Genetic dissection of acentrosomal spindle formation: the role of the Cdc2 kinase pathway

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Declaration of authenticity

I declare that this thesis was composed by myself and that the research presented within is my own work, except where explicitly stated and acknowledgement is given.
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Abbreviations

Cdk = Cyclin dependent kinase
K-MTs = Kinetochore microtubules
MCC = Mitotic checkpoint complex
FEAR = Cdc fourteen early anaphase
MEN = Mitotic exit network
DSB = Double strand break
HRR = Homologue recognition region
SC = Synaptonemal complex
APC/C = Anaphase promoting complex/Cyclosome
MAPK = Mitogen activated protein kinase
MAPs = Microtubule associated proteins
Ncd = Non-claret disjunction
MTOC = Microtubule organising centre
γ-TuRC = γ-Tubulin ring complex
γ-TuSC = γ-Tubulin small complex
PCM = Pericentriolar material
Plk1 = Polo-like kinases
Msps = Mini spindles
SAFs = Spindle assembly factors
NLS = Nuclear localisation signal
CPC = Chromosomal passenger complex
GVB = Germinal vesicle breakdown
rem = remnants
PCR = Polymerase chain reaction
RNAi = RNA interference
wisp = wispy
Summary

In many animals, female meiotic spindles lack centrosomes and their formation is instead driven by chromosomes. This process is not well understood so I examined two mutants from a screen for female sterile mutations with acentrosomal spindle defects. I found that *remnants* (*rem*) disrupted spindle morphology, chromosome alignment and microtubule dynamics in non-activated oocytes. *rem* encodes Cks30A, a conserved subunit of Cdc2. I also found that essential pole proteins, Msps and D-TACC were often mislocalised to the equator and that Cks30A is involved in the modification of D-TACC. The second mutant, *msps-like*, had tripolar spindles in non-activated oocytes. I found that *msps-like* encodes Dweel, a negative regulator of Cdc2 activity. Dweel is also required for the modification of D-TACC in a Cdc2 dependent manner. I found that Cdc2 itself is required for correct spindle morphology in female meiosis. I have shown that Cdc2, the Cks30A subunit and positive and negative regulators of Cdc2 are all required for acentrosomal spindle formation and that Cdc2 is a central figure in a kinase pathway required for multiple facets of acentrosomal spindle formation.

To gain new insights in the formation of female meiotic spindles I took part in a screen for metaphase I arrested spindle mutants in female meiosis on the X chromosome of *Drosophila*. I found mutants defective in chromosome alignment, spindle morphology, spindle unification and microtubule association around individual chromosomes. I characterised several of the mutants found in this screen and established complementation groups for all of the mutants discovered.
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1. Introduction

1.1 The cell cycle

1.1.1 Cell cycle control

The eukaryotic cell cycle consists of four phases, G₁, S, G₂ and M (Fig 1.1). The gap phases of G₁ and G₂ allow the cell to grow and increase in mass before S phase and M phase respectively. S phase is the stage where DNA is replicated and M phase includes the segregation of duplicated chromosomes and cell division.

Progression through the cell cycle is orchestrated by cyclin dependent kinases (Cdks) and Cyclins. The Cdk is activated when bound to a Cyclin. Each Cyclin is expressed and destroyed at particular stages in the cell cycle, regulating the activity and substrate specificity of the Cdks. D type Cyclin (D₁, D₂ and D₃) levels are controlled by the extracellular mitogenic environment and act as links between the extracellular environment and the core cell cycle machinery. E type cyclins (E₁ and E₂) are expressed from late G₁ until the end of S phase. Cyclins A and B rise during interphase and fall during mitosis. Cyclins are targeted for proteasome mediated destruction through ubiquitination by the SCF complex in G₁ and by the Anaphase promoting complex (APC) in mitosis. The irreversibility of protein degradation ensures the cell cycle moves in one direction only.

In yeast, there is only one Cdk, fission yeast Cdc2 (Nurse and Thuriaux, 1980) or budding yeast cdc28p (Reed et al., 1982). In fission yeast, Cdc2/Cyclin B can initiate S phase at low activity and mitosis at high activity in the absence of other G₁ cyclins (Fisher and Nurse, 1996). In mammals, there are a family of Cdks that can bind to specific cyclins. Cdk4 and Cdk6 bind Cyclin D, Cdk2 can bind Cyclin E and Cyclin A, and Cdk1 can bind Cyclin A and Cyclin B.
In a mammalian cell, Cdk4 and Cdk6 are activated by Cyclin D in early G1. Cdk4 and/or Cdk6 complexed with Cyclin D can phosphorylate the Retinoblastoma protein, relieving the inhibition of Cyclin E transcription (Sherr and Roberts, 1999) (Fig 1.1A). Cdk2/Cyclin E complex can then complete the hyperphosphorylation of Retinoblastoma protein, permitting E2F mediated transcription of genes required for entry into S phase (Fig 1.1B). Cdk2/Cyclin A regulates progression through S phase and Cdk1/Cyclin A allows transition into G2 (Fig 1.1C and Fig 1.1D). Cdk1 exists in an inactive phosphorylated form and an active unphosphorylated form. Activation of the phosphatase, Cdc25, and inactivation of the kinase, Wee1, results in activation of Cdk1. This, in conjunction with high Cyclin B levels, results in peak Cdk1/Cyclin B activity and promotes entry into mitosis (Fig 1.1E). The destruction of Cyclin B by the APC allows exit from mitosis and completion of the cell cycle (Fig 1.1F). While this sequence of events is widely accepted, experiments with knockout mice have demonstrated that most cell cycle proteins are dispensable for mouse development and cell proliferation (Sherr and Roberts, 2004).

Cyclin dependent kinase substrate (Cks) is the third subunit of the Cdk/Cyclin complex that plays a role in the regulation of the cell cycle called (Brizuela et al., 1987; Draetta and Beach, 1988). SucI in fission yeast and CKSI in budding yeast are essential for cell viability (Hadwiger et al., 1989; Hayles et al., 1986; Hindley et al., 1987) and can physically associate with Cdks and active Cdk1/Cyclin complexes (Brizuela et al., 1987; Hadwiger et al., 1989). In humans and budding yeast, CKSI is required for the G1/S transition (Ganoth et al., 2001; Harper, 2001; Spruck et al., 2001; Tang and Reed, 1993). CKSI is an essential cofactor for SCFSkp2 ubiquitin ligase for p27kip1. Destruction of p27kip1, drives cells into S phase as p27 binds, and
negatively regulates, the controllers of S phase; Cyclin E/Cdk2 and Cylin A/Cdk2 (Pagano et al., 1995; Shirane et al., 1999; Vlach et al., 1997).

Cks also has a role in mitotic entry. Overexpression of *suc1* in fission yeast and of *CKS1* in budding yeast delays entry into mitosis (Hayles et al., 1986; Hindley et al., 1987; Richardson et al., 1990). Removal of *Xenopus* Cks, Xe-p9, at interphase prevents entry into mitosis as Xe-p9 strongly stimulates the regulatory phosphorylations of Cdc25, Myt1 and Wee1 that are carried out by the Cdc2/Cyclin B complex leading to further activation of Cdc2/Cyclin B (Patra and Dunphy, 1996; Patra et al., 1999). Conversely, excess Xe-p9 prevents the dephosphorylation of Cdc2 on Tyr15 keeping Cdc2 inactive and blocking entry into mitosis (Dunphy and Newport, 1989; Patra and Dunphy, 1996). Cks1 has a conserved anion binding domain consisting of Arg33, Arg42, Ser82, Trp85 and Arg102 which may represent recognition sites for Cdks or other phosphorylated protein targets with the phospho-epitope Ser/pSer/pThr/X (Bourne et al., 2000).

Regulation of mitotic exit also involves Cks. Cks appears to play a role in the destruction of Cyclin B in fission yeast, budding yeast, *Xenopus*, and *C. elegans* (Basi and Draetta, 1995; Kaiser et al., 1999; Moreno et al., 1989; Patra and Dunphy, 1996; Polinko and Strome, 2000).

Cks/Cdk1 can even promote transcription in budding yeast by recruiting the proteasome to promoter regions, facilitating transcription by remodelling transcriptional complexes or chromatin (Morris et al., 2003; Yu et al., 2005). One such gene is CDC20 which is the substrate specific activator of APC/C.

The concerted efforts of Cdks, Cyclins and Cks ensure that the cell cycle occurs in an orderly and accurate fashion.
1.1.2 Comparing mitosis and meiosis

Mitosis and meiosis are the two forms of cell division that occur in eukaryotic cells (Fig 1.3). Mitosis occurs in somatic cells where the cell divides to produce two genetically identical cells. Meiosis is a specialised cell division, required for sexual reproduction, in which the cell undergoes two successive rounds of nuclear division, with only one round of DNA replication to produce four haploid daughter cells.

There are several differences between mitosis and meiosis;

1. Mitotic division involves one round of DNA duplication and nuclear division, while meiotic division involves two rounds of nuclear division and only one round of DNA duplication.

2. In meiosis homologous chromosomes can pair and recombine; unlike mitosis, where homologous chromosomes segregate independently.

3. Mitosis results in two diploid cells that are identical to the original cell, whereas meiosis results in four genetically unique haploid cells.

Despite these differences there are common mechanisms by which cell division occurs in mitosis and meiosis. In meiosis II and mitosis, sister chromatids are aligned and segregated into opposite cells. I will discuss mitosis and meiosis in more detail separately.

1.1.3 Cell division in mitosis

The process of cell division is a highly ordered event with several distinct phases that must occur in sequence. Mitotic cell division involves only one round of nuclear division so I will explain it first.
Prophase marks the transition from G2 into mitosis (Fig 1.2A). At this stage the nuclear membrane is still intact and the duplicated DNA has begun to condense from their diffuse state in interphase to their chromosome appearance. Chromosomes undergo a 10,000 fold compaction that is essential for segregation during mitosis. Chromosome condensation involves the V-shaped Condensin complex, which binds to the DNA and introduces supercoils in an ATP-dependent manner (Hirano et al., 2001; Kimura and Hirano, 1997; Strick et al., 2004). It is believed that the V shaped arms of the Condensin complex may loop and clamp the DNA in place (Hirano and Hirano, 2002; Bazett-Jones et al., 2002). In eukaryotes, Histone H3 phosphorylation at Ser10 correlates with chromosome condensation during mitosis (Hendzel and Bazett-Jones, 1997; Van Hooser et al., 1998). Aurora B is the major physiological kinase for histone H3 phosphorylation in mitosis in eukaryotes, such as budding yeast and C. elegans (Hsu et al., 2000; Speliotes et al., 2000).

Topoisomerase II allows the formation of cytologically distinguishable sister chromatids in prometaphase (Gimenez-Abian et al., 1995; Losada et al., 2002). Each sister chromatid has a sequence of DNA known as the centromere. The centromere, in conjunction with centromeric proteins, is essential for chromosome segregation and holding sister chromatids together. Sister chromatid cohesion is maintained by the cohesin complex, which consists of Smc1, Smc3, Scc1 and Scc3 (Huang and Laurent, 2004; Lengronne et al., 2004). The interphase microtubule network disassembles and begins to form the mitotic spindle. The mitotic spindle is a bipolar structure consisting of two centrosomes, microtubules and microtubule-associated proteins (MAPs).
In higher eukaryotes, dissolution of the nuclear membrane marks the entry into prometaphase (Fig 1.2B). Nuclear envelope breakdown is believed to be triggered by Cdk1/Cyclin B phosphorylating lamins and other nuclear membrane proteins (Enoch et al., 1991; Collas, 1999). Phosphorylation of lamins results in their depolymerisation (Peter et al., 1990; Ward and Kirschner, 1990). Dynein may also contribute to breakdown by generating invaginations in the nuclear envelope, resulting in permeabilisation of the membrane (Salina et al., 2002).

In prometaphase, microtubules of the mitotic spindle must capture chromosomes. The capture of chromosomes is facilitated by kinetochores. The animal kinetochore is composed of an inner and outer layer. The inner kinetochore normally forms on highly repetitive DNA sequences in the centromere of the DNA (Zinkowski et al., 1991; Brinkley et al., 1992). The outer kinetochore forms on the chromosome during nuclear envelope breakdown (Brinkley and Stubblefield, 1966; Ris and Witt, 1981; McEwen et al., 1993). In metazoans, CENP-A binds to the centromere and directs the assembly of outer kinetochore proteins.

In prometaphase of animal cells, spindle microtubules search the cytoplasm for chromosomes. Microtubules that encounter a kinetochore become stabilised whereas those that do not soon depolymerise (Hayden et al., 1990). Once attached the microtubules are referred to as kinetochore microtubules (K-MTs). The alignment of chromosomes to the spindle equator is also mediated by the kinetochore. The kinetochore can switch K-MTs from a state of plus-end depolymerization to polymerization. This allows poleward and anti-poleward chromosome movement during alignment in budding yeast and mammalian cell lines (He et al., 2001; Pearson et al., 2001; Skibbens et al., 1993). Chromosome alignment
is likely to be mediated by the minus-end-directed motor activity of cytoplasmic Dynein, which is abundant in unattached kinetochores (Echeverri et al., 1996; Sharp et al., 2000b; Vaisberg et al., 1993). In yeast and *C. elegans*, the Ndc80 complex is crucial in maintaining kinetochore-microtubule attachments during the tensions involved in achieving chromosome alignment (DeLuca et al., 2002; Desai et al., 2003; Howe et al., 2001).

Metaphase is the stage when both kinetochores of sister chromatids are attached to microtubules from opposite poles and aligned at the centre of the spindle (Fig 1.2C). The tension generated from both poles holds the chromosomes in place. The spindle checkpoint is a surveillance mechanism that prevents transition from metaphase to anaphase until sister chromatids have amphitelic (bipolar) attachments to the spindle. The core spindle checkpoint proteins are Mad1, Mad2, BubR1 (Mad3 in yeast), Bub1, Bub3 and Mps1 (May and Hardwick, 2006). Rod, Zw10 and CENP-E are additional checkpoint proteins in higher eukaryotes (Karess, 2005; Mao et al., 2003). The spindle checkpoint is activated in response to a lack of microtubule attachment at kinetochores and/or a lack of microtubule tension (Clute and Pines, 1999; Rieder et al., 1995; Skoufias et al., 2001). The Mad and Bub proteins accumulate on unattached kinetochores and are ideally placed to monitor microtubule-kinetochore attachments. The downstream target of the spindle checkpoint is the APC/C\(^{Cdc20}\) which can ubiquitinate Securin releasing Separase, which destroys Cohesin allowing sister chromatids to be pulled to opposite poles (Fig 1.4). Cdc20 is the key target of the spindle checkpoint and most models involve the sequestration of Cdc20 by forming complexes with checkpoint proteins such as
the mitotic checkpoint complex (MCC) (Fang, 2002; Hwang et al., 1998; Kim et al., 1998; Sudakin et al., 2001).

Once all the kinetochores have bipolar attachments, it is vital that the checkpoint is switched off. Mechanisms involve the removal of Mad1/Mad2 and BubR1 from the kinetochore by Dynein (Howell et al., 2001) and disruption of MCC formation through the phosphorylation of Mad2 (Mao et al., 2003). In mammalian cells, the binding of CENP-E downregulates BubR1 kinase activity, resulting in checkpoint silencing (Wassmann et al., 2003).

Anaphase consists of two phases. In anaphase A, chromosomes move towards opposite poles, via the kinetochore, as the kinetochore microtubules shorten (de Gramont and Cohen-Fix, 2005; Ganem and Compton, 2006). Anaphase B involves elongation of polar microtubules to increase the distance between the spindle poles (Brust-Mascher et al., 2004; Higuchi and Uhlmann, 2005).

In budding yeast, the Cdc fourteen early anaphase (FEAR) network is required for completion of chromosome separation (D'Amours et al., 2004) and partial release of Cdc14 phosphatase from the nucleolus (Azzam et al., 2004; Stegmeier et al., 2002). This triggers the mitotic exit network (MEN), which is believed to promote and maintain Cdc14 in a released state (Stegmeier et al., 2002). Cdc14 dephosphorylates and activates Cdh1, as well as causing an accumulation of Cdk inhibitor Sic1 (Prinz and Amon, 1999). The combined activities of APC/C\textsuperscript{Cdh1} and Sic1 eliminate Cdk1 activity, leading to mitotic exit (D'Amours and Amon, 2004).

Telophase is where the separated daughter chromosomes have reached their respective poles (Fig 1.2F). The kinetochore microtubules have disappeared and the
polar microtubules continue to elongate. The chromosomes start to decondense and this exposes binding sites for membrane vesicles, which accumulate on chromatin. Lamin B receptor can bind chromatin in vitro and target membrane vesicles to chromatin (Collas et al., 1996; Pyrpasopoulou et al., 1996). RanGTPase induces chromosome condensation and nuclear envelope assembly (Zhang and Clarke, 2000; Zhang et al., 2002).

The final stage, cytokinesis, involves the division of the cytoplasm to form two individual daughter cells (Fig 1.2G). The first stage of cytokinesis is determination of the division plane by astral microtubules, in C. elegans and mammals (Dechant and Glotzer, 2003; Matuliene and Kuriyama, 2002; Mollinari et al., 2002), or by the central spindle in Drosophila (Adams et al., 1998; Giansanti et al., 1998). The central spindle consists of microtubule bundles with overlapping plus ends. Once the division plane is determined, a cleavage furrow must form by assembling an actomyosin-based contractile ring. The small GTP-binding protein RhoA, induces contractile ring assembly through nucleation and polymerization of actin (Li and Higgs, 2003; Sagot et al., 2002) and the activation of the motor activity of myosin (Kawano et al., 1999; Totsukawa et al., 1999). The mitotic exit network is required for a fully functional actomyosin ring that contracts (Bi, 2001; Schmidt et al., 2002).

Once the contractile ring is assembled it constricts, leading to furrow ingression. As furrow ingression progresses, new membrane is added to allow separation of the daughter cells. In animals, new membrane is assembled by targeted secretion of membrane transport machinery, including Syntaxins and RabGTPases (Burgess et al., 1997; Conner and Wessel, 1999; Skop et al., 2001).
Cytokinesis needs to be co-ordinated with the nuclear cycle. There are several factors required for cytokinesis that are inhibited in metaphase, such as the light chain of myosin (Yamakita et al., 1994) and formation of the central spindle (Mishima et al., 2004). This could explain why cytokinesis does not initiate until anaphase onset.

The completion of mitosis results in two separate daughter cells that are genetically identical. These cells can now enter their own cell-cycle and repeat the process.

1.1.4 Cell division in meiosis

The goal of meiotic division is to produce haploid germ cells. There is a maternal and paternal copy of each chromosome, except for the sex chromosomes. Meiotic cell division results in the separation of maternal and paternal copies.

In prophase of the first meiotic division (Meiosis I), homologous chromosomes must be able to identify each other and pair. There are two classes of chromosome pairing mechanisms, double strand break (DSB) dependent and DSB-independent. In budding yeast, DSB and early recombination intermediates are required for stable pairing between homologues (Peoples et al., 2002). DSB machinery is thought to play a structural role in pairing, as catalytically inactive Spo11p, the protein required for generating meiotic DSB, can still have pairing of homologues (Cha et al., 2000; Lichten, 2001).

Examples of DSB-independent homologue pairing can be seen in *C. elegans* and *Drosophila*, which can pair with no DSBs (Dernburg et al., 1998; MacQueen et al., 2002; McKim et al., 1998; Vazquez et al., 2002). In *Drosophila* there is a 240 bp
repeat sequence clustered on the X and Y chromosomes, which in multiple copies facilitates the pairing and segregation of X and Y chromosomes in male meiosis (McKee, 1996). There are several internal chromosome sites in *Drosophila* females that may also play a role in facilitating chromosome pairing (Hawley, 1980).

*C. elegans* has a homologue recognition region (HRR) at the end of each chromosome that can stabilize pairing even in the presence of mutants that block synaptonemal complex formation (MacQueen et al., 2002; McKim et al., 1988). In both *C. elegans* and *Drosophila* it is believed that sequence-specific binding proteins recognize these regions and the aggregation of these proteins leads to pairing.

The clustering of telomeres during the leptotene to zygotene transition in prophase, called the bouquet (Zickler and Kleckner, 1998) has been observed in all organisms except *C. elegans* and *Drosophila* (McKee, 2004). In maize, humans and mice, telomeres attach to the nuclear envelope and then move around the inner surface of the nuclear envelope until they come into contact with one another (Bass et al., 1997; Scherthan et al., 1996). The bouquet has been implicated in chromosome pairing and initiation of synapsis (Bass et al., 2000; Davis and Smith, 2001; Pfeifer et al., 2001; Yamamoto and Hiraoka, 2001).

In most organisms homolog pairing is stabilised by a tight axial association called synapsis, in which the four chromatids are brought into intimate alignment. Synapsis is defined by the formation of the synaptonemal complex (SC), which holds the homologues together (Zickler and Kleckner, 1998). DSBs are essential for SC formation in budding yeast, *Arabidopsis* and mammalian spermatocytes (Lichten, 2001). Sumoylation has also been implicated in the regulation of SC assembly (Cheng et al., 2006).
The SC is composed of a pair of lateral elements connected by transverse filaments that form the central region of this structure. The lateral elements are derived from the axial elements of meiotic chromosomes and the cohesin complex (Eijpe et al., 2003; Klein et al., 1999). The transverse filaments of the SC are involved in homolog exchanges, as mutants in transverse filament genes abolish exchange in *Drosophila* and *C. elegans* (MacQueen et al., 2002; Page and Hawley, 2001).

Another important structure in chromosome cohesion are the chiasmata, the physical manifestation of meiotic chromosome exchange, which locks the homologues together and maintains sister chromatid cohesion until anaphase when they are resolved (Nicklas et al., 1995; Ross et al., 1996).

The cohesin complex also plays a crucial role in maintaining chromosome cohesion throughout meiosis. The cohesin complex has meiosis specific isoforms of Smc1, RAD21 and SA1/SA2, which are SMC1β, Rec8 and STAG3 respectively (Parisi et al., 1999; Prieto et al., 2001; Revenkova et al., 2001). Cohesin binds to specific regions of chromosome arms and pericentric regions. Cohesin binding at pericentric regions requires specific centromere sequences (Megee et al., 1999; Tanaka et al., 1999).

Nuclear envelope breakdown marks the transition from prophase to prometaphase I. The bivalents congress to the spindle equator through the attachment of two kinetochores of each bivalent to opposite poles of the spindle. In order to separate homologous chromosomes to opposite poles in anaphase I, each pair of sister kinetochores must first function as a single unit and establish attachments to the same pole, “Co-oriented” (Kapoor and Compton, 2002). In budding yeast,
monopolar attachment of sister kinetochores is facilitated by proteins called monopolins (Rabitsch et al., 2003; Toth et al., 2000).

Anaphase I is initiated by cleavage of cohesin by Separase (Buonomo et al., 2000; Siomos et al., 2001). The removal of cohesin is selective so that cohesion at chromosome arms is lost, while centromeric cohesion is retained until the second meiotic division (Eijpe et al., 2003; Klein et al., 1999; Prieto et al., 2001; Revenkova et al., 2001). Centromeric cohesion allows sister chromatids to remain attached during anaphase. Centromeric cohesin is protected by Shugoshin protein Sgo1p, which localises to kinetochores, locally protecting cohesin from cleavage by Separase in fission yeast (Katis et al., 2004; Kiburz et al., 2005; Kitajima et al., 2004). In Drosophila, the Shugoshin MEI-S332 also protects centromeric cohesion but can be displaced by Polo kinase (Clarke et al., 2005; Kerrebrock et al., 1992). Mnd2, a meiosis specific antagonist of APC/C and Spo13 also contribute to maintenance of centromeric cohesion (Katis et al., 2004; Lee et al., 2004; Oelschlaegel et al., 2005; Penkner et al., 2005).

DNA replication has to be suppressed between meiosis I and meiosis II. Cyclin B appears to be required for this suppression, as Cyclin B is destroyed in late mitosis but only halved during the transition from meiosis I to meiosis II. Cyclin B levels are sustained by Mos, which activates the Mitogen activated protein kinase (MAPK) and the downstream Rsk kinase (Furuno et al., 1994; Gross et al., 2000). Rsk promotes Cyclin B synthesis and partially inhibits APC/C activity (Gross et al., 2000; Taieb et al., 2001).

Meiosis II is a very similar process to that of mitosis. Sister kinetochores make amphitelic attachments to microtubules and are segregated to opposite poles.
when centromeric cohesion is destroyed at the metaphase to anaphase transition. Cytokinesis results in the formation of four haploid gametes.

The final fate of the meiotic products differs depending on the organism and gender. In the case of spores in fungi, pollen tetrads in plants, or spermatozoa in animals, all four haploid gametes give rise to four different cells. However, in the female meiosis of animals one or two haploid nuclei are discarded and form polar bodies and only one haploid nucleus ends up in the oocyte (Schmidt et al., 2006).

Despite its apparent complexity, there is no clear evidence that meiosis evolved later than mitosis in eukaryotes (Cavalier-Smith, 2002).

1.1.5 The importance of accurate cell division

It is essential that mitosis and meiosis are fully functional in eukaryotes. If mitosis fails, it leads to death for unicellular and multicellular organisms. If meiosis fails the organism is rendered sterile and cannot pass on its genes to successive generations. If a cell divides but fails to segregate its chromosomes accurately (non-disjunction or chromosome loss) the consequences can be as serious as failure. Non-disjunction causes aneuploidy, a state in which cells have an abnormal chromosome number. Monosomy is 2n - 1 (only 1 copy of a chromosome) and trisomy is 2n + 1 (3 copies of a chromosome).

In mitosis, aneuploidy is seen in the majority of solid cancers (Brinkley, 2001). It is still debated whether aneuploidy is a consequence or cause of tumorigenesis. There are several lines of evidence for aneuploidy playing an active role in tumorigenesis. One is that the loss or gain of chromosomes in each generation of aneuploid cell lines is higher than that of diploid colon cancer lines (Duesberg et
al., 1998). Additional evidence is that mutations in spindle checkpoint occur in cancers such as BubR1 in colorectal cancers (Cahill et al., 1998), Mad2 in breast cancer (Li and Benezra, 1996) and Mad1 in leukaemia (Jin et al., 1998). Causes of aneuploidy, other than spindle checkpoint mutations, can be the formation of defective spindles as a result of supernumerary centrosomes or mutations in spindle assembly factors (Brinkley, 2001; Pihan and Doxsey, 1999). Aneuploidy may contribute to the progression of aggressive tumours through polysomy of chromosomes containing oncogenes and genes that affect the balance of spindle formation apparatus (Dey, 2004).

The effects of non-disjunction and aneuploidy in meiosis are even further reaching. In humans, 50% of spontaneous abortions are chromosomally aneuploid with trisomies accounting for 50% of those abortions (Hassold et al., 1980). In addition, almost 1 in 300 liveborn infants and 1 in 20 clinically recognised pregnancies are aneuploid (Hassold and Hunt, 2001). Non-disjunction in maternal meiosis I is the most common cause of trisomies such as trisomy 21, causing “Down syndrome”, which occurs in 1-2:1000 livebirths (Hassold and Jacobs, 1984; Juberg and Mowrey, 1983). Defects in meiosis II can cause trisomies, such as trisomy 18 “Edward’s syndrome” (Eggermann et al., 1996; Fisher et al., 1995). Non-disjunction in meiosis II is associated with increased recombination occurring in meiosis I, suggesting that defects in meiosis I are the underlying cause of aneuploidy (Lamb et al., 1996).

Some women have a high risk of producing aneuploid oocytes (Mahmood et al., 2000; Pujol et al., 2003), suggesting that genetic factors are responsible (Brown et al., 2000). A two-hit model has been proposed for aneuploidy in meiosis (Lamb et
al., 1996). The first hit is a mutation that makes chromosomes susceptible to non-disjunction such as aberrant recombination resulting in susceptible chiasmate structures. The second hit could involve any number of meiotic structures, such as spindle defects, causing non-disjunction in the prone chiasma.

*Drosophila* has become an excellent model for the study of meiotic aneuploidy, and mutations in genes for spindle assembly and sister chromatid cohesion have shown that aneuploidy increases with oocyte age as observed in humans (Hawley et al., 1994; Jeffreys et al., 2003).

### 1.2 The spindle orchestrates mitosis and meiosis

A major feature of mitosis and meiosis is the spindle, which is a polar structure involved in the capture and movement of chromosomes. The following section will describe the spindle, its formation and role in cell division.

#### 1.2.1 The spindle is a microtubule based structure

The spindle is a large complex of microtubules and microtubule associated proteins (MAPs). To understand how the spindle works we first need to understand the dynamics of a microtubule.

Microtubules are cylindrical polymers of tubulin heterodimers of α-tubulin and β-tubulin. Heterodimers form protofilaments which associate laterally to form a microtubule. Heterodimers arrange so that the α-tubulin in one heterodimer associates with β-tubulin of another heterodimer (Fig 1.5A). This makes a microtubule inherently polar with α-tubulin at one end (the minus end) and β-tubulin at the other (plus end) (Dammermann et al., 2003). This polarity is what establishes
the bipolarity of the spindle, with minus ends of microtubules at the spindle pole and plus ends going into the centre of the spindle (Fig 1.5B). Microtubules act as “tracks” along which motor proteins can travel along. The polarity of a microtubule is exploited, as microtubule motors can often move in one direction, for example; Non-claret disjunction (Ncd) is a minus-end directed motor (Hatsumi and Endow, 1992b) and Eg5 is a plus-end directed motor (Sawin et al., 1992). In the case of the spindle, this allows other proteins to be delivered to specific regions; for example Dynein is required for the transport of NuMA to the poles (Merdes et al., 2000).

1.2.2 Microtubules are dynamically unstable

The other quality of microtubules that are essential to spindle function is dynamic instability. Dynamic instability is a behaviour involving repeated cycles of growth and shrinkage (Fig 1.5C) (Desai and Mitchison, 1997; Howard and Hyman, 2003). A “catastrophe” is when a microtubule changes from sustained polymerization to depolymerization. Conversely, a “rescue” is when a microtubule changes from sustained depolymerization to polymerization. GTP hydrolysis underpins this dynamic instability. GTP is able to bind β-tubulin when a tubulin subunit is added to the end of a microtubule. Hydrolysis of GTP allows the microtubule to depolymerise. GTP bound tubulin dimers bind one another stronger and can encourage microtubule growth. When growth is faster than the rate of GTP hydrolysis a GTP cap forms on the end of the microtubule. If the rate of growth slows down however, GTP hydrolysis occurs such that the microtubule will shrink.

There are MAPs that can influence microtubule growth positively and negatively. The ability to stabilise and destabilise microtubules is essential for
spindle formation and function. The transition from interphase into mitosis sees a
dramatic increase in microtubule turnover, corresponding with an increase in
Cdc2/Cyclin B activity (Verde et al., 1990). The decrease in microtubule stability is
thought to aid the rapid rearrangements required for interphase microtubules to form
the spindle.

The dynamic instability of microtubule plus ends aids the search and capture
of kinetochores of sister chromatids in the cytoplasm. Once captured, the kinetochore
microtubule is stabilised. This stabilisation could be aided by localised microtubule
growth promoters EB1 and CLASP, which are located on kinetochores (Kline-Smith
and Walczak, 2004). Kinetochore microtubule stabilisation may also be due to the
Ran gradient which promotes stabilisation near the chromatin (see section 1.4).

During metaphase the spindle microtubules may appear static but in actual
fact are still highly dynamic. This is due to a phenomenon known as treadmilling,
where the rate of polymerisation at the plus end is equal to the rate of
depolymerisation at the minus end (Fig 1.6A). Alongside treadmilling is the
movement of the microtubule lattice to the minus ends/spindle poles called
"Poleward Flux" (Fig 1.6A).

At anaphase, the kinetochore microtubules need to be stabilised, as
detachment would result in loss of the chromatids. In yeast, this is achieved by the
phosphatase Cdc14 which, at anaphase, promotes localisation of microtubule
stabilising proteins to the spindle and silences Ask1, a microtubule destabiliser
(Higuchi and Uhlmann, 2005).

Microtubule dynamics are also the driving force for polar movement of
chromosomes. Upon anaphase onset, polymerisation at the plus ends halts while
depolymerisation continues at the minus end. This generates poleward flux and the kinetochore microtubule shrinks from the plus end, bringing the sister chromatid towards the spindle pole (Fig 1.6B). In *Drosophila* KLP10A depolymerises microtubules at the poles to cause poleward flux (Rogers et al., 2005). The kinetochore also actively depolymerises the plus end of the microtubule in what is called the “Pacman” model (Fig 1.6C). In *Drosophila* KLP59C depolymerises microtubules for Pacman assisted chromatid movement (Rogers et al., 2005).

In anaphase B, flux based microtubule depolymerisation at the minus ends stops while plus-end motors (KLP61F in *Drosophila* embryos) cross link microtubules at the spindle midzone and push the microtubules out towards the poles, causing the spindle poles to elongate (Brust-Mascher et al., 2004) (Fig 1.6D).

In conclusion, microtubule dynamics and their manipulation underpin all of the major events in chromosome segregation.

### 1.3 Centrosomal spindle formation

I have discussed how microtubules are key components of the spindle. In the following section I will describe how the spindle is formed from microtubules and spindle associated proteins in the presence of the microtubule organising centre (MTOC) the centrosome.

#### 1.3.1 Microtubule nucleation in animals

At high concentrations (20-40 μM), tubulin is able to assemble itself into a microtubule filament (Mitchison and Kirschner, 1984). The tubulin concentration is usually below that level *in vivo* and requires microtubule nucleation factors for
assembly to occur. γ-tubulin, another member of the Tubulin family, is a key
microtubule nucleation factor. γ-tubulin exists in two complexes, the γ-tubulin ring
complex (γ-TuRC) and the γ-tubulin small complex (γ-TuSC) (Fig 1.7). γ-TuSC is a
tetramer consisting of two γ-tubulin subunits and (in mammals) GCP2 and GCP3
(Dgrip84 and Dgrip91 in Drosophila) (Oegema et al., 1999; Zhang and Clarke, 2000).
γ-TuRC consists of γ-TuSCs and several additional proteins including GCP4, GCP5
and GCP6 in humans (Murphy et al., 2001) or Dgrip75, Dgrip128, Dgrip163, and
Dgrip71 WD in Drosophila (Gunawardane et al., 2001). γ-TuRCs have been purified
and shown to nucleate microtubules in vitro in Xenopus, Drosophila and humans
(Murphy et al., 2001; Oegema et al., 1999; Zheng et al., 1995). There are two models
for how γ-TuRC aids microtubule nucleation. The template model has the γ-TuRC
resembling the first turn of the three-turn helix of a microtubule and acts as a direct
template for the addition of tubulin (Zheng et al., 1995) (Fig 1.7A). The
protofilament model has the γ-TuRC uncoil to form the first protofilament on which
tubulin associates laterally to form a sheet that then closes to form a hollow tube
(Erickson and Stoffler, 1996; Zheng et al., 1995) (Fig 1.7B).

In vertebrate somatic cells, 80% of the γ-tubulin is present in the cytoplasm,
the rest is concentrated at the centrosome. In mammals, a component of the
centrosome, Pericentrin A, binds to GCP3 of γ-TuRC (Takahashi et al.,
2002; Zimmerman et al., 2004).

1.3.2 The centrosome

The centrosome was first named by Theodor Boveri in 1888, who observed that they
grew into distinct spheres during mitosis in Ascaris megalcephala eggs. It is now
regarded as the principal MTOC in most somatic animal cells and also has other cellular functions in cell-cycle regulation (Doxsey et al., 2005).

The centrosome is a large complex of proteins comprising of two major components, the centrioles and the pericentriolar material (PCM) (Fig 1.8A). The centrioles are a pair of non-identical barrel-shaped arrays, each containing nine sets of triplet microtubules arranged as a pinwheel (Blagden and Glover, 2003). The centrioles are believed to be instrumental in organizing centrosomal components into a structurally stable organelle (Bobinnec et al., 1998; La Terra et al., 2005).

Surrounding the centrioles is the PCM. The PCM is a lattice-like structure (Dictenberg et al., 1998; Schnackenberg and Palazzo, 1999), which anchors other PCM components (Blagden and Glover, 2003). The PCM is the main site of nucleation of cytoplasmic and spindle microtubules requiring γ-tubulin (Euteneuer and McIntosh, 1981; Zimmerman et al., 1999).

Centrosome-nucleated microtubules have their minus ends at the centrosome while their plus ends extend out into the cytoplasm, ensuring that the spindle is a bipolar structure when microtubules are nucleated from two centrosomes.

1.3.3 The centrosome cycle

In interphase, there is only one centrosome present in the cell. In mid G1, duplication of the centrioles is triggered and the paired centrioles break apart and a new centriole is replicated next to each parental centriole (Hinchcliffe and Sluder, 2001). During S phase, the daughter centrioles elongate and by G2 there are two pairs of centrioles sharing the same PCM (Fig 1.8B). Entry into M-phase results in increased radial nucleation of microtubules around the centrosome forming asters. At prophase, the
cohesion between the parental centrioles is lost and the centrosomes split into two. The centrosomes then migrate to opposite sides of the nucleus in a dynein-dependent manner (Sharp et al., 2000a). The Kinesin-like plus-end motors, Klp61F (Drosophila) and Eg5 are also required for centrosome separation (Heck et al., 1993; Whitehead et al., 1996). During this separation, the microtubules that interact between centrosomes elongate and form polar microtubules. The separated centrosomes function as spindle poles upon nuclear envelope breakdown. Upon cytokinesis, the centrosomes are segregated into separate cells (centrosome cycle summarised in Fig 1.8C).

This cycle of events is essential with regards to spindle formation as it ensures that the correct number of centrosomes is present. Extra centrosomes or problems with their separation can result in multipolar or monopolar spindles.

1.3.4 Kinases in centrosomal spindle formation

There are other proteins that are required for spindle formation in the presence of centrosomes. These include motor proteins, kinases and MAPs. Some kinases are important in the maturation of centrosomes. Polo-like (Plk1) kinase in Drosophila recruits γ-TuRC and activates Asp at the centrosome facilitating, microtubule nucleation (do Carmo and Glover, 1999; do Carmo et al., 2001; Glover, 2005) (Fig 1.9A). Mutant phenotypes and the localisation of Plk1 to the kinetochores and spindle midzone also suggests a function in metaphase to anaphase and cytokinesis (Glover, 2005).

Aurora A kinase is also required for centrosome maturation as well as bipolar spindle formation/maintenance and chromosome segregation (Fig 1.9C). Aurora A
can bind and phosphorylate Eg5 (Giet et al., 1999), recruiting the kinesin to the spindle where it facilitates spindle formation. Aurora A is also required for the recruitment of D-TACC to the centrosome and phosphorylation of D-TACC is believed to activate D-TACC/Msp5 complex in pole maintenance (Barros et al., 2005; Giet et al., 2002).

A relative of Aurora A, Aurora B kinase, is localised to the centromeric regions before metaphase to anaphase transition and is thought to correct inappropriate attachments of microtubules to the kinetochore (Adams et al., 2001; Giet and Glover, 2001) (Fig 1.9E). My lab has shown that NHK-1 is required for spindle formation and chromosome segregation in mitosis (Cullen et al., 2005) (Fig 1.9D).

In *Drosophila* Dwee1, the inhibitory kinase of Cdc2/Cyclin B, is required for bipolar spindle formation (Stumpff et al., 2004) (Fig 1.9B). There is evidence suggesting this is independent of elevated Cdc2 activity, as Dwee1 is able to bind γ-tubulin. γ-tubulin is also phosphorylated in a Dwee1-dependent manner (Stumpff et al., 2005).

### 1.3.5 MAPs in centrosomal spindle formation

MAPs also play an essential role in spindle formation. Mini spindles (Msp5) belongs to the dis1-TOG family of MAPs. Msp5 can localise to microtubules and spindle poles, and can stabilise microtubule minus ends at the poles (Cassimeris and Morabito, 2004; Cullen et al., 1999; Lee et al., 2001) (Fig 1.10A). In *Xenopus*, XMAP215 (Msp5 homologue) is believed to stabilise microtubules by competing with XKCM1, a microtubule destabiliser (Pesar et al., 2005; Tournebize et al., 2000).
An important binding partner of Msps is D-TACC (Maskin in *Xenopus*), which anchors Msps to the centrosome and stabilises centrosomal microtubules (Lee et al., 2001; Peset et al., 2005).

NuMA is another MAP which is essential for spindle formation and maintenance of spindle poles (Gaglio et al., 1995; Merdes et al., 1996). NuMA acts as a tethering factor that bundles microtubules and anchors them at spindle poles (Gordon et al., 2001) (Fig 1.10A).

TPX2 activity in vertebrates is essential for spindle assembly in the presence or absence of centrosomes in egg extract and in tissue culture (Gruss et al., 2001; Gruss et al., 2002). TPX2 interacts with Aurora A and is required for the targeting of the kinase to spindle microtubules (Kufer et al., 2002). TPX2 also targets Xklp2 to microtubule minus ends and participates in the organisation of spindle poles (Wittmann et al., 1998; Wittmann et al., 2000).

1.3.6 Motor proteins in mitotic spindle formation

The mechanism underlying the integrity of a bipolar spindle appears to be a balance of opposing forces generated by cross-linking motors. Minus end directed motors, such as cytoplasmic Dynein and Ncd, are able to cross-link parallel (from the same pole) microtubules and focus the microtubules into bundles at the poles (Goshima and Vale, 2003; Merdes et al., 2000) (Fig 1.10B). *Xenopus* XCTK and human HSET are also minus end directed motors with pole focusing ability (Mountain et al., 1999; Walczak et al., 1997).

Opposing the force generated towards the poles are the plus-end directed motors, such as Eg5 and Klp61F (Sharp et al., 1999; Zhu et al., 2005) (Fig 1.10B).
These kinesin harbouring N-terminal (Kin N) motors form tetramers with motor domains on each end. By cross-linking parallel microtubules they form bundles, while cross-linking of anti-parallel (from opposite poles) microtubules results in the microtubules being pushed apart. This force prevents the spindle poles collapsing in on each other at metaphase and drives spindle elongation at anaphase B.

A third force for maintaining the spindle is present at the astral microtubules reaching the cell cortex. A fraction of cytoplasmic Dynein linking the astral microtubules to the cortex could serve to pull the poles apart (Heald, 2000).

Dynein has other functions that are essential to spindle formation, such as transporting spindle assembly factors (SAFs), NuMA and TPX2 to the centrosome (Merdes et al., 2000; Wittmann et al., 2000). Cytoplasmic Dynein tethered to the kinetochore also contributes to chromosome movement (Savoian et al., 2000; Sharp et al., 2000b).

Some KinI proteins can attach to and depolymerise microtubule ends. Examples of these include MCAK, XKCM1, KLP67A, KLP10A and KLP59C (Gandhi et al., 2004; Kline-Smith and Walczak, 2002; Walczak et al., 1996).

Chromosome alignment is thought to require a polar ejection force that decreases the further away the chromosomes are from the poles. This force is believed to be produced by plus end directed kinesins that localise to the chromosomes (chromokinesins). Xkid (Xenopus), Kid (human) and Klp3A (Drosophila) are chromokinesins that are thought to act in this way (Antonio et al., 2000; Funabiki and Murray, 2000; Goshima and Vale, 2005; Levesque and Compton, 2001). CENP-E is a plus end motor present at kinetochores that aids chromosome
alignment by keeping the kinetochore at the plus ends of kinetochore microtubules (Wood et al., 1997; Yucel et al., 2000).

Although centrosomes provide nucleation sites and spatial cues for spindle bipolarity, they cannot form a functioning spindle by themselves. The formation of a bipolar spindle requires the concerted actions of motor proteins and MAPs, in which kinases play an important regulatory role.

**1.4 Acentrosomal spindle formation**

**1.4.1 Spindle formation in the absence of centrosomes**

The centrosomes appear to be essential with regard to spindle formation however there are many cells that can form spindles in the absence of centrosomes (acentrosomal). Higher plants do not possess a centrosome-like structure yet still form bipolar spindles (Schmit, 2002). The oocytes of many animals also lack centrosomes as observed in *Drosophila, Xenopus*, pig, sheep, cows and humans (Gueth-Hallonet et al., 1993; Hertig and Adams, 1967; Kim et al., 1996; Le Guen and Crozet, 1989; Long et al., 1993; Manandhar et al., 2005; Matthies et al., 1996; Merdes and Cleveland, 1997). In mouse and *Xenopus* oocytes, γ-tubulin can still be found at the poles of spindles (Gard et al., 1995; Gueth-Hallonet et al., 1993) but not in others, such as *Drosophila* (Matthies et al., 1996).

It could be argued that the above examples are special cases that do not apply to cells with centrosomes but eliminating the centrosome by laser ablation (Khodjakov et al., 2000) or by genetic mutation (Basto et al., 2006; Bonaccorsi et al., 1998; Megraw et al., 2001) does not prevent the formation of a bipolar spindle. In cultured *Drosophila* cells, the formation of some kinetochore fibers are initiated by
the kinetochore even in the presence of centrosomes (Maiato et al., 2004). How then are microtubules nucleated in these cases without centrosomes? In *Drosophila* and *Xenopus*, microtubules are nucleated around the meiotic chromosomes and then organised into a bipolar spindle suggesting that the chromosomes may act as microtubule nucleation sites. Indeed, chromatin-coated beads in the absence of kinetochores and centrosomes can form spindles in *Xenopus* egg extracts (Heald et al., 1996).

In the following sections I will review current knowledge regarding chromatin induced microtubule nucleation and how these microtubules are organised into a bipolar spindle in the absence of centrosomes.

### 1.4.2 The RanGTP pathway facilitates spindle assembly

Ran is a small GTPase that has an established role in the regulation of nucleocytoplasmic trafficking (Mattaj and Englmeier, 1998) and has also been shown to have roles in nuclear envelope formation and kinetochore function (Arnaoutov and Dasso, 2003; Hetzer et al., 2000). The GTP form of Ran (RanGTP) at high concentrations has been shown to be sufficient to induce microtubule aster formation in the absence of chromatin and centrosomes (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). It may appear therefore that RanGTP microtubule nucleation is independent of chromatin. However, chromatin is able to locally generate a high concentration of RanGTP. Ran can exist as either RanGTP or RanGDP and these states are dependent on the nucleotide exchange factor RCC1 and the GTPase activating protein RanGAP (Cole and Hammell, 1998) (Fig 1.11A). Phosphorylation of the nuclear localisation signal of RCC1 by Cdc2
promotes the interaction of RCC1 with chromatin during mitosis (Hutchins et al., 2004; Li and Zheng, 2004). Chromatin bound RCC1 results in a high concentration of RanGTP in the vicinity of the chromosomes (Hinkle et al., 2002; Kalab et al., 2002; Trieselmann and Wilde, 2002) (Fig 1.11B).

Upon nuclear envelope breakdown, the nuclear compartment mixes with the cytoplasm and proteins with nuclear localisation signals (NLS) can be bound by Importin α and Importin β. This has been shown to inhibit the function of several spindle assembly factors such as TPX2 (Trieselmann et al., 2003), Kid (Gruss et al., 2001; Trieselmann et al., 2003), NuMA (Nachury et al., 2001; Wiese et al., 2001) and XCTK2 (Ems-McClung et al., 2004). High concentrations of RanGTP release SAFs from Importin α/β allowing them to promote microtubule nucleation and spindle assembly in the vicinity of the chromosomes (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001).

Other SAFs that are regulated by RanGTP are XRHAMM, Aurora A, HURP and Rae1. RanGTP also affects the balance of motor activities by increasing the amount of motile Eg5, promoting spindle assembly (Wilde et al., 2001).

XRHAMM is a MAP that associates with γ-TuRC and TPX2, and plays a role in focusing spindle poles and is essential for microtubule nucleation in the absence of centrosomes in *Xenopus* (Groen et al., 2004).

The association of TPX2 with Aurora A is RanGTP dependent and results in TPX2 activation of Aurora A (Trieselmann et al., 2003; Tsai et al., 2003) (TPX2 and Aurora A function are discussed in section 1.3.4). While TPX2 is required for the formation of asters, the MAP, HURP is required for the transition from aster-like to spindle-like formation (Koffa et al., 2006).
Rae1 exists as a ribonucleoprotein (RNP) complex and can bind microtubules and is required for Ran-dependent aster formation in *Xenopus* (Blower et al., 2005).

It has also been found that the chromosomal passenger complex (CPC) is able to stabilise chromatin induced microtubules through the inhibition of the microtubule destabiliser MCAK in a RanGTP independent manner (Sampath et al., 2004).

The fact that RanGTP regulates all of these SAFs (and presumably others) so that they are active in the vicinity of chromatin makes the nucleation of spindles around chromosomes much more likely and frequent. It is believed that chromatin induced spindle formation consists of three important stages (Gruss and Vernos, 2004; Kahana and Cleveland, 1999). First, nucleation by RanGTP-mediated factors like TPX2, followed by organization of microtubules into 2 half spindles by motor proteins Eg5 and Xklp1, and finally establishment of spindle bipolarity through minus end motor pole focusing.

*In vivo* studies of the role of Ran in mitosis of *Drosophila* has shown that as well as having a role in spindle-assembly, Ran is required throughout mitosis for chromosome alignment, chromosome segregation and spindle midbody organisation (Silverman-Gavrila and Wilde, 2006).

It is important to note that the RanGTP pathway is essential even in the presence of centrosomes (Carazo-Salas et al., 2001; Gruss et al., 2002), suggesting that this is the underlying mechanism in which spindles form.

### 1.4.3 Acentrosomal spindle formation in *Drosophila oocytes*

A lot of the discoveries made in the field of acentrosomal spindle formation have used the *Xenopus* system, usually in the form of egg extracts. *Drosophila*
*melanogaster* is another model system that is amenable to the study of acentrosomal spindle formation. The female meiotic spindles of *Drosophila* are acentrosomal and lack centrosome components, γ-tubulin, CP-60 and CP-190, at their poles (Matthies et al., 1996). The female meiotic spindle is relatively easy to visualise making cytological studies possible. Another advantage is that *Drosophila* oocytes arrest at metaphase I unlike several other model organisms that arrest at metaphase II. This feature allows for easier identification of protein roles at an earlier stage in spindle assembly as well as isolating spindle formation from chromosome segregation. The final, and perhaps most important, advantage of using *Drosophila* is that it is genetically tractable with a rich collection of mutants available from almost a century's worth of studies.

The maturation process of the *Drosophila* oocyte has been divided into 14 stages with stage 1 being the earliest in development. In prophase I of female meiosis, the chromosomes are condensed and held in a single unit called the karyosome within the nucleus (Fig 1.12A). The karyosome can be seen from stage 3 of oocyte development. Upon germinal vesicle breakdown (GVB) the chromatin appears to capture or nucleate microtubules by itself (Theurkauf and Hawley, 1992) (Fig 1.12B). Motor associated microtubule asters have also been visualised migrating towards the chromatin from the nuclear envelope after GVB (Skold et al., 2005). Microtubules elongate around the karyosome and the microtubules are organised into a bipolar spindle through the action of cross-linking motor proteins and MAPs. The microtubules are denser in the centre of the spindle, with the chromosomes, while the poles are tapered. The meiotic spindle arrests in metaphase I. The bivalent chromosomes (I-III) are symmetrically held at the metaphase plate, while the
smallest achiasmatic fourth chromosomes are situated closer to the poles. Despite being arrested, the metaphase I spindles remain highly dynamic with extensions, contractions and rotations with no net change over time (Endow and Komma, 1997). Upon fertilisation, or passage through the oviduct, meiosis is resumed. The spindle elongates and rotates 90 degrees so that it is perpendicular to the oocyte surface. Chromosome segregation occurs and the spindle reorganises itself to form a pair of meiosis II tandem spindles with a central MTOC between them. Whilst this central MTOC has no centrioles, it does possess centrosomal proteins including γ-tubulin (Endow and Komma, 1998) and CP190 (Riparbelli and Callaini, 1996).

### 1.4.4 Proteins involved in acentrosomal spindle assembly of *Drosophila*

Several proteins have been discovered to play vital roles in *Drosophila* female meiosis in the last 15 years. There are two known forms of γ-tubulin in *Drosophila*, γ-TUB23C and γ-TUB37C. γ-TUB37C is only detected in ovaries and embryos and while it cannot be visualised on the meiotic spindle, mutant analysis shows it is required for bipolar spindle formation and chromosome alignment (Tavosanis et al., 1997) (Fig 1.13E).

Nucleosomal histone kinase-1 (NHK-1) when mutated has the unique phenotype of each bivalent being able to form its own spindle, resulting in three meiotic spindles (Cullen et al., 2005). Cullen et al (2005) showed that NHK-1 is required for the maintenance of the karysome which may explain individual chromosomes being able to form separate spindles. They suggest NHK-1 may also have downstream roles in spindle unification as well as in maintenance of bipolarity (Fig 1.13A).
Subito has a role in organising chromatin nucleated microtubules. Subito is a kinesin-like protein from the kinesin 6/MKLP1 family and is required for the establishment and/or maintenance of the central spindle, possibly through the localization of central spindle components, Aurora B and INCENP (Jang et al., 2005) (Fig 1.13B). The central spindle may act as a scaffold/template on which the bipolar spindle can form.

Subito may also have a secondary role similar to that of Ncd (Giunta et al., 2002). Ncd has been proposed to function by cross-linking microtubules and moving to their minus ends focusing the ends into poles (Hatsumi and Endow, 1992b; Hatsumi and Endow, 1992a; Matthies et al., 1996) (Fig 1.13C). Ncd may also mediate lateral interactions between microtubule associated bivalent chromosomes (Skold et al., 2005). There is also genetic evidence that Ncd is required for the polar localisation of Mini spindles (Msps) (Cullen and Ohkura, 2001) (Fig 1.13D). Cullen et al (2001) also showed that Msps and D-TACC are both acentrosomal pole proteins that are essential for the maintenance of spindle bipolarity. D-TACC can physically interact with Msps (Cullen and Ohkura, 2001), possibly anchoring Msps to the poles so that it can stabilise microtubules at the pole (Fig 1.13D).

A novel mediator of meiotic spindle assembly is Aberrant X segregation (AXS), the founding member of transmembrane family of proteins (Kramer and Hawley, 2003). AXS colocalises with the endoplasmic reticulum and is present in a structure sheathing the meiotic spindle. Dominant AXS mutants disrupt meiotic cell cycle and meiotic chromosome segregation (Kramer and Hawley, 2003).

Nod is a chromokinesin that is believed to provide the anti-poleward force that ensures non-exchange chromosomes are maintained in the half spindle.
(Theurkauf and Hawley, 1992). Nod may provide this force by binding to microtubule plus ends and promoting polymerisation (Cui et al., 2005) (Fig 1.13E). A similar type of phenotype is observed in *wispy* mutants where the fourth chromosome is often lost from the main chromosome and forms its own mini spindle (Brent et al., 2000). In *wispy* mutants, the bivalent chromosomes are often separated and spread across the spindle. This could reflect a reduction in meiotic recombination (McKim and Hawley, 1995).

Although studies in *Drosophila* oocytes have greatly enhanced the general understanding of how acentrosomal spindle formation occurs, it is clear that there are still many gaps in our overall knowledge. Further studies of *Drosophila* female meiosis, accompanied by research in other model organisms, will increase our knowledge of acentrosomal spindle formation. It is also important to reiterate how an understanding of acentrosomal meiosis has implications for our understanding of centrosomal mitosis. Many of the proteins involved in acentrosomal spindle formation are also involved in centrosomal spindle formation. Msps also has roles in *Drosophila* mitosis (Cullen et al., 1999) as does NHK-1 (Cullen et al., 2005). My lab's work on NHK-1 is a particularly good example, as its role in acentrosomal female meiosis was discovered before its mitotic role was investigated.

In order to find new mutants with defects in acentrosomal female meiosis, C.F. Cullen from my lab conducted a screen in female sterile mutants. The screen included a collection of mutants where eggs laid by the mutant fail to develop beyond the blastoderm stage (Schupbach and Wieschaus, 1989). The screen uncovered several mutants with defects in metaphase I female meiotic spindles. One
such mutant was the triplet NHK-1 mutant (Cullen et al., 2005). The other mutants from this screen are currently being analysed.

1.5 Aims and approaches

My aim is to understand the molecular mechanisms of acentrosomal spindle formation in female meiosis using Drosophila by;

1. Studying the molecular and cytological defects of metaphase I mutant remnants.
2. Studying the molecular and cytological defects of metaphase I mutant msps-like.

I will approach these aims as follows;

1. I will start by molecular identification of the remnants mutation to gain an insight into the molecular nature of the mutation. Cytological analysis of fixed and live non-activated remnants oocytes to characterise the nature of the metaphase I defects in the remnants mutation. Finally, I intend to establish remnants in existing acentrosomal spindle formation pathways by investigating molecular and genetic interactions with candidate spindle formation genes.

2. I will start by molecular identification of the msps-like mutation to gain an insight into the molecular nature of the mutation. Cytological analysis of fixed and live non-activated msps-like oocytes to characterise the nature of the metaphase I defects in the msps-like mutation. Finally, I intend to establish msps-like in existing acentrosomal spindle formation pathways by investigating molecular and genetic interactions with candidate spindle formation genes.

3. Designing and implementing the mutagenesis, and cytological screening of mutants on the X chromosome for defects in metaphase I of female meiosis. I also
intend to classify new metaphase I mutants on the X chromosome, through cytological analysis and complementation testing.
Fig 1.1 The mammalian cell cycle. The cell spends most of its time in interphase, unless it is undergoing cell division in M phase. Interphase consists of S phase, in which the DNA of the cell is replicated, G₁, the interval between M phase and S phase, and G₂, the interval between S phase and M phase. A) In early G₁, Cdk4/Cyclin D and Cdk6/Cyclin D phosphorylate Retinoblastoma protein (Rb), relieving inhibition of Cyclin E transcription. B) Cdk2/Cyclin E hyperphosphorylates Rb permitting E2F mediated transcription of genes required for entry into S phase. C) Cdk2/Cyclin A allows progression through S phase and D) Cdk1/Cyclin A allows transition into G₂. E) Peak activity of Cdk1/Cyclin B, results in entry into mitosis. F) Destruction of Cyclin B, by the Anaphase promoting complex (APC) allows re-entry into G₁.
**Fig 1.2 Phases of mitosis**

A) In prophase, the duplicated chromosomes are condensing and sister chromatids are held together at centromeres. B) Upon nuclear envelope breakdown the cell enters prometaphase, where spindle microtubules try to capture the kinetochores of chromosomes. C) In metaphase, both kinetochores of sister chromatids have attached to microtubules from opposite poles and are aligned at the spindle equator. D) Sister chromatids are separated and pulled towards opposite poles at anaphase A and in E) anaphase B the poles separate further apart. F) At telophase, the chromatids have reached the poles and start to decondense and the nuclear membrane starts to reform. G) Cytokinesis is the process of splitting the cell in two, resulting in separate daughter cells that have the full complement of DNA.
Fig 1.3 Meiotic and mitotic cell division. In Meiosis I, cells undergo two rounds of nuclear division with only one round of DNA duplication. Mitosis undergoes one round of DNA duplication and nuclear division. In both types of cell division, chromosomes condense and sister chromatids pair, while in meiosis homologous chromosomes also pair. Recombination can occur between homologous chromosomes in meiosis I. In meiosis I, homologous chromosomes are aligned at the spindle equator and then separated to opposite poles. The events of meiosis II are similar to the events in mitosis, where sister chromatids are aligned at the spindle equator and chromatids separated to opposite poles. Cytokinesis results in four haploid gametes in meiosis, and two diploid daughters in mitosis.
Cohesins hold sisters together

Separase

Ub

microtubule

Cdc20

APC

Securin

Cohesin degradation

Sister chromatids separate

Fig 1.4 Metaphase to anaphase transition. Sister chromatids are held together by Cohesins. When chromosomes are aligned with bipolar attachments, the chromosome signals Cdc20 to activate APC/C. APC/C^Cdc20 ubiquitinates Securin, targeting it for destruction by the proteasome. The destruction of Securin releases Separase, which is then able to cleave Cohesin. This results in a loss of cohesion between chromatids and they separate towards opposite poles in anaphase.
Fig 1.5 Microtubule structure and behaviour A) Tubulin is a heterodimer consisting of α-tubulin and β-tubulin. Heterodimers form protofilaments that associate to form a hollow microtubule cylinder. Heterodimers associate so that β-tubulin of one heterodimer associates with α-tubulin from another heterodimer. This makes the microtubule intrinsically polar with the β-tubulin as the plus end and α-tubulin as the minus end. B) The spindle is a polar structure due to the organisation of microtubules. Spindle poles are where the minus ends of microtubules are situated, while the plus ends are in the centre of the spindle or at the ends of astral microtubules. C) Dynamic instability of microtubules. A catastrophe is a change from sustained polymerization to sustained depolymerization. A rescue is a change from sustained depolymerization to sustained polymerization. When growth is faster than GTP hydrolysis, a GTP cap forms at the plus ends of microtubules and encourages growth.
**Fig 1.6 Microtubule dynamics in mitosis**  
A) During metaphase, the kinetochore microtubules are treadmilling. The rate of minus end depolymerization, by proteins like Klp10A is equal to plus end polymerization. The highlighted tubulin molecule demonstrates poleward flux. B) At anaphase, polymerization at plus ends ceases and poleward flux drags the chromatid to the pole. C) Chromatids can also be pulled towards the pole by the kinetochore depolymerizing the plus ends. D) In anaphase B, interpolar microtubules are no longer depolymerized. Plus-end directed motors, such as Eg5, bundle microtubules, pushing them out towards the poles and increasing spindle pole separation.
Fig 1.7 Models for γ-Tubulin assisted microtubule nucleation. γ-TuRC consists of γ-TuSC subunits and several additional proteins (for clarity only γ-tubulin is shown). A) In the template model, γ-TuRC acts as a template on which tubulin subunits can build upon. B) In the protofilament model, γ-TuRC uncoils to form a protofilament on which tubulin subunits can laterally associate and eventually fold into a hollow microtubule cylinder.
Fig 1.8 The centrosome cycle. A) The centrosome consists of a pair of Centrioles, surrounded by pericentriolar material (PCM). B) Centrioles separate in G₁ and replicate daughter centrioles, which elongate throughout S phase until G₂ where two pairs of centrioles have formed. C) The centrosome cycle cartoon shows a single centrosome duplicating in G₁-G₂, followed by centrosome separation and migration to opposite sides of the nucleus in prophase. After nuclear envelope Breakdown, the bipolar spindle is fully formed. In cytokinesis, centrosomes are divided so that each daughter cell has a single centrosome.
Fig 1.9 The role of kinases in spindle formation. Several kinases have essential roles in spindle formation. A) Plk1 is important in recruiting γ-tubulin and Asp to the centrosomes. B) Wee1 is required for spindle formation, possibly through regulation of γ-tubulin. C) Aurora A is required for localisation of D-TACC to the centrosomes and Eg5 to the spindle. D) NHK-1 is required for spindle formation and chromosome segregation. E) Aurora B is present at the chromosomes at metaphase and is thought to correct microtubule-kinetochore attachments.
Fig 1.10 The role of MAPs and motor proteins in spindle pole maintenance. A) MAPs are important in spindle pole formation. NuMA forms a lattice that cross-links and anchors microtubules at the poles, while Msps is important for stabilization of microtubules at the poles. D-TACC binds and anchors Msps at the poles. B) Motors are essential in spindle formation. Minus-end directed motors like Ncd, cross-link microtubules and focus them into the poles as they travel towards the minus ends. Plus-end motors, such as Eg5, can cross-link anti-parallel microtubules, generating a sliding force that pushes microtubules apart. The combination of MAPs and motors contributes to spindle maintenance.
Fig 1.11 RanGTP pathway in spindle formation. A) Ran can exist as either RanGTP or RanGDP and these states are dependent on the nucleotide exchange factor RCC1 and the GTPase activating enzyme RanGAP. B) In Mitosis, RCC1 binds to chromatin inducing a high concentration of RanGTP in the vicinity of the chromosomes. Spindle assembly factors (SAFs), such as TPX2 and NuMA are inhibited by the binding of Importin alpha/beta. High concentrations of RanGTP displace Importin from SAFs, resulting in spindle formation around the chromosomes.
Fig 1.12 Acentrosomal spindle formation in *Drosophila* oocytes. A) In prophase, chromosomes condense and form a single unit called the karyosome. B) After nuclear envelope breakdown, the chromatin is able to nucleate short Microtubules, C) which are elongated and organised to form a bipolar spindle. D) Meiosis arrests at metaphase I until fertilisation.
Fig 1.13 Establishment of metaphase I of female meiosis in *Drosophila*. Several proteins are known to be essential for establishing metaphase I of female meiosis. A) NHK-1 is required for maintenance of the karysome and spindle unification. B) Subito is required for central spindle assembly and pole focusing. C-D) Ncd can cross-link and focus minus ends of microtubules into poles and is required for the localisation of Mspss to the poles. D-TACC binds Mspss and both are required for maintenance of spindle poles. E) Nod and γ-tubulin are involved in chromosome alignment.
2. Materials and methods

2.1 Supplier information

All chemical reagents used in this work, except where stated otherwise, were purchased from Invitrogen, BDH and Sigma. Restriction enzymes, DNA polymerases and other enzymes used in this work, unless otherwise stated, were purchased from New England Biolabs, Promega and Roche.

2.2 Buffers and stock solutions

Standard buffers and solutions were used (Sambrook et al., 1989).

PBS used in this study contains; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$ pH 7.5. For DEPC treated water, 0.01 % DEPC was added to water, stirred, left overnight and autoclaved.

2.3 PCR oligonucleotides

All PCR oligonucleotides were purchased from MWG-Biotech. Primers are listed in Table 2.1

2.4 Antibodies

All primary and secondary antibodies and their typical working dilutions are described in Table 2.2.

2.5 Fly lines

The *Drosophila melanogaster* lines that were used in experiments are listed in Table 2.3. Fly lines were obtained from Bloomington unless stated otherwise.
2.6 Fly growth media

Flies were grown on standard yeast-cornmeal agar medium (Ashburner, 1989). For establishment of X-chromosome mutagenised lines, an enhanced media, described as “Semi-defined Medium” (Backhaus et al., 1984). Fly media was supplied by the University of Edinburgh ICMB kitchen staff.

2.7 DNA techniques

2.7.1 DNA extraction

Fly genomic DNA was extracted by homogenisation of 1-3 male flies in 40 µl/fly homogenisation buffer (10 mM Tris, 1mM EDTA pH 8, 25 mM NaCl, 200 µg/ml freshly added Proteinase K, Roche). Homogenised flies were then incubated at 37 °C for 30 min and at 95 °C for 5 mins. DNA samples were stored at -20 °C. 1 µl was used for a single PCR reaction.

2.7.2 Quantification of DNA

DNA quantification was calculated by measuring the light absorption at λ= 260 nm using the following equation; [DNA] in µg/µl = (50 x dilution factor x absorbance at 260 nm) divided by 1000.

2.7.3 Agarose gel electrophoresis

For agarose gel electrophoresis, 0.7 % - 1 % gels were prepared by boiling agarose (Cambrex) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1mM EDTA pH 8) until
dissolved. Ethidium bromide was added to a final concentration of 0.5 μg/ml, for visualisation of DNA. Gels were run at 90-120 V in TAE buffer. Hyperladder (Bioline) was run in parallel to samples to estimate the size of DNA fragments. DNA was visualised on a transilluminator (Herolab UVT-28 M) and recorded with a digital camera (Herolab E.A.S.Y. 429 K).

2.7.4 PCR
A typical PCR reaction mix (30 μl) consisted of 1 μl Genomic Fly DNA preparation, 100 pmol/μl of each primer, 10 mM dNTPs, 10X PCR buffer (Roche) and 0.5 μl Taq polymerase 5U/μl (Roche). The PCR program: 94 °C 1 min + 30X [94 °C 30 sec, 55 °C 1 min, 72 °C (1 min per kb)] in a Hybaid PCR thermal cycler.

2.7.5 DNA sequencing
Before sequencing of PCR products, 0.5 μl of Exonuclease I (10 U/μl) and 0.5 μl of Shrimp Alkaline Phosphatase (1 U/μl) was added to 3 μl of PCR product and incubated at 37 °C for 15 min and 80 °C for 15 min. Sequence primer (0.8 pmol/μl) and 4 μl BigDye Terminator v3.1 (ABI PRISM) was added and then ran through the following sequencing program, 25X [96 °C 30 sec, 50 °C 15 sec, 60 °C 4 min] in a Hybaid PCR thermal cycler.

2.8 Drosophila methods
Standard Drosophila handling techniques were used (Ashburner, 1989). Stocks were maintained at 18 °C or grown at 25 °C for experiments. $W^{118}$ flies were used as wild
type unless stated otherwise. Temperature sensitive strains were grown at permissive 22 °C and restrictive 28 °C for experiments.

2.8.1 Generation of synthetic deficiencies

Drosdel deficiencies, $Df(2L)ED653$ and $Df(2L)ED673$, were generated using the RS elements $5$-$SZ$-$4012$ and $CB$-$5878$-$3$, and $CB$-$5433$-$3$ and $5$-$SZ$-$3139$ respectively in a described procedure (Golic and Golic, 1996). Activation ("Flip-Out") of RS3r and RS5r elements were mediated by the expression of FLP. To express FLP under the control of $hs$ promoter, heatshock was performed on larvae at 37 °C for 1 hour 48-72 hours after egg deposition. Successful recombination resulted in the loss of 5′-exon of the white gene in the RS3 element and loss of the 3′-exon of the white gene in the RS5 element. This resulted in a loss of pigment in fly eyes. Crossing the activated RS3 and RS5 element carrying flies, followed by another round of FRT recombination resulted in the deletion of the sequence between the two RS elements and the restoration of the white gene. Selection of successful deficiencies was based on the return of pigment to the eye. The presence of deletions in $Df(2L)ED653$ and $Df(2L)ED673$ was confirmed by PCR and failure to complement other deficiencies in the same regions.

2.8.2 Mutagenesis of X chromosome

For mutagenesis of the X chromosome, $y$ $w$ $v$ $FRT18E[w^{+}]$ flies were isogenised and newly eclosed males were collected. Males were starved for half a day before feeding them on an EMS solution (35 mM EMS, 2 % glucose) overnight. Males were given a day to recover and then individually crossed to $FM7c$, $ActGFP[w^{+}]$. 
Df(1)RA74 virgins on “semi-defined” media. Individual lines were established by crossing female offspring with genotype y w v FRT18E[w⁺]/FM7c, ActGFP[w⁺] to FM7cActGFP[w⁺]/Y male offspring (* = EMS mutagenised line). FM7c has the Bar marker and allowed selection based upon eye morphology.

2.8.3 Generation of germ-line clones

In order to observe oocytes of homozygous lethal mutants from heterozygous females, a FLP-FRT “germ-line clone” recombination technique was used as previously outlined (Chou and Perrimon, 1996). Female flies with the desired mutation insertion eg y w v FRT18E[w⁺]/FM7c, ActGFP[w⁺] were crossed to ovoDv FRT18E; hsFLP males. Eggs were collected for 2 days and larvae were heat-shocked on two consecutive days at 37°C. y w v FRT18E[w⁺]/ovoDv FRT18E females can be selected by absence of FM7c Bar eye phenotype.

2.8.4 Immunostaining of fixed Drosophila oocytes

In order to observe fixed Drosophila oocytes, the following procedure was used. Newly eclosed females (24-30 flies) were selected and matured for 3 days with males (~10) in yeasted fly media bottles. Females were dissected in methanol and ovaries were sonicated to remove the chorion from mature oocytes. Selected mature oocytes are taken through a series of PBST (PBS + 0.1% Triton)/methanol mixtures into PBST. Oocytes were blocked for 30 min in PBST containing 10% fetal calf serum (FCS). The primary antibodies were usually incubated with oocytes for 4 hrs/overnight. Usual primary antibody mixture contains anti α-tubulin (DmA mouse monoclonal antibody: Sigma-Aldrich, 1:250) and anti-Msp2 (264 raised in rabbit,
Cullen et al 1999, 1:100) or anti-D-TACC (raised in rabbit, Gergely et al 2000, 1:1000), in PBST containing 10 % FCS. Primary antibodies were removed and oocytes washed 3 times in PBST before incubation with secondary antibodies for 2 hours. Secondary antibody mixture consists of anti-rabbit Cy5 (1:1000) and anti-mouse Alexa 488 (1:250). In order to visualise DNA, oocytes were stained with DAPI (20 μg/ml; Sigma-Aldrich) and Propidium Iodide (1:1000; Sigma-Aldrich). Oocytes were mounted onto cover slips (Coverglass 18x18 mm thickness no.1; VWR International) in mounting media (85 % glycerol, 2.5 % propyl gallate). Oocytes were visualised using confocal microscopy.

2.8.5 Live imaging of *Drosophila* oocytes

Live imaging of *Drosophila* oocytes was conducted on fly lines containing GFP-tagged proteins such as Msp5, D-TACC and α-tubulin. Newly eclosed females (~10 flies) were selected and matured for 2-3 days with males (5-10 flies) in yeasted fly media bottles. The ovaries of 3 female flies were dissected under halocarbon oil (series 700 Halocarbon Products Corporation) and oocytes were separated out so that stage 12-14 oocytes were at the bottom of the slide. Images were observed and recorded using confocal microscopy.

2.9 Imaging methods

Oocyte/spindle images taken using a 63X Plan-Apochromat lens (1.4NA; Carl Zeiss) attached to an Axiovert 200M (Carl Zeiss Microimaging, Inc.) with a confocal scan head (LSM510 meta Carl Zeiss). Confocal images were presented as a maximum
intensity projection of the Z-stacks. Digital images were imported to Photoshop (Adobe) and adjusted for brightness and contrast.

2.10 Protein techniques

2.10.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE (Sambrook et al., 1989). Resolving gels (8 %–12 % acrylamide) and stacking gels (5 % acrylamide) were assembled in a Mini-PROTEAN II (BioRad) system.

Whole fly protein samples were made by homogenising 2 whole flies in 200 µl of 1x loading buffer (150 mM TrisCl pH 6.8, 6 % SDS, 3 % glycerol and 0.1 % bromophenol blue) and 5 % mercaptoethanol followed by boiling for 5 min. Ovaries were dissected in methanol and homogenised in 300 µl of 1x loading buffer and 5 % mercaptoethanol followed by boiling for 5 min. Ovaries from 5 flies were considered to contain the equivalent amount of protein to 2 whole flies.

Gels were ran in running buffer (25 mM Tris, 250 mM Glycine, 0.1 % SDS) at 150 V until protein marker (Prestained Protein Marker, Broad Range, New England Biolabs) reached the bottom of the gel. Gels were stained with Bio-safe Coomassie (Biorad), following manufacturer’s protocol, for visualisation of proteins.

2.10.2 Immunoblotting

For immunoblotting, Nitrocellulose membrane (Schleicher & Schuell) was placed on the gel and sandwiched between filter paper and assembled into the Mini-Trans Blot cell (BioRad) with an ice cooling unit. Proteins were transferred onto the membrane with transfer buffer (25 mM Tris, 250 mM glycine) at 80 V for 50 mins or until the
ice melted. Successful transfer could be assessed with Ponceau S (Sigma) staining (1 g Ponceau S, + 1 ml acetic acid + 98 ml H2O) of membrane. Ponceau S staining could be removed by washing membrane in PBS containing 0.05 % Tween 20 (PBST). The membrane was then blocked for 1 hr in blocking solution (PBST + 3 % skimmed milk powder). The membrane was incubated with the primary antibody (concentrations in Table 2.2), diluted in blocking solution, for 2 h/overnight. After 3 washes in PBST secondary antibodies, conjugated with Horseradish-peroxidase, were incubated with the membrane for 1-2 hrs (concentrations in Table 2.2). Following 3 washes in PBST, ECL reagent (Amersham) was added to membrane for 1 min and exposed to Hyperfilm ECL chemiluminescent film (Amersham). The exposure time was adjusted according to strength of signal in each experiment. The film was developed using a SRX-101A developer (Konica).

2.10.3 Affinity purification of antibodies

For affinity purification of antibodies, approximately 20 ng of antigen was run on an acrylamide gel and blotted to a nitrocellulose filter as described in 2.10.1 and 2.10.2. The membrane was Ponceau stained and the band cut out. The band was washed in PBST and then blocked in PBST + skimmed milk. The band was incubated at 4 °C overnight with 5-10 % antiserum in PBST + skimmed milk. After several washes with PBST, the antibody was eluted by three 30 s washes with 400 µl Elution buffer (50 mM glycine-HCl pH 2.3, 500 mM NaCl, 0.5 % Tween, 100 µg/ml of bovine serum albumin, 0.1 % azide). Eluates were pooled and immediately neutralised by the addition of Na2HPO4 solution to a final concentration of 50 mM. The purified antibody is stored at 4 °C.
2.11 RNA methods

When handling RNA, plastic items were soaked in 0.5 M NaOH, rinsed in DEPC treated water and autoclaved before use in experiments.

2.11.1 RNA extraction from whole flies

For RNA extraction of whole flies, 100 mg of female flies were homogenised in 1 ml of TRIzol. RNA was extracted as described in manufacturer’s protocol. The homogenised fly sample was centrifuged at maximum speed for 10-25 mins at 2-8°C. The RNA containing supernatant was removed and incubated for 5 mins at 15-30°C. 0.2 ml chloroform was added for every 1 ml TRIzol, agitated, and incubated at room temperature for 2-3 mins. The RNA sample was centrifuged at max speed for 15-20 mins at 2-8°C and the upper aqueous phase (containing RNA) was transferred to a clean tube. 0.5 ml of isopropanol was added for every 1 ml of TRIzol and incubated for 10 mins at room temperature before centrifugation for 10-15 mins at 2-8°C. Removal of supernatant should leave an RNA pellet. The pellet was washed 1-3 times with 75% ethanol. The pellet was left to air dry before resuspending in 20 μl RNase free water (DEPC treated water). The RNA sample was stored a -80°C.

2.11.2 Quantification of RNA

RNA quantification was calculated by measuring absorption of light at λ 260 nm using the following equation; [RNA] in μg/μl = (40 x dilution factor x absorbance at 260 nm) divided by 1000.
2.11.3 Agarose/formaldehyde RNA gels

RNA samples were run on agarose/formaldehyde gels for separation of RNA species. A 1.2 % formaldehyde gel was made by boiling 1.8 g agarose, 20 ml 10x HEPES (0.5 M HEPES pH 7.8, 10 mM EDTA) and 150 ml H₂O. Upon cooling, 32 ml formaldehyde was added, mixed and poured into the gel tank kit. RNA samples were heated at 65 °C in a 1:3 ratio with loading buffer (50 % formamide, 6 % formaldehyde, 0.1 x SSC, 0.025 % Xylene Cyanol, 0.025 % Bromophenol blue, 10 % glycerol and 200 μg/ml Ethidium Bromide), snap chilled on ice, quick spun and loaded onto the gel. Gels were run in 1x HEPES at 80 V until bromophenol blue reached bottom of the gel. Gels were visualised/photographed, alongside a ruler, on a transilluminator (Herolab UVT-28 M) and recorded with a digital camera (Herolab E.A.S.Y. 429 K). The gel was washed for 10 mins in DEPC-treated water, 20 mins in 75 mM NaOH, 2x 15 mins in Tris/salt buffer (0.5 M TrisCl pH 7.4, 1.5 M NaCl), and 20 mins in 6 x SSC. RNA on the gel was transferred to hybond-N⁺ (Amersham) using capillary action with 6 x SSC overnight.

2.11.4 Northern blot hybridisation

For Northern blot hybridisation, Hybond-N⁺ (Amersham Biosciences) membrane was cross-linked on an automatic Stratalinker (Stratagene) with RNA side face up. The membrane was incubated with hybridisation buffer (0.1 % SDS, 50 % formamide, 5 x SSC, 50 mM NaPO₄ pH 6.8, 5 x Denhardt’s solution, 50 μg/ml sheared salmon sperm DNA) for at least 1 hr at 42 °C in a hybridisation tube (Hybaid). To make the probe, ~25 ng of DNA template was made up to 11 μl with
water and boiled for 5 mins, and then put on ice. 4 μl of high prime solution (Roche) and 2 μl [α³²P]dCTP (1.11 MBq) (Amersham Biosciences) were added to DNA template mixture and incubated at 37 °C for at least 1 hr. 100 ml of hybridisation buffer was added and boiled before incubation with the membrane overnight.

The hybridisation probe was removed and the membrane was washed twice in 6 x SSE/0.1 % SDS for 30 mins at 45 °C, and then washed at least three times in 2x SSC/0.1 % SDS for 15 mins. The membrane was wrapped in Saran wrap and exposed to XLS or XAR film (Kodak) overnight at 80 °C. The film was developed using a SRX-101A developer (Konica).
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rem sequencing (CG3752)  
rem sequencing (CG17005)  
rem sequencing (tai)  
rem sequencing (CGAMMA30A)
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IS = immunostaining, W = Western blot

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Table 2.3 Fly lines

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**Mapping of RET48 mutation**

**cdc2 mutant**

**Mapping of rem mutation**

**MAPPING OF rem mutation**

**Mapping of rem mutation**

**Mapping of rem mutation**

**Making Df(2L)E665**

**Making Df(2L)E653**

**Making Df(2L)E667**

**Making Df(2L)E653**

**Mapping of rem mutation**

**P-element rem mapping**

**X chromosome germ-line mosaics**

**Mutagenesis of X chromosome**

**Mutagenesis of X chromosome**

**X chromosome germ-line mosaics**

**Mutagenesis of X chromosome**

**Mutagenesis of X chromosome**

**X-chromosome mutants for spindle defect screen**

**Klp61F**

**Klp61F germ-line mosaics**

**Klp61F germ-line mosaics**

**Klp61F germ-line mosaics**

**Klp61F germ-line mosaics**
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<td>2&lt;sup&gt;nd&lt;/sup&gt; chromosome crosses</td>
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<td>3&lt;sup&gt;rd&lt;/sup&gt; chromosome recombination</td>
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3. *remnants* gene encodes a Cks30A protein with defects in acentrosomal spindle formation

### 3.1 History of *remnants* gene

In this chapter I will discuss the characterisation of *remnants* gene and its role in acentrosomal spindles of *Drosophila* female meiosis. The *remnants* (*rem\textsuperscript{RA74}* ) gene was first discovered in a screen for female sterile mutations on the second chromosome of *Drosophila* (Schupbach and Wieschaus, 1989). *rem* was one of the class 1 and 2 mutants where eggs laid by mutants fail to develop beyond the blastoderm stage (Schupbach and Wieschaus, 1989). These two classes include known mutants in meiotic spindle formation, such as *fs(2)TW1* γ-tubulin 37C mutant (Tavosanis et al., 1997) and *subito* (Giunta et al., 2002; Tavosanis et al., 1997).

C.F.Cullen, from my lab, screened these two classes of mutant for acentrosomal spindle defects. Her screen identified several mutants that had defects in the acentrosomal spindle of *Drosophila* female meiosis. One such mutant was *rem\textsuperscript{RA74}* , which shows an abnormal spindle morphology and chromosome misalignment. The *rem\textsuperscript{RA74}* gene was initially mapped to 30A-C using a deficiency *Df(2L)30A-C* (Schupbach and Wieschaus, 1989). Further deficiency mapping by C.F.Cullen located the *rem\textsuperscript{RA74}* mutation between the breakpoints of *Df(2L)N22-3* and *Df(2L)gamma7*. This region is approximately 1Mb in length and contains 87 candidate genes.
3.2. Molecular identification of remnants gene

Determination of the gene affected by the rem\textsuperscript{RA74} mutation will aid in the dissection of how the mutant fails to establish metaphase I female meiotic spindles. The following section describes the process of identifying the rem gene.

3.2.1 Defining chromosomal deficiency breakpoints around remnants

The breakpoints of Df(2L)N22-3 and Df(2L)gamma7 were determined cytologically, and molecular determination of these breakpoints would potentially narrow the region in which rem\textsuperscript{RA74} lies. In order to determine the breakpoints of Df(2L)N22-3 and Df(2L)gamma7, I developed a new technique making use of P-elements and PCR. This technique involves making a heterozygote between a deficiency chromosome and a viable P-element containing chromosome. Primers that flank the P-element will only produce a PCR product if the deficiency lies outside the location of the P-element (Fig 3.1A).

Viable P-element containing lines (which I will call PI-P7 in order of their physical position on the chromosome) around regions between the cytologically determined breakpoint were selected and crossed to Df(2L)N22-3 and Df(2L)gamma7 (Details in 2.3). Genomic DNA from heterozygotes for the deficiency and P-element chromosomes were used for PCR with primers flanking the P-element as described above.

As this technique relies on lack of PCR products, controls had to be used to differentiate real negative results from a failed PCR reaction. To this end, all primers
were tested using wild-type genomic DNA and all heterozygote genomic DNA was tested with primers outside of the region.

The PCR results (Fig 3.1B) show that flanking primers were able to amplify the genomic DNA of heterozygotes between *Df(2L)N22-3* and the chromosomes containing *P1* and *P2*, but failed to amplify *P3*-*P7* (Fig 3.1Biii). To confirm the lack of PCR product from the *P3* heterozygote, another set of primers flanking *P3* P-element was used. This also failed to produce a PCR product (Fig 3.1Biii). The 5' breakpoint of *Df(2L)N22-3* must therefore lie between the location of *P2* and *P3* P-elements.

Flanking primer pairs were able to amplify the genomic DNA from heterozygotes between *Df(2L)gamma7* and chromosomes containing P-elements *P1*-*P5* but were unable to amplify those containing *P6* and *P7* (Fig 3.1Bvi). The 5' breakpoint of *Df(2L)gamma7* must be between the location of P-elements *P5* and *P6*.

Molecular determination of the breakpoints of *Df(2L)gamma7* and *Df(2L)N22-3* reduced the *remR474* region from 1 Mb to 400 kb and from 87 to 32 candidate genes.

### 3.2.2 Creation of synthetic deficiencies around remnants

To further narrow the region of *remR474* by deficiency mapping, I created synthetic deficiencies with molecularly defined breakpoints using site-specific recombination.

As part of the *Drosophila* genome project, P-elements containing Flipase recombination targets (FRTs) have been introduced throughout the *Drosophila* genome. Deficiencies and duplications can be created when two FRTs at different
sites on the same chromosome recombine on expression of Flipase (FLP) (Fig 3.2) in a method outlined by Golic and Golic (1996).

I made synthetic deficiencies, \( Df(2L)ED653 \), which deletes 29E4-30A2, and \( Df(2L)ED673 \), which deletes 30A4-30B5, using the method outlined by Golic and Golic (1996). Crossing of these synthetic deficiencies to \( Df(2L)N22-5 \) (a deletion spanning both synthetic deletions) produced no heterozygous offspring (\( Df(2L)N22-5/Df(2L)ED653 \) or \( Df(2L)N22-3/Df(2L)ED673 \)), indicating they were lethal and the desired deletion had occurred in both synthetic deficiencies.

3.2.3 The deficiency \( Df(2L)ED673 \) uncovers the \( rem^{RA74} \) mutation

To decrease the region in which \( rem^{RA74} \) lies, further deficiency mapping was carried out. If \( rem^{RA74} \) is present within the deficiency, then the heterozygous female between \( rem^{RA74} \) and the deficiency will be sterile.

The Exelixis deletion collection (Thibault et al., 2004) was released in 2004, after I began mapping the \( rem^{RA74} \) mutation. I selected suitable Exelixis deficiencies to include in further deficiency mapping. The deficiencies, \( Df(2L)Exel6021 \), \( Df(2L)Exel8022 \), \( Df(2L)Exel7040 \), \( Df(2L)ED673 \) and \( Df(2L)ED653 \), were crossed to \( rem^{RA74} \) and only \( Df(2L)ED673/ rem^{RA74} \) heterozygous females were sterile.

Initial studies of \( Df(2L)ED673/ rem^{RA74} \) heterozygous oocytes show a spindle phenotype recapitulating that seen in \( rem^{RA74} \) hemizygotes, indicating that the \( rem^{RA74} \) mutation must lie within the deleted region of \( Df(2L)ED673 \). The deficiency mapping data places the \( rem^{RA74} \) mutation in a region \( \sim 180 \) kb in length, containing 12 genes.
3.2.4 Genomic sequencing identifies \textit{rem}^{RA74} as a cks30A mutation

In order to establish which gene contained the \textit{rem}^{RA74} mutation, several approaches were employed. I sequenced the coding regions of the 12 candidate genes by comparing the genomic DNA of \textit{rem}^{RA74} with a wild type control. In this case I used the genomic DNA of a mutant (\textit{RE48}) from the same screen as \textit{rem}^{RA74} (Schupbach and Wieschaus, 1989) as a control to minimise the risk of polymorphisms giving false positives.

Of all the candidate genes, only one gene contained a mutation (Fig 3.3). It was gene \textit{CG3738}, also known as \textit{cks30A}. No other mutations were found within the other coding regions and splicing junctions in this region. To rule out the possibility that the amount and size of transcripts of other candidate genes were affected by mutations outside the coding regions, transcripts of candidate genes known to be expressed in adult females were compared between mutant and wild type by Northern blot analysis. The results indicated that none of these transcripts were affected in the mutant.

\textit{Cks30A} is one of two \textit{Drosophila} homologues of \textit{Saccharomyces cerevisiae} \textit{Cks1/Schizosaccharomyces pombe} Suc1, a subunit of the Cdc2 (Cdk1)/Cyclin B complex. The \textit{rem}^{RA74} mutation results in a conversion of the 61\textsuperscript{st} amino acid from proline to leucine. This proline is completely conserved in all Cks homologues indicating that the mutation is unlikely to be a polymorphism. Crystal structure analysis has indicated that this residue lies in the interactive surface with Cdc2 (Bourne et al., 1996). It has also been shown that a mutation of the equivalent proline
in human Cks1 inhibits binding of Cyclin-dependent kinases (Sitry et al., 2002). Cks binding to Cdc2 is thought to alter substrate specificity of Cdc2/Cyclin B as shown for the APC/C (Patra and Dunphy, 1998; Shteinberg and Hershko, 1999), and Cdc2 regulators Wee1, Myt1 and Cdc25 (Patra et al., 1999; Shteinberg and Hershko, 1999).

3.3 Cks30A is essential for establishment of metaphase I in female meiosis

The rem RA74 mutation in cks30A results in female sterility. Preliminary observations by C.F. Cullen showed a problem with chromosome alignment and spindle morphology in metaphase I arrested oocytes (Section 3.1). In this section I intend to define the rem RA74 phenotype more thoroughly by examination of a larger number of rem RA74 spindles, looking at D-TACC and Cyclin B localisation and investigation of spindle dynamics via GFP-tubulin.

3.3.1 Cks30A is required for chromosome alignment and spindle bipolarity in female meiosis

To investigate the role of Cks30A, I decided to look at rem RA74 mutant non-activated mature oocytes, which arrest at metaphase I.

I dissected the ovaries of rem RA74 hemizygotes (rem RA74/Df(2L)N22-3) and homozygotes. The oocytes were fixed and stained for DNA, α-tubulin and D-TACC protein. The stained oocytes were visualised by confocal microscopy. In wild type, non-activated mature oocytes contain a single bipolar spindle with D-TACC and MspS localised at both poles. The chromosomes are symmetrically aligned along the equator of the spindle with achiasmate chromosomes nearer the poles (Fig 3.4A).
The \textit{rem}^{RA74} mutant is able to enter meiosis, condense its chromosomes and assemble microtubules around the chromosomes but has several defects upon arrest at metaphase I. The most striking defect in \textit{rem}^{RA74} is chromosome misalignment (Fig 3.4B). The arrangement of chiasmatic chromosomes is non-symmetrical and unorganised and was observed in over half the spindles. The second type of defect was abnormal spindle morphology. Aberrant morphology varied but ectopic poles often formed around the spindle equator (Fig 3.4C). In many cases a mixture of chromosome alignment and spindle defects were observed in a single spindle indicating the phenotypes are not mutually exclusive.

\textit{rem}^{RA74} hemizygotes and homozygotes showed significant differences in spindle phenotype from the wild type oocytes studied (p \textless 10^{-2}). Comparison of \textit{rem}^{RA74} hemizygous and homozygous oocytes showed no significant difference in spindle phenotype (p = 0.8849) indicating the \textit{rem}^{RA74} mutation is a functional null.

These results demonstrated that Cks30A is required before the metaphase-anaphase transition for spindle morphology and chromosome alignment. This is the first time that a Cks protein has been implicated in having a role prior to the metaphase to anaphase transition in meiosis.

\subsection*{3.3.2 Live-imaging of \textit{rem}^{RA74} oocytes}

In order to examine spindle dynamics and the nature of the spindle and chromosome defects in the \textit{rem}^{RA74} mutant, a \textit{rem}^{RA74}/CyO; \textit{GFP-tubulin}/MRS fly line was established to allow for live-imaging of oocytes. The crossing scheme is summarised in Fig 3.5C.
Curiously, I was unable to obtain double homozygous \( \text{rem}^{RA74} \) \( \text{GFP-tubulin} \) flies, despite single homozygotes being viable. This suggests that GFP-tubulin is not fully functional, however with other mutants it seems to be perfectly viable (\( \text{spol;} \) \( \text{GFP-tubulin} \) and \( \text{triplet;} \) \( \text{GFP-tubulin} \)) indicating there may be a genetic interaction between Cks30A and \( \alpha \)-tubulin. As the presence of a balancer chromosome affects chromosome alignment, homozygous \( \text{rem}^{RA74} \) males were crossed to \( \text{rem}^{RA74}/\text{CyO;} \) \( \text{GFP-tubulin}/\text{MRS} \) virgins to obtain viable \( \text{rem}^{RA74}; \text{GFP-tubulin}^{+} \) females.

Live analysis of \( \text{rem}^{RA74}; \text{GFP-tubulin}^{+} \) mature oocytes showed that spindle morphology appeared normal in the majority of oocytes examined. There were some examples where ectopic poles formed. In one movie the formation of ectopic spindle outgrowths was observed over the space of 2 minutes (Fig 3.6A and Appendix 7.1). The movie revealed that these outgrowths are stable for some time (at least 2 minutes) and are then gradually destabilised (around 2 minutes) only for the process to repeat on the opposite side of the metaphase plate (Fig 3.6B and Appendix 7.2). This suggests that microtubules at the spindle equator are not being well maintained in the absence of Cks30A allowing for the temporary growth of ectopic poles at the spindle equator.

Visualisation of chromosomes in \( \text{rem}^{RA74}; \text{GFP-tubulin}^{+} \) oocytes were difficult as they can only be seen by the “shadow” they cast within the GFP signal of the spindle. In the cases where chromosomes could be visualised, misalignment was often observed.

Microtubule dynamics were measured in \( \text{rem}^{RA74}; \text{GFP-tubulin}^{+} \) metaphase I arrested spindles via FRAP analysis. \( \text{rem}^{RA74}; \text{GFP-tubulin}^{+} \) female meiotic spindle microtubules had a half-recovery time of 38.5 s +/- 10.4 s which is
significantly longer ($p = 0.004$) than $GFP$-tubulin/+ microtubules, 26.2 s +/- 5.4 s (Fig 3.6C). This means that the turnover of Tubulin in female meiotic spindles is slower in $rem^{RA74}$ implying that Cks30A has a role in regulating microtubule dynamics. The percentage of intensity recovery after photobleaching was not significantly different between $rem^{RA74}; GFP$-tubulin/+ (74 % +/- 14 %) and $GFP$-tubulin/+ (68 % +/- 14 %) metaphase I arrested spindles (Fig 3.6D).

Live-analysis of $rem^{RA74}$ spindles has shown that chromosomes stay misaligned during arrest and that the spindle defect is a dynamic, as opposed to static, phenotype explaining the variety of spindle defects observed in fixed $rem^{RA74}$ oocytes. The reduction of Tubulin turnover in $rem^{RA74}$ female meiotic spindles and the observation that $rem^{RA74}; GFP$-tubulin is synthetic lethal, suggests that Cks30A plays a role in regulating microtubule dynamics.

### 3.3.2 Cks30A is essential for proper localisation of Msp/D-TACC

When C.F.Cullen first identified defects of $rem^{RA74}$ in female meiosis, she also observed that Mini spindles (Msp) was often mislocalised. In the $rem^{RA74}$ mutant, although the Msp protein is still concentrated at the spindle poles, it is often accumulated around the equator of the spindle. This mislocalisation is often coincident with the formation of the ectopic poles found near the spindle equator.

Msp belongs to a conserved family of microtubule regulators, including XMAP215, and was the first protein identified at the acentrosomal poles in *Drosophila* (Cullen and Ohkura, 2001; Ohkura et al., 2001). The mutation $msps^{208}$ leads to the formation of tripolar spindles in female meiosis I.
It has also been shown that Msps localisation is dependent on another pole protein, D-TACC, which binds to Msps (Cullen and Ohkura, 2001). I decided to investigate whether D-TACC is also mislocalized in rem^{R474} oocytes. In wild type, D-TACC is localised to the poles (Fig 3.7A). In the rem^{R474} mutant D-TACC, like Msps, often accumulates at the spindle equator, although it can still be found at the poles to some degree (Fig 3.7B).

To try and study whether Msps/D-TACC dynamics were affected in live oocytes, rem^{R474} msps-GFP and rem^{R474} D-TACC-GFP lines were established (Fig 3.5C).

Unfortunately it was not possible to identify any spindles with Msps-GFP or D-TACC-GFP present at the spindles. This is made difficult by the lack of labelled chromosomes in live oocytes, and without the condensed chromosomes as a marker it is impossible to distinguish a real signal from a background one.

In summary Cks30A protein is required for the correct localisation of essential pole proteins Msps and D-TACC in female meiotic spindles.

3.3.3 Cyclin B is concentrated at the spindle equator

As Cks30A is part of the Cdc2/Cyclin B complex, I decided to investigate Cdc2 and Cyclin B localisation in metaphase I spindles of female meiosis. I was unable to observe any Cdc2 staining, but was successful in visualising the localisation of Cyclin B.

Cyclin B is considered to be the main determinant of Cdc2 activity and cellular localisation. Immunostaining of non-activated oocytes revealed that Cyclin B localises to the spindle, with a high concentration at the spindle equator (Fig 3.7C).
This suggests that Cdc2/Cyclin B has a possible regulatory role that allows localisation of Msps and D-TACC to the spindle poles. The localisation of Cyclin B is unaffected in the \textit{rem}^{RA74} mutant, suggesting that Cks30A mainly affects substrate specificity of Cdk1 complex as shown in other systems (Patra and Dunphy, 1996; Patra and Dunphy, 1998; Patra et al., 1999), rather than localisation of the Cdc2/Cyclin B complex.

3.4 Dissecting a Cks30A specific kinase pathway in the formation of acentrosomal spindles

A simple model for the role of Cks30A in acentrosomal spindle formation is that when Cks30A is part of the Cdc2/Cyclin B complex, Cdc2 phosphorylates pole proteins Msps/D-TACC at the spindle equator. This modification allows localisation of Msps/D-TACC to the spindle poles, permitting formation/maintenance of a bipolar spindle (Fig 3.8A). In the \textit{rem}^{RA74} mutation, Cks30A is either no longer present or unable to bind Cdc2, resulting in a lack of Msps/D-TACC modification. This results in Msps/D-TACC accumulating at the spindle equator, leading to formation of ectopic poles and chromosome misalignment in this region (Fig 3.8B). I tested this model by examining mutants of other proteins believed to be involved in D-TACC localisation and investigating \textit{rem}^{RA74} \textit{msps}^{208} and \textit{rem}^{RA74} \textit{D-TACC}^{stella592} double mutants.

3.4.1 \textit{rem}^{RA74} \textit{msps}^{208} double mutants are synthetic lethal

To investigate the relationship between Cks30A and Msps proteins in female meiosis, I investigated whether there was a genetic interaction between the \textit{rem}^{RA74}
and mspS208 mutations. The remRA74 mutation has been described (Section 3.3). Msps is essential for viability (Cullen et al., 1999), and mspS208 is hypomorphic and results in depletion of Msps and the occurrence of tripolar spindles in metaphase I of female meiosis (Cullen and Ohkura, 2001). remRA74 is located on the 2nd chromosome while mspS208 is located on the 3rd chromosome. remRA74/CyO; mspS208/MRS balanced fly lines were established using a series of crosses (Fig 3.5A).

My intention was to study non-activated metaphase I arrested oocytes of remRA74 mspS208 double homozygotes but these flies never emerged, although single homozygotes in this line were still viable (Table 3.1). This indicates that there is a genetic interaction between mspS208 and remRA74 that results in synthetic lethality. remRA74 mspS208 lethality is likely to be due to non-functional mitosis. This differs from my previous view that Cks30A and Cks85A have exclusive roles in meiosis and mitosis respectively (Pearson et al., 2005).

The possibility that Cks30A has a role in mitosis warrants further investigation. Despite being unable to observe remRA74 mspS208 spindles in female meiosis, the lethality gives a strong indication that Cks30A and Msps both have a vital role in the same pathway, possibly via spindle maintenance/microtubule dynamics.

3.4.2 Genetic interaction between remRA74 D-TACCstella592 double mutants in the establishment of metaphase I of female meiosis

In remRA74, the pole protein D-TACC is mislocalised to the metaphase plate of acentrosomal spindles. D-TACC has been shown to interact with Msps and both are required for the maintenance of acentrosomal spindles (Cullen and Ohkura, 2001). In
mutants less than 1% of D-TACC is produced but the flies are completely viable (Lee et al., 2001). D-TACC\textsuperscript{stella592} mutant has tripolar metaphase I spindles resembling msp208 mutants. I predict that in rem\textsuperscript{RA74} D-TACC\textsuperscript{stella592} oocytes the D-TACC\textsuperscript{stella592} phenotype will be epistatic to rem\textsuperscript{RA74}. This is based on the assumption that mislocalisation of D-TACC causes chromosome misalignment and ectopic pole formation in rem\textsuperscript{RA74}, therefore the severe reduction of D-TACC in rem\textsuperscript{RA74} D-TACC\textsuperscript{stella592} may remove the rem\textsuperscript{RA74} defects and spindles will be tripolar (Fig 3.9). The results in section 3.4.1 show that rem\textsuperscript{RA74} msp208 is lethal, so I expect that rem\textsuperscript{RA74} D-TACC\textsuperscript{stella592} could also be lethal, due to the shared role of Msp and D-TACC in spindle bipolarity in metaphase I of female meiosis (Cullen and Ohkura, 2001).

I established rem\textsuperscript{RA74}/CyO; dtacc\textsuperscript{stella592}/MRS fly lines using a crossing scheme outlined in Fig 3.5B. Unlike rem\textsuperscript{RA74} msp208, rem\textsuperscript{RA74} D-TACC\textsuperscript{stella592} flies are viable, but female sterile. Double homozgous females were dissected and fixed for examination of metaphase I arrested oocytes.

The rem\textsuperscript{RA74} D-TACC\textsuperscript{stella592} double mutant is able to enter meiosis, condense its chromosomes and assemble microtubules around the chromosomes but has several defects upon arrest at metaphase I. The most common defect (48%) observed was in spindle formation/maintenance. Defects included rem\textsuperscript{RA74}-like defects, such as the formation of ectopic poles (18%) (Fig 3.10A), and tripolar spindles (15%) reminiscent of D-TACC\textsuperscript{stella592} single mutants (Fig 3.10B). Interestingly, there was a third type of spindle defect distinct from individual rem\textsuperscript{RA74} and D-TACC\textsuperscript{stella592} spindle defects. This was the formation of 1-2 smaller spindles (15%) containing a
chromosome, which was presumably the fourth chromosome ("mini-spindles"). The extra spindles only occurred in conjunction with a \( \text{rem}^{RA74} \)-like or \( D-TACC^{\text{stella592}} \)-like main spindle (Fig 3.10C and Fig 3.10D). 27% of the spindles observed had chromosome defects, the majority of which were chromosome misalignment. Chromosomes also appeared slightly decondensed, but this could be an artefact caused during fixation.

These results are different from what I had predicted (Fig 3.9), as I was expecting to see the \( D-TACC^{\text{stella592}} \) phenotype being epistatic over \( \text{rem}^{RA74} \), if mislocalisation of D-TACC/Msps to the spindle equator was indeed the cause of the \( \text{rem}^{RA74} \) phenotype. The fact the \( \text{rem}^{RA74} \) phenotype is observed in \( \text{rem}^{RA74} D-TACC^{\text{stella592}} \) shows that D-TACC mislocalisation by itself cannot explain the \( \text{rem}^{RA74} \) phenotype. The occurrence of tripolar spindles also indicates that D-TACC and Cks30A can function in a shared pathway with regards to spindle formation/maintenance. The mini-spindle phenotype does suggest a genetic interaction between \( \text{rem}^{RA74} \) and \( D-TACC^{\text{stella592}} \) and possibly a pathway involving Cks30A and D-TACC is required for spindle cohesion.

3.4.3 D-TACC which cannot be phosphorylated by Aurora A does not recapitulate \textit{remnants} phenotype in female meiosis

It has been shown in \textit{Drosophila} that Aurora A kinase can phosphorylate D-TACC on Serine 863 (p-D-TACC) at the centrosomes, resulting in an accumulation of p-D-TACC at the poles of mitotic spindles (Barros et al., 2005). In female meiosis, Cks30A may be involved in the Aurora A/D-TACC pathway for localisation of D-TACC to the spindle poles (Fig 3.11). If this is the case, disruption of D-TACC
phosphorylation by Aurora A in metaphase I spindles of female meiosis should resemble the phenotype seen in \textit{rem}^{Ra74}. To test this, I used \textit{GFP-D-TACC} and \textit{GFP-D-TACC}^{S863L} flies (D-TACC where Aurora A can no longer phosphorylate Ser863 in a \textit{D-TACC}^{stella592} background (gift from Jordan Raff).

I initially looked at \textit{GFP-D-TACC} mature oocytes and found that metaphase I spindles were normal. I could not detect localisation of D-TACC in GFP-D-TACC oocytes with anti-D-TACC antibody. Addition of GFP to D-TACC may result in a loss of recognition with D-TACC antibody.

\textit{GFP-D-TACC}^{S863L} homozygous females are not sterile, but I thought it was still worth investigating oocytes for defects in metaphase I spindles. Observation of \textit{GFP-D-TACC}^{S863L} metaphase I arrested oocytes showed no defects. Localisation of D-TACC could not be assessed, due to D-TACC antibody not recognising GFP-D-TACC^{S863L} protein.

I decided to take advantage of the GFP tag by looking at live images of metaphase I spindles in oocytes. Visualisation of GFP-D-TACC was successful and in some cases clearly showed particles of D-TACC moving from the metaphase spindle to the poles (Fig 3.12 and Appendix 7.3). The speed at which D-TACC-GFP particles moved towards the poles was 2.6 \textmu m/min +/- 0.27 \textmu m/min.

Unfortunately, I was not able to visualise GFP-D-TACC^{S863L} in live oocytes. Whether this was due to GFP-D-TACC^{S863L} being different from normal D-TACC behaviour or not present at all in the oocyte could not be concluded.

These results suggest that even if Aurora A phosphorylation of D-TACC has a function in female meiosis, it does not result in sterility and has no apparent defect in metaphase I spindles in female meiosis.
This is the first time that D-TACC particles have been visualised moving from the spindle equator to the poles in female meiotic spindles. This observation gives strength to the hypothesis that Cks30A could be involved in the transport of D-TACC from the spindle equator to the poles in the establishment of a metaphase I acentrosomal spindle.

3.4.4 D-TACC protein is modified in a Cks30A dependent manner in oocytes

I had used anti-p(Ser863)-D-TACC antibody (gift from Jordan Raff) on fixed oocytes to see if and where p(Ser863)-D-TACC was localised on the metaphase I arrested spindle. I did not observe any localised signals. The antibody may not have worked well under oocyte fixing conditions or no p(Ser863)-D-TACC was localised on the metaphase I spindle.

As an alternative I used anti-p(Ser863)-D-TACC antibody to investigate p(Ser863)-D-TACC levels in \textit{rem}^{RA74} and wild type mature oocytes by immunoblotting. To avoid a loss of phosphorylation, I dissected ovaries of \textit{rem}^{RA74} and wild type in methanol and made protein preparations. The protein samples of \textit{rem}^{RA74} and wild type were analysed by SDS-PAGE and immunoblotted with anti-D-TACC and anti-p(Ser863)-D-TACC antibodies.

In wild type samples, D-TACC and p(Ser863)-D-TACC antibodies recognise different forms of D-TACC protein (Fig 3.13). Anti-D-TACC antibody recognises at least three bands, the uppermost of which (over 175 kDa) seems most abundant. Anti-p(Ser863)-D-TACC antibody recognises a single band just below 175 kDa that corresponds to the second band observed with anti-D-TACC antibody. In \textit{rem}^{RA74}
oocytes, anti-D-TACC recognises three bands as in wild type, although the faster running bands are at lower levels than in wild type. In $\text{rem}^{RA74}$ anti-p(Ser863)-D-TACC recognises a band that runs faster than the band in wild type oocytes.

This indicates that in the $\text{rem}^{RA74}$ mutant, Aurora A phosphorylation of Ser863 is unaffected but another modification has occurred or disappeared. The simplest interpretation is that Cks30A is required for Cdc2 phosphorylation of D-TACC, and in the $\text{rem}^{RA74}$ mutant this modification is absent (Fig 3.13C and Fig 3.13D). This is an important result as it provides molecular evidence that Cks30A is required for regulation of the modification state of D-TACC. This modification could be what allows D-TACC to be localised to the spindle poles from the equator.

### 3.4.5 Cdc2 is essential for the establishment of metaphase I in female meiosis

To investigate whether Cks30A is acting through Cdc2 complex, metaphase I spindles in female meiosis of a $\text{Cdc2}$ mutant were examined. Examination of acentrosomal spindles in the $\text{cdc2}$ mutant can be difficult due to most mutations being lethal. There is a temperature sensitive mutant allele of Cdc2 ($\text{cdc2}^{E1-24}$) which in combination with another Cdc2 allele ($\text{cdc2}^{B47}$) resulted in female sterility (Stern et al., 1993). I raised $\text{cdc2}^{E1-24} \;/\text{cdc2}^{B47}$ transheterozygous females at 22 °C, which I then incubated at the restrictive temperature of 28 °C. The flies were incubated for three days, under the assumption that during this time Cdc2 function should be depleted in the oocytes. Fixed metaphase I spindles were then examined (Fig 3.14).

Just under half of the spindles observed were normal with chromosomes symmetrically organised along the equator of the spindle with achiasmate
chromosomes nearer the poles (Fig 3.14C). The rest had abnormalities that ranged from spindles that were not fully bipolar (Fig 3.14B) to spindles that appeared to have fewer microtubules (Fig 3.14C). Many of the spindles had hardly formed at all and the chromosomes which they had formed around were badly organised. The range in abnormalities may reflect the level of Cdc2 depletion at the restrictive temperature. The severe spindle phenotypes may be from oocytes with fully depleted Cdc2 levels, while the weaker spindle phenotypes may be from oocytes with some functional Cdc2 present.

This result demonstrated that Cdc2 is essential for the proper establishment of metaphase I spindles in female meiosis. None of the spindle phenotypes strongly resembled those observed in rem\textsuperscript{RA74}, which does not appear to fit with the hypothesis that Cks30A acts through Cdc2. However Cks30A may only affect a subset of Cdc2 substrates and the fact that disruption of Cdc2 affects all Cdc2 substrates may well explain this difference in phenotype.

### 3.5 Summary of Cks30A function in acentrosomal spindle formation

The rem\textsuperscript{RA74} mutant has a mutation in cks30A gene. I showed that Cks30A is important in chromosome alignment and maintenance of a bipolar spindle in metaphase I. The spindle pole proteins, Msps and D-TACC, are mislocalised to the spindle equator in rem\textsuperscript{RA74} metaphase I of female meiosis. Imaging of rem\textsuperscript{RA74}; GFP-tubulin/+ live oocytes shows that ectopic pole formation is a dynamic and transient event and that microtubule dynamics are decreased in the rem\textsuperscript{RA74} mutant.

It is likely that Cks30A functions as part of the Cdc2/Cyclin B complex by giving specificity to a subset of substrates (Fig 3.15A). Analysis of temperature
sensitive cdc2 mutants at a restrictive temperature revealed defects in the establishment of metaphase I spindles, although it did not recapitulate the rem$^{RA74}$ phenotype. This could be explained by the rem$^{RA74}$ mutant only affecting a subset of Cdc2 substrates.

Cks30A is involved in the regulation of D-TACC modification and this modification may be essential for the localisation of D-TACC and Msps to the poles of acentrosomal spindles (3.15B). I have shown, for the first time, that D-TACC particles move from the spindle equator to the poles in metaphase I female meiotic spindles. The fact that Cyclin B is localised to the spindle equator suggests this is where Cdc2 is active in metaphase I spindles. Active Cdc2/CyclinB/Cks30A may be required for signalling Msps/D-TACC localisation, maintenance of spindle bipolarity, and chromosome alignment in metaphase I spindles of female meiosis.

Analysis of rem$^{RA74}$ D-TACC$^{stella592}$ double mutants suggests that the mislocalisation of D-TACC is not solely responsible for the spindle morphology and chromosome alignment defects observed in rem$^{RA74}$ mutants. This suggests there must be other substrates of Cks30A/Cdc2/Cyclin B complex that are involved in the establishment of metaphase I acentrosomal spindles. The synthetic lethality of rem$^{RA74}$ msps$^{208}$ double mutants suggests that Cks30A and Msps are involved in an essential pathway, possibly in mitotic spindle assembly (Fig 3.15C).

In conclusion Cks30A is a vital subunit of Cdc2/Cyclin B that affects the regulation of multiple spindle assembly factors required for acentrosomal and mitotic spindle maintenance.
Fig 3.1 Mapping deficiency breakpoints with P-element PCR

A) *Df*(2L)*N22-3* and *Df*(2L)*gamma7* were crossed to P-element lines (P1-P7). Primers that flank the P-element will only produce a PCR product if the deficiency lies outside the location of the P-element. B) Genomic DNA from heterozygotes for the deficiency and P-element chromosomes was used for PCR with primers flanking the P-element. iii) Flanking primers were able to amplify the genomic DNA of heterozygotes between *Df*(2L)*N22-3* and the chromosomes containing *P1* and *P2*, but failed to amplify *P3*-P7. To confirm the lack of PCR product from *P3* heterozygote, another set of flanking *P3* primers (*P3.2*) were used. The 5’ breakpoint of *Df*(2L)*N22-3* must be located between *P2* and *P3*. Flanking primer pairs were able to amplify the genomic DNA from heterozygotes between *Df*(2L)*gamma7* and chromosomes containing P-elements *P1*-P5 but were unable to amplify those containing *P6* and *P7*. The 5’ breakpoint of *Df*(2L)*gamma7* must be located between *P5* and *P6*.
Fig 3.2 Generation of synthetic deficiencies using site-specific recombination. A) Activation of RS3r and RS5r elements involves recombination between FRTs (red arrows) resulting in incomplete white genes. B) Crossing of activated RS3r and RS5 flies results in heterozygotes, which upon FLP expression can undergo site specific recombination. Recombination can produce deficiencies where the white gene is restored (w+), or duplications where the white gene is lost. Deficiency flies can be selected based on red eye colour.
Fig 3.3 Summary of molecular identification of rem gene

A) Deficiency Mapping of the rem gene located rem to a region of 175 kb (red box).
B) Sequencing of the rem gene. The arrows represent genes in the 175 kb region.
One gene (cks30A) contains a missense mutation. C) Sequence comparison among Cks homologues shows that the mutated Proline is completely conserved.
Fig 3.4. Cks30A is essential for spindle morphogenesis and chromosome alignment. The spindle and chromosomes in non-activated oocytes were visualized by immunostaining in; A) wild type. B,C) the rem^{R474} mutant. The arrowheads indicate chromosome misalignment and ectopic pole formation respectively. D) Quantification of spindle and chromosome defects in wild type, rem^{R474} homozygotes (rem) and hemizygotes (rem/Df). Scale bar, 10 μm. Between 29 and 135 spindles were counted for each genotype.
**Fig 3.5 Combining rem^{RA74} with msp{sup}^{208}, dtauca^{stella592} and GFP-tubulin lines.** A double chromosome balancer line (w; L^2/CyO; MRS/TM6B) was crossed to rem^{RA74} (rem) and the 3^{rd} chromosome mutants A) msp{sup}^{208}, B) dtauca^{stella592} and C) GFP-tubulin. msp{sup}^{208}-GFP and DTACC-GFP lines were established in the same way as in C) replacing GFP-tubulin line with msp{sup}^{208}-GFP or DTACC-GFP. Male offspring of rem with balanced 3^{rd} chromosomes (rem/L^2; +/MRS) could then be crossed to females carrying the desired allele and balanced 2^{nd} and 3^{rd} chromosomes eg (+/CyO; GFP-tubulin/TM6B). By following the markers, it was possible to select flies carrying rem and the desired 3^{rd} chromosome allele.
Fig 3.6 Dynamic growth/shrinkage of ectopic poles in rem metaphase I female meiotic spindles. A) Formation of an ectopic pole in a rem female meiotic spindle over the course of 2 mins. Arrowheads show tip of ectopic pole. B) Dissolution of another ectopic pole in the same rem female meiotic spindle as in A. It takes 2.5 mins for the ectopic pole to disappear. C) Microtubule dynamics are affected in rem female meiotic spindles half recovery time after photobleaching of GFP-tubulin in wild type and rem female meiotic spindles shows that rem female meiotic spindles recover more slowly than in wild type (p = 0.004, n = 10). D) % intensity recovery after photobleaching of GFP-Tubulin in wild type and rem female meiotic spindles are the same (p = 0.4, n = 10). Scale bar, 10 μm.
Fig 3.7 DTACC mislocalization in cks30A mutant

A) Localisation of D-TACC to the poles (arrowheads) in wild-type. B) Mislocalization of D-TACC to the spindle equator (arrowhead) localisation in \(rem^{474}\) mutant. C) Concentration of Cyclin B at the spindle equator. Scale bar, 10 \(\mu\)m.
Fig 3.8 Model explaining $rem^{RA74}$ phenotype. A) In my proposed model, Msp/C-D-TACC is present at the spindle equator where Cks30A-bound Cdc2/Cyclin B phosphorylates Msp. P-Msp is then localised to the poles, possibly via a minus end motor protein. B) A cartoon representing the defects observed in a $rem^{RA74}$ spindle as opposed to wild type.
Fig 3.9 Predicting \(rem^{RA74} \cdot DTACC^{stella592}\) phenotype. A) In metaphase I female meiosis of \(rem^{RA74}\) Msp/D-TACC is mislocalised to the spindle equator and defects in chromosome misalignment and spindle morphology are observed. B) In \(dtacc^{stella592}\), D-TACC is severely diminished, Msp is not localised to poles and tripolar spindles are observed. C) If the mislocalisation of D-TACC is responsible for Msp mislocalisation, spindle defects and chromosome misalignment observed in \(rem^{RA74}\), then \(dtacc^{stella592}\) should be epistatic to \(rem^{RA74}\).
Fig 3.10 \textit{rem}^{RA74} \textit{dtacc}^{stella592} acentrosomal spindle phenotype. A-D) The spindle and chromosomes in non-activated oocytes were visualized by immunostaining in \textit{rem}^{RA74} \textit{dtacc}^{stella592} double mutant. A, C) Ectopic poles were observed, indicated by arrows. B, D) Tripolar spindles were observed. B, C) chromosome misalignment. C, D) Extra mini spindles were also observed, indicated by arrowheads. E) Quantification of defects in \textit{rem}^{RA74} \textit{dtacc}^{stella592} metaphase I female meiosis. F) Quantification of the spindle defects observed in \textit{rem}^{RA74} \textit{dtacc}^{stella592}. Scale bar, 10 μm.
Fig 3.11 Model for Aurora A in D-TACC localisation. A) In mitosis, Aurora A phosphorylates D-TACC at the centrosomes and this maintains D-TACC at the poles. B) My proposed model in female meiosis, is that Cdc2/CyclinB/Cks30A may phosphorylate D-TACC directly or via activation of Aurora A. C) In remRA74, Cdc2/Cyclin B can no longer phosphorylate D-TACC and D-TACC accumulates at the spindle equator.
Fig 3.12 Polewards D-TACC movement in metaphase I spindles of female meiosis. Time lapse confocal imaging of live D-TACC-GFP wild type oocytes shows movement of D-TACC-GFP particles (indicated by arrows) towards the poles of acentrosomal spindles. The speed at which D-TACC-GFP particles move towards the poles is 2.6 μm/min +/- 0.27 μm/min. Scale bar, 10 μm.
Fig 3.13 Cks30A is required for D-TACC modifications. A) Immunoblot with D-TACC antibody showing the amount of D-TACC in wild type and \textit{rem}^{Ra74} oocytes. B) The same blot was then incubated with p(Ser863)-DTACC antibody. The p(Ser863)-D-TACC in \textit{rem}^{Ra74} runs faster than the D-TACC in wild type oocytes. C) My hypothesis is that in wild type oocytes, D-TACC is phosphorylated on Ser863 and on residue "X" (C). In \textit{rem}^{Ra74} mutant oocytes residue "X" is no longer phosphorylated but p-Ser863 is still recognised by p(Ser863)-DTACC Ab.
Fig 3.14 Cdc2 is essential for spindle morphogenesis and chromosome alignment. A-C) The spindle and chromosomes in non-activated oocytes were visualized by immunostaining in cdc2B47/Cdc2E1-24 transheterozygotes that had been incubated at the restrictive temperature of 28 °C for 3 days. A) Almost half the spindles were bipolar with chromosomes aligned at the spindle equator. B) The rest of the spindles had defects in spindle morphology and/or C) chromosomes alignment. D) Quantification of spindle and chromosome defects observed in cdc2 mutant. E) Quantification of spindle and chromosome defects observed in wild type (wt) and cdc2B47/cdc2E1-24 (cdc2). Scale bar, 10 μm.
Fig 3.15 Cks30A has multiple roles in the establishment of metaphase I in female meiosis. A) When Cks30A is bound to Cdc2/Cyclin B complex Cdc2 can phosphorylate specific substrates involved in the establishment of metaphase I spindles in female meiosis. D-TACC is modified in a Cks30A dependent manner. B) My hypothesis is that Cdc2/Cyclin B/Cks30A complex can phosphorylate D-TACC allowing D-TACC to be localised at spindle poles. Cks30A is required for polar localisation of Msps, possibly via Cdc2 phosphorylation of Msps. C) Double mutants of rem^{RA74} mps^{208} are synthetic lethal suggesting that Cks30A and Msps are in a shared pathway in mitosis.
### 3.7 Chapter 3 Tables

**Table 3.1 Frequency of rem$^{RA74}$/CyO; msps$^{208}$/MRS offspring**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>rem$^{RA74}$/CyO; msps$^{208}$/MRS</td>
<td>82</td>
<td>100</td>
</tr>
<tr>
<td>rem$^{RA74}$/msps$^{208}$/MRS</td>
<td>41</td>
<td>34</td>
</tr>
<tr>
<td>rem$^{RA74}$/msps</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>rem$^{RA74}$/CyO; msps$^{208}$</td>
<td>41</td>
<td>50</td>
</tr>
</tbody>
</table>

The observed frequencies of the various genotypes of rem$^{RA74}$/CyO; msps$^{208}$/MRS offspring compared to the expected frequencies. The difference between observed and expected frequencies is statistically significant $p \leq 7 \times 10^{-6}$. 
4. Wee1 in acentrosomal spindle formation of female meiosis

4.1 History of *msps-like* mutant

I decided to investigate another mutant from C.F.Cullen’s screen for acentrosomal spindle defects (as described in section 3.1) called *msps-like*. The *msps-like* mutation was originally found in the Schupbach and Wieschaus screen as *mat(2)synRE48* (*RE48*) (Schupbach and Wieschaus, 1989). The name *msps-like* was given due to the presence of tripolar metaphase I spindles in oocytes, that were similar to those seen in the *msps*²⁰⁸ mutant (Cullen and Ohkura, 2001).

The gene mutated in *msps-like* could encode a protein that is involved with Msps and D-TACC in the establishment of bipolar spindles in metaphase I of female meiosis. I feel the study of *msps-like*, in conjunction with my results that Cks30A is required for polar localisation of Msps and D-TACC, would enhance the understanding of how Msps and D-TACC behave in the establishment of bipolar spindles in female meiosis.

Complementation mapping by C.F.Cullen (unpublished data) found the *Df(2L)Dweel-W05* deficiency (Price et al., 2000) uncovered the *msps-like* mutation. *Df(2L)Dweel-W05* has a deletion of 20 kb, containing 8 genes (Fig 4.1A). Of these genes, *dweel* was a good candidate as *dweel/msps-like* transheterozygotes are female sterile. However, initial observations of heterozygous *dweel/msps-like* oocytes did not reveal spindle defects (C.F.Cullen, unpublished data).
4.2 Molecular identification of the msps-like gene

To clarify whether msps-like (which I will refer to from now on as RE48) is a mutation in dweel, I sequenced the 20 kb region defined by Df(2L)Dweel-W05. The 20 kb region was amplified into 2 kb segments by PCR. The amplified 2 kb segments were then sequenced. Genomic DNA of Hi10 (Schupbach and Wieschaus, 1989), another mutant from the same screen as RE48, was sequenced for comparison with RE48 genomic DNA.

Sequencing revealed that dweel was the only mutated gene in the 20 kb region defined by Df(2L)Dweel-W05. dweel sequence in RE48 differs from normal dweel sequence in that 118 bp has been deleted and has a TCCTCC insertion before returning to normal dweel sequence (Fig 4.1A). This causes a frame-shift resulting in a premature STOP codon in dweel (Fig 4.1B). This mutation should produce a truncated Dweel protein in which kinase domain IX-XI is missing, resulting in a loss of kinase activity (Fig 4.1C). As the kinase domain is essential to Dweel function (Stumpff et al., 2004). I concluded that RE48 is a mutation of dweel and will call the mutant dweel<sup>RE48</sup>.

Dweel is a conserved kinase involved in cell-cycle control. It phosphorylates Cdc2 on tyrosine 15, inactivating the Cdc2/Cylin B complex (Campbell et al., 1995). Whether Dweel acts in female meiosis directly or via Cdc2 requires further investigation.
4.3 Dweel is essential for spindle bipolarity in female meiosis of *Drosophila*

The initial observation of $dweel^{RE48}$ mutant having tripolar spindles in metaphase I of female meiosis (C.F. Cullen, unpublished data) was based on a low sample number. The frequency of observed tripolar spindles was low (20%). Examination of a greater number of metaphase I arrested oocytes will give me a more accurate description and frequency of the $dweel^{RE48}$ phenotype.

I dissected ovaries from hemizygous $dweel^{RE48}/Df(2L)Dweel-W05$ females. After fixation in methanol, oocytes were stained for DNA, α-tubulin and D-TACC and examined by confocal microscopy.

The $dweel^{RE48}$ mutant is able to enter meiosis, condense its chromosomes and assemble microtubules around the chromosomes but has defects at metaphase I. The majority (63%) of oocytes I examined had normal bipolar spindles with D-TACC/Msps present at the poles and chromosomes aligned at the spindle equator (Fig 4.3A). The major defect was abnormal spindle morphology. 28% of spindles examined exhibited a tripolar spindle morphology (or very bent spindle) in which D-TACC/Msps were often localised to all three poles (Fig 4.3B). The other spindles observed (8%) had misaligned chromosomes at the spindle equator but this was not significant compared to the frequency seen in wild type spindles.

Previously identified $dweel$ mutants, $dweel^{DS1}$ and $dweel^{ES1}$, were isolated from a screen for lethal and sterile mutations in genes uncovered by $Df(2L)Dweel-W05$ (Price et al., 2000). The authors showed that $dweel$ mutants have defects in the nuclear divisions in early embryogenesis. The $dweel^{ES1}$ mutation causes a truncation in kinase domain IV of Dweel. $dweel^{DS1}$ contains a missense mutation within the
ATP-binding site of Dweel and is likely to have low-level protein function, as homozygous embryos have a less severe phenotype to that of hemizygotes.

I visualised \textit{dweel}^{D51}/\textit{dweel}^{RE48} and \textit{dweel}^{ES1}/\textit{dweel}^{RE48} transheterozygous oocytes using confocal microscopy. \textit{dweel}^{D51}/\textit{dweel}^{RE48} adult females are sterile but metaphase I spindles appeared normal with no clear spindle morphology defects. \textit{dweel}^{ES1}/\textit{dweel}^{RE48} however, displayed tripolar spindles like that observed in \textit{dweel}^{RE48} mutants (Fig 4.3C).

These results suggest that there are different thresholds for Dweel activity with regards to female meiosis and embryogenesis. Fully functional Dweel is required for female meiosis and embryogenesis. Low level Dweel function (\textit{dweel}^{D51}/\textit{dweel}^{RE48}) is sufficient for bipolar spindle morphology in female meiosis but fails embryogenesis. Complete loss of Dweel function (\textit{dweel}^{ES1}/\textit{dweel}^{RE48}) results in abnormal spindle morphology in female meiosis and failure in embryogenesis.

These results indicate that Dweel is required for the establishment and/or maintenance of bipolar spindles in \textit{Drosophila} female meiosis. Msps and D-TACC can still localise to the poles in \textit{dweel}^{RE48} mutants. A simple hypothesis for the role of Dweel in acentrosomal spindle formation is that it phosphorylates Msps/D-TACC protein, activating their ability to maintain spindle bipolarity (Fig 4.4C). The regulation of Msps/D-TACC or other spindle assembly factors could be mediated directly by Dweel phosphorylation, or indirectly via Cdc2/Cyclin B complex (Fig 4.4A and Fig 4.4B).
4.4 Investigation of Cdc2 regulation in spindle formation in female meiosis

I have shown that Cdc2 is essential for the establishment of metaphase I in female meiosis (Section 3.5). I have also shown that the negative regulator of Cdc2, Dweel, is also essential for acentrosomal spindle formation. I thought it would be interesting to investigate the effect of mutating both the positive (twine) and negative (dweel) regulators of Cdc2 on the establishment of metaphase I in female meiosis.

4.4.1 Cytological analysis confirmed that Twine is essential for the establishment of a bipolar spindle in female meiosis

Cdc25 phosphatase activates Cdc2 by removing the Wee1 phosphorylation on tyrosine 15 of Cdc2 (Fig 4.2). There are two homologues of Cdc25 in Drosophila, String and Twine. String has been identified as the homologue required for mitosis (Edgar and O'Farrell, 1990) and Twine has been identified as the homologue required for meiosis (Alphey et al., 1992; Courtot et al., 1992). The twine mutant I am studying also originated from the Schupbach and Wieschaus screen (Schupbach and Wieschaus, 1989). The twine mutant blocks entry into male meiosis and has severe defects in female meiosis (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993). In female meiosis, the spindle fails to arrest at metaphase I resulting in spindle defects such as kinks and splaying of poles and the loss of chromosomes from the spindle (White-Cooper et al., 1993).

To familiarise myself with the twine phenotype, mature oocytes of twine/Df(2L)RA5 females were dissected, fixed in methanol, stained for DNA and Tubulin and visualised by confocal microscopy. The majority had abnormal spindle
or chromosome arrangement. 46% of spindles had defects in chromosome alignment where some spindles appeared to have arrested in anaphase I rather than metaphase I (Fig 4.5A). Oocytes with multiple spindles were also observed (Fig 4.5A). Over a third of oocytes had more than one spindle, a total of 5 spindles were observed in some cases. The multiple spindles were usually quite small and contained a chromosome pair, although in some cases unpaired chromosomes may have been able to form spindles. This phenotype is quite distinct, and unlike nhk-1 mutants, which form multiple spindles due to defective karysome maintenance (Cullen et al., 2005), twine does not have any abnormalities in prophase (Courtot et al., 1992; White-Cooper et al., 1993).

In accordance with previous interpretations (White-Cooper et al., 1993) twe/Df(2L)RA5 spindles are probably failing to arrest and as they proceed through anaphase I into meiosis II the spindle becomes increasingly unstable and falls apart. This would explain both the chromosome misalignment and multiple spindle defects observed. The variety of defects may also be due to the different ages of oocytes when fixed, with the older oocytes having the most severe defects. In this regard live-imaging of twine oocytes could prove very informative in the progression of spindle defects.

These results reconfirm previous reports that Twine is essential for the establishment or maintenance of the metaphase I spindle. Twine may regulate spindle assembly factors required for chromosome alignment and spindle unification (Fig 4.6A). The other scenario is that Twine is acting through the activation of the Cdc2/Cylin B complex and the twine mutation results in the inhibition of Cdc2 causing a failure to arrest in metaphase I of acentrosomal female meiosis (Fig 4.6C).
4.4.2 Investigating $dwee1^{RE48}$ $twine$ in establishment of metaphase I female meiosis

To investigate double mutants of $dwee1^{RE48}$ and $twine$, I first established a double mutant line. The $dwee1^{RE48}$ mutation is located at 27C4 of the left arm of chromosome 2 and $twine$ is also located on the left arm of chromosome 2 at 35F1. Double mutants were made by recombination and the presence of both mutations in the recombinant was confirmed by complementation testing with the single mutants (Fig 4.7). Homozygous $dwee1^{RE48}$ $twine$ flies are viable but female sterile. The females produced significantly fewer mature oocytes than in wild type, $twine$ or $dwee1^{RE48}$ females.

Oocytes from $dwee1^{RE48}$ $twine$ homozygous females were dissected, fixed in methanol, stained for DNA, Tubulin and D-TACC and visualised using confocal microscopy. Out of hundreds of females only 7 spindles were observed; 2 appeared to be early prophase, 1 bipolar spindle, 2 monopolar spindles (Fig 4.8A), and 2 cases of chromosome misalignment (Fig 4.8B). None of the observed spindles bore a resemblance to either $dwee1^{RE48}$ or $twine$ single mutants. Due to the low number of spindles observed, it is impossible to say what the nature and degree of the abnormality is in $dwee1^{RE48}$ $twine$ metaphase I of female meiosis.

$dwee1^{RE48}$ $twine$ double mutants cause sterility with a reduction in oocyte production/maturation. The double mutant line can be used to investigate D-TACC modifications in oocytes (section 4.5.3).
4.4.3 *dwee1*<sup>RE48</sup> and *twine* mutants affect the phosphorylation of Cdc2

In the cell cycle, Dwee1 kinase phosphorylates tyrosine 15 of Cdc2, inhibiting Cdc2 function (Stumpff et al., 2004), while Twine phosphatase removes the phosphorylation, relieving Cdc2 inhibition (Edgar and Datar, 1996; Sigrist et al., 1995b) (Fig 4.2). Investigation of the phosphorylation state of tyrosine 15 in *dwee1*<sup>RE48</sup> and *twine* oocytes should give me an indication as to whether these mutations affect Cdc2.

I used two antibodies to detect the presence of Tyr15 phosphorylation of Cdc2 in *Drosophila*. Cdc2 p34 (PSTAIRE) antibody was raised against a peptide within the PSTAIRE domain of human Cdc2. It can detect Cdc2 and other cyclin dependent kinases containing the PSTAIRE motif of mouse, human, yeast, *Xenopus* and *Drosophila* regardless of the phospo-status. p-Cdc2 (Tyr15) antibody can detect Cdc2 phosphorylated at Tyr15.

To protect phosphorylation, ovaries of wild type, *dwee1*<sup>RE48</sup> and *twine* flies were dissected in methanol before preparing protein samples. Protein samples were analysed by SDS-PAGE and immunoblotted with Cdc2 (PSTAIRE) and p-Cdc2 (Tyr15) antibodies. The results showed that while the levels of Cdc2 remained relatively constant in wild type and mutant oocytes, there was a difference in the levels of Tyr15 phosphorylation (Fig 4.9). In *dwee1*<sup>RE48</sup> oocytes there was a reduction in the level of Tyr15 phosphorylation compared to that of wild type. Conversely it appeared that *twine* oocytes had elevated levels of Tyr15 phosphorylation.

The altered phosphorylation levels were consistent with the known function of *dwee1*<sup>RE48</sup> and *twine*. Although a kinase assay would have to be performed to confirm the activity of Cdc2 in *dwee1*<sup>RE48</sup> and *twine*, the level of phosphorylated
Tyr15 can be used as an estimate of Cdc2 activity. This means $dweel^{RE48}$ flies the may have increased Cdc2 activity and $twine$ flies may have decreased Cdc2 activity in oocytes.

4.5 Dissecting Dweel function in establishment of metaphase I spindles

As Dweel is a kinase, it is likely that the phenotype observed in $dweel$ mutants is due to an unphosphorylated substrate of Dweel. There is strong evidence suggesting that the main substrate may be Cdc2 (Section 4.4), although it may still be the case that Dweel directly regulates a spindle assembly factor.

4.5.1 Creating and examining $dweel^{RE48} msps^{208}$ and $dweel^{RE48} D-TACC^{stella592}$ flies

The $dweel^{RE48}$ phenotype has tripolar spindles, like those observed in $msps^{208}$ and $D-TACC^{stella592}$ mutants. $msps^{208}$ is hypomorphic and results in severe depletion of MspS protein resulting in reduced viability and the occurrence of tripolar spindles in metaphase I of female meiosis (Cullen and Ohkura, 2001). In $D-TACC^{stella592}$ mutants D-TACC levels are less than 1% of wild type but the flies are completely viable (Lee et al., 2001). $D-TACC^{stella592}$ mutant has tripolar metaphase spindles which resembles $msps^{208}$ (Cullen and Ohkura, 2001).

The relationship between Dweel and MspS/D-TACC proteins was investigated by looking for a genetic interaction between $dweel^{RE48}$ and $msps^{208}$, and $dweel^{RE48}$ and $D-TACC^{stella592}$ mutations. If Dweel only acts upon MspS/D-TACC, with regard to acentrosomal spindle bipolarity, then the double mutant spindles would recapitulate the penetrance of $msps^{208}$ and $D-TACC^{stella592}$ mutations. If Dweel
does not act through Msp5/D-TACC, or has additional substrates, a different metaphase I phenotype or stronger penetrance of tripolar spindle morphology may arise from combining these mutations.

The \textit{dwee1}^{RE48} mutation is located on the 2\textsuperscript{nd} chromosome while \textit{msps}^{208} and \textit{D-TACC}\textsuperscript{stellas592} mutations are located on the third. The strategy for making \textit{dwee1}^{RE48} \textit{msps}^{208} and \textit{dwee1}^{RE48} \textit{D-TACC}\textsuperscript{stellas592} flies involved a series of crosses using a double balancer line. The crossing scheme is outlined in Fig 4.10A and Fig 4.10B.

My initial attempt to create \textit{dwee1}^{RE48}/\textit{CyO}; \textit{D-TACC}\textsuperscript{stellas592}/\textit{TM6B} stocks failed and time did not allow another attempt. I was able to establish \textit{dwee1}^{RE48}/\textit{CyO}; \textit{msps}^{208}/\textit{TM6B} stocks. Double homozygotes of \textit{dwee1}^{RE48} \textit{msps}^{208} are viable but their observed frequency is significantly less than the expected frequency of 1/16 (p <= 0.006 Table 4.1), although single mutants are viable. Unfortunately the low frequency of \textit{dwee1}^{RE48} \textit{msps}^{208} made it impossible to gather enough females for the study of acentrosomal spindles.

These results show that there is a genetic interaction between Dwee1 and Msp5, as \textit{dwee1}^{RE48} is fully viable, but \textit{dwee1}^{RE48} \textit{msps}^{208} double mutant viability is dramatically reduced compared with single \textit{msps}^{208} mutant. It would be interesting to see what the spindle defect was in female meiotic spindles and mitotic spindles of \textit{dwee1}^{RE48} \textit{msps}^{208} flies.

4.5.2 Establishment of \textit{dwee1}^{RE48}/\textit{CyO}; \textit{GFP-tubulin/MRS} flies
In order to examine spindle dynamics and the nature of the spindle defects in the dweel<sup>R48</sup> mutant, I established a dweel<sup>R48</sup>/CyO; GFP-tubulin/MRS line of flies to allow for live-imaging of female meiotic spindles (Fig 4.10C).

Curiously, dweel<sup>R48</sup>; GFP-tubulin double homozygotes appear to be lethal although the single mutants are viable. This was also observed with rem GFP-tubulin double homozygotes (section 3.3.2). This shows that there is a genetic interaction between dweel and α-tubulin and a possible interpretation is that Dweel is involved in modifying microtubule dynamics in mitosis. This is evidence that Dweel has a role in mitosis.

It should be possible to look at dweel<sup>R48</sup>; +/GFP-tubulin oocytes, as dweel<sup>R48</sup>; GFP-tubulin/MRS flies are viable. Live imaging of spindle formation in dweel<sup>R48</sup>; +/GFP-tubulin oocytes would hopefully show how the tripolar spindles are formed. It may also be able to test whether microtubule dynamics are altered in dweel<sup>R48</sup> mutants.

4.5.3 Dweel kinase is required for modification of D-TACC in Drosophila oocytes

In section 3.4.4, I showed that the rem<sup>R474</sup> mutant affects D-TACC modification. The p(Ser863)-DTACC antibody that recognises Aurora A phosphorylation of Ser863 on D-TACC had shown different sized bands in the rem<sup>R474</sup> and wild type oocytes.

Oocytes of dweel<sup>R48</sup> and dweel<sup>R48</sup> twh were dissected and fixed in methanol before being used to prepare protein samples to maintain phosphorylation status. Oocyte protein samples were analysed using SDS-PAGE and immunoblotted with D-TACC (recognises all D-TACC) and p(Ser863)-D-TACC antibodies. The
membrane was incubated with p(Ser863)-D-TACC and visualised before incubating the same membrane with D-TACC antibody. This was because the signal from p(Ser863)-D-TACC is much weaker than the signal from D-TACC antibody.

Immunoblotting with D-TACC antibody revealed three bands/isoforms, with the uppermost band being the most abundant. The levels of these three forms of D-TACC are similar in wild type, *dwee1*^RE48^/Df(2L)*Dwee1-W05* and *dwee1*^RE48^ twe oocytes.

Immunoblotting with p(Ser863)-D-TACC revealed one clear band of p(Ser863)-D-TACC in wild type which corresponds to the middle of the three forms detected by D-TACC antibody (Fig 4.1B). *dwee1*^RE48^ twe oocytes give the same size as wild type but with increased levels. *dwee1*^RE48^/Df(2L)*Dwee1-W05* oocytes had 3 forms of p(Ser863)-D-TACC which roughly correspond to the 3 bands detected by D-TACC antibody. The slowest running p(Ser863)-D-TACC was at a reduced level compared to the corresponding band detected by D-TACC antibody. The middle band ran at the same speed as p(Ser863)-D-TACC in wild-type and *dwee1*^RE48^ twe oocytes. The fastest running form of p(Ser863)-D-TACC was unique to *dwee1*^RE48^/Df(2L)*Dwee1-W05* oocytes.

These results are very interesting as they show that there is a difference in D-TACC between *dwee1*^RE48^ mutant and wild type oocytes. This fits my hypothesis that Dweel is required for D-TACC activity in acentrosomal spindle bipolarity. The fact that the D-TACC band observed in wild type returns when both regulators of Cdc2 activity are mutated in *dwee1*^RE48^ twe oocytes suggests that Cdc2/Cyclin B activity is probably responsible for the altered forms of D-TACC.
The nature of the modification and identification of the affected residue of D-TACC remains to be discovered. Consensus sequences for Cdc2 phosphorylation sites are probably prime candidates for investigation. The fact that p(Ser863)-D-TACC runs faster in dweel^{RE48} than in wild type oocytes suggests p(Ser863)-D-TACC is lacking a modification in dweel^{RE48}.

4.6 Investigation of dweel^{RE48} rem^{RA74} double mutants

I now have molecular evidence that Dweel and Cks30A may be involved in the modification/regulation of D-TACC. I decided to investigate whether Dweel1 and Cks30A interacted genetically by investigating the phenotype of metaphase I spindles in dweel^{RE48} rem^{RA74} double mutant. According to my predictions, if Cks30A is required for polar localisation of D-TACC and Dweel1 is required for D-TACC activity, the dweel^{RE48} rem^{RA74} double mutant should result in mislocalisation of D-TACC to the spindle equator. However, if mislocalised D-TACC/Msps activity causes chromosome misalignment and ectopic pole formation in rem^{RA74}, then the dweel^{RE48} rem^{RA74} double mutant should not have these defects (Fig 4.12C). The other scenario is that either dweel^{RE48} or rem^{RA74} phenotype will be epistatic in the double mutant (Fig 4.12C). The establishment of dweel^{RE48} rem^{RA74} double mutant flies will allow me to test these hypotheses.

4.6.1 Creating dweel^{RE48} rem^{RA74} double mutant flies

Both dweel^{RE48} and rem^{RA74} mutations are present on the left arm of chromosome 2. Double mutants were made by recombination and the presence of both mutations in the recombinant was confirmed by complementation testing with single
Df(2L)Dweel-WQ5 and remRA74 mutants. The crossing scheme for the establishment of dweelRE48 remRA74 fly strains is described in Figure 4.13.

I was able to establish a dweelRE48 remRA74 fly line with viable double mutants that were female sterile. Oocytes from dweelRE48 remRA74 double homozygous females were dissected, fixed in methanol, stained for DNA, Tubulin and D-TACC and visualised using confocal microscopy.

Over half the spindles observed had defects in metaphase I spindle morphology (Fig 4.14E). Nearly half of the spindles examined had D-TACC mislocalised at the spindle equator (Fig 4.14A and Fig 4.14E). The D-TACC mislocalisation was often in conjunction with other spindle defects. Defects in spindle morphology included ectopic pole formation and tripolar spindles (Fig 4.14B and Fig 4.14D). The frequency of tripolar spindles was significantly less in dweelRE48 remRA74 (5%) than in dweelRE48 (24%) mutants (Fig 4.14E). A third of observed dweelRE48 remRA74 spindles had misaligned chromosomes at the spindle equator and bore a strong resemblance to the chromosome misalignment observed in remRA74 spindles (Fig 4.14C). The frequency of chromosome misalignment in dweelRE48 remRA74 (27%) was less than that observed in remRA74 (55.5%) but greater than that observed in dweelRE48 (8%) mutants (Fig 4.14E).

These results indicate that remRA74 is epistatic to dweelRE48 as remRA74 phenotypes are retained but dweelRE48 phenotypes have nearly disappeared in the dweelRE48 remRA74 double mutant. The presence of ectopic poles at the equator suggests that either Msp/D-TACC mislocalisation is not responsible for ectopic pole formation or that dweelRE48 mutation does not completely abolish Msp/D-TACC
activity in maintenance of spindle bipolarity. The fact that chromosome
misalignment in \textit{dweel^{RE48}} \textit{rem^{RA74}} occurs at a lower frequency than observed in
\textit{rem^{RA74}} suggests that the double mutation may rescue chromosome alignment defect
to a small degree.

4.7 Summary of Dweel in acentrosomal spindle assembly

I have shown that Dweel is essential for bipolar spindle formation in metaphase I
female meiosis. The \textit{dweel^{RE48}} mutation introduces a premature stop codon in
\textit{Dweel} that results in a loss of Dweel kinase activity. Transheterozygotes between
\textit{dweel^{RE48}} and other \textit{Dweel} mutants indicates that the kinase activity of Dweel is
essential in spindle formation.

I made \textit{dweel^{RE48}} \textit{twine} double mutants and found that homozygous females
are sterile with possible defects in oocyte production. I have also shown that Cdc2
phosphorylation at Tyr15 is reduced in \textit{dweel^{RE48}} and increased in \textit{twine} oocytes,
indicating a loss of kinase and phosphatase function on Cdc2 in Dweel and Twine
respectively.

\textit{dweel^{RE48}} oocytes have two extra isoforms of D-TACC from the single
isoform existing in wild type ovaries. \textit{dweel^{RE48}} \textit{twine} double mutants return D-TACC
to the single isoform present in wild type oocytes. This suggests that Dweel is
required for D-TACC modification in a Cdc2 kinase dependent manner. This
observation lends credibility to the hypothesis that Dweel is required to activate
Mps/D-TACC complex in maintenance of spindle bipolarity. The observation that
the \textit{dweel^{RE48}} \textit{mmps^{208}} double mutant has reduced viability provides genetic evidence
that Dweel acts in conjunction with Msp1s in an essential process. This process may be involved in the establishment or maintenance of mitotic spindles.

Further evidence that Dweel is required in mitosis was seen in the lethality of \textit{dweel}^{RE48} \textit{GFP-tubulin} double mutant flies. This suggests that Dweel kinase function is required for some aspect of microtubule function in mitosis.

I established \textit{dweel}^{RE48} \textit{rem}^{RA74} double mutant fly lines. Analysis of \textit{dweel}^{RE48} \textit{rem}^{RA74} metaphase I arrested oocytes suggest that the \textit{rem}^{RA74} mutant is epistatic to \textit{dweel}^{RE48} mutant.

In conclusion, my studies have shown that Dweel is required for acentrosomal spindle bipolarity in female meiosis. I have provided molecular and genetic evidence that the role of Dweel in spindle formation is probably through Cdc2-dependent regulation of the D-TACC/Msp1s complex.
Fig 4.1 Molecular identification of the RE48 mutation. A) Deficiency mapping of RE48 mutation. The RE48 mutation is located within the 20 kb region, containing 8 genes (represented by arrows). Sequencing of the region revealed that one gene, dweel, contains a 118 bp deletion (shaded red) plus a 6 bp insertion. B) The mutation causes a frameshift, which results in a premature STOP codon leading to truncated Dweel protein. C) The deletion and following truncation removes 3 regions of the kinase domain, which is essential for Dweel activity.
Fig 4.2 Dweel and Twine regulate Cdc2/Cyclin B activity. Dweel kinase phosphorylates Cdc2 on Tyr 15 inactivating Cdc2. Twine (Cdc25) phosphatase can remove the phosphorylation of Cdc2 on Tyr 15, activating the Cdc2/Cyclin B complex.
**Fig 4.3 Dwel1 is essential for spindle bipolarity.** The spindle and chromosomes in non-activated oocytes were visualised by immunostaining in *dweel*<sup>RE48</sup>/Df and *dweel<sup>ES1</sup>/RE48* mutants. A) The majority of *dweel<sup>RE48</sup>* spindles were normal with bipolar spindles, aligned chromosomes and D-TACC at the poles. B) Typical abnormalities were bent/tripolar spindle morphologies. D-TACC was often present at all three poles, indicated by arrow heads. C) The bent/tripolar spindle morphology was also observed in *RE48/Dweel<sup>ES1</sup>* mutants. D) Quantification of the spindle defects in wild-type (+/+) and *RE48* hemizygotes (*RE48/Df*). 88 *RE48* hemizygous and 105 wild type spindles were counted. Scale bar, 10 μm.
Fig 4.4 Proposed models for Dwee1 in acentrosomal spindle formation. A) Dwee1 inhibition of Cdc2/Cyclin B directly leads to spindle bipolarity, or B) activation of a protein required for spindle bipolarity. C) Alternatively, Dwee1 directly phosphorylates and activates a protein required for spindle bipolarity in metaphase I of female meiosis.
Fig 4.5 Twine is essential for metaphase I arrest in female meiosis. The spindle and chromosomes in non-activated oocytes were visualised by immunostaining in *twine* mutant. A) A third of spindles appeared normal with aligned chromosomes and a bipolar spindle. B) Chromosome misalignment was frequently observed in *twine* mutant as well as C) defects in spindle morphology and number. D) Quantification of the spindle and chromosome defects in wild type (+/+) and *twine* hemizygotes (twe/Df). Between 24 and 105 spindles were counted for each genotype. Scale bar, 10 μm.
Fig 4.6 Proposed models for Twine in acentrosomal spindle formation. 
A) One model is that Twine is required for regulation of spindle assembly factors directly or through activation of Cdc2/Cyclin B complex. B) An alternative model is that Twine activates Cdc2/Cyclin B inhibiting metaphase to anaphase transition. The *tw* mutant enters anaphase prematurely, causing chromosome misalignment and collapse of spindles.
Fig 4.7 Generating *dwee1*<sub>RE48</sub> *twe* double mutants. A) In order to establish *dwee1*<sub>RE48</sub> (*RE48*) *twe* double mutants *RE48/*twe transheterozygous females are obtained. Recombination can occur in the female germline. B,C) Recombinants (Rec cn bw) were tested for the presence of *dwee1*<sub>RE48</sub> and *twe* by complementation testing with *Df(2L)Dwee-W05* and *twe*. Crosses can be followed by dominant markers *CyO*, *Sco* and the recessive marker *cn* (eye colour). Homozygotes of *cn bw* result in white eyes.
**Fig 4.8** *dwee1RE48 twe* double mutant phenotypes in female meiosis. The spindle and chromosomes in non-activated oocytes were visualized by immunostaining in *dwee1RE48 twe* double mutants. A) Monopolar spindles and B) chromosome misalignment defects were observed out of a total of 7 spindles. Scale bar, 10 μm.
Fig 4.9 *dwee1*<sup>RE48</sup> and *twine* mutants affect the Cdc2 Tyr 15 phosphorylation. C,D) *dwee1*<sup>RE48</sup>/Df(2L)Dwee1-WO5 (RE48/DF) and *twe*/Df(2L)RA5 (*twe*/Df) mutant oocytes were run on SDS-PAGE and immunoblotted with Cdc2 and p-Cdc2 antibodies. A,B) Tubulin was used as a loading control. Cdc2 levels remain constant in wild type *RE48*/Df and *twine*/Df oocytes (C). There is a reduced amount of p-Cdc2 in *RE48*/Df compared to wild type (D). p-Cdc2 levels are elevated in *twe*/Df oocytes to that of wild type.
Fig 4.10 Combining dweel\textsuperscript{RE48} with msp\textsubscript{s208}, dtacc\textsubscript{sta592} and GFP-tubulin lines. A double chromosome balancer line (w; L\textsuperscript{2}/CyO; MRS/TM6B) was crossed to dweel\textsuperscript{RE48} (RE48) and the 3rd chromosome mutants A) msp\textsubscript{s208}, B) dtacc\textsubscript{sta592} and C) GFP-tubulin. Male offspring of RE48 with balanced 3rd chromosomes (RE48/L\textsuperscript{2}; +/-MRS) could then be crossed to females carrying the desired allele and balanced 2nd and third chromosomes eg (+/CyO; GFP-tubulin/TM6B). By following the markers, it was possible to select flies carrying RE48 and the desired 3rd chromosome allele.
**Fig 4.11 DTACC modifications in Cdc2 regulator mutants.** Immunoblot of oocytes from wild type (+/+), dwee1^RE48/Df(2L)Dwee1-WO5 (RE48/Df) and dwee1^RE48 twine (RE48/twe) lines. The oocyte protein membranes were first immunoblotted with p-D-TACC antibody and then with D-TACC antibody. A) Western blot with D-TACC antibody shows three D-TACC bands at similar concentrations in wild-type, RE48/Df and RE48 twe oocytes. B) Immunoblot with p-D-TACC antibody shows that wild type and RE48 twe oocytes have a single p-D-TACC of identical size although there is an increased amount in RE48 twe. RE48/Df has three forms of p-D-TACC; the slowest band is comparable to the slowest band in D-TACC antibody blot, the middle band is comparable to the single bands observed in wild-type and RE48 twe p-D-TACC, and the fastest band is the most abundant. The three coloured bands represent the three forms of D-TACC seen in both blots.
Fig 4.12 Proposed models for \textit{rem}^{RA74} \textit{dwee}^{RE48} double mutant phenotype. A) \textit{rem}^{RA74} mutants mislocalise Msps to the equator and has ectopic poles and chromosome misalignment in metaphase I of female meiosis. B) \textit{dwee}^{RE48} mutants have tripolar spindles and Msps at the poles that is possibly inactive in metaphase I of female meiosis. C) In \textit{rem}^{RA74} \textit{dwee}^{RE48} double mutants one of the single mutant phenotypes may be epistatic to the other single mutant phenotype. If mislocalization of active Msps in \textit{rem}^{RA74} is responsible for ectopic poles and chromosome misalignment, the double mutant may only have mislocalised Msps as \textit{dwee}^{RE48} Msps is “inactive”.

---

**A**

Wild type bipolar spindle

\textit{cks30A rem}^{RA74} \textit{mutant}

Chromosome misalignment

Msps at equator

Spindle defects

**B**

Wild type

\textit{dwee}^{RE48}

Tripolar spindle

Msps at poles

**C**

Wild type

\textit{rem}^{RA74} \textit{dwee}^{RE48}

Chromosome misalignment

Msps at equator and Spindle defects

\textit{rem}^{RA74} is epistatic to \textit{dwee}^{RE48}

\textit{rem}^{RA74} \textit{dwee}^{RE48}

"Inactive" Msps mislocalised but no associated spindle or chromosome defects
Fig 4.13 Generating \textit{dwee}^{RE48} \textit{rem}^{RA74} double mutants. B) In order to establish \textit{dwee}^{RE48} (\textit{RE48}) \textit{rem}^{RA74} (\textit{rem}) double mutants, \textit{RE48}/\textit{rem} transheterozygous females were obtained. Recombination can occur in the female germ-line. A) The desired recombination event is shown. C.D) Recombinants (\textit{rec cn bw}) were tested for the presence of \textit{RE48} and \textit{rem} by complementation testing with \textit{RE48} and \textit{rem} single mutants (C,D). Crosses can be followed by dominant markers \textit{CyO} (curly wings), \textit{Sco} (bristle defect) and recessive marker \textit{cn}. 

\begin{align*}
\textbf{A} & \quad \begin{array}{c}
\text{dwee}^{\text{RE48}} \\
2L \quad \begin{array}{c}
\text{rem} \\
\text{cn} \\
\text{bw}
\end{array}
\end{array} \\
\text{X} & \quad \begin{array}{c}
\text{rem} \\
\text{b} \\
\text{pr} \\
\text{c}
\end{array}
\end{align*}

\begin{align*}
\downarrow \\
\text{dwee}^{\text{RE48}} \\
\text{rem} \\
\text{cn} \\
\text{bw}
\end{align*}

\begin{align*}
\textit{RE48 rem} \text{ recombinant}
\end{align*}

\begin{align*}
\textbf{B} & \quad \begin{array}{c}
\text{RE48 cn bw} \\
\text{CyO cn}
\end{array} \quad \begin{array}{c}
\text{rem} \\
\text{CyOcn}
\end{array}
\end{align*}

\begin{align*}
\text{RE48 cn bw} & \quad \begin{array}{c}
\text{rem} \\
\text{CyO cn}
\end{array}
\end{align*}

\begin{align*}
\text{RE48 cn bw} & \quad \begin{array}{c}
\text{Sco} \\
\text{CyO cn}
\end{array}
\end{align*}

\begin{align*}
\text{rec cn}^{+} & \quad \begin{array}{c}
\text{Sco} \\
\text{CyO cn}
\end{array}
\end{align*}

\begin{align*}
\text{rec cn}^{+} & \quad \begin{array}{c}
\text{rem} \\
\text{CyO cn}
\end{array}
\end{align*}

\begin{align*}
\text{rec cn}^{+} & \quad \begin{array}{c}
\text{RE48 cn bw} \\
\text{CyO cn}
\end{array}
\end{align*}

\begin{align*}
\text{Female sterile?}
\end{align*}

\begin{align*}
\textbf{C} & \quad \begin{array}{c}
\text{rec cn}^{+} \\
\text{CyO cn}
\end{array} \quad \begin{array}{c}
\text{X} \\
\text{RE48 cn bw}
\end{array}
\end{align*}

\begin{align*}
\text{D} & \quad \begin{array}{c}
\text{rec cn}^{+} \\
\text{RE48 cn bw}
\end{array} \quad \begin{array}{c}
\text{X} \\
\text{rec cn}^{+}
\end{array}
\end{align*}

\begin{align*}
\text{Female sterile?} & \quad \text{Make stock}
\end{align*}
**Fig 4.14 dweel^RE48 rem^RA74 double mutant spindle phenotype.** The spindle and chromosomes in non-activated oocytes were visualised by immunostaining in dweel^RE48 rem^RA74 (RE48 rem) double mutant. A) D-TACC mislocalisation to the spindle equator, B) the formation of ectopic poles and C) chromosome misalignment were observed that were reminiscent of rem single mutant phenotypes. D) Tripolar spindles like those in RE48 single mutants were also observed but at a much lower frequency. D) Quantification of the spindle and chromosome defects observed in wild type (+/+), rem, RE48/Df(2L)Dweel-W05 (RE48/Df) and RE48 rem. Scale bar, 10 μm.
### Chapter 4 Tables

#### Table 4.1

**dweel\textsuperscript{RE48} msps\textsuperscript{208} double mutants have reduced viability**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>dweel\textsuperscript{RE48}/CyO;</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>msps\textsuperscript{208}/TM6B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dweel\textsuperscript{RE48};</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>msps\textsuperscript{208}/TM6B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dweel\textsuperscript{RE48}/CyO;</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>msps\textsuperscript{208}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dweel\textsuperscript{RE48} msps</td>
<td>4</td>
<td>18.5</td>
</tr>
<tr>
<td>\textsuperscript{208}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The observed and expected frequencies of \textit{dweel}\textsuperscript{RE48}/CyO; msps\textsuperscript{208}/TM6B offspring.

There is a significant difference between the observed and expected frequencies in \textit{dweel}\textsuperscript{RE48} msps\textsuperscript{208} (P value $\leq 0.006$)
5. Screening for mutants in the establishment of metaphase I arrested female meiosis on the X chromosome

5.1 Introduction

In this chapter I will describe a screen on the X chromosome for mutants defective in establishing female meiotic spindles in *Drosophila*.

The reason I wanted to carry out such a screen was to try and find novel mutants which may reveal new insights into the formation of a female meiotic spindle. The X chromosome seemed a good candidate, as there were fewer screens previously carried out for female sterile mutations on the X chromosome than on the second or third chromosome. I performed a trial screen by mutagenising male flies with EMS and setting up individual mutant fly lines. I screened those lines for eggs laid by mutant females that fail to develop beyond the blastoderm stage. Out of the 80 mutant lines I generated, *X-01* to *X-80*, there were 20 lines whose eggs fail to develop beyond the blastoderm stage. These 20 lines remain to be screened for spindle defects in metaphase I arrested oocytes.

During the trial run of my screen, my lab was able to acquire an extensive collection of mutant lines from C.Nusslein-Volhard (Tubingen), where laid eggs fail to develop beyond the blastoderm stage. These mutants came from screens for identification of new maternal genes required for oocyte or early embryonic patterning, including zygotically required ones (Luschnig et al., 2004) (personal communication, N. Vogt, Tubingen). They used the FLP-FRT technique and a germline clone marker, GFP, to engineer a system that allowed efficient screening for maternal effect phenotypes after only one generation of breeding. In their
scheme, mutant phenotypes are detected amongst eggs from single females, which are obtained as F₁ progeny of mutagenised EMS-treated males. They carried out a separate screen for each of the five major chromosome arms. On the X chromosome a total of 16,000 crosses were successful, 9,500 of which F₁ females produced germ-line clones. 149 mutants gave embryos that failed to hatch (personal communication, N. Vogt, Tubingen). Due to the extensive screen the Tubingen mutants came from, it was more sensible to screen these lines for spindle defects in female meiosis than to continue generating my own mutants for screening. The screen was divided between me and C.F. Cullen due to the large number of mutants at our disposal.

5.2 Screen design

5.2.1 Generating homozygous females for screening

Many of the Tubingen mutants were homozygous lethal. In order to examine homozygous non-activated mutant oocytes, I made use of the germ-line cloning strategy. This system uses the FLP-FRT recombination system to promote site-specific chromosomal exchange, making it possible to generate a homozygous germline from a heterozygous female (Chou and Perrimon, 1996) (Fig 5.1A). This system uses the X-linked germline-dependent dominant female sterile mutation, ovo⁰, to select for homozygous mutant oocytes.

Tubingen mutant (tubingen mutant/FM7c) virgin females were crossed to ovo⁰/Y males and site-specific recombination was activated, by heat-shock induction of Flipase, in the progeny 48 hours after egg deposition (Fig 5.1B).
C.F. Cullen carried out the FLP-FRT recombination and selected the female flies for dissection, while we both carried out the dissection, staining and examination of oocytes.

5.2.2 Screening strategy

The screening strategy is to look for cytological defects in the establishment of metaphase I in female meiosis from each Tubingen mutant.

Non-activated mature oocytes were dissected from adult flies and fixed in methanol. To visualize spindles and chromosomes using confocal microscopy, oocytes were stained with α-tubulin antibody and DAPI/PDI. In wild type, non-activated mature oocytes contain a single bipolar spindle with chromosomes symmetrically organised at the spindle equator, and achiasmate 4th chromosomes nearer the poles. Deviations in spindle bipolarity, the number of spindles and chromosome alignment were considered abnormal. If no abnormalities are found in a minimum of 5 spindles, the mutant was not examined further. Mutant lines showing abnormalities in the first round of screening were dissected and visualised once more to ensure the spindle phenotype was reproducible. If reproducible, the mutant line was regarded to have a defect in the establishment of metaphase I in female meiosis. This strategy is outlined in Fig 5.2.

5.3 Screening results

In this section I will summarise the screening results. I will only describe in detail the mutant lines which I found.
5.3.1 Overview of screen results

A total of 103 Tubingen mutants on the X-chromosome were screened for spindle defects in the establishment of metaphase I in female meiosis. I screened 34 mutant lines in total, the rest of which were screened by C.F.Cullen. The results of the screen are summarised in Fig 5.3 and Table 5.1.

38 (36.9%) of the mutants were unable to be examined due to insufficient mosaic females, and abnormal oocytes. 40 (38.8%) of the mutant lines were normal metaphase I arrested oocytes with bipolar spindles and chromosomes symmetrically aligned at the metaphase plate and achiasmate 4th chromosomes nearer the poles. The remaining 25 lines (24.3%) had defects in metaphase I of female meiosis. These mutants were grouped into the following phenotypic categories; defects in spindle morphology, chromosome alignment, and spindle morphology/ chromosome alignment. The most common type of metaphase I defect was chromosome misalignment (10.7 %). Other metaphase I defects were in spindle morphology (5.8 %) and a combination of chromosome misalignment and spindle morphology defects (7.8 %).

I was able to find 14 metaphase I mutants from the screen (Table 5.2). I will describe my mutants in further detail in the following sections by assigning them to three classes; chromosome, spindle, and chromosome/spindle defects.

5.3.2 Metaphase I mutants with defects in chromosome alignment

Seven of the 14 metaphase I mutants I discovered, X-036-19-1, X-133-31-1, X-245-11-1, X-268-04-3 (Fig 5.4), X-281-24-1, X-347-20-3 and X-388-35-3 (Fig 5.5), had a defect in chromosome alignment (Table 5.3).
In wild type non-activated mature oocytes, chromosomes are aligned symmetrically with the chiasmate chromosomes (I-III) grouped together at the spindle equator, and the achiasmate 4th chromosomes nearer to the spindle poles.

In all 7 mutants, the chromosomes are separated out along the length of the spindle (X-245-11-1 and X-268-04-03 in Fig 5.4 and X-347-20-3 in Fig 5.5 are typical examples). I also observed that the number of chromosomes is in excess in some cases, suggesting that chromosome cohesion or formation of bivalents is defective in some mutants (X-347-20-3 Fig 5.5). There were varying types of chromosome separation to be observed within these mutants. In some cases, particular chiasmate chromosomes were present at the poles while the rest of the chiasmate chromosomes remained near the spindle equator (X-133-31-1 in Fig 5.4 and X-281-24-1and X-388-35-3 in Fig 5.5). In other cases, the chromosome configuration appeared to be in anaphase, where the chromosomes were divided into two groups, with each group near opposite poles leaving the spindle equator devoid of chromosomes (X-036-19-1 Fig 5.4).

5.3.3 Metaphase I mutants with defects in spindle morphology

Four of the mutants I identified had defects in spindle morphology (Table 5.4). In wild type, non-activated mature oocytes form a bipolar spindle, with microtubules concentrated around the chromosomes at the spindle equator and tapering out into two well-defined poles.

X-033-11-4 and X-073-02-6 had very weak spindles, where the microtubules were barely covering the chromosomes and only just bipolar in shape (Fig 5.6A). In
many cases the spindles appeared round-shaped as both poles failed to taper suggesting problems in bipolar spindle formation.

X-069-36-6 and X-162-03-3, whilst able to form bipolar spindles, had a high occurrence of extra mini-spindles accompanying the main spindle (Fig 5.6B). The mini-spindle was bipolar with a chromosome aligned at the equator. The mini-spindle was usually positioned away from the main spindle pole, rather than near the main spindle equator. In X-162-03-3 the mini-spindles appeared to form exclusively around the 4th chromosome. In X-069-36-6, although most mini-spindles formed around a 4th chromosome there were some cases in which mini-spindles formed around a bivalent chromosome (Fig 5.6B).

5.3.4 Metaphase I mutants with defects in spindle morphology and chromosome alignment

Three of the spindle mutants I identified, X-347-25-2, X-287-16-4 and X-074-29-3, had defects in both spindle morphology and chromosome alignment (Table 5.5). All three mutants in this class had small ectopic poles coming out of the main spindle and misalignment of chromosomes at the spindle equator (Fig 5.7A). X-347-25-2 has an additional defect in focusing the spindle poles (Fig 5.7A).

X-074-29-3 and X-287-16-4 strongly resembled the rem\textsuperscript{R474} phenotype in terms of ectopic poles and chromosome misalignment around the spindle equator.
5.3.5 Metaphase I mutants with defects in spindle formation around individual chromosomes

One of the most interesting mutant phenotypes found in this screen was discovered by C.F. Cullen in the X-173-40-4 mutant. X-173-40-4 has bipolar spindles that often have individual chromosomes, with no associated microtubules, outside the main spindle ("naked" chromosomes). Due to the lost chromosome phenotype X-173-40-4 was renamed awol (absent without leave). Individual chromosomes are usually able to form their own spindles as seen in NHK-1 and wisp mutants (Brent et al., 2000; Cullen et al., 2005), raising the intriguing question of why these chromosomes are without associated microtubules.

Although not the strongest phenotype, several of the mutant lines I found had the "naked" chromosome defect (Table 5.6 and Fig 5.8). The mildest form of the "naked" chromosome phenotype was observed in X-281-24-1, X-347-25-2 and X-388-35-3 where chromosomes were at the very edge of the poles and partially outside the spindle as if they were being ejected (Fig 5.8). X-133-31-1 and X-162-03-3 had examples where more than one chromosome was partially naked along the length of the spindle (Fig 5.8). The most extreme case I found was in X-245-11-1 spindles where several chromosomes were without microtubules (Fig 5.8).
5.4 Complementation testing of acentrosomal spindle mutants from female sterile X chromosome screen

5.4.1 Complementation testing strategy

Screening by C.F. Cullen and myself detected 25 mutants with defects in the establishment of metaphase I in female meiosis. Considering the scale of the original screen, several of these mutants may be allelic.

Complementation testing is a method that aids in detecting mutants that are allelic. Complementation is a phenomenon where two mutations of two different genes in combination rescue the defect of each single mutant in a diploid. In most cases, two mutants in the same gene fail to complement (Fig 5.9A), whereas mutants in different genes will complement (Fig 5.9B). There are special exceptions called intragenic complementation and non-allelic non-complementation. Intragenic complementation is complementation between mutations in the same gene. Non-allelic non-complementation is a failure of complementation between mutations in two different genes (Yook et al., 2001).

I intend to cross all of the Tubingen mutants to each other and test the transheterozygous females for fertility. Six mutant lines, X-009-30-3, X-283-06-3, X-287-16-4, X-347-25-2, X-268-04-3 and X-281-24-1, have been omitted so far as they were too ill at the time of performing the complementation tests.

I decided to include the wispy (wisp) mutant in the complementation tests, as it is a previously identified metaphase I mutant on the X chromosome. wisp has not been molecularly identified. Spindle defects in metaphase I arrested wisp oocytes include abnormally shaped spindles, spindle spurs and ectopic spindles associated
with lost chromosomes (Brent et al., 2000). This phenotype resembles the mini-
spindle phenotype I described in X-069-36-6 and X-162-03-3 (see 5.2.3) mutants, and
it would be interesting to test whether they are allelic with wisp.

I judged complementation by the viability and fertility of transheterozygous females.

5.4.2 Acquiring X chromosome mutant males

In order to perform complementation tests, male flies carrying the mutation on the X
chromosome are required for at least one mutant of a test pair. As males have only
one copy of the X chromosome, males carrying lethal X chromosomes will not
emerge (Fig 5.1A). The majority of the metaphase I mutants do not have males
carrying the mutant X chromosome. wisp and 5 of the metaphase I mutants, X-010-
39-1, X-033-11-4, X-173-40-4, X-245-11-1 and X-388-35-3, have fertile males
carrying the mutant allele (Table 5.8). The males from these lines can be crossed to
the females of the other metaphase I mutants for complementation tests.

Even if the mutant X chromosome carries a lethal mutation, viable males
carrying the mutation could be generated using X-chromosome duplications that cover
the lethal mutation. These lines have duplicated X chromosome fragments attached
to the Y chromosome, autosomes or “free” chromosomes. A series of duplication
lines are estimated to cover 88 – 92 % of X chromosome euchromatin.

By crossing this series of duplication lines to the metaphase I mutants, it
should be possible to acquire male flies carrying the mutant allele. If the wild type
copy of the mutated gene is present in the duplicated X-chromosome fragment, the
male will be viable (Fig 5.10B). Mutant males then need to be tested for fertility, as
the duplication may have only rescued the lethal mutation but not the mutation causing sterility.

It is worth noting that although the duplicated segment may rescue mutant allele carrying males from lethality, the duplicated region does not necessarily contain the mutation responsible for the metaphase I defect in female meiosis.

Duplication line crosses generated mutant allele carrying males in 9 of the remaining metaphase I mutants (Table 5.8). Males carrying the mutant allele could then be crossed to other metaphase I mutants for complementation testing (Fig 5.10C).

This leaves 5 of the available 19 metaphase I mutants, \( X-036-19-1, X-027-06-1, X-074-29-3, X-270-13-1 \) and \( X-315-11-1 \) without mutant males. These mutants will be included in the complementation tests but it will not be possible to ascertain whether they can complement each other.

5.4.3 Complementation test results

I crossed \( \text{wisp} \) and the 14 mutant carrying male lines to the 19 metaphase I mutant females and tested the transheterozygous female offspring for fertility. If the female was fertile, the two mutant alleles complemented.

The results of the complementation tests can be seen in Table 5.9 and are summarised in Table 5.10. Seven of the metaphase I arrested mutants did not fall into any complementation groups (Table 5.10), suggesting the mutations are in genes unique among the metaphase I mutants. Another four metaphase I mutants, \( X-036-19-1, X-027-06-1, X-074-29-3 \) and \( X-270-13-1 \), did not fall into any complementation
groups as far as I can test. As these lines have no mutant carrying males I cannot rule out the possibility that these mutants fail to complement each other.

The rest of the metaphase I mutants fell into three complementation groups. \(X-010-39-1\) and \(X-315-11-1\) fail to complement each other, which I dubbed the “X-group 1” complementation group. Both mutants have a chromosome misalignment phenotype, making it reasonable to assume that \(X-010-39-1\) and \(X-315-11-1\) have mutations in the same gene.

The second complementation group includes \(wisp, X-069-36-6\) and \(X-162-03-3\), which I named the “wisp” complementation group. I had predicted that these lines may be mutations in the same gene based on their shared mini spindle phenotype.

The final complementation group included \(X-173-40-4\) (awol), \(X-069-36-6, X-133-31-1, X-162-03-3, X-245-11-1\) and \(X-388-35-3\) mutants, which I called the “awol” complementation group. All of these mutants, except \(X-069-36-6\), have evidence of the “naked” chromosome phenotype observed in awol (see section 5.2.5).

Curiously, \(X-069-36-6\) and \(X-162-03-3\) fail to complement both \(wisp\) and \(awol\), whereas \(wisp/awol\) females are fertile. \(X-069-36-6\) and \(X-162-03-3\) complement, adding a further complication (Fig 5.11).

5.5 Summary of the metaphase I mutant screen

In this chapter I have described a screen for defects in metaphase I arrested oocytes on female sterile mutants of the X chromosome. Screening by C.F.Cullen and myself uncovered 25 metaphase I mutants.
I placed the 14 mutants that I categorised into three groups. Seven mutants had defects in chromosome alignment, four had defects in spindle morphology, and three had defects in chromosome alignment and spindle morphology. Six of my mutants had the additional defect of having chromosomes, with no associated microtubules, outside the main spindle.

Complementation testing of 19 of the metaphase I mutants identified several complementation groups. Seven of the metaphase I mutants are unique among the metaphase I mutants. Four of the metaphase I mutants were unique, but as they do not have mutant males I cannot test for complementation within these mutants. X-group 1 contains X-315-11-1 and X-010-39-1 chromosome misalignment mutants. The “wisp” and “awol” complementation groups contain a total of 7 mutants and due to the complicated complementation results they form a “Super-complementation” group (Fig 5.11). I will discuss the Super-complementation group in more detail in 6.4.5.
Fig 5.1 Obtaining homozygous oocytes via germ-line clones A) cartoon outlying how germ-line cloning works. {\textit{ovo}^D} is a dominant mutant that results in failure to lay eggs, \( m = \text{mutant} \). Heat shock of larvae 48 hours after egg laying activates FLPase and induces site-specific recombination between FRTs (arrowheads). There are four outcomes of recombination and only the one homozygous for the mutant can lay eggs. B) Crossing scheme for acquiring flies that lay homozygous mutant eggs. Mutant virgins are crossed to \textit{ovo}^D males to obtain \textit{mutant/ovo}^D females. \textit{FM7c} carries \textit{bar}, allowing identification of females that lay homozygous eggs.
Tubingen mutant → Dissect and examine non-activated oocytes → 1 in 5 spindles with defects?

- No: Discard
- Yes: Is phenotype reproducible?

- No: Class of mutation?
  - Spindle defect
  - Chromosome misalignment
  - Other
- Yes:phenotype reproducible?

Fig 5.2 Screening strategy for spindle defects in female sterile mutants on the X chromosome. Non-activated oocytes were dissected and examined for defects in spindle formation. If no abnormalities are found in a minimum of 5 spindles, the mutant was discarded. Mutant lines showing abnormalities were dissected and visualised once more to see if the phenotype was reproducible. If reproducible, the mutant line was classed as having a defect in metaphase I of female meiosis. Metaphase I mutants were further categorised into mutant classes, such as spindle defects and chromosome misalignment.
Fig 5.3 Overview of screening for metaphase I mutants of female meiosis on the X chromosome. A) Quantification of Tubingen mutant screen for metaphase mutants. Many Tubingen mutants could not be examined for metaphase I phenotypes due to lack of mosaic females or mature eggs (“fail”). 38.8% of Tubingen mutants screened were normal with bipolar spindles and aligned chromosomes. The rest were metaphase I mutants. B) Quantification of classes of metaphase I mutants. Defects are presented as a percentage of the total screen. Numbers in brackets are as a percentage of total metaphase I mutants.
Fig 5.4 Metaphase I mutants with defects in chromosome alignment.
The spindle and chromosomes in non-activated oocytes were visualised by immunostaining in Tubingen mutants. Examples of chromosome misalignment in X-036-19-1, X-133-31-1, X-245-11-1 and X-268-04-03 Tubingen mutants. In X-133-31-1 some of the chiasmate chromosomes, indicated by arrowhead, are present at the poles with the 4th chromosomes, indicated by arrows. Chromosome misalignment was observed in X-268-04-3 by DAPI staining, but Propidium Iodide staining was very poor. The background “noise” for DNA staining is probably RNA, which Propidium Iodide also stains. RNA remains in some samples despite treatment with RNAsase in the staining solution. Scale bar, 10 µm
Fig 5.5 Metaphase I mutants with defects in chromosome alignment continued. The spindle and chromosomes in non-activated oocytes were visualised by immunostaining in Tubingen mutants. Examples of chromosome alignment defects in X-281-24-1, X-347-20-3 and X-388-35-3 Tubingen mutants. Scale bar, 10 μm
Fig 5.6 Metaphase I mutants with defects in spindle morphology. The spindle and chromosomes of non-activated oocytes were visualised by immunostaining. Examples of X-073-02-6 and X-033-11-4 (A) mutants have “weak” spindles with very few microtubules that are only just bipolar in shape. B) X-162-03-3 and X-069-36-6 mutants often have extra mini spindles accompanying the main spindle. Arrows indicate chromosome in extra spindle, while arrowheads indicate the extra spindle. Scale bar, 10 μm.
Fig 5.7 Metaphase I mutants with defects in spindle morphology and chromosome alignment. The spindles and chromosomes of non-activated oocytes were visualised by immunostaining. A) Examples of defects in chromosome alignment and spindle morphology in X-347-25-2, X-287-16-4 and X-074-29-3 mutants. Arrowheads show ectopic poles. Scale bar, 10 µm.
Fig 5.8 Metaphase I mutants with defects in microtubule association with chromosomes. The spindle and chromosomes of non-activated oocytes were visualised by immunostaining. Although not the strongest phenotype, these Tubingen mutants displayed varying degrees of the "naked" chromosome phenotype. The Arrows indicate the chromosomes that are not fully associated with microtubules. Scale bar, 10 μm
Fig 5.9 Explanation of complementation test results.
A) Non-complementation between two individual mutants usually occurs when both mutations are in the same gene. A transheterozygote of the two mutants will not have a normal copy of the gene and no complementation can occur. B) Complementation between two individual mutants usually occurs when both individual mutations occur in 2 different genes. A transheterozygote of the two mutants will still have a single normal copy of each gene and they complement.
**Fig 5.10 Acquiring X chromosome mutant males for complementation testing**  
A) As males have only one copy of the X chromosome, males carrying lethal X chromosomes will not emerge. Males carrying the mutant chromosome can be identified by lack of FM7c, Bar marker which affects eye morphology.  
B) If the wild type copy of the mutated gene is present on Y chromosome + duplicated X chromosome fragment the male will be viable. X duplication lines that give fertile mutant males can be used in complementation tests (C). Transheterozgous females are the only females with wild type eye morphology. These females can then be tested for fertility. Fertile females have complementation between both mutants. Sterile females are where both genes non-complement.
Fig 5.11 “wisp” and “awol” super-complementation group. A network of complementations between several metaphase I mutants. The double bars indicate a failure to complement. *awol* and *X-388-35-3* can be regarded as equivalent, as can *X-245-11-1* and *X-133-31-1*. For example; *X-162-03-3* cannot complement *awol*, *X-388-35-3* or *wisp* but can complement all the other mutants.
5.7 Chapter 5 Tables

Table 5.1 Overview of screen for spindle mutants in female meiosis

<table>
<thead>
<tr>
<th>No. Mutant lines</th>
<th>Insufficient mosaic females</th>
<th>Abnormal oocytes</th>
<th>Wild-type spindle</th>
<th>Chromosome misalignment</th>
<th>Spindle defects</th>
<th>Chromosome and spindle defects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 (8.7%)</td>
<td>29 (28.1%)</td>
<td>40 (38.8%)</td>
<td>11 (10.7%)</td>
<td>6 (5.8%)</td>
<td>8 (7.8%)</td>
</tr>
</tbody>
</table>

Table 5.2 My metaphase I mutants from female sterile screen on X chromosome

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Spindle defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-036-19-1</td>
<td>Double spindle</td>
</tr>
<tr>
<td>X-069-36-6</td>
<td>Double spindle</td>
</tr>
<tr>
<td>X-133-31-1</td>
<td>Double spindle</td>
</tr>
<tr>
<td>X-162-03-3</td>
<td>Double spindle</td>
</tr>
<tr>
<td>X-388-35-3</td>
<td>Double spindle</td>
</tr>
<tr>
<td>X-268-04-3</td>
<td>Multiple spindles</td>
</tr>
<tr>
<td>X-281-24-1</td>
<td>Naked chromosome, spread-out, double spindle</td>
</tr>
<tr>
<td>X-074-29-3</td>
<td>Ectopic poles</td>
</tr>
<tr>
<td>X-287-16-4</td>
<td>Ectopic poles</td>
</tr>
<tr>
<td>X-347-25-2</td>
<td>Ectopic poles</td>
</tr>
<tr>
<td>X-073-02-6</td>
<td>Weak/messy</td>
</tr>
<tr>
<td>X-033-11-4</td>
<td>Weak/messy</td>
</tr>
<tr>
<td>X-245-11-1</td>
<td>Alignment</td>
</tr>
<tr>
<td>X-347-20-3</td>
<td>Messy</td>
</tr>
</tbody>
</table>

Table 5.3 Metaphase I mutants with defects in chromosome alignment

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Chromosome defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-036-19-1</td>
<td>Spread out (double spindle)</td>
</tr>
<tr>
<td>X-133-31-1</td>
<td>Spread out (double spindle)</td>
</tr>
<tr>
<td>X-245-11-1</td>
<td>Naked chromosomes</td>
</tr>
<tr>
<td>X-268-04-3</td>
<td>Alignment, naked chromosomes</td>
</tr>
<tr>
<td>X-347-20-3</td>
<td>Misaligned/unpaired (multiple spindles)</td>
</tr>
<tr>
<td>X-388-35-3</td>
<td>Alignment</td>
</tr>
<tr>
<td>X-281-24-1</td>
<td>Spread out/misaligned</td>
</tr>
<tr>
<td></td>
<td>Naked chromosome, spread-out, double spindle</td>
</tr>
</tbody>
</table>
Table 5.4 Metaphase I mutants in spindle morphology

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Spindle defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-033-11-4</td>
<td>Weak</td>
</tr>
<tr>
<td>X-069-36-6</td>
<td>Double spindle (wispy)</td>
</tr>
<tr>
<td>X-073-02-6</td>
<td>Weak</td>
</tr>
<tr>
<td>X-162-03-3</td>
<td>Double spindle (wispy)</td>
</tr>
</tbody>
</table>

Table 5.5 Metaphase I mutants with defects in spindle morphology and chromosome alignment

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Chromosome/spindle defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-074-29-3</td>
<td>Alignment, ectopic poles (rem-like)</td>
</tr>
<tr>
<td>X-287-16-4</td>
<td>Alignment, ectopic poles (rem-like)</td>
</tr>
<tr>
<td>X-347-25-2</td>
<td>Alignment, naked chromosome</td>
</tr>
<tr>
<td></td>
<td>Ectopic poles</td>
</tr>
</tbody>
</table>

Table 5.6 Metaphase I mutants with “naked” chromosome phenotype

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Chromosome/spindle defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-133-31-1</td>
<td>Spread out (double spindle) naked chromosomes</td>
</tr>
<tr>
<td>X-162-03-3</td>
<td>Double spindle (wisp)</td>
</tr>
<tr>
<td>X-245-11-1</td>
<td>Alignment, naked chromosomes</td>
</tr>
<tr>
<td>X-281-24-1</td>
<td>Naked chromosome, spread-out, double spindle</td>
</tr>
<tr>
<td>X-347-25-2</td>
<td>Alignment, naked chromosome</td>
</tr>
<tr>
<td></td>
<td>Ectopic poles</td>
</tr>
<tr>
<td>X-388-35-3</td>
<td>Spread out/misaligned</td>
</tr>
</tbody>
</table>

Table 5.7 Viable metaphase I mutant males

<table>
<thead>
<tr>
<th>Tubingen line</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-010-39-1</td>
</tr>
<tr>
<td>X-033-11-4</td>
</tr>
<tr>
<td>X-173-40-4 (awol)</td>
</tr>
<tr>
<td>X-245-11-1</td>
</tr>
<tr>
<td>X-388-35-3</td>
</tr>
</tbody>
</table>
Table 5.8 X chromosome duplication line screening for viable metaphase I mutant males

<table>
<thead>
<tr>
<th>Tubingen line</th>
<th>Rescuing Duplication(s) and cytology.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-016-35-3</td>
<td>Multiple (semi-lethal)</td>
</tr>
<tr>
<td>X-027-06-1</td>
<td>None</td>
</tr>
<tr>
<td>X-036-19-1</td>
<td>None</td>
</tr>
<tr>
<td>X-074-29-3</td>
<td>None</td>
</tr>
<tr>
<td>X-069-36-6</td>
<td>$Dp(1;2)sn^+72d,(7A8;8A5)$ and $Dp(1;Y)BSC1,(1A1;1B1-2 + 10C1-2; 11D3-8)$</td>
</tr>
<tr>
<td>X-133-31-1</td>
<td>$Dp(1;3)sn^{13al},(6C;7C9-D1)$ and $Dp(1;2;Y)w^*,(2D1-2;3D3-4 + 1B1;1B2 + 20B;20F + 21A1;22E4)$</td>
</tr>
<tr>
<td>X-162-03-3</td>
<td>Multiple (semi-lethal)</td>
</tr>
<tr>
<td>X-201-11-5</td>
<td>$Dp(1;3)sn^{13al}$ $Dp(1;2)4FRDup,(3C2;3F + 3F;4E3 + 20Fh;20Fh + 4E3;5A1-2;26D7)$ and $Dp(1;Y)BSC1$</td>
</tr>
<tr>
<td>X-223-12-3</td>
<td>$Dp(1;Y)W39,(16F1-4;18A5-7 + 1A1;1B2 + 19E5-7;20Fh)$</td>
</tr>
<tr>
<td>X-268-02-2</td>
<td>$Dp(1;2)4FRDup$ and $Dp(1;4)r^+1,(13F1-4;16A1 +$ probably X tip segment $1A1;1A)$</td>
</tr>
<tr>
<td>X-270-13-1</td>
<td>$Dp(1;J)LJ9$ but sterile</td>
</tr>
<tr>
<td>X-315-11-1</td>
<td>None</td>
</tr>
<tr>
<td>X-322-18-5</td>
<td>$Dp(1;2)4FRDup$</td>
</tr>
<tr>
<td>X-347-20-3</td>
<td>$Dp(1;Y)FF1,(8C-D;9B + 1A1;1B2)$</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>wispδ</td>
<td></td>
</tr>
<tr>
<td>X-010-39-1</td>
<td>✓</td>
</tr>
<tr>
<td>X-016-35-3</td>
<td>✓</td>
</tr>
<tr>
<td>X-027-06-1</td>
<td>✓</td>
</tr>
<tr>
<td>X-033-11-4</td>
<td>✓</td>
</tr>
<tr>
<td>X-036-19-1</td>
<td>✓</td>
</tr>
<tr>
<td>X-069-36-6</td>
<td>✗</td>
</tr>
<tr>
<td>X-074-29-3</td>
<td>✓</td>
</tr>
<tr>
<td>X-106-03-1</td>
<td>✓</td>
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<tr>
<td>X-162-03-3</td>
<td>✗</td>
</tr>
<tr>
<td>X-173-40-4</td>
<td>✓</td>
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<td>✓</td>
</tr>
<tr>
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<td>X-245-11-1</td>
<td>✓</td>
</tr>
<tr>
<td>X-298-02-2</td>
<td>✓</td>
</tr>
<tr>
<td>X-370-13-1</td>
<td>✓</td>
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<tr>
<td>X-315-11-1</td>
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<tr>
<td>X-322-18-5</td>
<td>✓</td>
</tr>
<tr>
<td>X-347-20-3</td>
<td>✓</td>
</tr>
<tr>
<td>X-368-33-3</td>
<td>✓</td>
</tr>
</tbody>
</table>

Key ✓ = fertile females/complements  ✗ = sterile females/fails to complement  
✓ = Reciprocal test complements  ✗ = reciprocal test fails to complement  
? = result not yet determined  
/ = Not required if allelic to an allele that has been tested
Table 5.9 Complementation tests of metaphase I mutants (continued)

<table>
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Key ✓ = fertile females/complements × = sterile females/fails to complement
✓= Reciprocal test complements × = reciprocal test fails to complement
? = result not yet determined
/ = Not required if allelic to an allele that has been tested
Table 5.10 Complementation groups of metaphase I mutants

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6. Discussion

6.1 Cks30A

6.1.1 Cks function in eukaryotes

The results I have presented suggest that Cks30A has an essential role in acentrosomal spindle formation and chromosome alignment in female meiosis of *Drosophila*. The mislocalisation of Msps and D-TACC, and possible lack of D-TACC phosphorylation, in *Cks30A<sup>rem</sup>* mutant provides a molecular insight into the role of Cks30A in spindle morphogenesis.

Cks1/Suc1 is conserved across all eukaryotes and is a subunit of the Cdc2/Cyclin B complex (Brizuela et al., 1987; Draetta and Beach, 1988). Cks proteins were originally identified as suppressors of mutations in *cdkl/cdc2* genes in fission and budding yeast (Hadwiger et al., 1989; Hayles et al., 1986). Suc1 and CKS1, in fission yeast and budding yeast, are essential for cell viability (Hadwiger et al., 1989; Hayles et al., 1986; Hindley et al., 1987). Cks in budding and fission yeast were shown to physically associate with Cdks and active Cdk1/Cyclin complexes (Brizuela et al., 1987; Hadwiger et al., 1989).

Cks has been implicated to have roles in various stages of the cell cycle. In humans, CKS1 is required for the G1/S transition (Ganoth et al., 2001; Harper, 2001; Spruck et al., 2001). CKS1 is an essential cofactor for SCF<sup>Skp2</sup> ubiquitin ligase for p27<sup>Kip1</sup>. This leads to destruction of p27<sup>Kip1</sup>, driving cells into S phase as p27 binds and negatively regulates the controllers of S phase; Cyclin E/Cdk2 and Cyclin A/Cdk2 (Pagano et al., 1995; Shirane et al., 1999; Vlach et al., 1997). It has also been shown that *CKS1* mutants in budding yeast display defects at the G1/S transition (Tang and Reed, 1993).
There are several lines of evidence that implicate Cks in entry into mitosis. Overexpression of *suc1* in fission yeast and of *CKSI* in budding yeast delays entry into mitosis (Hayles et al., 1986; Hindley et al., 1987; Richardson et al., 1990). Paradoxically, overexpression and immunodepletion of *Xenopus* Cks, Xe-p9, prevents entry into mitosis. Removal of Xe-p9 at interphase prevents entry into mitosis as Cdc2/Cyclin B is not active (Patra and Dunphy, 1996). This is because *Xenopus* Cks1 strongly stimulates the regulatory phosphorylations of Cdc25, Myt1 and Wee1 that are carried out by the Cdc2/Cyclin B complex leading to further activation of Cdc2/Cyclin B (Patra et al., 1999). Conversely, excess Xe-p9 prevents the dephosphorylation of Cdc2 on Tyr15 keeping Cdc2 inactive and blocking entry into mitosis (Dunphy and Newport, 1989; Patra and Dunphy, 1996). Cks1 has a conserved anion binding domain consisting of Arg33, Arg42, Ser82, Trp85 and Arg102 (Bourne et al., 2000). This domain might represent recognition sites for Cdk's or other phosphorylated protein targets with the phospho-epitope Ser/pSer/pThr/X (Bourne et al., 2000).

Regulation of mitotic exit also involves Cks. *suc1* deletion mutants of fission yeast cause anaphase arrest due to elevated levels of Cyclin B (Basi and Draetta, 1995; Moreno et al., 1989). It has also been shown that immunodepletion of Xe-p9 in *Xenopus* mitotic extracts prevents exit from mitosis because of failure to destroy Cyclin B (Patra and Dunphy, 1996). RNA interference (RNAi) of *CKSI* in *C. elegans* also causes an accumulation of B-type Cyclins (Polinko and Strome, 2000). In budding yeast it has been shown that *CKSI* plays a role in helping target ubiquitinated Cyclin B to the proteasome for degradation (Kaiser et al., 1999).
It even appears that Cks is required for the efficient transcription of a subset of genes in budding yeast, such as CDC20 which is the substrate specific activator of APC/C (Morris et al., 2003; Yu et al., 2005).

As can be seen, the role of Cks in the cell cycle is quite complex. It appears that Cks functions by directing Cdc2 or ubiquitin ligase to particular substrates.

6.1.2 Cks function in meiosis

In addition to a mitotic role, Cks also has roles in meiosis. In fission yeast, depletion or overexpression of *suc1* caused a frequent failure of meiosis II resulting in two-spored asci (Hayles et al., 1986). Depletion of *Xe-p9* in *Xenopus* egg extracts also results in metaphase II arrest (Patra and Dunphy, 1996).

Animal genomes encode two Cks homologues and *Drosophila* has a second Cks homologue called *cks85A* (Pearson et al., 2005; Swan et al., 2005). *cks85A* and *cks30A* have a stronger similarity to each other than they do to *cks1/cks2* homologues in other organisms. It has been shown in *C. elegans* and mice that the homologues can have different roles (Polinko and Strome, 2000; Spruck et al., 2003).

In *C. elegans*, RNAi of *CKS-2* has no apparent defects, while RNAi of *CKS-1* in eggs has defects in meiosis and mitosis (Polinko and Strome, 2000). RNAi *CKS-1* embryos are able to initiate but not correctly exit meiotic divisions.

In mice *CKS2* is required for the metaphase/anaphase transition in the first meiotic division (Spruck et al., 2003). *CKS2*−/− males and females are sterile and have elevated levels of Cyclin B in males suggesting metaphase I arrest. In wild type, oocytes arrest at metaphase II but *CKS2*−/− oocytes arrest at metaphase I. A small percentage of oocytes arrested at metaphase I exhibited chromosome misalignment.
as I have observed in *rem*<sup>Ra74</sup>. Spruck et al (2003) also showed that CKS1 is excluded or not expressed in male and female germlines potentially explaining why CKS1 cannot compensate for loss of CKS2 in *CKS2<sup>−/−</sup>* mice.

These studies show that Cks homologues have different functions in various organisms, which fits with the observations that Cks30A and Cks85A are required for female meiosis and mitosis respectively (Pearson et al., 2005; Swan et al., 2005).

### 6.1.3 *Drosophila cks30A* has roles in pre-anaphase I and post-anaphase I of female meiosis

Independent to my studies, a paper was published discussing the role of Cks30A in female meiosis and the mitotic divisions of the early embryo (Swan et al., 2005). They studied the *rem* mutants and found consistent defects in meiosis II where there was often a kink and/or separation of the two tandem spindles. *rem* embryos either arrested with a single spindle or progressed through a number of aberrant cell cycles. *cks30A* deletion embryos and ovaries showed elevated levels of Cyclin A. The increased levels of Cyclin A were involved in metaphase arrest as reduction of Cyclin A levels by 50% caused a significant partial rescue of metaphase II arrest phenotype in *rem* mutants. Cortex is a female germline specific CDC20 homologue and *cortex* mutants show a metaphase II arrest and increase in Cyclin A levels as observed in *rem*. It is proposed that Cks30A promotes the Cdc2-dependent activation of an APC/C<sup>Cortex</sup> complex that targets Cyclin A for destruction.

These observations present evidence for why *rem* mutants die as early embryos. It is most likely that the metaphase II arrest, not the defects observed in metaphase I oocytes, is the cause of female sterility.
Curiously Swan et al. (2005) only observed 7% of metaphase I arrested oocytes with defects in spindle morphology and chromosome alignment in an unspecified rem allele. This is in contrast to my observations that over half the remRA74 (Cks30A) metaphase I arrested oocytes have defects in spindle morphology and chromosome alignment.

Swan et al. (2005) used two alleles of rem, the weaker allele remHG24, and the stronger allele remRA74. Although Swan et al. (2005) state that both alleles are similar in all phenotypes tested, it appears from their figures that they mainly used remHG24 homozygotes in their studies of metaphase I arrested oocytes. I suspect the use of different alleles has caused the difference in our observed frequencies of metaphase I defects in female meiosis.

Swan et al. (2005) also showed that cks30A mutants have a defect in abdominal cuticle deposition indicative of a partial disruption of larval abdominal histoblast development. They found that this phenotype was enhanced with loss of a single copy of Cdc2, suggesting Cks30A interacts with Cdc2 to prevent polyploidy in larval histoblasts. I have shown that remRA74 msp208 and remRA74 GFP-tubulin homozygotes are synthetically lethal, suggesting Cks30A interacts with Msp and microtubules in the establishment of mitotic spindles. This means that both our studies show that Cks30A functions in mitosis and that it has genetic interactions with several other genes.

I predicted that the remRA74 mutation inhibits Cdc2 interaction as the mutation is a conserved amino acid in a region implicated in Cdc2 binding. Swan et al. (2005) confirmed this by showing that FLAG-tagged-Cks30Arem was severely impaired in Cdc2 binding compared to wild type FLAG-tagged-Cks30.
Cks1 has a conserved anion binding domain that is thought to recognise phosphorylated substrates (Bourne et al., 2000). Swan et al (2005) also showed that a mutant in the anion-binding domain of Cks30A could not rescue a Cks30A deletion mutant, indicating the anion-binding domain is essential for Cks30A function. This confirms my hypothesis that Cks30A functions by mediating Cdc2 targeting, as the anion-binding domain can aid substrate recognition. It has also been shown in *Xenopus* that phosphorylation of Cdc2 substrates is enhanced in the presence of Cks (Patra and Dunphy, 1998; Patra et al., 1999).

Cks85A was identified as the second homologue of Cks in *Drosophila* in both of our papers (Pearson et al., 2005; Swan et al., 2005). Swan et al (2005) generated a null mutant of Cks85A and showed the mutant grew more slowly than wild type and was lethal at the third instar/pupal transition but they did not observe any obvious defects in zygotic tissues. In the Pearson et al (2005) paper, N. Dzhindzhev showed that RNAi of Cks85A in *Drosophila* S2 culture cells results in a significant increase in chromosome misalignment/mis-segregation and spindle abnormalities in mitosis.

Both of our papers showed that Cks85A and Cks30A have different roles in *Drosophila*. Swan et al (2005) used the FRT-ovo^D^ system to generate Cks85A null germline clones and found that Cks85A was not required maternally and that double mutants with *cks30A* produced a *cks30A* mutant phenotype. In the Pearson et al (2005) paper, N. Dzhindzhev used quantitative PCR to show that Cks85A is expressed uniformly throughout *Drosophila* development and is present in male and female adults while Cks30A is only strongly expressed in female adults and embryos. This, along with the observation that RNAi of Cks30A *Drosophila* S2 culture cells
had no significant defects, lead us to the conclusion that Cks85A and Cks30A were required for mitosis and female meiosis respectively.

An important additional finding by Swan et al (2005) was that both genes cannot functionally replace each other, even if they were ubiquitously expressed, suggesting it is not just restrictive expression of the two genes that determines their roles, as shown in mice (Spruck et al., 2003).

In conclusion I feel that both papers complement each other in characterisation of Cks30A and Cks85A in Drosophila, with a focus on post metaphase I defects, by Swan et al (2005), and pre-anaphase I defects, by myself, in female meiosis. Swan et al (2005) showed that defects in metaphase II arrest of rem are probably due to an inability to destroy Cyclin A through the APC/C\textsuperscript{Cortex} complex. A failure to destroy Cyclin A is unlikely to explain metaphase I defects, as expression of non-degradable Cyclin A does not affect chromosome alignment and spindle morphology in mitotic metaphase spindles of Drosophila (Sigrist et al., 1995a).

6.1.4 Role of Cks30A in metaphase I of female meiosis

Cks1, as part of the Cdc2/Cyclin B complex, has been shown to increase substrate specificity, resulting in the regulation of several cell-cycle proteins in Xenopus (Patra and Dunphy, 1996;Patra and Dunphy, 1998;Patra et al., 1999). As the rem mutation inhibits the binding of Cks30A to Cdc2 (Swan et al., 2005), it is plausible that this results in reduced activity toward a subset of Cdc2 targets causing the observed defects in acentrosomal spindle formation.
One possible substrate is D-TACC, as I have shown that in \textit{rem}^{RA74} mutant oocytes a modification is absent (see 3.4.4). The concentration of Cyclin B at the spindle equator in metaphase I spindles strengthens the hypothesis that Cks30A-dependent Cdc2 phosphorylation of D-TACC is required for translocation of D-TACC to the poles. D-TACC can bind Msps (Cullen and Ohkura, 2001) and this interaction may result in Msps also being mislocalised to the spindle equator in the \textit{rem}^{RA74} mutant.

The Msps homologue, XMAP215, can be phosphorylated by Cdc2 (Cassimeris and Spittle, 2001) raising a possibility that Msps localisation may also require Cks30A-dependent Cdc2/Cyclin B phosphorylation in \textit{Drosophila}.

The mislocalisation of D-TACC is not solely responsible for the chromosome misalignment and ectopic pole phenotypes in \textit{rem}^{RA74} metaphase I oocytes, as the \textit{rem}^{RA74} \textit{dtacc}^{stella592} double mutant still has ectopic poles forming at the equator and chromosome misalignment. I was unable to confirm the localisation of Msps in \textit{rem}^{RA74} \textit{dtacc}^{stella592} double mutant spindles, but if Msps is still mislocalised to the spindle equator it may be responsible for ectopic pole formation.

The cause of chromosome misalignment in metaphase I arrested \textit{rem}^{RA74} spindles is currently unknown, although there are several potential candidates. Mutants in \textit{γ-tubulin} have been shown to affect chromosome alignment in \textit{Drosophila} female meiosis (Tavosanis et al., 1997). In \textit{Drosophila}, mutants/inhibition of the RanGTP pathway and the chromokinesin Klp3A exhibit chromosome alignment defects in mitosis (Goshima and Vale, 2003; Silverman-Gavrila and Wilde, 2006; Tavosanis et al., 1997). They may have a similar function in meiosis, making them potential candidates.
Cdc2 can phosphorylate RCC1, and RCC1 phosphorylation is necessary to generate RanGTP on mitotic chromosomes in mammalian cells (Li and Zheng, 2004). If RCC1 generation of RanGTP on meiotic chromosomes in Drosophila requires Cks30A-dependent Cdc2/Cyclin B phosphorylation, it could result in chromosome misalignment as shown in Ran mutants in mitosis (Silverman-Gavrila and Wilde, 2006).

The discovery of two “rem-like” phenotypes, X-074-29-3 and X-287-16-4, from the screen for metaphase I female meiotic mutants on the X chromosome may well be substrates of Cdc2/Cyclin B/Cks30A and warrants further investigation.

6.2 Dweel

6.2.1 Dweel function in Drosophila

Wee1 kinases are responsible for inhibitory phosphorylation of Cdc2, preventing mitosis during S phase. Wee1 inhibition of Cdc2 can also delay mitosis in G2 in response to DNA damage or developmental signals (Rhind and Russell, 2001).

*Drosophila* Wee1 kinase (Dweel) was identified in a screen for cDNA clones that rescue fission yeast wee1 mikl mutants from lethal mitotic catastrophe (Campbell et al., 1995). Metazoans have two distinct Wee1-like kinases, the nuclear Wee1 which phosphorylates Tyr15 exclusively, and the cytoplasmic Myt1 that can phosphorylate Thr14 and Tyr15 of Cdc2.

Dweel is required maternally for inhibitory phosphorylation of Cdc2 in the early syncytial cycles of embryogenesis (Price et al., 2000). In dweelESI mutants Cdc2/Cylin B activity is 4.4 fold higher than in wild type syncitial embryos (Stumpff et al., 2004). Stumpff et al (2004) showed that dweelESI mutant embryos have
multiple defects in the syncytial cycles, suggesting that dweel is required for coordinating the timing of multiple events in syncitial mitosis.

These experiments seem to indicate that Dweel regulates the timing of mitosis. However, it was shown that the spindle abnormalities of dweel<sup>ESI</sup> mutant embryos could not be explained by premature entry into mitosis or by elevated levels of Cdc2 activity (Stumpff et al., 2005). These dweel<sup>ESI</sup> specific spindle defects included formation of multipolar spindles, ectopic foci of microtubule organisation and promiscuous interactions between neighbouring spindles. Dweel can physically interact with members of the γ-TuRC, and γ-tubulin is phosphorylated in a Dweel-dependent manner.

These experiments show that Dweel has an essential mitotic role in early embryogenesis of Drosophila. My experiments have also shown that Dweel has an essential role in mitosis due to the reduced viability and synthetic lethality of double homozygous dweel<sup>RE48</sup> msps<sup>208</sup> and dweel<sup>RE48</sup> GFP-tubulin flies respectively. Msp is known to affect microtubule dynamics and stability (Brittle and Ohkura, 2005; Cullen et al., 1999; Cullen and Ohkura, 2001). One possible explanation is that Dweel phosphorylates Msp in the regulation of microtubule dynamics.

The most recent observations that Dweel can affect spindle morphology and interact with and regulate known spindle assembly factors (Stumpff et al., 2004; Stumpff et al., 2005), is consistent with similar spindle morphology defects observed in metaphase I female meiosis with the dweel<sup>RE48</sup> mutants.
6.2.2 Dweel in metaphase I of female meiosis

I have shown that Dweel is essential for acentrosomal spindle bipolarity in female meiosis. The \(dweel^{RE48}\) mutant is kinase dead and its tripolar phenotype strongly resembles that of Msps and D-TACC mutants (Cullen and Ohkura, 2001). Dweel is required for D-TACC modification, providing a molecular insight into Dweel function in acentrosomal spindle formation.

My results suggest that Dweel-mediated D-TACC modification is via Cdc2/Cyclin B as the \(dweel^{RE48}\) \(twine\) double mutant has the wild type form of D-TACC. This modification may explain the \(dweel^{RE48}\) tripolar spindle phenotype as D-TACC/Msps complex may be inactive and unable to maintain bipolar spindles.

It remains to be determined whether the different forms of D-TACC in wild type and \(dweel^{RE48}\) oocytes are due to the presence/absence of phosphorylation. This will determine how Dweel regulates D-TACC as Dweel may directly phosphorylate and activate D-TACC (Fig 6.1A). Alternatively, Dweel may be required to prevent Cdc2/Cyclin B from phosphorylation and inhibition of D-TACC (Fig 6.1B).

The \(dweel^{RE48}\) \(rem^{RAFT}\) double mutant still has ectopic poles present at the equator of acentrosomal spindles despite the mislocalisation of Msps/D-TACC. This may contradict my hypothesis that mislocalisation of active Msps/D-TACC to the spindle equator causes ectopic pole formation. However, it could be that Msps/D-TACC activity is not completely abolished in \(dweel^{RE48}\) mutants. Evidence for this is the lower penetrance of tripolar spindle morphology in \(dweel^{RE48}\) compared to \(D-TACC^{astele592}\) and \(Msps^{208}\) oocytes. It is perhaps more accurate to say that Dweel is required for efficient maintenance of spindle bipolarity by the Msps/D-TACC complex.
Dweel has been shown to bind γ-tubulin and *Drosophila* Eg5 homologue, Klp61F, and both are dependent on Dweel for phosphorylation (Stumpff et al., 2005). In *Xenopus*, Cdc2 phosphorylation of Eg5 localises Eg5 to the spindle, leading to spindle bipolarity (Nigg et al., 1996). Dweel mutants may have overactive Cdc2, leading to over-recruitment of Klp61F to the spindle resulting in the formation of tripolar spindles.

### 6.3 Cdc2 Kinase pathway is essential for acentrosomal spindle formation

I have shown that the role of Cks30A and Dweel in acentrosomal spindle formation is probably through the regulation of Cdc2 function. Cdc2 temperature sensitive mutants, when incubated at the restrictive temperature, revealed that Cdc2 is essential for the establishment of metaphase I in female meiosis.

Cdc25 phosphatase removes the phosphorylation of Cdc2 on Tyr15, activating Cdc2 (Edgar and Datar, 1996; Sigrist et al., 1995b). Twine is the meiosis specific form of Cdc25 phosphatase in *Drosophila*. Mutants of *twine* show a failure to arrest in metaphase I, resulting in chromosome segregation and spindle defects in acentrosomal spindles of female meiosis (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993). My investigations into the twine mutant confirm these observations and show that active Cdc2 is required for metaphase I arrest in female meiosis in *Drosophila* (Fig 6.2A).

Cdc2 regulation by Dweel1 and Cks30A may be essential in the localisation and activation of Msps and D-TACC. In *rem*<sup>RA74</sup> mutants, Msps and D-TACC are mislocalised to the spindle equator. The different sized forms of D-TACC in wild...
type and rem$^{R474}$ oocytes suggest that Cdc2 may modify D-TACC and this modification may aid in localisation of D-TACC to the poles (Fig 6.2C). Cks30A function indicates that Cdc2 is essential for the regulation of factors required for chromosome alignment at the spindle equator in female meiosis of Drosophila (Fig 6.2D and 6.2E).

The dweel$^{RE48}$ mutant has a tripolar spindle phenotype that resembles msp$^{D208}$ and d-tacc$^{stella592}$ mutants. Msp$^{s}$ and D-TACC can be found at all three poles in dweel$^{RE48}$ mutants, suggesting that Dweel is required for efficient Msp$^{s}$/D-TACC activation rather than localisation (Fig 6.2B).

These results suggest that Cdc2 regulation by Cks30A is essential for Msp$^{s}$/D-TACC polar localisation and Cdc2 regulation by Dweel1 is essential for Msp$^{s}$/D-TACC activity.

Cdc2/Cyclin B has multiple essential functions in; the control of meiotic progression, localisation and regulation of Msp$^{s}$/D-TACC, chromosome alignment and bipolar spindle formation/maintenance in metaphase I of female meiosis. Mutant studies have shown that the regulation of Cdc2 function by Dweel, Twine and Cks30A is essential in orchestrating acentrosomal spindle formation.

It remains to be discovered what the substrates of Cdc2 are, with regards to acentrosomal spindle formation. It is possible that known spindle assembly factors are substrates of Cdc2 and this will give valuable insights into their regulation. Identification of Cdc2 substrates may also unveil spindle assembly roles for novel proteins. I feel that elucidation of the Cdc2 kinase pathway for acentrosomal spindle formation will solve a large piece of the puzzle in understanding how the spindle is established and functions in meiosis and mitosis.
6.4 Discussion of the screen for metaphase I mutants

In this section, I would like to discuss the classes of mutant phenotype I found in the screen for metaphase I mutants. I will also comment on possible explanations for the super-complementation group results.

6.4.1 Mutants in metaphase I chromosome alignment

Seven of the metaphase I mutants I found had defects in chromosome alignment. Rather than the normal configuration of chromosomes being aligned at the spindle equator, with achiasmate 4th chromosomes nearer the poles, the mutants had chromosomes that were separated out along the length of the spindle.

These chromosome alignment mutants may give an insight into how chromosomes are aligned at the spindle equator or how chromosomes are maintained at the equator during metaphase I arrest. The mutants could be in genes encoding motor proteins, kinetochore components, regulators of chromosome movement, meiotic recombination or metaphase I arrest. Mutants affecting the RanGTP pathway in embryotic mitosis of *Drosophila* exhibit chromosome alignment defects (Silverman-Gavrila and Wilde, 2006). If the perturbation of the Ran pathway has similar defects in female meiosis, some of the chromosome alignment mutants may have defects in the RanGTP pathway.

6.4.2 Metaphase I spindle morphology mutants

Four of the metaphase I mutants I found had defects in spindle morphology. Two mutants, X-033-11-4 and X-073-02-6, had very weak spindles, where the
microtubules were barely covering the chromosomes and only just bipolar in shape, with spindles being barrel shaped rather than having tapered poles.

The X-033-11-4 and X-073-02-6 mutations may occur in genes required for chromosome driven spindle formation or the establishment of bipolar spindles after initial microtubule nucleation.

X-069-36-6 and X-162-03-3 mutants had mini-spindles that formed around a chromosome outside the main spindle. The mini-spindle phenotype of X-069-36-6 and X-162-03-3 bears a strong resemblance to the wisp phenotype in metaphase I arrested spindles (Brent et al., 2000). It was suggested that the wisp gene may encode a kinesin or kinesin regulator. wisp has not been molecularly identified but is present on the X chromosome, so X-069-36-6 and X-162-03-3 may be allelic to wisp. This was confirmed by complementation testing in which X-069-36-6 and X-162-03-3 could not complement wisp with regards to female fertility. However X-069-36-6 and X-162-03-3 could complement each other. I will discuss this complication further in section 6.4.5

In X-069-36-6 mutants, mini-spindles can form around bivalent chromosomes. The mutant of NHK-1 is unable to maintain the karyosome and spindles form around each bivalent chromosome, resulting in three spindles (Cullen et al., 2005). It will be worth investigating if the karyosome is formed and/or maintained in prophase of X-069-36-6 mutant oocytes.

Future investigation of these two distinct classes of spindle morphology defect promises to give valuable insights into acentrosomal formation and organisation of metaphase I spindles.
6.4.3 Metaphase I mutants with chromosome alignment and spindle defects

Three of the mutants I found, X-347-25-2, X-287-16-4 and X-074-29-3, had defects in chromosome alignment and spindle morphology. As these mutants affect chromosome alignment and spindle formation, it could be that a regulator of spindle assembly is being mutated, as seen with Cks30A (Chapter 3). These mutations could be in a kinase or the RanGTP pathway, affecting multiple aspects in the establishment of metaphase I in female meiosis.

The "rem-like" phenotypes of X-074-29-3 and X-287-16-4 are particularly exciting as they could be mutants in downstream substrates of Cks30A-bound Cdc2/Cyclin B. Alternatively they may be mutants in Cks30A or Cdc2 regulation. Further investigation into a connection between X-287-16-4, X-074-29-3 and rem could provide valuable insights into Cdc2/Cks30A function in the establishment of metaphase I in female meiosis.

6.4.4 Metaphase I mutants with individual chromosomes lacking associated microtubules

The phenotype of chromosomes without associated microtubules in metaphase I of female meiosis ("naked" chromosomes), I believe, is a novel phenotype. It was first found in the awol mutant phenotype by C.F.Cullen as part of the screen for metaphase I mutants on the X chromosome. Six of my metaphase I mutants exhibited this "naked" chromosome phenotype (Table 5.6). They ranged in severity from a single chromosome being partly naked within the main spindle, to several chromosomes being completely naked and isolated from the main spindle. I was only
able to perform complementation testing with four of my “naked” chromosome mutants but all four fell into the same complementation group as *awol*. This suggests that the “naked” chromosome phenotypes are caused by mutations in a single gene.

This “naked” chromosome phenotype is incredibly interesting as it raises many new questions with regards to chromatin driven microtubule nucleation. Do these “naked” chromosomes ever have microtubules associated with them, and if they don’t, why is only one chromosome affected? If the chromosomes somehow lose associated microtubules it would be of interest to find out how, and at what stage, they are lost. Another valuable insight would be to know whether the lost chromosomes are ejected from the main spindle or if they are always on their own. Based on my observations of “naked” chromosome mutant phenotypes, it seems likely that the chromosomes are lost from the main spindle as opposed to never being a part of the spindle.

A lot of these questions could be addressed through live imaging of spindle formation in female meiosis using fluorescently labelled tubulin and DNA.

The fact that there are at least 3 other mutant alleles of *awol* from the complementation tests allows for thorough genetic and molecular analysis of the “naked” chromosome phenotype by C.F.Cullen in the future.

**6.4.5 Interpretation of complementation test results**

Complementation testing of 19 of the metaphase I mutants identified several complementation groups. Seven of the metaphase I mutants are unique among the metaphase I mutants. Four of the metaphase I mutants are unique but could not be tested for complementation within themselves as they don’t have mutant males, but
they were unique from the other metaphase I mutants. X-group 1 contains X-315-11-1 and X-010-39-1 metaphase I mutants, which both have chromosome misalignment phenotypes. The “wisp” complementation group includes wisp, X-069-36-6 and X-162-03-3 and the “awol” complementation group contains 7 mutants. X-069-36-6 and X-162-03-3 are in both “awol” and “wisp” complementation groups, yet X-069-36-6 /X-162-03-3 and awol/wisp can complement each other (Fig 5.11). Due to these complications, the “awol” and “wisp” groups can be considered a “super-complementation group”.

The super-complementation group is quite intriguing and, while this scenario can be directly answered by molecularly identifying the genes and the molecular nature of the mutation in each allele, I would like to discuss some possible explanations.

These complementation results cannot be resolved using the standard interpretation that mutants in different genes complement and mutants in the same gene do not complement. An alternative interpretation involves intragenic complementation which occurs when mutations in the same gene can complement and non-allelic non-complementation when mutations in two different genes fail to complement.

One mechanism of intragenic complementation can occur when a protein has two or more functional domains that act independently. In this scenario, two mutants with mutations in different functional domains could still complement each other (Fig 6.3). A classic example is HIS4 which encodes a single multifunctional polypeptide with 3 distinct, independent mutable catalytic domains (Fink, 1966). Another example of this can be seen in Drosophila EGF receptor, where several
genetically defined classes of alleles cluster in subdomains of the receptor protein (Clifford and Schupbach, 1994). Intragenic complementation can also occur in hybrid multimers where mutant proteins may not be able to function as homodimers but compensate for each other’s defects in heterodimers. An example of this can be seen in Glutamate dehydrogenase of *Neurospora crassa* (FINCHAM and CODDINGTON, 1963). A more recent example is in arginosuccinate lyase, which is made up from three monomers (Howell et al., 1998). It could be the case that the super-complementation group mutants all exist within the same gene, but this gene has at least two functional domains that act independently of each other or form multimers (Fig 6.3).

The alternative interpretation of the super-complementation group is that the mutants are in different genes yet fail to complement. Non-allelic non-complementation is a failure of complementation between mutations in two different genes (Yook et al., 2001). There are two proposed mechanisms explaining non-allelic complementation known as the dosage (Stearns and Botstein, 1988) and poison (Fuller et al., 1989) models. The dosage model occurs when two different gene products have related functions. Mutations in both genes could result in activity that is below the viable threshold, resulting in non-complementation. For example, null mutations in *slit*, a ligand required for proper neuronal migration during *Drosophila* development, fails to complement null alleles of *robo*, a receptor for Slit protein (Kidd et al., 1999). It may be the case that the super-complementation group mutants encode gene products with related functions. In this scenario, the super-complementation results could be due to a complicated pathway.
In the poison model, an altered gene must bind and impair the protein complex with which it is normally associated. Non-complementation only occurs when in the presence of a second mutation in another member of the protein complex. This can occur in α-tubulin and β-tubulin, where a mutation in α-tubulin sequesters β-tubulin or disrupts microtubule polymerization. In conjunction with a mutant in β-tubulin this will result in non-complementation (Fuller et al., 1989; Hays et al., 1989; Stearns and Botstein, 1988). It could be that X-069-36-6 and X-162-03-3 mutants disrupt complex formation, “poisoning” wisp and “awol” complementation group mutants.

It is likely that a combination of some or all of these mechanisms of intragenic complementation and non-allelic non-complementation are involved in explaining the super complementation group. I will discuss two possible scenarios.

The first scenario assumes that all of the super-complementation group mutants occur in the same “super-complementation group” gene (SCG). This gene has two functionally independent domains, the WISP domain and the AWOL domain. The WISP domain is required for spindle maintenance and the AWOL domain is required for microtubule association with chromosomes. For female fertility the WISP and AWOL domains must each have a minimum of 100% activity. We have to assume that the super-complementation group mutants have different activities in WISP and AWOL domains. Wisp mutant has 0% WISP domain activity but still has 100% AWOL domain activity. The “awol” complementation group mutants all have 100% WISP domain activity but 0% AWOL domain activity, except for X-245-11-1 which has 100% WISP activity and...
40% AWOL activity. These mutations could be due to disruption of catalytic regions.

X-069-36-6 has 40% activity in both WISP and AWOL domains and X-162-03-3 has 60% activity in both WISP and AWOL domains. This could be due to these mutants affecting the expression level of SCG gene.

Using these rules and scores for super-complementation group alleles, it is possible to explain the observed complementation results (Table 6.1). For example X-069-36-6 with wisp or “awol” complementation group mutants will fail to complement as the total AWOL activity will always be less than 100%. X-069-36-6/X-162-03-3 can complement because they have a total activity of 100% for both WISP and AWOL domains (40% + 60% for X-069-36-6 and X-162-03-3). This system also explains how X-245-11-1 can complement X-162-03-3 but not X-069-36-6 as X-245-11-1/X-162-03-3 has 100% (40 + 60) WISP and 100% (40 + 60) AWOL activity, while X-245-11-1/X-069-36-6 only has 80% (40 + 40) WISP and 80% (40 + 40) AWOL activity.

The only caveat is that X-162-03-3 homozygotes have to be female sterile despite having 120% WISP and AWOL domain activity. This could be due to background mutations. If this model is correct then all the mutants in the super-complementation group are mutations in a single gene. This may actually fit with the phenotypes, as the only difference between the mini-spindle phenotype of wisp and the “naked” chromosome phenotype of awol is the association of microtubules with the lost chromosome.

An alternative explanation of the super-complementation group results is based on non-allelic complementation. In this scenario the super-complementation
group mutants are in at least 3 genes, the products of which form a “Super-complementation group” complex. This complex has two active domains that mediate two independent functions. The WISP subunit is encoded in the *wisp* gene and is required for spindle maintenance while the AWOL subunit, encoded by the “awol” complementation group mutants, is required for microtubule association with chromosomes. The third subunit, encoded by the gene mutated in *X-069-36-6* and *X-162-03-3*, exists as a dimer within the complex (Fig 6.4A). *X-069-36-6* and *X-162-03-3* mutants prevent the complex from forming, abolishing spindle maintenance and microtubule association with chromosomes (Fig 6.4B). *X-069-36-6* and *X-162-03-3* mutants can complement, as both mutations in conjunction can restore dimer formation and complex formation, through heterodimer intragenic complementation (Fig 6.4C). This would explain many of the observed super-complementation results.

Molecular identification of the genes affected by the super-complementation group mutants will determine which of these possible mechanisms are closest to reality. The super-complementation results are an excellent example of how genetic interactions can be used to build models for potential pathways and complexes.

In conclusion the mutants I have found, in the screen for metaphase I mutants in female meiosis on the X chromosome, should provide a valuable resource for my lab in the future. The range of mutant defects in chromosome alignment and spindle morphology could potentially elucidate a wide range of pathways involved in the establishment of metaphase I in female meiosis.
6.5 Future prospects

The mutants from the metaphase I mutant screen are probably the most exiting feature of my work at the moment, as they potentially represent new genes involved in the establishment of metaphase I in female meiosis. Having completed the complementation tests of metaphase I mutants, the next step is to try and molecularly identify the mutations. Mapping through recombination between a metaphase I mutant chromosome and a multiply recessive marked 1st chromosome, should narrow the region on the 1st chromosome containing the mutation. This can then be further defined by complementation mapping using chromosomes with deficiencies covering the region of the 1st chromosome in question. Finally sequencing of candidate genes should identify the gene in which the mutation has occurred.

A prime target for molecular identification of metaphase I mutants is awol due to the novel "naked" chromosome phenotype. awol is part of the super-complementation group, meaning members may all have mutations in the same gene. For this reason, it is probably more practical to molecularly identify awol first and then cross-check the rest of the super-complementation group. If any of the super-complementation group fails to map to the awol locus, then they will have to be molecularly identified separately.

The other two types of mutant that would be interesting to molecularly identify are the X-group 1 metaphase I mutants, X-315-11-1 and X-010-39-1, and the rem-like mutants, X-287-16-4 and X-074-29-3. The X-group 1 metaphase mutants are interesting as they have a chromosome misalignment defect. Molecular identification of the mutated gene, will shed light onto whether chromosome misalignment is due to problems in meiotic recombination/homolog pairing, kinetochore/motor function,
the Ran pathway or metaphase I arrest. If the mutated gene is a novel gene, it will be interesting to see which pathway it has a role in.

My interest in the *rem-like* mutants is that they may be involved in the same pathway as Cks30A and molecular identification of the genes affected by these mutants may reveal substrates/regulators of Cks30A function in metaphase I of female meiosis.

The next important step with regards to the roles of Dwee1 and Cks30A is to identify the nature of the modifications of D-TACC in metaphase I of female meiosis (Section 3.4.4 and 4.5.3). With the acquisition of additional p(Ser863) antibody, I would like to check for the presence/absence of D-TACC modification in mature oocytes of *twine/Deficiency, cdc2<sup>B47</sup>/cdc2<sup>E1-24</sup>*(heatshocked) and the *dweel<sup>RE48</sup> cks30A<sup>rem</sup>* double mutants as this should help clarify whether the modification is dependent on Cdc2 activity. Investigation of the Cdc2 phosphorylation motif of S/T-P-(X-R/K) in D-TACC reveals 4 weak (S-P) Cdc2 phosphorylation sites that are conserved in fly species. Mutation of these potential target Serines to Alanine may prevent phosphorylation of D-TACC by Cdc2. This could be confirmed by kinase assays or by 2D-gel analysis. If mutation of any of these 4 sites results in loss of Cdc2 phosphorylation, transgenic flies could be made in a *dtacc<sup>stella592</sup>* mutant background. The transgenic flies could then be studied for defects in metaphase I of female meiosis, and in D-TACC localisation in mitotic cells, in order to determine whether these phosphorylations have a role in D-TACC regulation. Msps protein also has four strong Cdc2 phosphorylation sites that are conserved in fly species. The possible function of these sites could be examined as described for D-TACC.
I think the future of Dweel and Cks30A is to investigate their roles in mitosis. The observation that Cks30A\(^{rem}\) mps\(^{208}\) and Dweel\(^{RE48}\) mps\(^{208}\) double mutants are lethal or have reduced viability is one that needs to be investigated further. It may be interesting to look at the metaphase I female meiotic spindle phenotypes of Cks30A\(^{rem}\) mps\(^{208}/+\), Cks30A\(^{rem/+}\) mps\(^{208}\), Dweel\(^{RE48}\) mps\(^{208}/+\) and Dweel\(^{RE48/+}\) mps\(^{208}\). The phenotypes in these genotypes may give some indication to the lethality in double mutants. It may also be interesting to determine the stage these double mutants die and examine mitotic spindles in actively dividing cell tissues to try and ascertain the nature of the lethality.

Pressing experiments that still need to be performed regarding Dweel function in female meiosis are to examine Dweel\(^{RE48}\) spindle formation and microtubule dynamics in Dweel\(^{RE48};\) GFP-Tubulin/+ flies. This should indicate how tripolar spindles form and whether this has a basis in altered microtubule dynamics. I would also like to observe a spindle phenotype in Dweel\(^{RE48}\) twine double mutants. Although examination of fixed metaphase I oocytes has been difficult, it may be that examination of Dweel\(^{RE48}\) twine; GFP tubulin live oocytes may be more rewarding. Determining the stage in embryogenesis where eggs laid by Dweel\(^{RE48}\) twine females die and staining for spindles in embryogenesis may provide an insight into a mitotic spindle phenotype. It could be the case that the double mutant rescues mutant spindle morphology and that sterility is due to some other factor.

Although I feel that Cks30A acts through Cdc2 in metaphase I of female meiosis, due to the mutation severely reducing Cks30A binding Cdc2, stronger evidence may still be required. In fission yeast Cdc2, several mutations at sites involved in Suc1 binding have been described (Ducommun et al., 1991). Some of
these mutations do not affect Cyclin binding but they are still non-functional when overexpressed in fission yeast. If these sites were conserved in *Drosophila Cdc2* it could be possible to make mutant *Drosophila Cdc2*. If this mutant Cdc2 could no longer bind Cks30A, it could be that transgenic mutant Cdc2 flies could phenocopy *Cks30A<sup>rem</sup>* metaphase I female meiosis. If this were the case it would be convincing evidence that Cks30A does act through Cdc2 in the establishment of metaphase I female meiosis.

To conclude, further clarification of Dweel and Cks30A in D-TACC modification could provide a valuable insight into how MAPS are regulated during the cell cycle. Exploring the role of Dweel and Cks30A in mitosis with regards to Msps may also establish them as key regulators of spindle formation in mitosis as well as in meiosis. Molecular identification of the metaphase I mutants should provide new insights into proteins and the mechanisms involved in the establishment of metaphase I of female meiosis and will most likely form the basis of many future investigations.
Fig 6.1 Proposed models for Dweel modification of D-TACC. There are at least two models for how Dweel regulates D-TACC modification. A) Dweel may directly phosphorylate and activate D-TACC. B) Alternatively, Dweel may be required to prevent Cdc2/Cyclin B from phosphorylating and inhibiting D-TACC.
Fig 6.2 Cdc2 kinase pathway in the establishment of metaphase I of female meiosis. A summary of Cdc2 function in the establishment of metaphase I of female meiosis. A) Twine is required for Cdc2 activation and metaphase I arrest. B) Dweel inhibits Cdc2 activity and is involved in the activation of D-TACC/Msps complex in the maintenance of spindle bipolarity. C) Cks30A bound Cdc2 is required for the localisation of Msps/D-TACC to the poles, spindle formation and chromosome alignment. D) Candidate substrates for chromosome alignment are γ-tubulin and members of the Ran pathway for spindle assembly. E) X-074-29-3 and X-287-16-4 have spindle phenotypes to the Cks30A mutant and may represent additional substrates of Cdc2/Cyclin B in spindle formation.
Fig 6.3 Explanation of Super-complementation group by intragenic complementation. In the domain theory, wisp and awol are mutants in two functional domains that act independently of each other in the same gene. This would allow wisp/awol to complement but awol cannot complement other mutations in the "AWOL" domain, such as X-245-11-1. Similarly, wisp cannot complement other mutations in the "WISP" domain.

X-069-36-6 and X-162-03-3 are null mutants of the gene, and cannot complement wisp or awol mutants.
Fig 6.4 Explanation of super-complementation group by non-allelic non-complementation. The super-complementation group mutants may encode several genes whose products form a complex. A) For example, the “awol” complementation group encodes the AWOL subunit, *wisp* encodes WISP subunit and *X-162-03-3* and *X-069-36-6* encode a subunit that exists as a dimer in the complex. The AWOL and WISP subunits have independent functions and one can function, even if the other is mutated, as long as the complex can form. B) Mutations in *X-069-36-6* and *X-162-03-3* disrupt complex formation, therefore failing to complement *wisp* and *awol* mutations. C) In combination, *X-069-36-6* and *X-162-03-3* mutations complement and restore complex formation, possibly by heterodimer intragenic complementation.
### Table 6.1 score matrix explaining super-complementation results

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<th>Wisp WI=60 AW=60</th>
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<td>AW=40</td>
<td>AW=80</td>
<td>AW=100</td>
<td>AW=80</td>
</tr>
<tr>
<td>X-245-11-1</td>
<td>Fertile</td>
<td>Sterile</td>
<td>Sterile</td>
<td>Sterile</td>
<td>Sterile</td>
</tr>
</tbody>
</table>

Table 6.1 Score matrix explaining super-complementation results

WISP domain activity (WI) and AWOL domain activity (AW) are listed below each mutant allele. WI and AW activity must be greater than 100 in a transheterozygote to complement and have fertile females. In this scenario, the complementation results match the scores for a particular pairing of mutants. The inclusion of X-245-11-1 as a partial lack of function mutant in AW domain activity gives a possible explanation for why X-245-11-1 complements X-162-03-3 but fails to complement X-069-36-6. The only exception is in X-162-03-3 homozygotes and the assumption, in this case, is that a recessive background mutation reduces the activity resulting in a failure to complement.
7. Appendix

7.1 Movie of ectopic pole formation in \textit{rem}^{RA74} oocytes

7.2 Movie of ectopic pole dissolution in \textit{rem}^{RA74} oocytes

7.3 Movie showing polewards movement of D-TACC-GFP in an acentrosomal spindle

7.4 Copy of "A pre-anaphase role for a Cks/Suc1 in acentrosomal spindle formation of Drosophila female meiosis" (Pearson et al., 2005)
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A pre-anaphase role for a Cks/Sucl in acentrosomal spindle formation of Drosophila female meiosis

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Conventional centrosomes are absent from a female meiotic spindle in many animals. Instead, chromosomes drive spindle assembly, but the molecular mechanism of this acentrosomal spindle formation is not well understood. We have screened female sterile mutations for defects in acentrosomal spindle formation in Drosophila female meiosis. One of them, remnants (rem), disrupted bipolar spindle morphology and chromosome alignment in non-activated oocytes. We found that rem encodes a conserved subunit of Cdc2 (Cks30A). As Drosophila oocytes arrest in metaphase I, the defect represents a new Cks function before metaphase-anaphase transition. In addition, we found that the essential pole components, Msps and D-TACC, were often mislocalized to the equator, which may explain part of the spindle defect. We showed that the second cks gene cks85A, in contrast, has an important role in mitosis. In conclusion, this study describes a new pre-anaphase role for a Cks in acentrosomal meiotic spindle formation.

Keywords: Cdk; chromosome; meiosis; microtubule; Msps

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INTRODUCTION

Spindle formation in female meiosis is unique in terms of the absence of conventional centrosomes (McKim & Hawley, 1995). Instead, chromosomes have a central role in the assembly of spindle microtubules. This acentrosomal (also called acenoriolar or anastral) spindle formation is common in female meiosis for many animals including mammals, insects, and worms. Despite potential medical implications, this spindle formation is much less studied than centrosome-mediated spindle formation in mitosis. Drosophila provides a valuable tool to study the acentrosomal spindle formation in vivo. Unlike many other species, mature non-activated Drosophila oocytes arrest in metaphase of meiosis I until ovulation, which coincides with fertilization (Mahowald & Kambysellis, 1980). This provides a unique opportunity to study spindle formation, without interference from chromosome segregation or meiotic exit.

We have previously identified two components of acentrosomal spindle poles, Msps and D-TACC, which physically interact and are crucial for spindle bipolarity (Cullen & Ohkura, 2001). Other studies have identified essential components for spindle formation, such as kinesin-like proteins (Ncd and Sub; Endow et al., 1990; McDonald et al., 1990; Giunta et al., 2002), γ-tubulin (Tavosanis et al., 1997) and a membrane protein surrounding the spindle (AxS; Kramer & Hawley, 2003). Some of these spindle components are probably modulated by cell-cycle regulators, but our knowledge of the regulation is limited. To identify essential components and regulators, we carried out a cytological screen for mutants defective in acentrosomal spindle formation of non-activated oocytes.

Through the screen, we identified remnants and that it is a mutant of a Drosophila Cks/Suc1 homologue, Cks30A. Cks is the third subunit of the Cdc2 (Cdk1)—cyclin B complex, but the role of Msps is less clearcut than that of other subunits of the complex. It is implicated in entry into mitosis/meiosis, metaphase—anaphase transition, exit from mitosis/meiosis and inactivation of Cdk inhibitors (Patra & Dunphy, 1996; Polinko & Strome, 2000; Ganoth et al., 2001; Spruck et al., 2001, 2003). Here, we show that Cks30A is required for spindle morphogenesis and chromosome alignment in the metaphase I spindle in arrested mature oocytes. This requirement of a Cks before metaphase—anaphase transition represents a new function that has not previously been identified. Furthermore, we found that essential spindle pole components Msps and D-TACC mislocalize in the mutant, which may be partly responsible for the spindle defects.

RESULTS

Screening for spindle mutants in female meiosis

For molecular analysis of the acentrosomal spindle in Drosophila female meiosis, we screened female sterile mutants for spindle defects in non-activated oocytes. Female sterile mutants on the second chromosome have previously been isolated (Schupbach & Wieschaus, 1989). We focused on classes of mutants that lay eggs that do not develop beyond the blastoderm stage (class 1 and 2 in Schupbach & Wieschaus, 1989). This category of mutants...
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A \mbox{Complement}

Df(2L)N22-3
Df(2L)gamma-7
Df(2L)ED665
Df(2L)ED653
Df(2L)ED673
Df(2L)Exel6021

Cks30A

L (in rem<sup>1</sup>)

DmCks30A 54 HVMHPEPHILLFRRP 71
HsCks 1 HVMHPEPHILLFRRP
AtCks1 HYAVHPEPHILLFRRP
ScCks1 HYBCHPEPHILLFRRP
SpSuc1 MYEVHVPEPHILLFRRP

includes known meiotic mutants affecting spindle formation, such as fs(2)TW1 (α-tubulin 37C; Tavosanis et al., 1997) and subito (a kinesin-like protein; Giunta et al., 2002).

As many of the stocks gained background lethal mutations, we recombined them out or placed them over appropriate deficiencies (large chromosomal deletions) before examination. Non-activated oocytes from each mutant were dissected from mature adult females, and spindle and chromosomes were stained using α-tubulin antibody and DAPI. This study focused on one of the mutants, remnants<sup>1</sup> (rem<sup>1</sup> or rem<sup>1A2A</sup>), which showed an abnormal spindle and chromosome misalignment (see below).

Molecular identification of the remnants gene

For understanding the molecular role, we determined the identity of the remnants (rem<sup>1</sup>) gene. The rem<sup>1</sup> gene was previously mapped to 30A-C using a deficiency (Df(2L)30AC; Schupbach & Wieschaus, 1989). We confirmed spindle defects in rem<sup>1</sup> over the deficiency. Further deficiency mapping located the rem<sup>1</sup> mutation between the left breakpoints of two deficiencies, Df(2L)N22-3 and Df(2L)gamma-7 (Fig 1A). As these breakpoints had been mapped only cytologically, we physically mapped them more precisely (data not shown). To further narrow the region, we used synthetic deficiencies that have defined breakpoints (Golic & Golic, 1996). They were created either by us—using stocks obtained from the Drosdel project—or by Exelixis. Complementation tests using these deficiencies limited the location of the rem<sup>1</sup> gene to within a 175 kb region (Fig 1A).

We amplified and sequenced all predicted coding regions and splicing junctions within the region from the rem<sup>1</sup> mutant. We found one missense mutation in the gene CG3738 (cks, hereafter called cks30A; Finley & Brent, 1994). There were no other mutations within coding sequences and splicing junctions in the region. In addition, we tested the amount and size of the transcripts that are known to be expressed in adult females, and found no differences between rem and wild type (data not shown).

Cks30A is one of two Drosophila homologues of S. cerevisiae Cks1/S. pombe Suc1, a conserved subunit of the Cdc2 (Cdk1)/cyclin B complex, and has been shown to interact with Cdc2 (Finley & Brent, 1994). The mutation in rem<sup>1</sup> results in a conversion of the 61st amino acid from proline to leucine. This proline is completely conserved among all Cks homologues (Fig 1C), further confirming that the

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mutation is not a polymorphism. Crystal structure analysis has indicated that this residue forms part of the interaction surface with Cdc2 (Bourne et al, 1996). Immunoblots using an anti-human Cks1 antibody indicated that this mutation disrupts the stability of the Cks30A protein (see below).

Cks30A is essential for metaphase I in female meiosis
To explain the role of Cks30A, we focused our analysis of the rem\(^1\) mutant on non-activated oocytes, which arrest in metaphase I. Non-activated oocytes were dissected from wild type and the rem\(^1\) mutant, and chromosomes and spindles were visualized by immunostaining.

In wild type, non-activated mature oocytes contain a single bipolar spindle around chromosomes. Bivalent chromosomes align symmetrically with chiasomatic chromosomes at the equator and acentromeric chromosomes that are located nearer the poles (Fig 2A). The rem\(^1\) mutant was able to enter meiosis, condense chromosomes and assemble microtubules around chromosomes. However, only a minority of spindles showed normal spindle morphology and chromosome alignment.

The most prominent defect in the rem\(^1\) mutant was chromosome misalignment. This defect was observed in about half of the spindles (Fig 2D). Even in the cases in which the spindle remained well organized, chiasomatic chromosomes often moved away from the equator and lost overall symmetrical distribution (Fig 2B). The second class of defect in the rem\(^1\) mutant was abnormal spindle morphology. Although the abnormality varied from spindle to spindle in the rem\(^1\) mutant, the most typical defect was the formation of ectopic poles near the spindle equator (Fig 2C). The focusing of spindle poles seemed to be unaffected.

Further quantitative analysis showed no significant difference between the phenotypes of rem\(^1\) homozygotes (rem\(^1\)/rem\(^1\)) and hemizygotes (rem\(^1\)/Df). This indicates that the rem\(^1\) mutation is genetically amorphic. A recent independent study has indicated that another weaker allele rem\(^2\)
(Schupbach & Wieschaus, 1989) shows similar abnormalities at a lower frequency (Swan et al, 2005).

These results indicate that Cks30A is required before the metaphase–anaphase transition for spindle morphology and chromosome alignment.

Cks30A is essential for proper localization of Msps
To gain an insight into the spindle defects in female meiosis, we examined the localization of Msps. Msps protein belongs to a conserved family of microtubule regulators, including XMAP215, and is the first protein identified at the acentromeral poles in Drosophila (Cullen & Ohkura, 2001; Ohkura et al, 2001). An msps mutation often leads to the formation of a tripolar spindle in female meiosis I.

In wild type, Msps protein is accumulated at the acentromeral poles of the metaphase I spindle in female meiosis (Fig 3A), although the localization sometimes spreads to the spindle microtubules. In the rem\(^1\) mutant, although the Msps protein is still concentrated at the poles, it is often accumulated around the equator of the spindle (Fig 3B). Mislocalization of this important pole protein to the equator in the rem\(^1\) mutant may sometimes lead to the formation of ectopic spindle poles near the equator.

We have previously shown that Msps localization is dependent on another pole protein D-TACC, which binds to Msps (Cullen & Ohkura, 2001). To test whether D-TACC also mislocalizes, we examined the localization of D-TACC in the rem\(^1\) mutant. In wild type, D-TACC is highly concentrated at the acentromeral poles (Fig 3C). In the rem\(^1\) mutant, D-TACC often accumulated at the spindle equator (Fig 3D), although it is still concentrated around the poles to some degree.

In summary, Cks30A is required for correct localization of the essential pole proteins, Msps and D-TACC.

Cyclin B is concentrated at the spindle equator
To gain an insight into how the defect in the Cdc2 complex leads to Msps or D-TACC mislocalization to the spindle equator, we...
studied the localization of cyclin B. Cyclin B is considered to be the main determinant of the activity and cellular localization of the Cdc2 complex. Immunostaining in non-activated oocytes showed that cyclin B is localized to the metaphase I spindle, with a concentration around the spindle equator (Fig 3E). This cyclin B localization could suggest a possible regulatory role of the Cdc2 complex in the transport of Msps and D-TACC from the spindle equator to the poles. The cyclin B localization is not affected in the rem mutant, suggesting that Cks30A mainly affects the substrate specificity of the Cdc2 complex, as shown in other systems (Patra & Dunphy, 1998).

Two Drosophila Cks homologues

The Drosophila genome contains one more predicted cks homologue (CG9790), which we called cks85A. Although mammalian genomes also have two Cks genes, they are more similar in sequence to each other than to either of the two cks genes in Drosophila (Fig 4A).

We next examined the gene expression pattern of the two cks genes during Drosophila development. RNAs were isolated from various stages of development and analysed by reverse transcription–PCR (RT–PCR) using primers that correspond to each of the cks genes. cks30A gave strong signals in adult females and embryos, whereas it gave only weak signals in adult males, larvae and pupae (Fig 4B). This maternal expression pattern is consistent with the observed female sterile phenotype of the cks30A (rem1) mutant. In contrast, cks85A signals were obtained more uniformly throughout the development without sex specificity in adults (Fig 4B). In S2 cultured cells, which originated from embryos, both genes were well expressed.

To identify the Cks proteins, we used an anti-human Cks1 antibody for immunoblots of protein extracts from embryos and S2 cells. Although the antibody recognized many proteins, two bands were detected within a range of molecular weights consistent with the Cks proteins (Fig 4C, D). In embryos laid by the rem1 mutant, the amount of the smaller band was greatly reduced. To further confirm their identity, S2 cells were subjected to RNA interference (RNAi) using double-stranded RNAs (dsRNAs) corresponding to the cks genes. We found that both of the bands disappeared when both genes were simultaneously knocked down by RNAi (Fig 4D). It indicated that, consistent with our RT–PCR results, S2 cells produced both the Cks proteins and that RNAi effectively depleted them.

Cytological analysis showed that cks85A RNAi resulted in a significant increase in chromosome misalignment/missegregation and spindle abnormality in mitosis after an extended time, whereas cks30A RNAi had a lesser impact on mitotic progression (Fig 5D). About a half of anaphase or telophase cells had lagging chromosomes or chromosome bridges after cks85A RNAi (Fig 5A). In some cases, spindles contained scattered chromosomes the sister chromatids of which were either attached or detached (Fig 5B). The frequency of multipolar spindles was also increased (Fig 5C).

The genetic and RNAi results indicated that cks85A has an important function in mitotic progression, whereas cks30A mainly functions in female meiosis.

**DISCUSSION**

In this study, we showed a new pre-anaphase function of a Cks protein in acentrosomal spindle formation during Drosophila female meiosis. Through a cytological screen, we found spindle defects in remnants among female sterile mutants and discovered that remnants encodes one of two Cks proteins (Cks30A) in Drosophila. Cytological analysis showed that Cks30A is required for correct formation of the acentrosomal spindle and...
has Cdk-independent functions (Ganoth et al., 2001; Spruck et al., 2001). Even if Cks is limited to roles in mitosis/meiosis, Cks proteins are implicated in entry into mitosis/meiosis, metaphase–anaphase transition and also exit from mitosis/meiosis (Patra & Dunphy, 1996; Polinko & Strome, 2000; Spruck et al., 2003). Furthermore, the roles of Cks were further complicated by the fact that animal genomes encode two Cks homologues.

Studies in Caenorhabditis elegans and mice showed that one of two cks genes is required for female fertility (Polinko & Strome, 2000; Spruck et al., 2003). Similarly, our results indicated that one of two Drosophila cks homologues, cks30A, is expressed maternally and is required for female meiosis. Further analysis indicated that Cks30A is required for proper bipolar spindle formation and chromosome alignment in mature oocytes arrested in metaphase I. In C. elegans, deletion of one of the Cks proteins by RNAi results in a failure to complete meiosis I (Polinko & Strome, 2000). Similarly, in mice, oocytes from a Cks2 knockout cannot progress past metaphase I and a small percentage of oocytes show chromosome congression failure (Spruck et al., 2003). In both cases, the defects were interpreted mainly as post-metaphase defects. As Drosophila non-activated oocytes are arrested in metaphase I until ovulation, we can distinguish pre-anaphase function of Cks30A from possible post-metaphase function. Our study clearly showed that Drosophila Cks30A has a function in establishing metaphase I, in addition to later functions that were reported recently (Swan et al., 2005).

At the moment, we do not know how the cks30A mutation disrupts spindle formation and chromosome alignment in female meiosis. It has been thought that a loss of Cks function affects the Cdc2 activity towards certain substrates. We found that the essential pole components, MspS and D-TACC, mislocalize to the spindle equator in the mutant. Previously, we hypothesized that MspS is transported by the Ncd motor and anchored to the poles by D-TACC (Cullen & Ohkura, 2001). D-TACC localizes to the poles independently from Ncd, but may also be transported from the spindle equator along microtubules by other motors. Cks30A-dependent Cdc2 activity may be required for activating the transport system at the onset of spindle formation in female meiosis. Consistently, we found that cyclin B is concentrated around the equator of the metaphase I spindle. MspS is the XMAP215 homologue and belongs to a family of conserved microtubule-associated proteins (Cullen et al., 1999; Ohkura et al., 2001). It is a major microtubule regulator, both in mitosis/meiosis and interphase (Gard & Kirschnner, 1987; Ohkura et al., 2001; Brittle & Ohkura, 2005). The mislocalization of this microtubule-regulating activity could lead to the disruption of spindle organization in the mutant.

METHODS

Drosophila genetics. Standard techniques for fly manipulation were followed (Ashburner, 1989). All stocks were grown at 25 °C in standard cornmeal media. w1118 flies were used as wild type. Details of mutations have been described previously (Lindsley & Zimm, 1992). Female sterile chromosomes were recombined with rucuca chromosome to remove background lethal mutations. rem1 mutation was kept over CyO.

Molecular and immunological techniques. Standard molecular techniques were followed throughout (Harlow & Lane, 1988; Sambrook et al., 1989). One-step RT–PCR was carried out using
Taql polymerase and a reverse transcriptase (Invitrogen, Paisley, UK). Non-activated oocytes were prepared for immunostaining, as described previously (Cullen & Ohkura, 2001). RNAi was carried out as described previously (Brittle & Ohkura, 2005; Dzhindzhev et al., 2005). Mitotic abnormalities were counted after incubating with dsRNA for 11–18 days. A human Cks1 antibody was purchased from Santa Cruz Biotechnology (CA, USA). A Drosophila cyclin B antibody (Whitfield et al., 1990) was obtained from Dr W. Whitfield (Dundee, UK). Immunofluorescence microscopy was carried out, as described (Brittle & Ohkura, 2005).

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