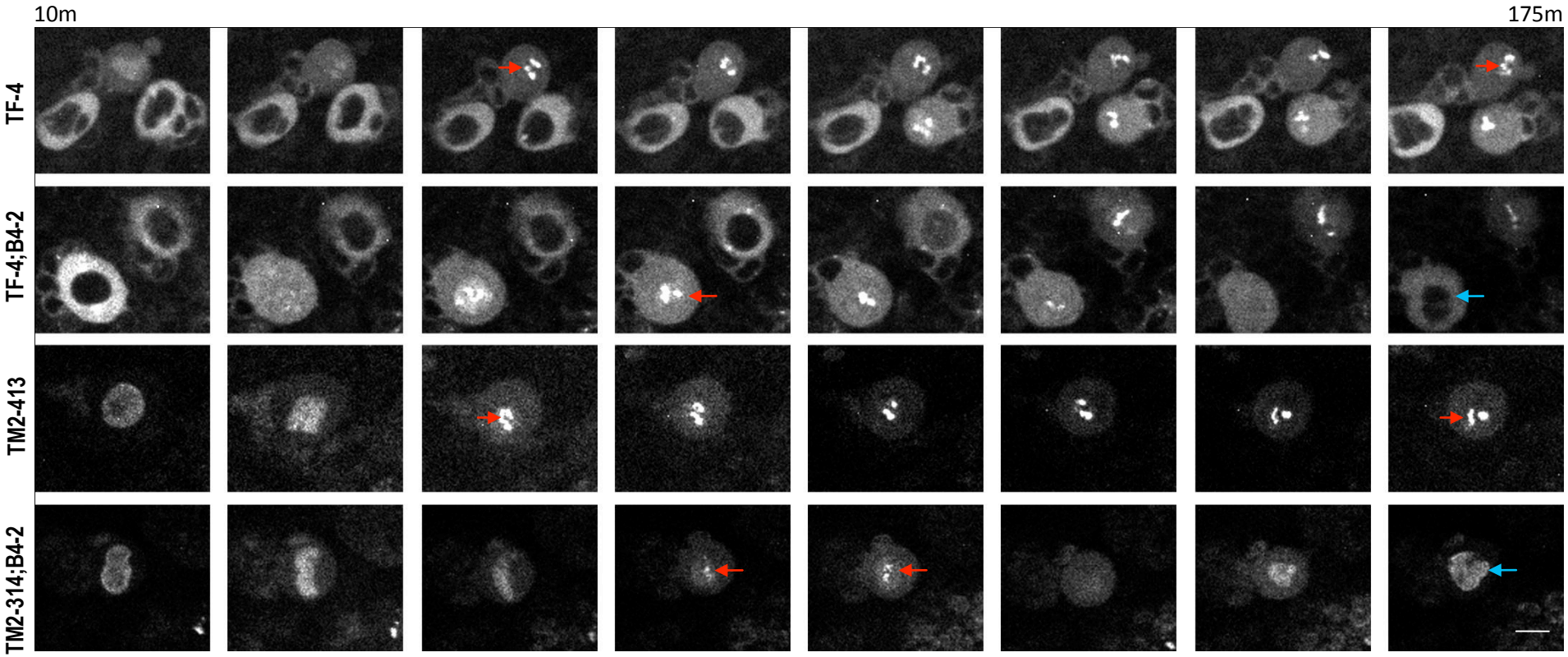

10m
175m
TF-4
TF-4;B4-2
TM2-413
TM2-314;B4-2

Figure 5.8. With depletion of Mps1, neuroblasts treated with colchicine fail to arrest at metaphase, indicating that the SAC is ablated.

Almost immediately after colchicine treatment, TF4 and TM2-413 neuroblasts display cells arresting at metaphase, with visible kinetochore localisation of Cdc20 (Fizzy) and Mad2, respectively (see red arrows, rows 1 and 3). In the B4-2 background, cells fail to arrest with 10μM colchicine after 175 min. Kinetochore recruitment of Cdc20 can be observed in the TF-4;B4-2 line (red arrows), but cells progress through metaphase to anaphase, indicative of a defective SAC (second row, white arrows). Weak Mad2 kinetochore recruitment can be observed in the TM2-413; B4-2 line (forth row, red arrows), but again, even after 175 min, cells are not arrested by colchicine, and are able to bypass the SAC (forth row, white arrows). This indicates that in the presence of only ~16% Mps1, and even with normal levels of Cdc20 kinetochore recruitment, and weak levels of Mad2 recruitment, the SAC is not capable of a normal response, and cells bypass the SAC under the treatment of colchicine. Cells were prepared for imaging as described in 2.7.10, with time-lapse movies captured using confocal microscopy incorporating Visitech spinning disk technology. A minimum of five neuroblasts per line were colchicine treated and observed for checkpoint response. Scale bar represents 2μ.

5.3.Discussion

These data suggest that with a significant reduction in Mps1 expression, the *Drosophila* spindle assembly checkpoint is unable to function. Although approximately 16% endogenous Mps1 is expressed in the B4-2 fly lines used in this study, it is unable to support the SAC response, even if it is able to sustain normal kinetochore localisation of Fizzy, and a proportion of Mad2 kinetochore localisation. Until now no other reports as to the effect of Mps1 depletion on Cdc20 localisation have been made.

Although the original B4-2 fly line was originally generated by Page et al, (2005) the line itself was never characterised fully. The 4% viability of B4-2 lines was reported (consistent with the findings of this study), but the actual Mps1 status was never confirmed using biochemical analysis. These data illustrate that the B4-2 lines, as well as those derived from it for use in this chapter, have approximately 16% endogenous Mps1 expression, a reduction of 84% in comparison to wild-type.

The inability of B4-2 cells to retain a functional checkpoint with an 84% reduction of Mps1 is consistent with observations in other systems, as well as the observed reduction in Mad2 kinetochore recruitment. Previous reports indicated that without Mps1, Mad2 kinetochore localisation is abolished. This complete loss of Mad2 recruitment to kinetochores at metaphase was observed in the apparent absence of Mps1, not one simply subject to a vast reduction in its expression. The fact that the B4-2 lines still express approximately 16% endogenous Mps1 may provide an explanation for the weak Mad2 kinetochore signals observed in this study. Because a reduction in Mps1 expression appears to result in a reduction of Mad2 kinetochore recruitment, it cannot be ruled out that the relationship between Mps1 and Mad2 localisation is dose-dependent, rather than *Drosophila* proteins behaving slightly different to human and *Xenopus* Mps1 and Mad2. In order to determine whether the weak Mad2 kinetochore signals were a result of the small amount of Mps1 remaining in B4-2 cells, fly lines completely null for Mps1 would have to be generated or Mps1 knockdown could be attempted in *Drosophila* S2 cells by dsRNAi. Either approach was too time consuming to be carried out as part of this thesis, as no

null fly lines are available at present, and as the Mad2 antibody available is unsuitable for immunostaining, S2 cells would need to be generated which express GFP-Mad2 before dsRNAi could be performed. Even then, a total knockdown of Mps1 would not be guaranteed.

It should be noted that previous reports into the loss of SAC function in the absence of Mps1 in *Xenopus* suggested that this could be attributed to loss of proper localisation of checkpoint proteins including Mad2 and Bub1. It could be speculated that without Mps1, if Mad2 and BubR1 are not recruited to kinetochores, formation of the MCC would not occur. This hypothesis was tested with the use of co-immunoprecipitation experiments, which implied that MCC formation was disturbed in the absence of Mps1. With regard to the role of failure of Mad2 to localise to kinetochores, as a potential cause of loss of checkpoint arrest, colchicine treatment of the TM2-413;B4-2 line display that, in *Drosophila*, the defective checkpoint is not coupled with a total loss of Mad2 kinetochore localisation. The exact level of Mad2 required to be recruited to unattached kinetochores in *Drosophila* cells in order to allow SAC arrest in the presence of colchicine is not known. It can therefore not be ruled out that the decreased Mad2 kinetochore localisation observed in B4-2 lines is not enough to allow sufficient MCC formation. It also, however, cannot be ruled out that the decreased kinetochore localisation of Mad2 with the depletion of Mps1 is not the cause of the loss of checkpoint function.

Another possible explanation for the ability of Mad2 association with kinetochores to a lesser extent in the relative absence of Mps1, relates to a recent publication using chemical inhibition of Mps1, in which it was speculated that only the recruitment of O-Mad2 to kinetochores was affected (Hewitt et al, 2010). Without further investigation it cannot be ruled out that this is also the case in *Drosophila*, and that the Mad2 observed at kinetochores in the B4-2 background relates to C-Mad2.

A previous publication from our lab has provided evidence that, in *Drosophila*, it is BubR1 and not Mad2 that is required for kinetochore localisation of Fizzy. This paper proposes that Mad2 may act to retain Fizzy localisation on kinetochores rather than be required for its localisation there (Li et al 2010). The mechanism by which Mad2 may act to sustain Fizzy localisation at kinetochores

is yet to be investigated, but it could be presumed that a physical presence of Mad2 at kinetochores is likely. If this were to be the case, could the small amount of Mad2 which localises to kinetochores be capable of retaining Fizzy localisation? It would be interesting to see whether in an instance whereby Mad2 kinetochore localisation is abolished, rather than reduced, Fizzy kinetochore levels would still be comparable to wild-type. Again, it is possible that a fly line completely null for Mps1 could provide this scenario, allowing us to determine whether, if Mps1 expression was completely abolished, Mad2 kinetochore localisation would also be abolished in a dose-dependent type fashion, and the result this may have on the ability of Fizzy to remain at the kinetochore. Studies in humans and *Xenopus* which claim to have knocked out Mps1 and observed abolished Mad2 kinetochore localisation and Mad2-Cdc20 and BubR1-Cdc20 interaction (Xu et al, 2009) failed to investigate any effects this may have on Cdc20 localisation to, or sustenance on, kinetochores.

The generated B4-2 lines did not allow for analysis of BubR1 kinetochore localisation. Unfortunately, the BubR1 antibody generated for use in this thesis, is not pure enough to be used in the immunostaining of neuroblast cells, and so this technique could not be used to determine whether BubR1 localises to kinetochores in the absence of 84% endogenous Mps1. Genetic techniques were used to attempt to cross RFP-BubR1 into the B4-2 background, but unfortunately the generation of this line was unsuccessful. It would be interesting to see whether BubR1 recruitment to kinetochores in the B4-2 background is disturbed. If BubR1 and the majority of Mad2 is not recruited to kinetochores, but Fizzy kinetochore recruitment is not perturbed, co-immunoprecipitation experiments to detect the binding of checkpoint proteins to Fizzy could be carried out to determine if protein-protein interactions have been abolished. This may also provide data into the ongoing debate as to whether Mad2 is actually part of the MCC complex (as proposed by Pines et al, 2008) and whether MCC can form in the absence of direct kinetochore interaction of all components.

In conclusion, fly lines have been developed which allow for an initial investigation into the role of Mps1 in SAC signalling. Mps1 appears to be essential for a functional spindle checkpoint in *Drosophila*. With only 16% endogenous Mps1 present, Fizzy can effectively localise to kinetochores

whereas Mad2 kinetochore levels are decreased (although not abolished), yet cells do not display a checkpoint-induced metaphase arrest in response to colchicine treatment. It is unclear as to whether BubR1 kinetochore recruitment is affected by depletion of Mps1 in *Drosophila*, and whether the reduction of Mad2 at kinetochores is Mps1 dose-dependent. It cannot, therefore, be ruled out that the remaining 16% Mps1 in the B4-2 lines may be sufficient for enough Mad2 to be recruited to kinetochores to sustain the kinetochore recruitment of Fizzy. Further investigation must be carried out in the future to determine the effects of a null-Mps1 background on SAC protein kinetochore localisation, and how this can account for the observed checkpoint failure through biochemical analysis of disruption to protein-protein interactions within the MCC. As this aspect has been recently covered using other models, focussing on the potential dose-dependent effect of Mps1 on Mad2 localisation, or the previously proposed stabilisation role of Mad2 of Fizzy at kinetochores, may be of use in our current understanding of SAC signalling.

Chapter 6. Discussion

The role of Cdc20 kinetochore localisation in formation of the MCC is a topic within the SAC response which remains poorly understood. Whether the complex is capable of forming in part or in full before localising to the kinetochore, or whether all components must localise to and directly interact with kinetochores for complex formation, is yet to be established. The interactions between Cdc20 and other MCC components has been linked to a number of conserved domains, such as the BubR1 KEN box, and phosphorylation sites within Cdc20 itself, as well as the localisation of the other MCC components and the requirement of other upstream factors in the signalling cascade. The importance of these conserved residues/domains within key SAC proteins is yet to be verified in *Drosophila*.

Transgenic approaches in the investigation of specific residues, domains or upstream proteins which are implicated in the interaction between Cdc20 and other MCC components, could provide useful in establishing the link between their ability to interact with one another in relation to their ability to localise to kinetochores during mitosis. By potentially generating fly lines whereby the localisation of Cdc20 and other MCC proteins prevents their physical interaction at kinetochores, the kinetochore-dependent model of MCC formation (see 1.17) could be elegantly tested, with the localisation of proteins of interest available for imaging within living cells.

A recent publication from our lab (Li et al 2010) demonstrated that, in *Drosophila,* BubR1 appears to be more prominent than Mad2 in the localisation of Cdc20 to kinetochores, with depletion of BubR1 resulting in loss of Cdc20 kinetochore association and ablation of the SAC response. Although these findings provided a system whereby Cdc20 kinetochore localisation was disrupted, potentially as a result of the loss of kinetochore-dependent MCC formation, the specific mechanisms and domains involved in the key interactions required for the incorporation of Cdc20 into this complex had not been investigated in this model organism.

For the first time in *Drosophila*, this study builds upon current understanding of the key SAC proteins required in the checkpoint response, confirming a role for

specific residues within Cdc20 in its interaction with other MCC components, particularly BubR1, which could potentially be attributed, at least in part, to its phosphorylation by Cdk1. In addition to the role of Cdc20 phosphorylation in its incorporation into the MCC, we verify the requirement of the single conserved N-terminal BubR1 KEN-box motif in the Cdc20-BubR1 interaction. We also highlight the importance of Mps1 in the SAC response, and establish *Drosophila* lines capable of providing evidence of the requirement for Mps1 in checkpoint activation, as well as any role it may have in the localisation of Cdc20 and Mad2.

It is widely accepted that in human cells, the phosphorylation of Cdc20 by protein kinases including MAPK, Cdc2 protein kinase Cdk1 and the SAC protein Bub1 (Tang et al., 2004a) are required for an active SAC. Studies in *Xenopus* confirmed that conserved sites within the N-terminal of Cdc20 are phosphorylated by Cdk1, mutation of which renders the checkpoint inactive. Ablation of these sites also decreased the interactions between Cdc20-BubR1 and Cdc20-Mad2 (Chen et al 2003, Kramer et al 2000). Although these studies provide evidence of the role of Cdc20 phosphorylation in MCC formation, whether this is a result of failure of non-phosphorylatable Cdc20 to localise to unattached kinetochores was never explored. Three of the sites investigated in human and *Xenopus* studies are also conserved in *Drosophila* Cdc20, although whether they behave in a similar fashion to their human and *Xenopus* counterparts has until now, never been tested. The data presented in Chapter 3 confirms that the three conserved residues (Thr63, Thr78, and Thr82) within *Drosophila* Cdc20 are phosphorylated by Cdk1. Mutation of these three residues reduced the overall phosphorylation of Cdc20 by approximately 69% (Fig. 3.3), implicating Cdk1 as a key kinase in the phosphorylation of Cdc20, specific to the three aforementioned conserved threonine residues.

Immunoprecipitation experiments which probed for interaction of SAC proteins including Mad2 and BubR1 with the triple mutant Cdc20 (Figs. 3.4 & 3.5) are consistent with that observed in other systems, in that with ablation of Cdk1 phosphorylation, the interaction with other MCC components is reduced. This was particularly apparent in BubR1, in keeping with previous implications into its prominent role in Cdc20 function (particularly localisation) as opposed to Mad2 in *Drosophila* (Li et al 2010). The fact that the effect on the Cdc20-Mad2

interaction is less significant in embryos synchronised in mitosis (Fig. 3.5) is potentially interesting, in that it could correlate to the hypothesis that Mad2 may not be required for Cdc20 localisation to kinetochores in response to SAC activation, or potentially be required as part of the initial MCC. Instead, it may assume a role in maintaining Cdc20 at kinetochores (Li et al 2010, Pines et al, 2009). In order to form a more specific hypothesis as to the incorporation of Mad2 into the MCC in *Drosophila* mitosis, further investigation would be required. With regard to the interaction of Cdc20 with the APC/C, these data suggest that the phosphorylation of Cdc20 is not required for the APC/C to interact with its activator, and that phosphorylation of Cdc20 at Thr63, 78, and 82 are not required for normal mitotic progression.

We were unable to verify whether the 69% reduction in Cdc20 phosphorylation caused by ablation of Thr63, 78 and 82 will disrupt the kinetochore localisation of Cdc20. Consequently, we were unable to verify whether any of the interaction results observed are due to the physical location of Cdc20 protein. However, this chapter still provides strong evidence for a role in Cdk1 phosphorylation in the formation of the MCC within the *Drosophila* SAC response.

Although these data strongly suggest that Cdc20 phosphorylation by Cdk1 is required SAC for signalling, many other conserved regions exist within SAC proteins which are equally as likely to have a key role in protein function, and subsequently, the SAC response. Other key residues implicated in the incorporation of Cdc20 into the MCC, with regard to the Cdc20-BubR1 interaction, reside within the single N-terminal *Drosophila* BubR1 KEN-box conserved from humans to yeast. Our lab has already determined that BubR1 is required for the localisation of Cdc20 to the kinetochore, however, the essential region of BubR1 required for this was undetermined. To be able to generate transgenic fly lines in which BubR1 lacks an intact N-terminal KEN-box would provide an opportunity to determine the effect of the loss of this domain in the localisation and behaviour of Cdc20 in the future. Before embarking on a transgenic approach, we developed bacterial and yeast three hybrid systems whereby the importance of the BubR1 KEN-box can first be verified. During the course of these studies, the requirement of an intact N-terminal KEN box in the Cdc20-BubR1 interaction was proven in yeast (Hardwick et al 2010), but until now the role of the BubR1 KEN-box in *Drosophila* had never been tested.

In support of recently published findings in yeast, the data presented within Chapter 4 demonstrates a role for the N-terminal BubR1 KEN-box in the interaction between Cdc20 and BubR1, implicating it as a potentially essential motif in MCC formation in *Drosophila*. The requirement of Mad2 in this interaction is not apparent (Fig. 4.11), but without further investigation could not be ruled out as previous reports in other systems into the Cdc20-BubR1 interaction have implicated Mad2 as essential (Davenport et al, 2006). Based upon these findings, the generation of BubR1 KEN-box mutant fly lines, carrying GFP-Cdc20 for localisation analysis, are in progress by other lab members.

A transgenic approach to the role of Mps1 in the SAC response with regard to localisation of Cdc20 and Mad2 was employed in the data presented throughout Chapter 5. With the incorporation of GFP-Fizzy and GFP-Mad2 fusion proteins into the Mps1 B4-2 weak allele line, a system was established, characterised and employed to verify the essential nature of Mps1. Previously, the effect of Mps1 depletion on SAC signalling had not been explored in *Drosophila*, but these data show that with depletion to approximately 16% wild-type levels of endogenous Mps1, the SAC is ablated. This was not unexpected, due to the lethal nature of the generated lines. It is clearly observed that when neuroblast cells which exist in the B4-2 background are treated with colchicine, the cell cycle fails to arrest at metaphase. Colchicine-induced metaphase arrest is clearly observed in control cells, consequently it can be assumed that Mps1 is essential in the *Drosophila* SAC, in accordance with research in *Xenopus*. Cdc20 localisation is unaffected by reduction in Mps1. It is likely that this would also be the case if Mps1 levels were completely abolished, but it cannot be ruled out that 16% endogenous Mps1 is sufficient to support Cdc20 localisation. No data as to the requirement of Mps1 in Cdc20 localisation in *Drosophila* or other models has been published. In order to verify this, dsRNAi experiments in S2 cells, staining for Cdc20 protein could be carried out, although this is not something currently available in our lab. Other approaches, including chemical inhibition of Mps1, could be used, but the specificity of such inhibitors has never been tested in *Drosophila*.

Mad2 kinetochore localisation is greatly decreased with reduced levels of Mps1 (Fig. 5.7), although, not completely abolished. Approximately 20.5% Mad2 is capable of localising to kinetochores in the presence of 16% endogenous Mps1.

Whether this is indicative of a dose-dependant relationship between Mps1 and Mad2, or whether Mps1 is only required for the localisation of one of the two forms of Mad2 (Hewitt *et* al, 2010) would require further investigation. It would also be interesting to establish the behaviour of BubR1 in this B4-2 weak allele background. Unfortunately, attempts to generate this line (J. Huang) were unsuccessful.

Overall, the data presented in this thesis present some novel results with regard to essential domains potentially required for MCC formation in *Drosophila*, having established systems which can be used for further investigation into whether *Drosophila* MCC forms in a kinetochore-dependent manner. Future direction includes the confirmation of the effect of mutation to the three conserved potential Cdk1 phosphorylation sites (Thr63, 78 and 82) on the localisation of Cdc20, as well as the role of the BubR1 KEN-box motif in localisation of both BubR1 itself and Cdc20. Once determined, current data into the interaction between SAC components under these conditions will become more meaningful with regard to the requirement of direct kinetochore localisation. The role of Mps1 in the *Drosophila* SAC is apparent, and it would be interesting to include BubR1 analysis in this system. As verification of the Mad2 template model is key in the understanding of MCC formation, and the potential exists in the Mps1-null background to investigate differences in the localisation or maintenance of kinetochore signal between the different forms of Mad2, there appears to be a place for further research into verification of this hypothesis within investigation of the SAC response in *Drosophila*.

References

A., U. A. a. D. (1983) 'Role of cyclic AMP in bacteria', *Adv. Cyclic Nucleotide Res,* 15, pp. 1-53.

Abrieu, A., Magnaghi-Jaulin, L., Kahana, J. A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D. W. and Labbe, J. C. (2001) 'Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint', *Cell,* 106, (1), pp. 83-93.

Acquaviva, C., Herzog, F., Kraft, C. and Pines, J. (2004) 'The anaphase promoting complex/cyclosome is recruited to centromeres by the spindle assembly checkpoint', *Nat Cell Biol,* 6, (9), pp. 892-8.

Acquaviva, C. and Pines, J. (2006) 'The anaphase-promoting complex/cyclosome: APC/C', *J Cell Sci,* 119, (Pt 12), pp. 2401-4.

Agarwal, R., Tang, Z., Yu, H. and Cohen-Fix, O. (2003) 'Two distinct pathways for inhibiting pds1 ubiquitination in response to DNA damage', *J Biol Chem,* 278, (45), pp. 45027-33.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) *Molecular Biology of the Cell.* Garland Science.

Andrews, P. D., Ovechkina, Y., Morrice, N., Wagenbach, M., Duncan, K., Wordeman, L. and Swedlow, J. R. (2004) 'Aurora B regulates MCAK at the mitotic centromere', *Dev Cell,* 6, (2), pp. 253-68.

Au, S. W., Leng, X., Harper, J. W. and Barford, D. (2002) 'Implications for the ubiquitination reaction of the anaphase-promoting complex from the crystal structure of the Doc1/Apc10 subunit', *J Mol Biol,* 316, (4), pp. 955-68.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W. and Elledge, S. J. (1996) 'SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box', *Cell,* 86, (2), pp. 263-74.

Bain, J., McLauchlan, H., Elliott, M. and Cohen, P. (2003) 'The specificities of protein kinase inhibitors: an update', *Biochem J,* 371, (Pt 1), pp. 199-204.

Baker, D. J., Dawlaty, M. M., Galardy, P. and van Deursen, J. M. (2007) 'Mitotic regulation of the anaphase-promoting complex', *Cell Mol Life Sci,* 64, (5), pp. 589-600.

Barr, S. M., Leung, C. G., Chang, E. E. and Cimprich, K. A. (2003) 'ATR kinase activity regulates the intranuclear translocation of ATR and RPA following ionizing radiation', *Curr Biol,* 13, (12), pp. 1047-51.

Bashir, T. and Pagano, M. (2004) 'Don't skip the G1 phase: how APC/CCdh1 keeps SCFSKP2 in check', *Cell Cycle,* 3, (7), pp. 850-2.

Braunstein, I., Miniowitz, S., Moshe, Y. and Hershko, A. (2007) 'Inhibitory factors associated with anaphase-promoting complex/cylosome in mitotic checkpoint', *Proc Natl Acad Sci U S A,* 104, (12), pp. 4870-5.

Buffin, E., Emre, D. and Karess, R. E. (2007) 'Flies without a spindle checkpoint', *Nat Cell Biol,* 9, (5), pp. 565-72.

Burke, D. J. and Stukenberg, P. T. (2008) 'Linking kinetochore-microtubule binding to the spindle checkpoint', *Dev Cell,* 14, (4), pp. 474-9.

Burton, J. L. and Solomon, M. J. (2001) 'D box and KEN box motifs in budding yeast Hsl1p are required for APC-mediated degradation and direct binding to Cdc20p and Cdh1p', *Genes Dev,* 15, (18), pp. 2381-95.

Buster, D. W., Zhang, D. and Sharp, D. J. (2007) 'Poleward tubulin flux in spindles: regulation and function in mitotic cells', *Mol Biol Cell,* 18, (8), pp. 3094-104.

Camasses, A., Bogdanova, A., Shevchenko, A. and Zachariae, W. (2003) 'The CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20', *Mol Cell,* 12, (1), pp. 87-100.

Chan, G. K., Jablonski, S. A., Sudakin, V., Hittle, J. C. and Yen, T. J. (1999) 'Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC', *J Cell Biol,* 146, (5), pp. 941-54.

Cheeseman, I. (2009) 'Iain Cheeseman: a strong attachment to kinetochores. [An interview by Ben Short]', *J Cell Biol,* 186, (6), pp. 770-1.

Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. and Desai, A. (2006) 'The conserved KMN network constitutes the core microtubule-binding site of the kinetochore', *Cell,* 127, (5), pp. 983-97.

Cheeseman, I. M. and Desai, A. (2008) 'Molecular architecture of the kinetochore-microtubule interface', *Nat Rev Mol Cell Biol,* 9, (1), pp. 33-46.

Chen, R. H. (2002) 'BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1', *J Cell Biol,* 158, (3), pp. 487-96.

Chen, R. H. (2007) 'Dual inhibition of Cdc20 by the spindle checkpoint', *J Biomed Sci,* 14, (4), pp. 475-9.

Chen, R. H., Waters, J. C., Salmon, E. D. and Murray, A. W. (1996) 'Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores', *Science,* 274, (5285), pp. 242-6.

Chien, C. T., Bartel, P. L., Sternglanz, R. and Fields, S. (1991) 'The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest', *Proc Natl Acad Sci U S A,* 88, (21), pp. 9578-82.

Chung, E. and Chen, R. H. (2002) 'Spindle checkpoint requires Mad1-bound and Mad1-free Mad2', *Mol Biol Cell,* 13, (5), pp. 1501-11.

Chung, E. and Chen, R. H. (2003) 'Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint', *Nat Cell Biol,* 5, (8), pp. 748-53.

Ciferri, C., De Luca, J., Monzani, S., Ferrari, K. J., Ristic, D., Wyman, C., Stark, H., Kilmartin, J., Salmon, E. D. and Musacchio, A. (2005) 'Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore', *J Biol Chem,* 280, (32), pp. 29088-95.

Cimini, D., Moree, B., Canman, J. C. and Salmon, E. D. (2003) 'Merotelic kinetochore orientation occurs frequently during early mitosis in mammalian tissue cells and error correction is achieved by two different mechanisms', *J Cell Sci,* 116, (Pt 20), pp. 4213-25.

Cimprich, K. A. and Cortez, D. (2008) 'ATR: an essential regulator of genome integrity', *Nat Rev Mol Cell Biol,* 9, (8), pp. 616-27.

Cleveland, D. W., Mao, Y. and Sullivan, K. F. (2003) 'Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling', *Cell,* 112, (4), pp. 407-21.

Clute, P. and Pines, J. (1999) 'Temporal and spatial control of cyclin B1 destruction in metaphase', *Nat Cell Biol,* 1, (2), pp. 82-7.

D'Angiolella, V., Mari, C., Nocera, D., Rametti, L. and Grieco, D. (2003) 'The spindle checkpoint requires cyclin-dependent kinase activity', *Genes Dev,* 17, (20), pp. 2520-5.

Davenport, J., Harris, L. D. and Goorha, R. (2006) 'Spindle checkpoint function requires Mad2-dependent Cdc20 binding to the Mad3 homology domain of BubR1', *Exp Cell Res,* 312, (10), pp. 1831-42.

De Antoni, A., Pearson, C. G., Cimini, D., Canman, J. C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E. D. and Musacchio, A. (2005) 'The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint', *Curr Biol,* 15, (3), pp. 214-25.

De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. and Kim, S. H. (1993) 'Crystal structure of cyclin-dependent kinase 2', *Nature,* 363, (6430), pp. 595-602.

Ditchfield, C., Johnson, V. L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N. and Taylor, S. S. (2003) 'Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores', *J Cell Biol,* 161, (2), pp. 267-80.

Emanuele, M., Burke, D. J. and Stukenberg, P. T. (2007) 'A Hec of a microtubule attachment', *Nat Struct Mol Biol,* 14, (1), pp. 11-3.

Fang, G. (2002) 'Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex', *Mol Biol Cell,* 13, (3), pp. 755-66.

Fang, G., Yu, H. and Kirschner, M. W. (1998a) 'The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation', *Genes Dev,* 12, (12), pp. 1871-83.

Fang, G., Yu, H. and Kirschner, M. W. (1998b) 'Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1', *Mol Cell,* 2, (2), pp. 163-71.

Fang, S., Lorick, K. L., Jensen, J. P. and Weissman, A. M. (2003) 'RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer', *Semin Cancer Biol,* 13, (1), pp. 5-14.

Feldman, M. D. (1997) 'Pandora's Box', *West J Med,* 166, (2), pp. 157-8.

Fields, S. and Song, O. (1989) 'A novel genetic system to detect protein-protein interactions', *Nature,* 340, (6230), pp. 245-6.

Fisk, H. A., Mattison, C. P. and Winey, M. (2004) 'A field guide to the Mps1 family of protein kinases', *Cell Cycle,* 3, (4), pp. 439-42.

Fraschini, R., Beretta, A., Sironi, L., Musacchio, A., Lucchini, G. and Piatti, S. (2001) 'Bub3 interaction with Mad2, Mad3 and Cdc20 is mediated by WD40 repeats and does not require intact kinetochores', *Embo J,* 20, (23), pp. 6648-59.

Friedman, D. B., Kern, J. W., Huneycutt, B. J., Vinh, D. B., Crawford, D. K., Steiner, E., Scheiltz, D., Yates, J., 3rd, Resing, K. A., Ahn, N. G., Winey, M. and Davis, T. N. (2001) 'Yeast Mps1p phosphorylates the spindle pole component Spc110p in the N-terminal domain', *J Biol Chem,* 276, (21), pp. 17958-67.

Gerlich, D., Hirota, T., Koch, B., Peters, J. M. and Ellenberg, J. (2006) 'Condensin I stabilizes chromosomes mechanically through a dynamic interaction in live cells', *Curr Biol,* 16, (4), pp. 333-44.

Giniger, E. and Ptashne, M. (1988) 'Cooperative DNA binding of the yeast transcriptional activator GAL4', *Proc Natl Acad Sci U S A,* 85, (2), pp. 382-6.

Giniger, E., Varnum, S. M. and Ptashne, M. (1985) 'Specific DNA binding of GAL4, a positive regulatory protein of yeast', *Cell,* 40, (4), pp. 767-74.

Gorr, I. H., Boos, D. and Stemmann, O. (2005) 'Mutual inhibition of separase and Cdk1 by two-step complex formation', *Mol Cell,* 19, (1), pp. 135-41.

Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J. M. and Pines, J. (2002) 'Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1', *J Cell Biol,* 157, (7), pp. 1125-37.

Hardwick, K. G. (2005) 'Checkpoint signalling: Mad2 conformers and signal propagation', *Curr Biol,* 15, (4), pp. R122-4.

Hardwick, K. G., Johnston, R. C., Smith, D. L. and Murray, A. W. (2000) 'MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p', *J Cell Biol,* 148, (5), pp. 871-82.

Harris, L., Davenport, J., Neale, G. and Goorha, R. (2005) 'The mitotic checkpoint gene BubR1 has two distinct functions in mitosis', *Exp Cell Res,* 308, (1), pp. 85-100.

Harvey, S. L. and Kellogg, D. R. (2003) 'Conservation of mechanisms controlling entry into mitosis: budding yeast wee1 delays entry into mitosis and is required for cell size control', *Curr Biol,* 13, (4), pp. 264-75.

Hassold, T. and Hunt, P. (2001) 'To err (meiotically) is human: the genesis of human aneuploidy', *Nat Rev Genet,* 2, (4), pp. 280-91.

Hauf, S. (2003) 'Fine tuning of kinetochore function by phosphorylation', *Cell Cycle,* 2, (3), pp. 228-9.

Haugwitz, U., Tschop, K. and Engeland, K. (2004) 'SIRF--a novel regulator element controlling transcription from the p55Cdc/Fizzy promoter during the cell cycle', *Biochem Biophys Res Commun,* 320, (3), pp. 951-60.

He, X., Rines, D. R., Espelin, C. W. and Sorger, P. K. (2001) 'Molecular analysis of kinetochore-microtubule attachment in budding yeast', *Cell,* 106, (2), pp. 195-206.

Hershko, A. and Ciechanover, A. (1998) 'The ubiquitin system', *Annu Rev Biochem,* 67, pp. 425-79.

Hershko, A., Ciechanover, A. and Rose, I. A. (1981) 'Identification of the active amino acid residue of the polypeptide of ATP-dependent protein breakdown', *J Biol Chem,* 256, (4), pp. 1525-8.

Hershko, A., Heller, H., Elias, S. and Ciechanover, A. (1983) 'Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown', *J Biol Chem,* 258, (13), pp. 8206-14.

Hewitt, L., Tighe, A., Santaguida, S., White, A. M., Jones, C. D., Musacchio, A., Green, S. and Taylor, S. S. (2010) 'Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex', *J Cell Biol,* 190, (1), pp. 25-34.

Howell, B. J., Hoffman, D. B., Fang, G., Murray, A. W. and Salmon, E. D. (2000) 'Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells', *J Cell Biol,* 150, (6), pp. 1233-50.

Howell, B. J., Moree, B., Farrar, E. M., Stewart, S., Fang, G. and Salmon, E. D. (2004) 'Spindle checkpoint protein dynamics at kinetochores in living cells', *Curr Biol,* 14, (11), pp. 953-64.

Hoyt, M. A., Totis, L. and Roberts, B. T. (1991) 'S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function', *Cell,* 66, (3), pp. 507-17.

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A. and Murray, A. W. (1998) 'Budding yeast Cdc20: a target of the spindle checkpoint', *Science*, 279, (5353), pp. 1041-4.

Ibrahim, B., Diekmann, S., Schmitt, E. and Dittrich, P. (2008) 'In-silico modeling of the mitotic spindle assembly checkpoint', *PLoS ONE*, 3, (2), pp. e1555.

Ingham, R. J., Gish, G. and Pawson, T. (2004) 'The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture', *Oncogene*, 23, (11), pp. 1972-84.

Irniger, S., Piatti, S., Michaelis, C. and Nasmyth, K. (1995) 'Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast', *Cell*, 81, (2), pp. 269-78.

Iwanaga, Y. and Jeang, K. T. (2002) 'Expression of mitotic spindle checkpoint protein hsMAD1 correlates with cellular proliferation and is activated by a gain-of-function p53 mutant', *Cancer Res*, 62, (9), pp. 2618-24.

Janssen, A., Kops, G. J. and Medema, R. H. (2009) 'Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells', *Proc Natl Acad Sci U S A*, 106, (45), pp. 19108-13.

Jarviluoma, A., Child, E. S., Sarek, G., Sirimongkolkasem, P., Peters, G., Ojala, P. M. and Mann, D. J. (2006) 'Phosphorylation of the cyclin-dependent kinase inhibitor p21Cip1 on serine 130 is essential for viral cyclin-mediated bypass of a p21Cip1-imposed G1 arrest', *Mol Cell Biol*, 26, (6), pp. 2430-40.

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J. and Jackson, S. P. (2006) 'ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks', *Nat Cell Biol*, 8, (1), pp. 37-45.

Jeganathan, K. B., Baker, D. J. and van Deursen, J. M. (2006) 'Securin associates with APCCdh1 in prometaphase but its destruction is delayed by Rae1 and Nup98 until the metaphase/anaphase transition', *Cell Cycle*, 5, (4), pp. 366-70.

Jelluma, N., Brenkman, A. B., van den Broek, N. J., Cruijsen, C. W., van Osch, M. H., Lens, S. M., Medema, R. H. and Kops, G. J. (2008) 'Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment', *Cell*, 132, (2), pp. 233-46.

Jelluma, N., Dansen, T. B., Sliedrecht, T., Kwiatkowski, N. P. and Kops, G. J. (2010) 'Release of Mps1 from kinetochores is crucial for timely anaphase onset', *J Cell Biol*, 191, (2), pp. 281-90.

Joazeiro, C. A. and Weissman, A. M. (2000) 'RING finger proteins: mediators of ubiquitin ligase activity', *Cell*, 102, (5), pp. 549-52.

Johnson, V. L., Scott, M. I., Holt, S. V., Hussein, D. and Taylor, S. S. (2004) 'Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression', *J Cell Sci*, 117, (Pt 8), pp. 1577-89.

Jones, M. H., Huneycutt, B. J., Pearson, C. G., Zhang, C., Morgan, G., Shokat, K., Bloom, K. and Winey, M. (2005) 'Chemical genetics reveals a role for Mps1 kinase in kinetochore attachment during mitosis', *Curr Biol,* 15, (2), pp. 160-5.

Kallio, M., Mustalahti, T., Yen, T. J. and Lahdetie, J. (1998) 'Immunolocalization of alpha-tubulin, gamma-tubulin, and CENP-E in male rat and male mouse meiotic divisions: pathway of meiosis I spindle formation in mammalian spermatocytes', *Dev Biol,* 195, (1), pp. 29-37.

Kallio, M. J., Beardmore, V. A., Weinstein, J. and Gorbsky, G. J. (2002) 'Rapid microtubule-independent dynamics of Cdc20 at kinetochores and centrosomes in mammalian cells', *J Cell Biol,* 158, (5), pp. 841-7.

Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C. and Conaway, J. W. (1999) 'The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2', *Genes Dev,* 13, (22), pp. 2928-33.

Kang, J., Chen, Y., Zhao, Y. and Yu, H. (2007) 'Autophosphorylation-dependent activation of human Mps1 is required for the spindle checkpoint', *Proc Natl Acad Sci U S A,* 104, (51), pp. 20232-7.

Kapoor, T. M., Mayer, T. U., Coughlin, M. L. and Mitchison, T. J. (2000) 'Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5', *J Cell Biol,* 150, (5), pp. 975-88.

Karimova, G., Pidoux, J., Ullmann, A. and Ladant, D. (1998) 'A bacterial two-hybrid system based on a reconstituted signal transduction pathway', *Proc Natl Acad Sci U S A,* 95, (10), pp. 5752-6.

Kelly, A. E. and Funabiki, H. (2009) 'Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view', *Curr Opin Cell Biol,* 21, (1), pp. 51-8.

Kim, E. M. and Burke, D. J. (2008) 'DNA damage activates the SAC in an ATM/ATR-dependent manner, independently of the kinetochore', *PLoS Genet,* 4, (2), pp. e1000015.

Kim, J. C., Ou, Y. Y., Badano, J. L., Esmail, M. A., Leitch, C. C., Fiedrich, E., Beales, P. L., Archibald, J. M., Katsanis, N., Rattner, J. B. and Leroux, M. R. (2005) 'MKKS/BBS6, a divergent chaperonin-like protein linked to the obesity disorder Bardet-Biedl syndrome, is a novel centrosomal component required for cytokinesis', *J Cell Sci,* 118, (Pt 5), pp. 1007-20.

King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M. W. (1995) 'A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B', *Cell,* 81, (2), pp. 279-88.

Kline-Smith, S. L. and Walczak, C. E. (2004) 'Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics', *Mol Cell,* 15, (3), pp. 317-27.

Kobayashi, H., Stewart, E., Poon, R. Y. and Hunt, T. (1994) 'Cyclin A and cyclin B dissociate from p34cdc2 with half-times of 4 and 15 h, respectively, regardless of the phase of the cell cycle', *J Biol Chem,* 269, (46), pp. 29153-60.

Kops, G. J., Weaver, B. A. and Cleveland, D. W. (2005) 'On the road to cancer: aneuploidy and the mitotic checkpoint', *Nat Rev Cancer,* 5, (10), pp. 773-85.

Kotani, S., Tanaka, H., Yasuda, H. and Todokoro, K. (1999) 'Regulation of APC activity by phosphorylation and regulatory factors', *J Cell Biol,* 146, (4), pp. 791-800.

Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J. and Peters, J. M. (2003) 'Mitotic regulation of the human anaphase-promoting complex by phosphorylation', *Embo J,* 22, (24), pp. 6598-609.

Kraft, C., Vodermaier, H. C., Maurer-Stroh, S., Eisenhaber, F. and Peters, J. M. (2005) 'The WD40 propeller domain of Cdh1 functions as a destruction box receptor for APC/C substrates', *Mol Cell,* 18, (5), pp. 543-53.

Kramer, E. R., Gieffers, C., Holzl, G., Hengstschlager, M. and Peters, J. M. (1998) 'Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family', *Curr Biol,* 8, (22), pp. 1207-10.

Kramer, E. R., Scheuringer, N., Podtelejnikov, A. V., Mann, M. and Peters, J. M. (2000) 'Mitotic regulation of the APC activator proteins CDC20 and CDH1', *Mol Biol Cell,* 11, (5), pp. 1555-69.

Kudo, N. R., Wassmann, K., Anger, M., Schuh, M., Wirth, K. G., Xu, H., Helmhart, W., Kudo, H., McKay, M., Maro, B., Ellenberg, J., de Boer, P. and Nasmyth, K. (2006) 'Resolution of chiasmata in oocytes requires separase-mediated proteolysis', *Cell,* 126, (1), pp. 135-46.

Kulukian, A., Han, J. S. and Cleveland, D. W. (2009) 'Unattached kinetochores catalyze production of an anaphase inhibitor that requires a Mad2 template to prime Cdc20 for BubR1 binding', *Dev Cell,* 16, (1), pp. 105-17.

Kwiatkowski, N., Jelluma, N., Filippakopoulos, P., Soundararajan, M., Manak, M. S., Kwon, M., Choi, H. G., Sim, T., Deveraux, Q. L., Rottmann, S., Pellman, D., Shah, J. V., Kops, G. J., Knapp, S. and Gray, N. S. (2010) 'Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function', *Nat Chem Biol,* 6, (5), pp. 359-68.

Laine, D. I., McCleland, B., Thomas, S., Neipp, C., Underwood, B., Dufour, J., Widdowson, K. L., Palovich, M. R., Blaney, F. E., Foley, J. J., Webb, E. F., Luttmann, M. A., Burman, M., Belmonte, K. and Salmon, M. (2009) 'Discovery of novel 1-azoniabicyclo[2.2.2]octane muscarinic acetylcholine receptor antagonists', *J Med Chem,* 52, (8), pp. 2493-505.

Lampson, M. A., Renduchitala, K., Khodjakov, A. and Kapoor, T. M. (2004) 'Correcting improper chromosome-spindle attachments during cell division', *Nat Cell Biol,* 6, (3), pp. 232-7.

Lan, W. and Cleveland, D. W. (2010) 'A chemical tool box defines mitotic and interphase roles for Mps1 kinase', *J Cell Biol,* 190, (1), pp. 21-4.

Li, D., Morley, G., Whitaker, M. and Huang, J. Y. (2010) 'Recruitment of Cdc20 to the kinetochore requires BubR1 but not Mad2 in Drosophila melanogaster', *Mol Cell Biol,* 30, (13), pp. 3384-95.

Li, R. and Murray, A. W. (1991) 'Feedback control of mitosis in budding yeast', *Cell,* 66, (3), pp. 519-31.

Li, X. and Nicklas, R. B. (1995) 'Mitotic forces control a cell-cycle checkpoint', *Nature,* 373, (6515), pp. 630-2.

Li, Y. and Benezra, R. (1996) 'Identification of a human mitotic checkpoint gene: hsMAD2', *Science,* 274, (5285), pp. 246-8.

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. (1997) 'MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity', *Proc Natl Acad Sci U S A,* 94, (23), pp. 12431-6.

Lindberg, R. A., Fischer, W. H. and Hunter, T. (1993) 'Characterization of a human protein threonine kinase isolated by screening an expression library with antibodies to phosphotyrosine', *Oncogene,* 8, (2), pp. 351-9.

Lindqvist, A., van Zon, W., Karlsson Rosenthal, C. and Wolthuis, R. M. (2007) 'Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression', *PLoS Biol,* 5, (5), pp. e123.

Liu, D. and Lampson, M. A. (2009) 'Regulation of kinetochore-microtubule attachments by Aurora B kinase', *Biochem Soc Trans,* 37, (Pt 5), pp. 976-80.

Liu, D., Vader, G., Vromans, M. J., Lampson, M. A. and Lens, S. M. (2009) 'Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates', *Science,* 323, (5919), pp. 1350-3.

Liu, S. T., Chan, G. K., Hittle, J. C., Fujii, G., Lees, E. and Yen, T. J. (2003) 'Human MPS1 kinase is required for mitotic arrest induced by the loss of CENP-E from kinetochores', *Mol Biol Cell,* 14, (4), pp. 1638-51.

Lopes, C. S., Sampaio, P., Williams, B., Goldberg, M. and Sunkel, C. E. (2005) 'The Drosophila Bub3 protein is required for the mitotic checkpoint and for normal accumulation of cyclins during G2 and early stages of mitosis', *J Cell Sci,* 118, (Pt 1), pp. 187-98.

Lorca, T., Castro, A., Martinez, A. M., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Doree, M. and Labbe, J. C. (1998) 'Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts', *Embo J,* 17, (13), pp. 3565-75.

Lovejoy, C. A. and Cortez, D. (2009) 'Common mechanisms of PIKK regulation', *DNA Repair (Amst),* 8, (9), pp. 1004-8.

Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J. and Yu, H. (2004) 'The Mad2 spindle checkpoint protein has two distinct natively folded states', *Nat Struct Mol Biol,* 11, (4), pp. 338-45.

Maia, A. F., Lopes, C. S. and Sunkel, C. E. (2007) 'BubR1 and CENP-E have antagonistic effects upon the stability of microtubule-kinetochore attachments in Drosophila S2 cell mitosis', *Cell Cycle,* 6, (11), pp. 1367-78.

Maiato, H., DeLuca, J., Salmon, E. D. and Earnshaw, W. C. (2004) 'The dynamic kinetochore-microtubule interface', *J Cell Sci,* 117, (Pt 23), pp. 5461-77.

Malureanu, L. A., Jeganathan, K. B., Hamada, M., Wasilewski, L., Davenport, J. and van Deursen, J. M. (2009) 'BubR1 N terminus acts as a soluble inhibitor of cyclin B degradation by APC/C(Cdc20) in interphase', *Dev Cell,* 16, (1), pp. 118-31.

Mandell, J. W., Hussaini, I. M., Zecevic, M., Weber, M. J. and VandenBerg, S. R. (1998) 'In situ visualization of intratumor growth factor signaling: immunohistochemical localization of activated ERK/MAP kinase in glial neoplasms', *Am J Pathol,* 153, (5), pp. 1411-23.

Mapelli, M., Filipp, F. V., Rancati, G., Massimiliano, L., Nezi, L., Stier, G., Hagan, R. S., Confalonieri, S., Piatti, S., Sattler, M. and Musacchio, A. (2006) 'Determinants of conformational dimerization of Mad2 and its inhibition by p31comet', *Embo J,* 25, (6), pp. 1273-84.

Mapelli, M., Massimiliano, L., Santaguida, S. and Musacchio, A. (2007) 'The Mad2 conformational dimer: structure and implications for the spindle assembly checkpoint', *Cell,* 131, (4), pp. 730-43.

Maresca, T. J. and Salmon, E. D. (2009) 'Intrakinetochore stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity', *J Cell Biol,* 184, (3), pp. 373-81.

Martin-Lluesma, S., Stucke, V. M. and Nigg, E. A. (2002) 'Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2', *Science,* 297, (5590), pp. 2267-70.

Mattison, C. P., Old, W. M., Steiner, E., Huneycutt, B. J., Resing, K. A., Ahn, N. G. and Winey, M. (2007) 'Mps1 activation loop autophosphorylation enhances kinase activity', *J Biol Chem,* 282, (42), pp. 30553-61.

May, K. M. and Hardwick, K. G. (2006) 'The spindle checkpoint', *J Cell Sci,* 119, (Pt 20), pp. 4139-42.

McCleland, M. L., Farrell, J. A. and O'Farrell, P. H. (2009) 'Influence of cyclin type and dose on mitotic entry and progression in the early Drosophila embryo', *J Cell Biol,* 184, (5), pp. 639-46.

McCleland, M. L., Gardner, R. D., Kallio, M. J., Daum, J. R., Gorbsky, G. J., Burke, D. J. and Stukenberg, P. T. (2003) 'The highly conserved Ndc80 complex is

required for kinetochore assembly, chromosome congression, and spindle checkpoint activity', *Genes Dev,* 17, (1), pp. 101-14.

Millband, D. N. and Hardwick, K. G. (2002) 'Fission yeast Mad3p is required for Mad2p to inhibit the anaphase-promoting complex and localizes to kinetochores in a Bub1p-, Bub3p-, and Mph1p-dependent manner', *Mol Cell Biol,* 22, (8), pp. 2728-42.

Mills, G. B., Schmandt, R., McGill, M., Amendola, A., Hill, M., Jacobs, K., May, C., Rodricks, A. M., Campbell, S. and Hogg, D. (1992) 'Expression of TTK, a novel human protein kinase, is associated with cell proliferation', *J Biol Chem,* 267, (22), pp. 16000-6.

Minshull, J., Sun, H., Tonks, N. K. and Murray, A. W. (1994) 'A MAP kinase-dependent spindle assembly checkpoint in Xenopus egg extracts', *Cell,* 79, (3), pp. 475-86.

Mondal, G., Baral, R. N. and Roychoudhury, S. (2006) 'A new Mad2-interacting domain of Cdc20 is critical for the function of Mad2-Cdc20 complex in the spindle assembly checkpoint', *Biochem J,* 396, (2), pp. 243-53.

Moores, C. A., Cooper, J., Wagenbach, M., Ovechkina, Y., Wordeman, L. and Milligan, R. A. (2006) 'The role of the kinesin-13 neck in microtubule depolymerization', *Cell Cycle,* 5, (16), pp. 1812-5.

Morozov, A., Lee, S. J., Zhang, Z. K., Cimica, V., Zagzag, D. and Kalpana, G. V. (2007) 'INI1 induces interferon signaling and spindle checkpoint in rhabdoid tumors', *Clin Cancer Res,* 13, (16), pp. 4721-30.

Morris, M. C., Kaiser, P., Rudyak, S., Baskerville, C., Watson, M. H. and Reed, S. I. (2003) 'Cks1-dependent proteasome recruitment and activation of CDC20 transcription in budding yeast', *Nature,* 423, (6943), pp. 1009-13.

Morrow, C. J., Tighe, A., Johnson, V. L., Scott, M. I., Ditchfield, C. and Taylor, S. S. (2005) 'Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20', *J Cell Sci,* 118, (Pt 16), pp. 3639-52.

Munier, H., Gilles, A. M., Glaser, P., Krin, E., Danchin, A., Sarfati, R. and Barzu, O. (1991) 'Isolation and characterization of catalytic and calmodulin-binding domains of Bordetella pertussis adenylate cyclase', *Eur J Biochem,* 196, (2), pp. 469-74.

Musacchio, A. and Salmon, E. D. (2007) 'The spindle-assembly checkpoint in space and time', *Nat Rev Mol Cell Biol,* 8, (5), pp. 379-93.

Muster, C. J., Shapiro, J. A. and MacHattie, L. A. (1983) 'Recombination involving transposable elements: role of target molecule replication in Tn1 delta Ap-mediated replicon fusion', *Proc Natl Acad Sci U S A,* 80, (8), pp. 2314-7.

Nakayama, K. I. and Nakayama, K. (2006) '[Ubiquitin system regulating G1 and S phases of cell cycle]', *Tanpakushitsu Kakusan Koso,* 51, (10 Suppl), pp. 1362-9.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F. B., Mendenhall, M. D., Sicheri, F., Pawson, T. and Tyers, M. (2001) 'Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication', *Nature,* 414, (6863), pp. 514-21.

Nasmyth, K. (2005) 'How do so few control so many?' *Cell,* 120, (6), pp. 739-46.

Nezi, L., Rancati, G., De Antoni, A., Pasqualato, S., Piatti, S. and Musacchio, A. (2006) 'Accumulation of Mad2-Cdc20 complex during spindle checkpoint activation requires binding of open and closed conformers of Mad2 in Saccharomyces cerevisiae', *J Cell Biol,* 174, (1), pp. 39-51.

Nicklas, R. B. and Koch, C. A. (1969) 'Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes', *J Cell Biol,* 43, (1), pp. 40-50.

Nilsson, J., Yekezare, M., Minshull, J. and Pines, J. (2008) 'The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction', *Nat Cell Biol,* 10, (12), pp. 1411-20.

Ohta, T., Michel, J. J. and Xiong, Y. (1999) 'Association with cullin partners protects ROC proteins from proteasome-dependent degradation', *Oncogene,* 18, (48), pp. 6758-66.

Osborne, M. A., Dalton, S. and Kochan, J. P. (1995) 'The yeast tribrid system--genetic detection of trans-phosphorylated ITAM-SH2-interactions', *Biotechnology (N Y),* 13, (13), pp. 1474-8.

Page, S. L., Nielsen, R. J., Teeter, K., Lake, C. M., Ong, S., Wright, K. R., Dean, K. L., Agne, D., Gilliland, W. D. and Hawley, R. S. (2007) 'A germline clone screen for meiotic mutants in Drosophila melanogaster', *Fly (Austin),* 1, (3), pp. 172-81.

Passmore, L. A., Booth, C. R., Venien-Bryan, C., Ludtke, S. J., Fioretto, C., Johnson, L. N., Chiu, W. and Barford, D. (2005) 'Structural analysis of the anaphase-promoting complex reveals multiple active sites and insights into polyubiquitylation', *Mol Cell,* 20, (6), pp. 855-66.

Pereg, Y., Lam, S., Teunisse, A., Biton, S., Meulmeester, E., Mittelman, L., Buscemi, G., Okamoto, K., Taya, Y., Shiloh, Y. and Jochemsen, A. G. (2006) 'Differential roles of ATM- and Chk2-mediated phosphorylations of Hdmx in response to DNA damage', *Mol Cell Biol,* 26, (18), pp. 6819-31.

Peters, J. M. (2002) 'The anaphase-promoting complex: proteolysis in mitosis and beyond', *Mol Cell,* 9, (5), pp. 931-43.

Peters, J. M. (2006) 'The anaphase promoting complex/cyclosome: a machine designed to destroy', *Nat Rev Mol Cell Biol,* 7, (9), pp. 644-56.

Pfleger, C. M. and Kirschner, M. W. (2000) 'The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1', *Genes Dev,* 14, (6), pp. 655-65.

Pickart, C. M. (2001) 'Ubiquitin enters the new millennium', *Mol Cell,* 8, (3), pp. 499-504.

Pinsky, B. A. and Biggins, S. (2005) 'The spindle checkpoint: tension versus attachment', *Trends Cell Biol,* 15, (9), pp. 486-93.

Pinsky, B. A., Kung, C., Shokat, K. M. and Biggins, S. (2006) 'The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores', *Nat Cell Biol,* 8, (1), pp. 78-83.

Poddar, A., Stukenberg, P. T. and Burke, D. J. (2005) 'Two complexes of spindle checkpoint proteins containing Cdc20 and Mad2 assemble during mitosis independently of the kinetochore in Saccharomyces cerevisiae', *Eukaryot Cell,* 4, (5), pp. 867-78.

Prinz, S., Hwang, E. S., Visintin, R. and Amon, A. (1998) 'The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis', *Curr Biol,* 8, (13), pp. 750-60.

Raff, J. W., Jeffers, K. and Huang, J. Y. (2002) 'The roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time', *J Cell Biol,* 157, (7), pp. 1139-49.

Rieder, C. L., Cole, R. W., Khodjakov, A. and Sluder, G. (1995) 'The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores', *J Cell Biol,* 130, (4), pp. 941-8.

Rubin, G. M. and Spradling, A. C. (1983) 'Vectors for P element-mediated gene transfer in Drosophila', *Nucleic Acids Res,* 11, (18), pp. 6341-51.

Salmon, E. D., Cimini, D., Cameron, L. A. and DeLuca, J. G. (2005) 'Merotelic kinetochores in mammalian tissue cells', *Philos Trans R Soc Lond B Biol Sci,* 360, (1455), pp. 553-68.

Salmon, M. D. and Ahluwalia, J. (2009) 'Relationship between calcium release and NADPH oxidase inhibition in human neutrophils', *Biochem Biophys Res Commun,* 384, (1), pp. 87-92.

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K. and Linn, S. (2004) 'Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints', *Annu Rev Biochem,* 73, pp. 39-85.

Sancar, A. and Reardon, J. T. (2004) 'Nucleotide excision repair in E. coli and man', *Adv Protein Chem,* 69, pp. 43-71.

Santaguida, S. and Musacchio, A. (2009) 'The life and miracles of kinetochores', *Embo J,* 28, (17), pp. 2511-31.

Schmidt, M., Budirahardja, Y., Klompmaker, R. and Medema, R. H. (2005) 'Ablation of the spindle assembly checkpoint by a compound targeting Mps1', *EMBO Rep,* 6, (9), pp. 866-72.

Schramm, C., Janke, C. and Schiebel, E. (2001) 'Molecular dissection of yeast spindle pole bodies by two hybrid, in vitro binding, and co-purification', *Methods Cell Biol,* 67, pp. 71-94.

Sczaniecka, M., Feoktistova, A., May, K. M., Chen, J. S., Blyth, J., Gould, K. L. and Hardwick, K. G. (2008) 'The spindle checkpoint functions of Mad3 and Mad2 depend on a Mad3 KEN box-mediated interaction with Cdc20-anaphase-promoting complex (APC/C)', *J Biol Chem,* 283, (34), pp. 23039-47.

Shah, J. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M. and Cleveland, D. W. (2004) 'Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing', *Curr Biol,* 14, (11), pp. 942-52.

Shah, J. V. and Cleveland, D. W. (2000) 'Waiting for anaphase: Mad2 and the spindle assembly checkpoint', *Cell,* 103, (7), pp. 997-1000.

Shapiro, L., Heidenreich, K. A., Meintzer, M. K. and Dinarello, C. A. (1998) 'Role of p38 mitogen-activated protein kinase in HIV type 1 production in vitro', *Proc Natl Acad Sci U S A,* 95, (13), pp. 7422-6.

Shaw, S. L., Maddox, P., Skibbens, R. V., Yeh, E., Salmon, E. D. and Bloom, K. (1998) 'Nuclear and spindle dynamics in budding yeast', *Mol Biol Cell,* 9, (7), pp. 1627-31.

Shiloh, Y. (2003) 'ATM and related protein kinases: safeguarding genome integrity', *Nat Rev Cancer,* 3, (3), pp. 155-68.

Sironi, L., Mapelli, M., Knapp, S., De Antoni, A., Jeang, K. T. and Musacchio, A. (2002) 'Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint', *Embo J,* 21, (10), pp. 2496-506.

Skoufias, D. A., Andreassen, P. R., Lacroix, F. B., Wilson, L. and Margolis, R. L. (2001) 'Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints', *Proc Natl Acad Sci U S A,* 98, (8), pp. 4492-7.

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J. and Harper, J. W. (1997) 'F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex', *Cell,* 91, (2), pp. 209-19.

Steen, J. A., Steen, H., Georgi, A., Parker, K., Springer, M., Kirchner, M., Hamprecht, F. and Kirschner, M. W. (2008) 'Different phosphorylation states of the anaphase promoting complex in response to antimitotic drugs: a quantitative proteomic analysis', *Proc Natl Acad Sci U S A,* 105, (16), pp. 6069-74.

Stern, B. M. and Murray, A. W. (2001) 'Lack of tension at kinetochores activates the spindle checkpoint in budding yeast', *Curr Biol,* 11, (18), pp. 1462-7.

Stucke, V. M., Baumann, C. and Nigg, E. A. (2004) 'Kinetochore localization and microtubule interaction of the human spindle checkpoint kinase Mps1', *Chromosoma,* 113, (1), pp. 1-15.

Stucke, V. M., Sillje, H. H., Arnaud, L. and Nigg, E. A. (2002) 'Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication', *Embo J,* 21, (7), pp. 1723-32.

Sudakin, V., Chan, G. K. and Yen, T. J. (2001) 'Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2', *J Cell Biol,* 154, (5), pp. 925-36.

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V. and Hershko, A. (1995) 'The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis', *Mol Biol Cell,* 6, (2), pp. 185-97.

Tang, Z., Bharadwaj, R., Li, B. and Yu, H. (2001a) 'Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1', *Dev Cell,* 1, (2), pp. 227-37.

Tang, Z., Li, B., Bharadwaj, R., Zhu, H., Ozkan, E., Hakala, K., Deisenhofer, J. and Yu, H. (2001b) 'APC2 Cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex', *Mol Biol Cell,* 12, (12), pp. 3839-51.

Tang, Z., Shu, H., Oncel, D., Chen, S. and Yu, H. (2004a) 'Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint', *Mol Cell,* 16, (3), pp. 387-97.

Tang, Z., Sun, Y., Harley, S. E., Zou, H. and Yu, H. (2004b) 'Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis', *Proc Natl Acad Sci U S A,* 101, (52), pp. 18012-7.

Taylor, S. S., Ha, E. and McKeon, F. (1998) 'The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase', *J Cell Biol,* 142, (1), pp. 1-11.

Taylor, S. S., Hussein, D., Wang, Y., Elderkin, S. and Morrow, C. J. (2001) 'Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells', *J Cell Sci,* 114, (Pt 24), pp. 4385-95.

Taylor, S. S., Scott, M. I. and Holland, A. J. (2004) 'The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation', *Chromosome Res,* 12, (6), pp. 599-616.

Telzer, B. R., Moses, M. J. and Rosenbaum, J. L. (1975) 'Assembly of microtubules onto kinetochores of isolated mitotic chromosomes of HeLa cells', *Proc Natl Acad Sci U S A,* 72, (10), pp. 4023-7.

Thornton, B. R. and Toczyski, D. P. (2006) 'Precise destruction: an emerging picture of the APC', *Genes Dev,* 20, (22), pp. 3069-78.

Tighe, A., Staples, O. and Taylor, S. (2008) 'Mps1 kinase activity restrains anaphase during an unperturbed mitosis and targets Mad2 to kinetochores', *J Cell Biol,* 181, (6), pp. 893-901.

Topper, L. M., Campbell, M. S., Tugendreich, S., Daum, J. R., Burke, D. J., Hieter, P. and Gorbsky, G. J. (2002) 'The dephosphorylated form of the anaphase-promoting complex protein Cdc27/Apc3 concentrates on kinetochores and chromosome arms in mitosis', *Cell Cycle,* 1, (4), pp. 282-92.

Tulu, U. S., Fagerstrom, C., Ferenz, N. P. and Wadsworth, P. (2006) 'Molecular requirements for kinetochore-associated microtubule formation in mammalian cells', *Curr Biol,* 16, (5), pp. 536-41.

Tunquist, B. J., Schwab, M. S., Chen, L. G. and Maller, J. L. (2002) 'The spindle checkpoint kinase bub1 and cyclin e/cdk2 both contribute to the establishment of meiotic metaphase arrest by cytostatic factor', *Curr Biol,* 12, (12), pp. 1027-33.

Tyers, M. and Jorgensen, P. (2000) 'Proteolysis and the cell cycle: with this RING I do thee destroy', *Curr Opin Genet Dev,* 10, (1), pp. 54-64.

Uchida, K. S., Takagaki, K., Kumada, K., Hirayama, Y., Noda, T. and Hirota, T. (2009) 'Kinetochore stretching inactivates the spindle assembly checkpoint', *J Cell Biol,* 184, (3), pp. 383-90.

van Leuken, R., Clijsters, L. and Wolthuis, R. (2008) 'To cell cycle, swing the APC/C', *Biochim Biophys Acta,* 1786, (1), pp. 49-59.

Vigneron, S., Prieto, S., Bernis, C., Labbe, J. C., Castro, A. and Lorca, T. (2004) 'Kinetochore localization of spindle checkpoint proteins: who controls whom?' *Mol Biol Cell,* 15, (10), pp. 4584-96.

Vink, M., Simonetta, M., Transidico, P., Ferrari, K., Mapelli, M., De Antoni, A., Massimiliano, L., Ciliberto, A., Faretta, M., Salmon, E. D. and Musacchio, A. (2006) 'In vitro FRAP identifies the minimal requirements for Mad2 kinetochore dynamics', *Curr Biol,* 16, (8), pp. 755-66.

Visconti, R., Palazzo, L. and Grieco, D. (2010) 'Requirement for proteolysis in spindle assembly checkpoint silencing', *Cell Cycle,* 9, (3), pp. 564-9.

Visintin, R., Prinz, S. and Amon, A. (1997) 'CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis', *Science,* 278, (5337), pp. 460-3.

Vodermaier, H. C. (2004) 'APC/C and SCF: controlling each other and the cell cycle', *Curr Biol,* 14, (18), pp. R787-96.

Vodermaier, H. C. and Peters, J. M. (2004) 'APC activators caught by their tails?' *Cell Cycle,* 3, (3), pp. 265-6.

von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K. I., Soderberg, O., Kerppola, T. K. and Larsson, L. G. (2003) 'The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription', *Mol Cell,* 11, (5), pp. 1189-200.

Walczak, C. E. and Heald, R. (2008) 'Mechanisms of mitotic spindle assembly and function', *Int Rev Cytol,* 265, pp. 111-58.

Wang, M., Wang, T. and Liu, L. (1997) '[Expression of mitogen-activated protein kinase in hypertrophic myocardium in SHRsp]', *Zhonghua Yi Xue Za Zhi,* 77, (9), pp. 680-2.

Waters, J. C., Chen, R. H., Murray, A. W. and Salmon, E. D. (1998) 'Localization of Mad2 to kinetochores depends on microtubule attachment, not tension', *J Cell Biol,* 141, (5), pp. 1181-91.

Waters, J. C., Mitchison, T. J., Rieder, C. L. and Salmon, E. D. (1996) 'The kinetochore microtubule minus-end disassembly associated with poleward flux produces a force that can do work', *Mol Biol Cell,* 7, (10), pp. 1547-58.

Weaver, B. A. and Cleveland, D. W. (2006) 'Does aneuploidy cause cancer?' *Curr Opin Cell Biol,* 18, (6), pp. 658-67.

Weaver, B. A., Silk, A. D. and Cleveland, D. W. (2006) 'Cell biology: nondisjunction, aneuploidy and tetraploidy', *Nature,* 442, (7104), pp. E9-10; discussion E10.

Weinstein, J. (1997) 'Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1', *J Biol Chem,* 272, (45), pp. 28501-11.

Wigge, P. A. and Kilmartin, J. V. (2001) 'The Ndc80p complex from Saccharomyces cerevisiae contains conserved centromere components and has a function in chromosome segregation', *J Cell Biol,* 152, (2), pp. 349-60.

Winey, M. and Huneycutt, B. J. (2002) 'Centrosomes and checkpoints: the MPS1 family of kinases', *Oncogene,* 21, (40), pp. 6161-9.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J. and Harper, J. W. (1999) 'The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro', *Genes Dev,* 13, (3), pp. 270-83.

Wittmann, T., Hyman, A. and Desai, A. (2001) 'The spindle: a dynamic assembly of microtubules and motors', *Nat Cell Biol,* 3, (1), pp. E28-34.

Wong, O. K. and Fang, G. (2006) 'Loading of the 3F3/2 antigen onto kinetochores is dependent on the ordered assembly of the spindle checkpoint proteins', *Mol Biol Cell,* 17, (10), pp. 4390-9.

Wu, H., Lan, Z., Li, W., Wu, S., Weinstein, J., Sakamoto, K. M. and Dai, W. (2000) 'p55CDC/hCDC20 is associated with BUBR1 and may be a downstream target of the spindle checkpoint kinase', *Oncogene,* 19, (40), pp. 4557-62.

Xiong, H., Rivero, F., Euteneuer, U., Mondal, S., Mana-Capelli, S., Larochelle, D., Vogel, A., Gassen, B. and Noegel, A. A. (2008) 'Dictyostelium sun-1 connects the centrosome to chromatin and ensures genome stability', *Traffic,* 9, (5), pp. 708-24.

Xu, Q., Zhu, S., Wang, W., Zhang, X., Old, W., Ahn, N. and Liu, X. (2009) 'Regulation of kinetochore recruitment of two essential mitotic spindle checkpoint proteins by Mps1 phosphorylation', *Mol Biol Cell,* 20, (1), pp. 10-20.

Yamaguchi, S., Okayama, H. and Nurse, P. (2000) 'Fission yeast Fizzy-related protein srw1p is a G(1)-specific promoter of mitotic cyclin B degradation', *Embo J,* 19, (15), pp. 3968-77.

Yamano, H., Gannon, J., Mahbubani, H. and Hunt, T. (2004) 'Cell cycle-regulated recognition of the destruction box of cyclin B by the APC/C in Xenopus egg extracts', *Mol Cell,* 13, (1), pp. 137-47.

Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K. and Tai, T. (2002) 'E3 ubiquitin ligase that recognizes sugar chains', *Nature,* 418, (6896), pp. 438-42.

Yu, H. (2006) 'Structural activation of Mad2 in the mitotic spindle checkpoint: the two-state Mad2 model versus the Mad2 template model', *J Cell Biol,* 173, (2), pp. 153-7.

Yu, H. (2007) 'Cdc20: a WD40 activator for a cell cycle degradation machine', *Mol Cell,* 27, (1), pp. 3-16.

Yudkovsky, Y., Shteinberg, M., Listovsky, T., Brandeis, M. and Hershko, A. (2000) 'Phosphorylation of Cdc20/fizzy negatively regulates the mammalian cyclosome/APC in the mitotic checkpoint', *Biochem Biophys Res Commun,* 271, (2), pp. 299-304.

Yuen, K. W., Montpetit, B. and Hieter, P. (2005) 'The kinetochore and cancer: what's the connection?' *Curr Opin Cell Biol,* 17, (6), pp. 576-82.

Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. (1998) 'Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex', *Science,* 282, (5394), pp. 1721-4.

Zhai, Y., Kronebusch, P. J., Simon, P. M. and Borisy, G. G. (1996) 'Microtubule dynamics at the G2/M transition: abrupt breakdown of cytoplasmic microtubules at nuclear envelope breakdown and implications for spindle morphogenesis', *J Cell Biol,* 135, (1), pp. 201-14.

Zhang, P., Wong, C., DePinho, R. A., Harper, J. W. and Elledge, S. J. (1998) 'Cooperation between the Cdk inhibitors p27(KIP1) and p57(KIP2) in the control of tissue growth and development', *Genes Dev,* 12, (20), pp. 3162-7.

Zhang, Y. and Lees, E. (2001) 'Identification of an overlapping binding domain on Cdc20 for Mad2 and anaphase-promoting complex: model for spindle checkpoint regulation', *Mol Cell Biol,* 21, (15), pp. 5190-9.

Zhang, Y. W., Hunter, T. and Abraham, R. T. (2006) 'Turning the replication checkpoint on and off', *Cell Cycle,* 5, (2), pp. 125-8.

Zhao, Y. and Chen, R. H. (2006) 'Mps1 phosphorylation by MAP kinase is required for kinetochore localization of spindle-checkpoint proteins', *Curr Biol,* 16, (17), pp. 1764-9.

Zich, J. and Hardwick, K. G. (2010) 'Getting down to the phosphorylated 'nuts and bolts' of spindle checkpoint signalling', *Trends Biochem Sci,* 35, (1), pp. 18-27.