Exploring the roles of kinesin-8 in mitotic progression

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Declaration

I, Corinne Pinder, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

The mitotic spindle employs myriad proteins for chromosome segregation, in addition to monitoring the fidelity of this process and correcting mistakes before they become problematic for daughter cells. Key players in mitosis include the superfamily of kinesin motors, a variety of microtubule-associated proteins (MAPs) and members of the spindle assembly checkpoint; deregulation of these factors has been observed in multiple tumour types. Our interest lies in the conserved family of microtubule plus-end directed kinesin-8 motors, which have been implicated in some breast and colorectal cancers.

Klp5/Klp6 constitute the *S. pombe* kinesin-8 complex, and like the human counterpart, Kif18A, localise dynamically on the spindle, interact with kinetochores and regulate microtubule dynamics to allow a timely and coordinated metaphase-anaphase transition. Although these functions have been assigned in multiple species, exact mechanisms of the diverse kinesin-8 functions have not been elucidated, particularly in fission yeast.

In contrast to prior studies of Klp5/6, where mainly deletion or truncation mutants were used, we employ a targeted mutagenesis approach to look directly at kinesin-8 function and regulation. Though Klp5/6 are non-essential, we exploit their synthetic lethality with various MAPs to isolate temperature-sensitive alleles of *klp5*. We find that a specific mutation in the non-motor kinesin tail has a deletion-like phenotype, disrupting chromosome congression, bipolar kinetochore attachment, negative regulation of spindle length and overall control of microtubule dynamics. Additionally, the processivity of the mutant is greatly impaired *in vitro*. Using this loss-of-function mutant with the deletion of XMAP215/TOG family microtubule polymerase, *dis1Δ*, we find that both kinesin-8 and Dis1 synergistically negatively regulate pre-anaphase and anaphase A spindle elongation but promote bipolar kinetochore-microtubule attachments in distinct ways. The activity of both factors is required to prepare metaphase for a timely and concerted anaphase for high fidelity chromosome segregation.
**Impact Statement**

The research presented in this thesis have myriad benefits, both small and large. The results of kinesin-8 and XMAP215/TOG behaviour in fission yeast, in a field filled with lots of debate, will further understanding of these conserved proteins in processes universal to complex life. The investigation of cell division at the level of basic research underpins the current landscape of biomedical research into disease states. The characterisation of conserved eukaryotic proteins involved in cell division and the consequences of the loss of their function is vital in gaining the total picture, both in healthy states and diseased states, such as cancer. Medically, full understanding of how a protein or process works, supplemented by data from other organisms that may allude to previously unknown functions or interactions in human cells, can aid the development of targeted and non-toxic therapies. Academically, basic research using model organisms stimulates the community, improves future research and provides insight into the evolution and conservation of biological processes. Further, obtaining results from cheap and ethical lower eukaryotes in biomedical studies increases their attractiveness to future or outside researchers and can ultimately reduce time, costs and the number of model higher eukaryotes used in research.
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Δ</td>
<td>gene deletion</td>
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<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>APC/C</td>
<td>anaphase-promoting complex/cyclosome</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>aurf</td>
<td>aureobasidin resistance</td>
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<td>cyan fluorescent protein</td>
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<td>dsRed</td>
<td>Discosoma sp. red fluorescent protein</td>
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<td>EMM</td>
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<td>equatorial microtubule organising centre</td>
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<td>green fluorescent protein</td>
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<td>iMTOC</td>
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<td>IPTG</td>
<td>isopropyl B-D-1-thiogalactopyranoside</td>
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<td>MEA4S</td>
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<td>MAP</td>
<td>microtubule-associated protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MCC</td>
<td>mitotic checkpoint complex</td>
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<tr>
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<tr>
<td>TACC</td>
<td>transforming acidic coiled-coil</td>
</tr>
<tr>
<td>TBZ</td>
<td>thiabendazole</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>TOG</td>
<td>tumour overexpressed gene</td>
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<tr>
<td>ts</td>
<td>temperature-sensitive</td>
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<td>WT</td>
<td>wild-type</td>
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YE5S     yeast extract with five supplements  
YFM      yeast freezing media  
YFP      yellow fluorescent protein
Chapter 1. Introduction

1.1 Eukaryotic mitosis

The survival of a cell and development of progeny relies on precise control of the cell cycle for growth and division. Within this cycle, mitosis coordinates the segregation of duplicated genetic material into two new daughter cells. This process is ubiquitous in eukaryotes, from the reproduction and proliferation of unicellular organisms to the development and repair of tissues in metazoa. Accuracy of DNA transmission is imperative for the survival of daughter cells; such stringent mechanisms regulating mitosis are therefore highly conserved across eukaryotic species, including the fission yeast *Schizosaccharomyces pombe*.

The cell cycle coordinates cell growth with division through the execution of various processes in a specific order that can be separated into four distinct phases. The overall characteristics of these phases are conserved across eukaryotes but there are differences in the fine details and duration of each phase. All are driven by the activity of cyclin-dependent kinases (CDKs) in complex with phase-specific cyclins. Generally, G1 is a ‘gap’ period for cell growth where the cell is metabolically active and proteins are synthesised for S-phase. This phase is very short in fission yeast. S-phase involves the synthesis of the new DNA, termed replication, doubling the DNA content. G2 is a second ‘gap’ phase for cell growth and the production of proteins for the subsequent mitosis. The G2/M transition is a key point of regulation by CDK to ensure cells are ready and able to segregate their DNA with high fidelity. Mitosis is an exciting event in the cell cycle as it sees huge changes in signalling, cytoskeletal arrangement and ultimately the identity and morphology of the cell as one becomes two. In this section, an overview of the key steps of mitosis in fission yeast is laid out, however, the majority of components and processes are conserved in other eukaryotes. Descriptions of microtubules, their dynamics and proteins involved in mitosis are described in subsequent sections.
Figure 1.1 Stages of mitosis and the types of microtubules involved
(A) Schematic of fission yeast mitosis detailing SPB, chromosome and microtubule movements from interphase to cytokinesis. (B) Simplified layout of the mitotic spindle displaying kinetochore-microtubules, interpolar microtubules and astral microtubules. Note that astral microtubules only actually appear during anaphase in S. pombe and as such would not be present in a cell that retains bipolar kinetochore attachments.
The mitotic spindle is a macromolecular machine that orchestrates a wide array of processes, subject to various regulation and signalling, for the proper segregation of genetic material into two daughter cells. Interphase cells contain 2-5 bundles of microtubules in the cytoplasm, some nucleated from the spindle pole body (SPB) and some not (Sagulla et al., 2003) (Figure 1.1A, ‘interphase’). 5-7 microtubules form an antiparallel bundle (Hoog et al., 2007), where microtubule minus-ends are in the middle of the cell and plus-ends are located towards the cell cortex. Microtubule-plus ends are regulated by compression force when tips reach the cortex and the subsequent accumulation of factors such as kinesin-8 (Drummond and Cross, 2000). Cytoplasmic microtubules are vital in the establishment and maintenance of cell shape and polarity and rely on the plus end-directed kinesin-7 family motor Tea2 to maintain the cells as rod-shaped through the transportation of polarity factors, such as Tea1 and Tip1 (Browning et al., 2000, Busch et al., 2004).

When cells transition from G2 into mitosis, cytoplasmic microtubules disassemble and the mitotic spindle begins to form in the nucleus (Figure 1.1A, ‘prophase’). Importantly, fission yeast undergoes a closed mitosis where the nuclear envelope doesn't break down, requiring the active transport of mitotic proteins into the nucleus (Ding et al., 1997). Increasing the activation of Cdc2-Cdc13/Cdk1/cyclin B leads to phosphorylation of many microtubule-associated proteins (MAPs) allowing them to be imported to the nucleus using the Ran-GTPase transport machinery (Sato and Toda, 2010).

1.1.1 Pre-anaphase

The centromeres of chromosomes are clustered near to the SPB during G2 but this arrangement is disrupted at the onset of mitosis (Funabiki et al., 1993) For bipolar spindle assembly, the SPB duplicates and both SPBs insert into the nuclear envelope (NE) via an opening/fenestra where it must be anchored (Ding et al., 1997, West et al., 1998) (Figure 1.1A, ‘prophase’). In prophase, duplicated SPBs, as microtubule-organising centres (MTOCs), nucleate microtubules to form the spindle and search for chromosomes. Microtubule growth in early mitosis is promoted by the microtubule polymerase in complex with its adaptor Alp7-Alp14. The homo-tetrameric kinesin-5, Cut7, localises to overlapping interpolar-
microtubule plus-ends where it can cross-link this antiparallel array and slide them apart to extend spindle length (Hagan and Yanagida, 1990, Yukawa et al., 2017)(Figure 1.1B). To prevent excessive outward sliding over interpolar-microtubules, the kinesin-14 proteins, Pkl1 and Klp2, can cross-link parallel microtubules and uses its minus-end direct motility to antagonise Cut7-mediate spindle elongation (Syrovatkina and Tran, 2015, Yukawa et al., 2018). Additionally, Pkl1 brings Msd1 and Wdr8 to SPBs in a ternary complex to stably anchor microtubule-minus ends at the SPB (Yukawa et al., 2015). This initial period of spindle elongation in fission yeast is referred to as phase 1. During this time, kinetochore microtubules undergo rapid growth and shrinkage, in addition to pivoting, to search for and capture chromosomes at their kinetochores. This process is mediated and controlled by multiple proteins that regulate microtubule behaviour but also relies on the dynamic instability that is intrinsic to microtubules (Mitchison and Kirschner, 1984a). Kinetochore capture has been studied in detail in the budding yeast \textit{Saccharomyces cerevisiae}; microtubules first make contacts with kinetochores via their lattice as this has a larger surface area than the microtubule plus-end then this lateral attachment is subsequently converted to end-on attachment. While factors regulate microtubules for this process, kinetochore-associated proteins can also influence microtubules to promote their interaction. These initial spindle-chromosome attachments occur during prometaphase and are likely not bipolar, meaning they are not connected properly to microtubules from both poles (Figure 1.1A, ‘prometaphase’). Proteins of the outer kinetochore can make direct microtubule contact for this process and can also recruit microtubule-interacting factors as mediators.

After some time and outward sliding, the SPBs are directly opposite each other and thus the spindle spans the diameter of the nucleus (Figure 1.1A, ‘prometaphase’ and ‘metaphase’). The spindle length is maintained at around 2-3 µm through various processes (Syrovatkina et al., 2013). The kinesin-8 family Klp5-Klp6 complex actively reduces spindle length likely through microtubule-destabilising activity at microtubule-plus ends, although whether these are kinetochore- or interpolar-microtubules that are affected is unknown (Figure 1.1B) (West et al., 2001, Garcia et al., 2002b). Cut7 also plays an active role in the maintenance of spindle length (Syrovatkina et al., 2013). More passive responsibilities in this
process fall on Ase1, an interpolar-microtubule cross-linker to stabilise outward spindle forces (Yamashita et al., 2005) and Dam1 of the Dam1/DASH complex at kinetochore-microtubule plus-ends that translates inward spindle force (from sister chromatid cohesion) through stable kinetochore-microtubule interaction (Sanchez-Perez et al., 2005, Syrovatkina et al., 2013). Additionally, the integrity of the kinetochore also contributes to negative regulation of spindle length likely through maintaining stable kinetochore-microtubule attachments to keep the spindle under tension with sister chromatids (Goshima et al., 1999). These processes that contribute to spindle length regulation during metaphase also appear to contribute to metaphase alignment of chromosomes (Syrovatkina et al., 2013). The progression from prometaphase to metaphase relies on the stable attachment of kinetochores to microtubules and the generation of tension across these sites. The XMAP215/TOG proteins Dis1 and Alp14 (in complex with Alp7/TACC (Thakur et al., 2013)) connect microtubule plus-ends and their dynamics to kinetochores by interacting with the outer kinetochore Ndc80 complex (Hsu and Toda, 2011, Tang et al., 2013). Components of the spindle assembly checkpoint (SAC), Bub1 and Bub3, are implicated in the promotion of bipolar kinetochore attachment from monopolar attachments (Windecker et al., 2009). The Klp5-Klp6 complex also contributes to this process by regulating microtubule dynamics for congression of sister chromatids to the centre of the metaphase spindle (Mary et al., 2015). The SAC monitors the status of kinetochore-microtubule attachments, where a signalling cascade begins with unattached kinetochore phosphorylation and culminates in inhibition of the anaphase promoting complex/cyclosome (APC/C). The activation of the SAC halts cells in a metaphase-like state in that attachments may be made but may not be correct or under tension, so that microtubules can be turned over until the correct bipolar attachment is formed (see section 1.3.5). At this point Cdc2-Cdc13/Cdk1-cyclin B activity and levels are high.

1.1.2 Anaphase

When kinetochore-microtubule attachments are finally bipolar, the spindle assembly checkpoint is silenced as a result by dephosphorylation and delocalisation of its components away from kinetochores. The APC/C can then become activated after SAC-mediated inhibition is relieved. The APC/C is an E3
ubiquitin ligase that targets Cdc13/Cyclin B and Cut2/securin, amongst other proteins, for degradation by the proteasome. Phosphorylation of Cut2/securin by Cdc2-Cdc13 prevents its premature degradation. Cut2/securin protects sister chromatid cohesion by the interaction and thus inhibition of Cut1/separase. Cut1/separase is a protease that cleaves cohesin, a complex required for the physical maintenance of sister chromatids as a pair for organised and accurate chromosome segregation. When the APC/C is activated, it can ubiquitinate Cdc13/cyclin B for inactivation of Cdc2/Cdk1 and subsequently target Cut2/securin for degradation. Sister chromatids are then free to segregate to the poles in anaphase A (Figure 1.1A, ‘anaphase A’). The force for chromosome movement during this period is generated by microtubule depolymerisation (Grishchuk and McIntosh, 2006). From the beginning of metaphase to the end of anaphase A, when chromatids have finished segregating, the spindle remains at a constant length and corresponds to phase 2. Proper kinetochore-microtubule attachment facilitates equal and timely chromosome segregation (Tang and Toda, 2015). During anaphase, astral microtubules can be seen within the nucleus (Zimmerman et al., 2004)(Figure 1.1B).

Once sister chromatids reach SPBs, further chromosome segregation occurs via increase in the distance between the two poles in anaphase B (or phase 3) (Hagan and Hyams, 1988)(Figure 1.1A, ‘anaphase B’). For this, the kinesin-6 family member, Klp9, localises to the midzone where it uses plus-end directed motility to drive the outward sliding of interpolar microtubules in collaboration with Cut7 (Fu et al., 2009, Yukawa et al., 2017). Ase1 is also involved in stabilising these interdigitating interpolar-microtubule plus-ends during this process. This step occurs within the confines of the nuclear envelope and results in nuclear division.

1.1.3 Post-anaphase

Following anaphase B, the spindle disassembles for mitotic exit and appearance of the post-anaphase array, nucleated by equatorial MTOCs (eMTOCs) between the two daughter nuclei. For cytokinesis, the contractile actomyosin ring assembles at the cell centre during anaphase for generation of the septum that eventually divides the binucleated cell into to symmetrical mononucleate daughter cells.
The septation initiation network (SIN) controls cytokinesis in fission yeast (reviewed in (Simanis, 2015). This signalling network emanates from the SPBs. Briefly, the most upstream control in this cascade is the conserved polo kinase Plo1; its activity generates a protein scaffold at the SPB that allows recruitment of downstream kinases. In mitosis, Cdc7, Sid1 and Sid2 kinases and their activators function sequentially to ultimately assemble the contractile actomyosin ring for the execution of cytokinesis. Cdc2-Cdc13/Cdk1/Cyclin B inhibits the SIN in early mitosis and as such, inactivation of the kinase at anaphase onset by APC/C-mediated Cdc13/Cyclin B degradation is required for septum formation.

1.2 Microtubules

1.2.1 Structure

Microtubules are protein polymers with that form in a hierarchical manner. α-tubulin and β-tubulin come together to form heterodimers that polymerise in a head-to-tail configuration to form linear protofilaments. These protofilaments are inherently polar, with α-tubulin at one end and β-tubulin at the other, where the former is designated the more stable minus-end and the latter the more dynamic plus-end, due to its GTPase activity (Mitchison, 1993, Weisenberg et al., 1968). Lateral attachments between thirteen parallel protofilaments constitute the formation of the hollow cylindrical microtubule structure (Linck and Langevin, 1981, Inoue and Salmon, 1995). Assembly of microtubules occurs in a unidirectional manner through the addition of heterodimers (Dentler et al., 1974).

1.2.2 Nucleation

Microtubules are usually anchored by their minus ends at a structural platform called a microtubule-organising centre (MTOC). Microtubule nucleation relies on another form of tubulin, γ-tubulin. Together with other components, γ-tubulin forms a ring structure (γ-TuRC) as a template on which αβ tubulin heterodimers can polymerise from (Moritz et al., 2000). MTOCs are found mainly at centrosomes and in the cytoplasm of animal cells and at the SPBs in both interphase and mitosis in fission yeast. Multiple proteins contribute to γ-TuRC formation, as core and non-
core components. Core components include Gtb1/GCP1, Alp4/GCP2, Alp6/GCP3 and Mzt1/MOZART1 (Stearns et al., 1991, Vardy and Toda, 2000, Masuda et al., 2013). Gfh1/GCP4, Mod21/GCP5 and Alp16/GCP6 constitute the non-core factors (Venkatram et al., 2004, Anders et al., 2006, Fujita et al., 2002). Other proteins not involved in γ-TuC formation have been implicated in the incorporation of the complex to the SPB, including Mto1, Mto2, Msd1 and Pcp1, in addition to their other roles in microtubule nucleation (Samejima et al., 2008, Venkatram et al., 2004, Janson et al., 2005, Toya et al., 2007, Yukawa et al., 2015, Zimmerman and Chang, 2005, Fong et al., 2010).

Three distinct MTOCs function in fission yeast for microtubule nucleation. Interphase MTOCs (iMTOCs) nucleate cytoplasmic microtubules in antiparallel bundles from the cytoplasmic face of the SPB, the nuclear periphery and existing microtubules (Hagan and Hyams, 1988, Hoog et al., 2007). Bundles can be SPB- or non-SPB-associated, where those nucleated from the SPB tend to contain more individual microtubules, but on average contain 5-7 microtubules (Hoog et al., 2007). Interphase cells typically contain 2-5 bundles of cytoplasmic microtubules (Sagolla et al., 2003). For mitosis, cytoplasmic microtubules disassemble (Hagan and Hyams, 1988) and the SPB is inserted into the nuclear envelope (NE) for duplication and to nucleate microtubules for mitotic spindle formation inside the nucleus (Hagan and Hyams, 1988, Ding et al., 1997). The SPBs nucleate between 12-14 interpolar-microtubules and 3-4 microtubules per kinetochore each for proper spindle formation (Ding et al., 1993). After the spindle disassembles and cells exit mitosis, the post-anaphase array is nucleated at the centre of the dividing cell by equatorial MTOCs (eMTOCs) and is regulated by the SIN (Heitz et al., 2001).

1.2.3 Dynamics

The intrinsic polarity of tubulin heterodimers and hence protofilaments contributes to different characteristics at each end of the microtubule. The plus-end contains exposed β-tubulin subunits that has an exchangeable GTP-binding site (Mitchison, 1993) while the minus-end terminates in the less active α-tubulin, which cannot hydrolyse GTP (guanosine triphosphate) (Weisenberg et al., 1968, Mitchison, 1993). Microtubules are extremely dynamic structures and can undergo various
phases of behaviour (Salmon et al., 1984). Microtubule growth involves the incorporation of GTP-bound αβ-tubulin heterodimers at the plus end where a GTP-cap is formed (Caplow and Shanks, 1990). Within the GTP-cap, protofilaments are kept in a straight conformation (Mandelkow et al., 1991). After incorporation, hydrolysis of GTP converts the heterodimers into a GDP-bound form (Caplow et al., 1994, Nogales et al., 1998). GDP-bound heterodimers contribute to a more curved conformation due to instability but the microtubule remains stable due to weak lateral contacts between protofilaments and the presence of the straighter GTP-cap (Melki et al., 1989). When the GTP-cap is lost due to the faster rate of hydrolysis than incorporation, microtubules depolymerise through the loss of tubulin heterodimers from the plus-end and the energy stored in the lattice (Mandelkow et al., 1991, Desai and Mitchison, 1997). The bent conformation of GDP-tubulin contributes to microtubule depolymerisation in that the reduced lateral interactions allow protofilaments to peel away from each other. The switch from microtubule growth to shrinkage is termed catastrophe, while the change from shrinkage to growth is termed rescue. The ability of microtubules to undergo growth, shrinkage, catastrophe and rescue based on the rate of GTP hydrolysis versus the rate of tubulin incorporation gives microtubules intrinsic dynamic instability (Drechsel and Kirschner, 1994, Mitchison and Kirschner, 1984b).

1.3 Kinetochores

Kinetochores are proteinaceous structures that connect microtubules to chromosomes for segregation. By electron microscopy they appear to be three-layered in structure, composed of a loosely defined central structure flanked on each side by electron dense layers (Brinkley and Stubblefield, 1966, Jokelainen, 1967). Inner proteins of the constitutive centromere-associated network (CCAN) interact with the specially marked centromere while outer kinetochore proteins (Knl1, Mis12 and Ndc80 complexes, KMN network) contact structural and signalling components of the spindle. Kinetochores not only serve as the intermediate between chromosomes and microtubules, but also act as a recruitment and signalling platform for the SAC to control proper mitotic progression.
1.3.1 Assembly on the centromere

Centromeres are specialised regions of heterochromatic DNA that act as the template for kinetochore assembly. The centromere is primarily specified by the incorporation of the histone H3 variant CENP-A (Black et al., 2007, Westhorpe and Straight, 2013), however multiple other proteins determine the DNA site for kinetochore formation. CENP-B binds to alpha-satellite proteins but is non-essential in kinetochore assembly (Black and Cleveland, 2011, Perpelescu and Fukagawa, 2011). For the full assembly, kinetochores rely on the CCAN. The vast array of proteins within this network localise to the interface between the centromere and kinetochore and aid the incorporation of CENP-A.

1.3.2 Inner kinetochore

The vast majority of CCAN proteins are conserved from yeast to humans. CENP-C is recruited to centromeric DNA (Saitoh et al., 1992) and is required for structural integrity for kinetochore assembly (Przewloka et al., 2011). CENP-C localisation also relies on CENP-H and CENP-I (Fukagawa et al., 2001, Nishihashi et al., 2002). Proteomic analysis of CENP-A centromeric chromatin found components CENP-K, CENP-L/M/N and CENP-O/P/Q/R/U, all of which remain at the centromere throughout the cell cycle. The later discovery of CENP-T and CENP-W in addition to CENP-S and interacting CENP-X brought the total number of CCAN proteins to 16. CENP-C and CENP-T interact with components of the outer kinetochore for stabilisation of its assembly for the generation of stable kinetochore-microtubule attachments, mediated by the outer kinetochore (Screpanti et al., 2011).

1.3.2.1 Mis12 complex

Mis12/Mtw1, Mis13/Dsn1, Mis14/Nsl1 and Nnf1 constitute the conserved Mis12 complex (Obuse et al., 2004). Mis12 was identified as a key factor in faithful chromosome segregation due to the loss of mini-chromosomes in its absence in fission yeast (Takahashi et al., 1994). Mis12 mutant cells exhibit longer metaphase spindles due to loss of the generation of inward tension to oppose spindle growth in early mitosis, which is accompanied by increased mis-segregation of chromosomes in fission and budding yeasts (Goshima et al., 1999, Goshima and Yanagida,
Additionally, the absence of Mis12 in human cell lines causes mitotic delay and misalignment of chromosomes (Kline et al., 2006). Structurally, the Mis12 complex is key in linking the centromere-bound components of the kinetochore with those of the outer layer (Westermann et al., 2003). Mis12/Mtw1 links CENP-C/Mif2 and the Ndc80 complex via Spc24/Spc25 in budding yeast and binds CENP-C directly in human cells (Screpanti et al., 2011). At the outer kinetochore, Nsl1 can interact with both Knl1 and Spc24/25 of the Ndc80 complex (Petrovic et al., 2010).

In contrast to the Ndc80 complex and Knl1, the Mis12 complex does not interact with microtubules directly, but rather promotes the microtubule-binding activity of these other two outer kinetochore complexes and is involved in tension sensing in the Aurora B mediated SAC signal (Cheeseman et al., 2006, Welburn et al., 2010). Further than the outer kinetochore, Mis12 has been found to interact with the Ska complex on microtubule plus-ends (Chan et al., 2012).

### 1.3.3 KNL1 complex

Knl1/Spc105/Spc7 functions at the outer kinetochore to interact with microtubules for chromosome segregation in human cells, budding yeast and fission yeast, respectively (Petrovic et al., 2010, Nekrasov et al., 2003, Pagliuca et al., 2009, Jakopec et al., 2012). Knl1/Spc105/Spc7 heterodimerise with Zwint/Kre28/Sos7, respectively for recruitment to the outer kinetochore by the Mis12 complex via motifs in the C-terminus of Knl1. The interaction between Knl1 and microtubules is regulated by Aurora B-mediated phosphorylation of sites in the N-terminus during an activated SAC (Espeut et al., 2012). This begins the cascade of SAC component recruitment and signalling (discussed in section 1.4). The direct microtubule-binding activity of Knl1 was discovered through *in vitro* reconstitution of the KMN network and subsequently pinpointed to the extreme N-terminus (Cheeseman et al., 2006, Espeut et al., 2012). Additionally, Knl1/Spc7 recruits protein phosphatase 1 (PP1) for silencing of the SAC signal and progression into anaphase in multiple organisms (Liu et al., 2010, Welburn et al., 2010, Meadows et al., 2011, Rosenberg et al., 2011).
1.3.4 Ndc80/Hec1 complex

Ndc80/Hec1, Nuf2, Spc24 and Spc25 proteins make up the conserved heterotetrameric Ndc80/Hec1 complex (Rout and Kilmartin, 1990, Osborne et al., 1994, Wigge et al., 1998, Wigge and Kilmartin, 2001, Janke et al., 2001). Electron microscopy revealed that complex forms a dumbbell structure, where the Ndc80-Nuf2 dimer uses its C-terminal coiled-coil domain to interact with the N-terminal coiled-coil domain of the Spc24/Spc25 dimer (Wei et al., 2005, Ciferri et al., 2005). The globular head of Ndc80/Nuf2 contains calponin-homology (CH) domains that face towards microtubules whereas the Spc24/Spc25 globular end is located towards the inner kinetochore (Wei et al., 2007). The CH domains are relatively rich in positively-charged residues and as such form electrostatic interactions with the negatively-charged E-hooks of tubulin in the microtubule lattice (Ciferri et al., 2008, Wei et al., 2007). This function may largely pertain to the CH domain of Ndc80 while that of Nuf2 may play more of a structural role (Sundin et al., 2011). Further, the unstructured N-terminal tail of Ndc80 also contains several positively-charged residues that are implicated in microtubule-binding (Guimaraes et al., 2008, Miller et al., 2008, Tooley et al., 2011). This region has been found to be phosphorylated by Aurora B to disrupt the electrostatic charges (Cheeseman et al., 2006, DeLuca et al., 2006). The N-terminal tail may also contribute to cooperativity between Ndc80 complexes; Aurora B controls both the microtubule-binding function of the tail and the cooperativity role separately. Ndc80 in budding yeast also contributes to SAC signalling via its phosphorylation by Mps1 to recruit Mad1-Mad2 to kinetochores (Kemmler et al., 2009, Wei et al., 2011). Moreover, recent work from our lab found that the hairpin region of Ndc80, located between the CH domain and coiled-coil region, is essential for the recruitment of Mph1 (the Mps1 homologue) for proper establishment of SAC signalling (Chmielewska et al., 2016).

1.3.5 Types of kinetochore-microtubule attachments

Kinetochore-attachments must be bipolar in nature to facilitate the equal segregation of chromosomes. Bipolar attachment, also known as amphitelic attachment, involves the attachment of each sister kinetochore to one pole so that anaphase sees their segregation to opposite poles. The different types of attachment are summarised in Figure 1.2. Amphitelic attachments allow
microtubules to pull on sister kinetochores and thus generate tension (Figure 1.2A). However, due to the nature of chromosome capture by microtubules in early mitosis, other stages of kinetochore-microtubule attachments can arise. Monotelic attachments occur early in spindle formation as kinetochores are captured by microtubules from one pole (Figure 1.2B) (Rieder et al., 1995). Capture of both kinetochores by microtubules from one pole creates syntelic attachment (Figure 1.2C). The lack of attachment to both poles in monotelic and syntelic attachments means sister kinetochores are not under tension in these situations. Conversely, merotelic attachment involves the attachment of sister kinetochores to opposite poles and so can generate tension, as in the amphitelic situation but microtubules from one pole are in contact with the other kinetochore too (Figure 1.2D) (Cimini et al., 2003).

To ensure that problematic kinetochore-microtubule attachments do not cause problems with chromosome segregation, cells have evolved a checkpoint mechanism to recognise and amend incorrect attachments: this is known as the spindle assembly checkpoint (SAC).
1.4 The Spindle Assembly Checkpoint

The SAC involves the hierarchical recruitment of multiple proteins to prevent chromosome segregation in the presence of erroneous kinetochore-microtubule interactions. Upstream kinases recruit one set of proteins (Bubs) to improper kinetochore-microtubule attachments that in turn recruit another set (Mads) with the ultimate goal being generation of the correct form of Mad2 to form the mitotic checkpoint complex (MCC). The MCC inhibits activation of the APC/C and so delays the degradation of securin and cyclin B until proper bipolar spindle attachment is achieved for high-fidelity chromosome segregation.

1.4.1 Components of the SAC

Genetic screens in budding yeast uncovered the first proteins involved in the checkpoint by looking for mutants that could divide in the presence of microtubule drugs that would normally activate the checkpoint (Hoyt et al., 1991, Li and Murray, 1991). Mad1, Mad2 and Mad3 were isolated as mitotic arrest deficient genes (Li and Murray, 1991) while Bub1, Bub2 and Bub3 were found as budding uninhibited by benzimidazole (Hoyt et al., 1991). Further, Aurora B kinase (Tanaka et al., 2002, Hauf et al., 2003, Petersen and Hagan, 2003, Morrow et al., 2005) and Mps1 kinase (Weiss and Winey, 1996, Hardwick et al., 1996, Abrieu et al., 2001) are involved in activation of the SAC and amplification of the signal. Ipl1 and Ark1 constitute the budding yeast and fission yeast Aurora B, respectively, and Mph1 is the fission yeast counterpart of Mps1. All these proteins are recruited in a hierarchical manner to prevent the trigger of anaphase onset in the presence of improper kinetochore-microtubule attachments.

1.4.2 Checkpoint activation

Aurora B and Mps1 are kinases and represent the most upstream components in the SAC cascade. Aurora B localises to the centromeric region of chromosomes behind the inner kinetochore through its interaction with Sgo2 (Adams et al., 2000, Kawashima et al., 2010). Cells lacking proper Aurora B function cannot segregate their chromosomes equally (Biggins et al., 1999), due to an inability to correct monotelic (Tanaka et al., 2002) or syntelic attachments (Hauf et al., 2003). Aurora
B is able to correct kinetochore-microtubule attachments by sensing the lack of tension at monotelic and syntelic attachments (Biggins and Murray, 2001) and phosphorylate an array of substrates. In the absence of tension, Aurora B is close to its substrates at the outer kinetochore where it can promote microtubule attachment turnover as follows. Dsn1/KNL-3 of the Mis12 complex and KNL1 are phosphorylated by Aurora B to fine tune microtubule interaction by the KMN network (Welburn et al., 2010); this phosphorylation event also maintains checkpoint activation by preventing KNL1-PP1 interactions (Liu et al., 2010). Ndc80 interaction with microtubules plus-ends and MAPs is regulated by Aurora B. Phosphorylation of the CH domains and N-terminal tail introduces negative charges that disrupts the electrostatic interaction of Ndc80 with negatively-charge sites on microtubules (DeLuca et al., 2006, Cheeseman et al., 2006, Ciferri et al., 2008, Akiyoshi et al., 2009). Phosphorylation of Dam1/DASH components at the kinetochore-microtubule interface disrupt its interaction with Ndc80 which is normally required to promote the proper association of Ndc80 with microtubules (Gestaut et al., 2008, Keating et al., 2009, Lampert et al., 2010, Tien et al., 2010). Aurora B also recruits Mps1/Mph1 to kinetochores for propagation of the SAC signal (Nijenhuis et al., 2013). Thus, Aurora B at centromeres acts as a tension sensor for kinetochore-microtubule attachments: a lack of tension keeps Aurora B’s substrates in close enough range for their phosphorylation and as such SAC recruitment and microtubule turnover, but under tension the substrates move away where they can become dephosphorylated to further stabilise correct attachments (Wan et al., 2009, Wang et al., 2011).

Kinetochore-recruited Mps1/Mph1 phosphorylates MELT motifs in KNL1/Spc7/Spc105 to bring Bub1, Bub3 and BubR1/Mad3 to the kinetochore, all of which are highly conserved (Yamagishi et al., 2012, Heinrich et al., 2012, Shepperd et al., 2012, Krenn et al., 2014). While all are kinases, the yeast/worm/plant Mad3 lacks the catalytic domain at the C-terminus. Bub1 phosphorylates histone H2A for the localisation of Sgo1/Sgo2 to promote further recruitment of Aurora B to the inner centromeres to amplify the SAC signal (Kawashima et al., 2010, Yamagishi et al., 2012, Caldas et al., 2013). Bub1 is required for the centromere/kinetochore localisation of CENP-F, BubR1, CENP-E and Mad2 (Johnson et al., 2004). Bub1 can also bind to Mad1 in a process that depends on Mps1 and Mad2 (Brady and
Both Bub1 and BubR1 bind Bub3, yet these interactions are mutually exclusive as each binds on the same site on Bub3 (Larsen et al., 2007). Bub3 can also recruit Bub1 to phosphorylated MELT motifs in KNL1 (Primorac et al., 2013). BubR1/Mad3 and Bub3, in addition to their roles in the kinetochore-recruitment of SAC components, contribute to the formation of the mitotic checkpoint complex (MCC), in conjunction with Mad2 and Cdc20 (Han et al., 2013). Bub1 also plays a part in MCC function in the phosphorylation of Cdc20, but the consequence of this remains under debate (Tang et al., 2004). In contrast, fission yeast Bub3 appears to be less important for checkpoint activation as cells can maintain an activated SAC in its absence, although levels of checkpoint components are reduced at kinetochores (Tange and Niwa, 2008, Windecker et al., 2009, Vanoosthuyse et al., 2009) and plays a larger role in checkpoint silencing and the conversion of monopolar attachments to amphitelic attachments when mitosis is perturbed (Vanoosthuyse et al., 2009, Windecker et al., 2009). Moreover, Bub3 is also not a part of the MCC in fission yeast (Vanoosthuyse et al., 2009).

Additional to its role in SAC signalling, Bub1 plays a part in the establishment/maintenance of amphitelic kinetochore-microtubule attachment and chromosome alignment (Meraldi and Sorger, 2005). Additionally, BubR1 is required for chromosome alignment (Meraldi and Sorger, 2005).

The final components of the SAC to be recruited to unattached kinetochores are Mad1 and Mad2. Mad1 is recruited to these kinetochores by Bub1 (Johnson et al., 2004) and in turn recruits Mad2 where Mad1 and Mad2 form a heterotetramer complex (Luo et al., 2002) (Sironi et al., 2002). The absence of Mad2 in any model system renders cells unable to sustain checkpoint signalling and so cells undergo premature exit from mitosis. Mad2 is critically involved in the formation of the MCC through its interaction with Cdc20 (Kim et al., 1998). The interaction between Mad2 and Mad1 causes conformational changes in Mad2, from an inactive open form (O-Mad2) to an active closed form (C-Mad2), where the former interacts with Mad1 and the latter with Cdc20 (Slp1 in fission yeast) (Luo et al., 2002, Sironi et al., 2002). This conversion of Mad2 from open to closed is known as the Mad2 template model and allows the rapid activation and amplification of the SAC as the result of just one unattached kinetochore to halt mitotic progression into anaphase (De Antoni et al., 2005). The Mad1-C-Mad2 complex at the kinetochore then acts
as a platform for the conversion of more O-Mad2 into the closed form and amplifies the signal (Mapelli et al., 2007, Yang et al., 2008). The high levels of C-Mad2 are then sufficient to bind to Cdc20 and together with Bub3 (not in fission yeast) and BubR1/Mad3, form the MCC.

The binding of Cdc20 in the MCC is crucial in delaying APC/C activation in the presence of improper kinetochore-microtubule attachments. Cdc20 is an activator of the APC/C and also functions as a cofactor, therefore its interaction with the MCC prevents Cdc20 from binding and activating the APC/C (Kim et al., 1998, Hwang et al., 1998). The APC/C is a highly-conserved, multi-subunit E3 ubiquitin-ligase that, when activated by Cdc20 in the presence of tension-generating amphitelic kinetochore-microtubule attachments, targets securin and cyclin B for destruction to allow cells to enter anaphase (King et al., 1995, Holloway et al., 1993, Cohen-Fix et al., 1996, Funabiki et al., 1996).

**1.4.3 SAC silencing**

To ensure chromosome attachments remain stable and cells can progress through anaphase, the SAC must be silenced. After APC/C activation, Cdc20 becomes an APC/C substrate rather than activator and so is degraded to break up the MCC (Foster and Morgan, 2012, Lara-Gonzalez et al., 2012). The type 1 protein phosphatase (PP1) is highly important in this process through removal of the phosphorylation signals placed at kinetochores while the SAC was active, at least in yeast. Aurora B-phosphorylated KNL1 recruits PP1 upon dephosphorylation as the SAC becomes satisfied, creating feedback as satisfaction promotes removal of activating marks (Liu et al., 2010, Lesage et al., 2011, Espeut et al., 2012). This recruitment is dependent on the application of tension across sister chromatids (Meadows et al., 2011). PP1 reverses the phosho-dependent recruitment of Bub1, Bub3 and BubR1/Mad3 at KNL1 to prevent further SAC signalling (Pinsky et al., 2009, Vanoosthuyse and Hardwick, 2009, London et al., 2012, Zhang et al., 2014). In fission yeast, Klp5 and Klp6 have been found to interact with PP1 for efficient checkpoint silencing and timely anaphase onset though their kinetochore localisation, representing a separate pool of non-kinetochore-bound PP1 (Meadows et al., 2011, Tang et al., 2013). Interestingly Bub3 is also involved in
checkpoint silencing, at least in fission yeast, which may be indirect in that its recruitment to kinetochores promotes the enrichment of downstream SAC factors that ultimately feedback to correction of kinetochore-microtubule attachments (Vanoosthuyse et al., 2009). The exact mechanism of Bub3-mediated checkpoint silencing outside of the promotion of biorientation and checkpoint activation, if any, remains to be elucidated.

1.5 Microtubule-associated proteins

1.5.1 Dis1/XMAP215/TOG

Tumour overexpressed gene (TOG) proteins are a class of microtubule plus-tip-tracking proteins (+TIPS) that are important for many microtubule-based cellular processes and are found to be overexpressed in human colonic and hepatic tumour cell lines (Charrasse et al., 1995, Charrasse et al., 1998). This family was first identified in *Xenopus* - *Xenopus* microtubule assembly protein (XMAP215) in *Xenopus* eggs through the observation that extracts could promote plus-end assembly of axonemes or bovine brain tubulin (Gard and Kirschner, 1987a, Gard and Kirschner, 1987b). Independent studies around this time identified members of this family across a range of organisms; fission yeast (Dis1 and Alp14) (Ohkura et al., 1988), budding yeast (Stu2) (Wang and Huffaker, 1997, Chen et al., 1998), worm (ZYG9) (Matthews et al., 1998), fly (msps) (Cullen et al., 1999) and human (chTOG) (Charrasse et al., 1995, Charrasse et al., 1998).

XMAP215 was found to promote microtubule dynamicity in extracted axonemes by increasing elongation and shortening rates, but not catastrophe frequency, suggesting this family could promote both the assembly of microtubules and their turnover (Vasquez et al., 1994). XMAP215 localises to cytoplasmic microtubules during interphase and spindle microtubules during mitosis to increase the growth and shrinkage rates of the former but not the latter population of microtubules (Tournebize et al., 2000). Remarkably, XMAP215 is required to reduce the frequency of microtubule catastrophe in both interphase and mitosis, by antagonism of the kinesin-13 XKCM1 (Tournebize et al., 2000, Walczak et al., 1996). This behaviour is characteristic of Stu2 *in vitro* (Podolski et al., 2014).
Recently, the microtubule polymerase activity of Dis1 was confirmed \textit{in vitro} (Matsuo et al., 2016).

This family of MAPs is able to accelerate microtubule growth through conserved HEAT repeat-containing TOG domains in the N-terminus, though different members contain varying numbers of these domains (Neuwald and Hirano, 2000, Ohkura et al., 2001). The TOG domains allow these proteins to capture free tubulin heterodimers and its C-terminal non-TOG domains interact with microtubules, where the affinity for free tubulin correlates with microtubule polymerase activity (Al-Bassam et al., 2006, Widlund et al., 2011, Ayaz et al., 2012, Ayaz et al., 2014).

Dis1 localises to cytoplasmic microtubules in interphase and to the spindle in mitosis, specifically to microtubule plus-ends in contact with kinetochores before anaphase where it appears to move with segregating kinetochores (Nabeshima et al., 1995, Nakaseko et al., 2001, Hsu and Toda, 2011). Work from our lab found that Dis1 is efficiently recruited to kinetochores via the loop of Ndc80 where it coordinates microtubule attachment and dynamics with kinetochore movements. The \textit{ndc80-21} mutant showed pre-anaphase SAC-dependent arrest due to the inability to generate stable kinetochore-microtubule attachments when Dis1 could not interact with Ndc80 (Hsu and Toda, 2011). Additionally, Dis1 may be involved in the localisation of Dam1 to the kinetochore-microtubule interface (Kakui et al., 2013). These proteins are not motile but move to plus-ends via a diffusive mechanism and can track growing microtubule plus-ends, in addition to direct recruitment by other proteins and +TIPs \textit{in vivo} (Brouhard et al., 2008, Matsuo et al., 2016). Further, a non-canonical interaction between the fission yeast EB1 protein Mal3 and Dis1 is required for the synergistic increase in microtubule growth rate \textit{in vitro} and fidelity of chromosome segregation \textit{in vivo} (Matsuo et al., 2016).

This family of proteins is required for initial spindle assembly in \textit{Xenopus}, worm and fly (Tournebize et al., 2000, Matthews et al., 1998, Cullen et al., 1999), but Dis1 and Stu2 are not (Ohkura et al., 1988, Nakaseko et al., 2001, Severin et al., 2001). Some of the earliest work on this family identified Dis1 as a cold-sensitive mutant that was required for the segregation of sister chromatids (disjunction) in fission yeast (Ohkura et al., 1988). Intriguingly, in this study, \textit{dis1} mutants at low
temperature exhibited chromatid non-disjunction and extremely long spindles which contradicts the role of these proteins in microtubule assembly (Ohkura et al., 1988, Nabeshima et al., 1998). In contrast, \textit{dis1}\Delta cells or an \textit{ndc80} mutant that cannot interact with Dis1 show short spindles that are unstable (Hsu and Toda, 2011). The true phenotype of fission cells lacking Dis1 function at low temperature remains to be clearly characterised to establish if these differences are true. At permissive temperature, cells lacking Dis1 are viable as the gene is non-essential but little has been done to characterise these cells. Further, there may be redundancy of these factors in fission yeast as the genome encodes a second XMAP215/TOG family member, Alp14 (discussed in the next section).

Interestingly, under some conditions, these microtubule polymerases have been observed to destabilise microtubules \textit{in vitro}. XMAP215 is proposed to behave more like a catalyst of microtubule dynamics that promotes growth in the presence of tubulin dimers, as in the absence of free tubulin XMAP215 was able to depolymerise GMPCPP-microtubules (Brouhard et al., 2008). This phenomenon is also seen for human chTOG (Roostalu et al., 2015) and Dis1 (Matsuo et al., 2016).

XMAP215 is phosphorylated by CDK1 in metaphase during the mitotic cell cycle of oogenesis and similarly Dis1 is phosphorylated by Cdc2 to alter its localisation from interphase microtubules to kinetochores (Gard and Kirschner, 1987b) (Nabeshima et al., 1995, Aoki et al., 2006).

1.5.2 Alp14/XMAP215/TOG and Alp7/TACC

Alp14 is the other XMAP215/TOG family protein expressed in fission yeast, identified through a mutational screen for cells with altered polarity (Radcliffe et al., 1998, Garcia et al., 2001). As is the case for XMAP215/TOG proteins in other species, Alp14 complexes with the transforming acidic coiled-coil protein (TACC) Alp7 to mediate its functions (Lee et al., 2001, Sato et al., 2004).

Alp14 localises to the SPBs and exhibits microtubule-dependent localisation to kinetochores in metaphase (Garcia et al., 2001). Alp14 can also be seen moving with segregating chromosomes in anaphase (Nakaseko et al., 2001). The absence
of Alp14 produces monopolar spindles and activates the SAC, implicating it in proper kinetochore-microtubule attachments (Garcia et al., 2001). Alp14 and Alp7 are non-essential but their deletion and mutants are temperature-sensitive. Deletion of Alp7 shows a similar phenotype to cells lacking Alp14 as they function as a complex (Sato et al., 2004). Indeed, the coiled-coil domain of Alp7 interacts with the C-terminal region of Alp14: this interaction is required for their colocalisation to the SPB and to the spindle. Alp14 relies on Alp7 for its for its interaction with SPBs and spindle microtubules whereas Alp7 can localise to SPBs but not spindles without Alp14 (Sato et al., 2004). Alp7 must be recruited to SPBs first for the assembly of bipolar spindles by Alp14 (Tang et al., 2014). The identity of Alp14 as a member of the XMAP215/TOG family and its requirement for bipolar spindle formation suggested it possessed microtubule-polymerase activity. This activity was confirmed by the ability of purified Alp14 to directly track growing microtubule plus-ends and increase microtubule assembly in vitro and in vivo (Al-Bassam et al., 2012).

Later work found that the Alp7-Alp14 complex interacts with the outer loop of Ndc80 to mediate its recruitment to kinetochores (Tang et al., 2013). The ndc80-NH12 mutant could not interact with Alp7 and so the absence of the TACC-TOG complex at kinetochores prevents their stable capture and end-on attachment by microtubules for proper metaphase and satisfaction of the SAC and also causes mis-segregation of chromosomes during anaphase (Tang et al., 2013). Subsequent analysis of this recruitment event found that Alp7 interaction with Ndc80 was required to recruit the Klp5-Klp6 kinesin-8 complex to kinetochores for timely silencing the SAC, likely through turnover of microtubule attachments and the recruitment of PP1 (Tang and Toda, 2015).

1.5.3 DASH

The Dam1/DASH complex is made up of ten subunits – Dam1, Duo1, Ask1, Hsk3, Spc19, Spc34 and Dad1, 2, 3 and 4, many of which were first isolated in budding yeast (Hofmann et al., 1998, Jones et al., 1999, Enquist-Newman et al., 2001, Cheeseman et al., 2001, Janke et al., 2002). Mutation of dam1, duo1 and dad1 causes spindle defects in cells with increased mis-segregation of chromosomes.

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These subunits are all conserved in fission yeast where it is known as the DASH complex. These multi-subunit complexes appear to be yeast-specific with no Dam1 homologue identified in higher eukaryotes. However, a functional equivalent of the complex may take the form of the Ska1 complex, composed of Ska1-3 that are required for microtubule attachment and chromosome congression (Jeyaprakash et al., 2012). A large proportion of the structural and in vitro characterisation of the complex has been performed with budding yeast proteins.

The Dam1 complex forms ring structures on microtubules when the components are reconstituted (Westermann et al., 2005, Wang et al., 2007). These rings move processively on depolymerising microtubule ends which, in combination with the ability to interact with the kinetochore via Ndc80 (Tien et al., 2010, Kim et al., 2017), makes for an attractive mechanism for the coupling of chromosomes to depolymerising microtubules in mitosis. The formation of a ring in this situation provides a collar that slides along microtubules, pushed by the outward depolymerisation of the plus-end whilst maintaining attachment to chromosomes via kinetochores.

In fission yeast, the components of the DASH complex are non-essential but do play an important role in chromosome segregation by promoting bipolar kinetochore attachment to promote anaphase onset and to prevent mis-segregation (Sanchez-Perez et al., 2005, Griffiths et al., 2008). Dam1 recruitment to the kinetochore-microtubule interface has been found to rely on the presence of Dis1 for poleward movement of chromosomes during meiosis I (Kakui et al., 2013). Consistent with a role in kinetochore-microtubule attachment Dam1/DASH is also subject to regulation by Aurora B to destabilise Ndc80 interactions (Tien et al., 2010, Lampert et al., 2013).

1.6 Kinesins

Kinesin-1 (or conventional kinesin) was identified in squid giant axons as a microtubule translocator that was also present in the bovine brain (Vale et al., 1985). Kinesins constitute a super family of proteins that are molecular motors required for a variety of processes (Goldstein and Philp, 1999). So far, fourteen
major groups exist, based on phylogenetic analysis (Lawrence et al., 2004, Hirokawa et al., 2009). These functions include organelle and vesicle transport in the cytoplasm and the transport of microtubule-associated proteins, other microtubules, chromosomes and kinetochores during mitosis (Hirokawa, 1998).

1.6.1 Structure

Kinesins are characterised by a well conserved globular motor domain that contains ATPase and microtubule-binding activity (Meluh and Rose, 1990, Enos and Morris, 1990). Stalk/coiled-coil/neck regions mediate protein-protein interactions or oligomerisation and are also involved in kinesin movement. Non-motor domains are often termed ‘tails’, the function(s) of which vary between kinesin family and species. Some kinesins contain both heavy chains and light chains, however fungal kinesins do not contain associated light chains. Three main structural identities exist for kinesins, dependent on the location of the motor domain within the protein: N-kinesins contain an amino-terminal motor domain; M-kinesins have a central motor domain; C-kinesins have a C-terminal motor domain (Hirokawa et al., 2009, Vale and Fletterick, 1997).

1.6.1.1 Kinesin motor domain

The kinesin motor domain that characterises members of this superfamily take the form of an arrowhead, in that a central eight-stranded beta sheet is flanked on either site by three helices (Kull et al., 1996, Sablin et al., 1996, Vale, 1996, Peters et al., 2010). The motor domain possesses two key functions: catalysis and microtubule-binding. Early work characterised the enzymatic activity of kinesins as ATPases (Brady, 1985). This fold is also common to another type of motor protein, myosin, but the conserved ATP-binding pocket is more open and exposed in kinesin (Kull et al., 1996, Walker et al., 1982). The nucleotide-binding pocket is composed of three key loops that not only permit the generation of energy for kinesin movement but also couple the conformation of the motor domain to microtubule-binding ability. These loops are the P-loop and switch I and II. The P-loop is near to the α and β phosphates of the nucleotide that is bound, while switch I and switch II are close to the γ phosphate and as such detect its loss upon ATP hydrolysis (Amos and Hirose, 2007). In the empty or ATP-bound state microtubule-
binding is strong, whereas kinesin detaches from microtubules when ADP is bound (Amos and Hirose, 2007).

Microtubule-interacting residues are positively-charged and cluster in a patch on the motor surface with the core residues located in a highly conserved loop and helix (Woehlke et al., 1997). The microtubule-motor interface between negatively-charged tubulin and positively charged patches on kinesin is thus electrostatic, consistent with salt-sensitivity of kinesin motility (Vale et al., 1996).

1.6.1.2 Neck linker

Kinesins contain multiple or multifunctional coiled-coil regions that can facilitate homo or hetero-oligomerisation, dependent on the kinesin subclass and are also required for proper kinesin motility in conjunction with the motor domain (Hirokawa et al., 2009). The use of chimeras in the study of human conventional kinesin revealed that the neck and not the motor region dictates kinesin directionality (Case et al., 1997) and that it is involved in amplifying the movement of the protein (Case et al., 2000). This is achieved by microtubule-bound kinesin mediating a change in the neck region to an immobilised conformation that is extended towards the microtubule plus-end and docks on the catalytic motor region when ATP is bound (Rice et al., 1999).

1.6.1.3 Tail domains

While motor domains are on the whole conserved across the kinesin superfamily, and more so within kinesin subclasses, the tail domains are highly diverged, sometimes even within subclasses. Tail domains can bind cellular cargo in the cytoplasm (Hirokawa et al., 2009) and are involved in building and regulating the spindle during mitosis, in addition to the regulation of microtubule dynamics (Vicente and Wordeman, 2015). Some kinesins have been thought to rely on cargo-binding in the tail to regulate their motor activity, perhaps to prevent constant ATP usage. Namely, human kinesin-1 heavy chain is kept inactive through tail-motor interaction that is mediated by a coiled-coil hinge region. Truncation of the cargo-binding region alleviates inhibition and increases motility (Friedman and Vale, 1999). Further, the tail of drosophila kinesin has been found to inhibit the
ATPase activity of the motor but not microtubule binding (Coy et al., 1999). In contrast, tail-motor binding in the human kinesin-2 KIF17 impedes motor-microtubule interactions but also brings a more central coiled-coil region to the vicinity of the motor domain to inhibit motility (Hammond et al., 2010). Members of the kinesin-8 family amongst others have been found to possess secondary microtubule-binding activity towards the end of the tail region, however the exact motif has not been identified and this may not be conserved across the subfamily (Mayr et al., 2011, Stumpff et al., 2011, Weaver et al., 2011, Su et al., 2011).

1.6.1.4 Mechanism

Kinesins can be highly processive in their microtubule motility and take many 8 nm steps along the microtubule lattice. The step of each kinesin head requires the hydrolysis of one molecule of ATP (Hua et al., 1997, Schnitzer and Block, 1997). As introduced previously, ADP-bound motor (ADP-motor) has weak affinity for microtubules. The binding of ADP-motor to microtubules accelerates the release of ADP leaving the motor empty (apo-motor), a state that has strong microtubule-binding affinity. MgATP can now bind to the motor (ATP-motor) and trigger a closure of the two switch regions that flank the P-loop. This ATP-motor conformation has a high affinity for microtubules and promotes ATP hydrolysis to ADP.Pi (ADP.Pi-motor). ADP.Pi can still bind strongly to microtubules but subsequent phosphate release returns the kinesin to the weaker microtubule-binding ADP-motor state and detach from microtubules (Crevel et al., 1996, Cross, 2016). The closure of the nucleotide-binding pocket facilitates hydrolysis by organisation of the active site. In this way, microtubule-binding is coupled with ATP-hydrolysis via changes in the motor domain structure. In the currently favoured hand-over-hand model for dimeric kinesin movement, each motor domain is undergoing the above cycle in an orchestrated manner to move nanometer distances over the microtubule lattice, where domains must coordinate microtubule binding with release to ensure one remains attached the microtubule and one is stepping (Kaseda et al., 2003, Cross, 2016).
1.6.1.5 Regulation of microtubule dynamics

In addition to the motility function of kinesins, for cargo transport or microtubule organisation, many kinesins are capable of influencing microtubule dynamics. The nature of the influence exerted over microtubules groups kinesins into three rough sets: kinesin that promote microtubule growth by subunit addition, those that suppress or dampen the microtubule dynamicity and those that promote shrinkage by tubulin loss. These processes are not always ATP-dependent. However, these rules are not strict for the same family across different species, as is discussed briefly below. The kinesin-7, Kif10/CENP-E is required for chromosome alignment via their transport towards microtubule plus-ends (Wood et al., 1997) and is implicated in microtubule polymerisation (Sardar et al., 2010, Moschou et al., 2016). Kinesin-7 uses ATP for its role in microtubule growth but the exact molecular mechanism is not known (Sardar et al., 2010). The Drosophila kinesin-14, Nod, is a non-motile kinesin that promotes microtubule growth for its role in meiotic metaphase chromosome alignment (Cui et al., 2005). In contrast to kinesin-7, microtubule polymerisation by Nod does not require ATP and instead may stabilise plus-ends to promote incorporation of tubulin (Cui et al., 2005).

Kif4 of the kinesin-4 family is a chromokinesin that can relocate chromosomes that have deviated from the metaphase plate. Xenopus Kif4A possesses plus-end-directed motility that facilitates its role in the regulation of kinetochore oscillations through the suppression of microtubule dynamics (Wandke et al., 2012). The human kinesin-8, Kif18A is also seen to function by suppression of microtubule dynamics however this is currently under debate. Kif18A accumulates at the plus-ends of kinetochore-microtubules where it appears to dampen kinetochore oscillations for proper metaphase congression (Du et al., 2010, Stumpff et al., 2008, Stumpff et al., 2012).

To date, kinesin-13 and kinesin-8 (not all members) are the best studied groups of microtubule-depolymerising kinesins. Kinesin-13 are M-kinesins with the catalytic domain located centrally and as such are not processive. Xenopus kinesin-13s, XCKM1 and XKIF2 use ATPase activity to dissociate tubulin dimers from microtubule ends rather than translocation (Desai et al., 1999). Human MCAK also
uses ATP for depolymerisation of both ends of the microtubule and uses an ATP-independent one-dimensional diffusion method to reach microtubule ends rapidly (Wordeman and Mitchison, 1995, Helenius et al., 2006). The lower eukaryotes budding and fission yeasts do not possess a kinesin-13 family member, but do contain kinesin-8. In contrast to the human kinesin-8, Kif18A, the budding yeast orthologue Kip3 is a true microtubule depolymerase as it promotes the removal of tubulin subunits from the microtubule plus-end (Varga et al., 2006, Su et al., 2011, Varga et al., 2009). These members of the kinesin-8 family are discussed in greater detail below.

1.7 Kinesin-8 family

Kinesin-8 are a conserved family of plus-end-directed N-kinesins that function in the regulation of microtubule dynamics. Some family members are also involved in the orchestration of proper chromosome events in mitosis.

1.7.1 Human kinesin-8

The human genome encodes 3 kinesin-8 genes; Kif18A, Kif18B and Kif19 (Hirokawa et al., 2009). Little work exists on Kif19 and so it will not be discussed in any further detail.

1.7.1.1 Kif18B

Primary study of Kif18B found the protein level to be elevated at late G2/M phase in HeLa cells and drops during anaphase likely due to cell-cycle regulated degradation (Lee et al., 2010). Immunofluorescence experiments revealed that Kif18B localises predominantly to the nucleus during interphase, to microtubule plus-ends during pro/metaphase and disappears in anaphase and telophase (Lee et al., 2010). Closer examination of these microtubules revealed that Kif18B localised specifically to astral microtubule plus-ends (Lee et al., 2010). Subsequently, this localisation was found to rely on a direct interaction of Kif18B with EB1 (Stout et al., 2011). Kif18B is required at the plus-ends of astral microtubules to negatively regulate both their length and number (Stout et al., 2011, Walczak et al., 2016). The necessity of the motor domain for the localisation of Kif18B suggests it is able to processively walk towards the plus-end, like other
members of its sub-family, however the motor activity of Kif18B is only partly involved in microtubule shortening (Stout et al., 2011, Tanenbaum et al., 2011). A tail-less construct was still able to interact with the lattice of astral microtubules, suggestive of a potential auto-inhibitory role for the tail in the lattice localisation of full-length Kif18B. Intriguingly, the human kinesin-13 MCAK also binds to Kif18B in an interaction that increases each of their affinity for microtubule plus-ends and subsequent efficient microtubule depolymerisation (Tanenbaum et al., 2011). This interaction is mediated through a stretch of residues in tail of Kif18B and is negatively regulated by Aurora kinases to control astral microtubule dynamics (Tanenbaum et al., 2011).

1.7.1.2 Kif18A

The preliminary work characterising Kif18A provides a great deal of insight into the behaviour and function of this kinesin-8 (Mayr et al., 2007). Similar to Kif18B, Kif18A is also cell-cycle regulated, accumulating in mitosis up until anaphase, during which its levels are reduced (Mayr et al., 2007). Immunofluorescence against Kif18A revealed punctate localisation to the prophase spindle and accumulation at kinetochore plus-ends in metaphase; signals reduced after anaphase (Mayr et al., 2007, Stumpff et al., 2008). Although, the latter study actually saw Kif18A localise to the midbody during telophase and cytokinesis. Efficient enrichment of fluorescent Kif18A requires an active motor domain; a less motile mutant was found to decorate the length of kinetochore-microtubules (Stumpff et al., 2008, Woehlke et al., 1997). In line with these observations, the study of recombinant Kif18A in microtubule-gliding or kinesin motility assays clearly characterise this kinesin-8 as a plus-end-directed motor (Mayr et al., 2007, Du et al., 2010, Stumpff et al., 2012). Moreover, Kif18A only localises near kinetochores in the presence of microtubules, as the addition of the microtubule depolymerising drugs nocodazole or vinblastine abolished this localisation pattern (Mayr et al., 2007, Stumpff et al., 2008).

siRNA knockdown of Kif18A causes cells to accumulate in mitosis with doubled DNA content, dependent on the presence of Mad2, indicative of a role for Kif18A in timely progression through mitosis (Mayr et al., 2007, Stumpff et al., 2008). Indeed,
Mad2 can be observed on kinetochores in the absence of the kinesin-8 and chromosomes fail to congress for metaphase (Mayr et al., 2007, Stumpff et al., 2012). Additional defects were observed in the form of elongated spindles and reduction of tension across sister kinetochores in Kif18A knockdown cells (Mayr et al., 2007, Stumpff et al., 2008). Conversely, overexpression of Kif18A produces shorter spindles (Mayr et al., 2011). At metaphase chromosomes normally oscillate back and forth about the plate alignment after biorientation but Kif18A depletion causes the amplitude of these oscillations to increase dramatically, independent of total spindle length (Stumpff et al., 2008, Mayr et al., 2011, Weaver et al., 2011). The cause of these larger oscillations is both an increase in the velocity of their movement and a reduction in the frequency of directional switch (Stumpff et al., 2008, Stumpff et al., 2012). Consistently, microtubules were more resistant to depolymerisation by cold-treatment in cells lacking Kif18A. These data heavily inferred that Kif18A was required to negatively regulate kinetochore-microtubule dynamics. Intriguingly, variation in Kif18A distribution throughout the spindle permitted the observation that on kinetochore-microtubules displaying higher levels of Kif18A the amplitude of kinetochore oscillations was reduced, implying a relationship between the amount of Kif18A and kinetochore movement (Stumpff et al., 2008). Further, Kif18A is also required to limit chromosome segregation speeds in anaphase, as depleted cells saw quicker rates of poleward chromosome movement.

Recent studies have found that the suppression of kinetochore oscillations by attenuation of microtubule dynamics for proper chromosome alignment is a function specific to kinesin-8 due to the presence of a unique surface loop within the motor domain (Kim et al., 2014). The use of kinesin chimeras found that only the chimera containing the motor domain of Kif18A in conjunction with the Kif18A tail was able to stably accumulate at kinetochore-microtubule plus-ends (Kim et al., 2014). Sequence analysis revealed that Kif18A and Kif18B contain an extended loop2 region that is rich in positively-charged lysine residues that is necessary for plus-end accumulation at kinetochores (Kim et al., 2014).

Detailed dissection of how exactly Kif18A can efficiently accumulate at kinetochore-microtubule plus-ends revealed the requirement of a C-terminal (final 121 residues)
secondary microtubule-binding site, in addition to the motor domain (Mayr et al., 2011, Stumpff et al., 2011, Weaver et al., 2011). Moreover, this region is essential for both the processivity of recombinant Kif18A and its ability to regulate spindle length and chromosome alignment in vivo (Mayr et al., 2011, Stumpff et al., 2011). Recombinant tail-less Kif18A is less processive on microtubules, exhibiting shorter run lengths than the full-length construct (Mayr et al., 2011, Stumpff et al., 2011). Intriguingly, the tail-less can move faster on microtubules than full-length Kif18 (Mayr et al., 2011, Stumpff et al., 2011), reminiscent of a role auto-inhibition of motor activity similar to drosophila kinesin (Coy et al., 1999). However, the significance of the potential auto-inhibition of Kif18A remains to be addressed. Overall, the secondary microtubule-binding site confers processivity by increasing time on the microtubule and promotes kinetochore localisation by tethering of Kif18A at the microtubule plus end where it can regulate microtubule dynamics for proper metaphase alignment (Stumpff et al., 2011, Weaver et al., 2011). Regulation of the accumulation of Kif18A at kinetochores is implemented by the binding PP1 to the tail domain (De Wever et al., 2014). Kif18A contains the conserved R/KxVxF/W PP1-binding motif that is located N-terminal to Cdk1 phosphorylation sites (Hafner et al., 2014). When Cdk1 activity is high prior to anaphase onset, phosphorylation of serines 674 and 684 prevents efficient accumulation of Kif18A at kinetochore-microtubule plus ends, however the molecular mechanism for this remains unknown (Hafner et al., 2014). Non-phospho mutants of Kif18A accelerates the kinesin’s chromosome congression function by suppressing oscillations, linking this process with mitotic timing via Cdk1 activity. As cells approach the metaphase-anaphase transition, PP1 can be recruited to kinetochores as kinetochore-microtubule attachments become properly established. PP1 can dephosphorylates these serines, dependent on the PP1-binding motif, for proper metaphase plate thinning by congression for a timely and proper anaphase onset (Hafner et al., 2014).

Mayr et al., 2011 observed increasing depolymerisation of GMPCPP-stabilised microtubules in the presence of increasing concentrations of Kif18A, a process that was dependent on ATP. This result contrasts that of the in vivo findings from Stumpff et al., 2011, where Kif18A appears to be required to regulate microtubule dynamicity. Additionally, Du et al., 2010 could not detect depolymerisation of
GMPCPP- or taxol-stabilised microtubules. Recombinant Kif18A was able to prevent the growth of dynamic microtubules but not change the length of existing microtubules, favouring the role of Kif18A in the dampening of microtubule dynamics (Du et al., 2010). Further, mutation of Kif18A to relocalise it to the cytoplasm in HeLa cells does not have any effect on the length of cytoplasmic microtubules (Du et al., 2010). The mutant also did not stabilise microtubule plus-ends in vivo but cytoplasmic microtubule regrowth after nocodazole washout was suppressed (Du et al., 2010). Careful assessment of cytoplasmic microtubule dynamics revealed that Kif18A acts to limit the distance that plus-ends can grow and shrink and as such dampens microtubule dynamics (Du et al., 2010).

In support for Kif18A functioning in a microtubule-destabilising capacity that is not a depolymerase, Kif18A motor domains did not depolymerise stabilised microtubules in the presence of ATP, but in the presence of a non-hydrolysable analogue, free tubulin was released from the microtubules (Peters et al., 2010). The trapped form of Kif18A motor by the analogue likely has a very strong affinity for microtubules, compared to the rest of the ATPase cycle and so can remove tubulin from microtubule plus-ends when it dissociates. Indeed, observation of this situation by negative stain electron microscopy revealed tubulin rings coated in the Kif18A motor domain (Peters et al., 2010). However, this removal of tubulin may be more extreme than the role of Kif18A in vivo as in the presence of ATP full-length Kif18A would be more motile and so hydrolyse ATP and dissociate from plus-ends; therefore, it may be that Kif18A can stabilise curved microtubule intermediates to dampen plus-end dynamics. Additionally, the fact that the constructs used in this assay were monomeric yet could cause tubulin loss negates the requirement of motility in their control of microtubule dynamics. Recent work (Locke et al., 2017) reveals the presence of the neck linker sequence improves the microtubule-binding affinity, ATPase activity and gliding velocity of the monomeric motor domain, in line with the requirement of the neck for the mediation of ATPase activity to kinesin function. Remarkably, this longer construct can actually depolymerise stabilised microtubules and at a faster rate than the motor alone, but still via removal of tubulin rings by multiple kinesin constructs (Locke et al., 2017). Currently, there is no strong united view on exactly which mechanism Kif18A uses to influence dynamics as there are now multiple reports of their depolymerising activity and
multiple reports of the absence of this. Experiments using the whole range of constructs available, including full-length would shed light on this, as it could be hypothesised that the tail could be responsible for regulating the depolymerising activity in addition to enhancing processivity.

The motor domain of Kif18A shows microtubule-stimulated ATPase activity, consistent with its motility and exhibited tubulin-stimulated ATPase activity to a lesser extent (Peters et al., 2010). Kinesin-13 use an extended loop2 in the motor domain to depolymerise microtubules in an ATP-dependent manner but not to interact with the microtubule lattice or for tubulin stimulated ATPase activity (Ogawa et al., 2004, Shipley et al., 2004, Tan et al., 2008). Comparison of Kif18A to kinesin-13 revealed an even more extended but flexible loop2 but the kinesin-8 did not contain the functional residues of loop2 (Peters et al., 2010).

Kif18A overexpression in HeLa cells causes an increase in the number of mitotic cells that have multipolar spindles that lead to chromosome mis-segregation (Du et al., 2010, Zhang et al., 2010). In line with this observation, Kif18A is overexpressed in human breast cancer and correlates with tumour grade, metastasis and poor survival (Zhang et al., 2010).

1.7.2 Budding yeast kinesin-8

Kip3 is the sole member of the kinesin-8 family of motor proteins in *Saccharomyces cerevisiae*. Kip3 localises to both spindle and cytoplasmic microtubules and cells lacking Kip3 were seen to have long microtubules (Miller et al., 1998, Straight et al., 1998). Kip3 is required for nuclear migration in the proper segregation of genetic material in mitosis (Miller et al., 1998) and also to regulate spindle length and the timing of spindle disassembly at the end of anaphase (Straight et al., 1998).

Kip3 is a plus-end directed N-kinesin that can track the microtubule plus-end (Varga et al., 2006, Gupta et al., 2006). Recombinant Kip3 is able to depolymerise GMPCPP-stabilised microtubules in the presence of ATP (Varga et al., 2006). Visualisation of GMPCPP- or taxol-stabilised microtubules in the presence of Kip3 confirmed its role as a microtubule depolymerase as it rapidly shortens
microtubules from the plus-end (Varga et al., 2006). In this assay, the maximum depolymerisation rate is higher for longer microtubules than for shorter microtubules, revealing a length-dependent mechanism, as longer microtubules accumulate more Kip3 at their plus-ends (Varga et al., 2006). Subsequent experiments established the model for depolymerisation – Kip3 that has translocated to the plus-end pauses until another Kip3 molecule comes in behind it and pushes the first Kip3, in complex with tubulin heterodimer(s), off the end, therefore actively shrinking microtubules (Varga et al., 2009). Cooperativity of this nature therefore couples motor flux to the plus-end with depolymerisation rate. The ATPase activity of Kip3 is activated by both the presence of microtubules and tubulin heterodimers (Gupta et al., 2006). Recent work has challenged the model for microtubule depolymerisation by the bumping off of tubulin-bound Kip3. A recombinant monomeric, non-motile Kip3 truncation is able to depolymerise GMPCPP-stabilised microtubules, implying that intrinsic motility of the kinesin-8 is not directly required for this process (Arellano-Santoyo et al., 2017). Additionally, this group found that strong selective binding for microtubule plus-ends and not ATPase activity of Kip3 was sufficient for microtubule plus-ends (Arellano-Santoyo et al., 2017).

In cells, the absence of Kip3 causes the reduction of catastrophe frequency and pause time of spindle microtubules, leading to longer microtubules and the aberrant positioning of the spindle (Gupta et al., 2006). In contrast to the human and fission yeast kinesin-8 orthologs, Kip3 appears to play no role in the regulation of pre-anaphase spindle length or kinetochore-microtubule attachment, but instead is involved in chromosome congression in metaphase via regulation of kinetochore-microtubule length (Wargacki et al., 2010). In late anaphase however, Kip3 localises to spindle midzone to limit polymerisation and hence elongation of the spindle by Stu2/XMAP215 (Rizk et al., 2014) and later sustains interpolar-microtubule depolymerisation for spindle disassembly (Woodruff et al., 2010).

Similar to Kif18A, the tail region of Kip3 is also required for motility but in a dimerisation capacity (Su et al., 2011). This construct was made motility-competent by the introduction of a leucine zipper (Kip3ΔT-LZ) but cells are still resistant to the microtubule-destabilising drug benomyl, as are kip3Δ cells (Su et al., 2011). The tail
also functions in in kinetochore clustering in pre-anaphase cells and disassembly of late anaphase spindles (Su et al., 2011). Recombinant Kip3ΔT-LZ showed reduce run length and plus-end dwell time on microtubules but interestingly showed no change in run velocity (Su et al., 2011). The reduction of these parameters leads to lower amounts of Kip3 at microtubule tips and so microtubules are longer with slower shrinkage rates, implicating a role for the tail in the proper depolymerisation of microtubules (Su et al., 2011). Recombinant chimera studies fusing the tail of Kip3 to truncated human kinesin-1 confirmed its function in efficient recruitment to microtubules (Su et al., 2011). Remarkably, the kinesin-1-kip3-tail chimera could accumulate on microtubule plus-end in cells but specifically lead to long buckling spindles, as seen in kip3Δ cells, revealing some form of microtubule-stabilising role for the Kip3 tail (Su et al., 2011). This was corroborated by the ability of a recombinant tail-only construct to reduce microtubule-shrinkage rate. The Kip3 tail can bind both microtubules and tubulin dimers, reflective of its role in both depolymerisation and plus-end stabilisation (Su et al., 2011). Recent work has found that the loop11 structure is required for microtubule-plus-end recognition and depolymerase activity of Kip3 (Arellano-Santoyo et al., 2017). This function is mediated by the preference of loop11 for curved tubulin at microtubule plus-ends via recognition of an α-tubulin residue (Arellano-Santoyo et al., 2017).

Remarkably, the tail of Kip3 can cross-link and slide outward purified anti-parallel microtubules in a manner that is similar to those activities required for bipolar spindle formation (Su et al., 2013). The sliding activity depends on the tail but the microtubule-depolymerising activity of Kip3 relies on an intact neck domain (Su et al., 2013). The neck domain functions partially in Kip3 motility but can still move to the plus-end, however here it is deficient for depolymerase activity, as the neck is likely involved in conformational changes of kinesin for function (Su et al., 2013, Arellano-Santoyo et al., 2017).

Doubling the dose of the Kip3ΔT-LZ construct in vivo rescued the benomyl-resistance of these cells, suggesting spindle length and chromosome alignment are sensitive to the amount of Kip3 at plus-ends (Su et al., 2011). For proper spindle length, Kip3 must maintain a balance between its spindle-shortening microtubule
depolymerase activity and its spindle-lengthening antiparallel microtubule-sliding activity (Su et al., 2013).

1.7.3 Fission yeast kinesin-8

In fission yeast, the genome encodes two kinesin-8 genes, klp5 and klp6 (West et al., 2001) that work together as a heterodimer (Garcia et al., 2002b, Li and Chang, 2003, Unsworth et al., 2008). Klp5 is the larger of the gene products at 883 amino acids, forming a 99 kDa protein, whereas the 784 amino acids of Klp6 make a 88 kDa protein. Klp5 and Klp6 each contain an N-terminal kinesin motor domain, preceded by a stretch of non-motor residues, of which the function remains unknown (West et al., 2001). The homology between these two kinesin-8 is largely confined to the motor region, where they share 66% identity and 76% similarity of sequence (West et al., 2001). Following the motor domain, each protein contains a stretch of sequence that is predicted to form one turn of a coiled-coil (West et al., 2001) and that is required for heterodimerisation (Amy Unsworth’s thesis). Overall, Klp5 and Klp6 exhibit 50% identity and 69% similarity (Garcia et al., 2002b) due to the divergent nature of the sequence of the so-called tail regions that follow the neck domains. Many kinesins utilise these regions to bind and carry cargo proteins or organelles, but the true nature of these regions in Klp5 and Klp6 is not fully understood.

1.7.3.1 Subcellular localisation

Studies of the localisation of Klp5 and Klp6 found that it was dynamic throughout the cell cycle. In interphase, they bind to cytoplasmic microtubules (West et al., 2001) with preference for the microtubule plus ends that are located at cell ends (Garcia et al., 2002b, Tischer et al., 2009). In mitosis, Klp5 and Klp6 localise to the full length of short early spindles, additionally to astral microtubules after the onset of anaphase and to become restricted to the spindle midzone during anaphase B (West et al., 2001, Garcia et al., 2002b). Additionally, in metaphase arrested cells, these kinesins were observed to colocalise with Nuf2 of the outer kinetochore and to interact with the outer repeats of centromeres by ChIP, of which interaction depends on the presence of microtubules (Garcia et al., 2002b). Localisation of the
complex relies on the presence of Alp14 to localize Klp5-interacting Alp7 to the kinetochore (Sato et al., 2004, Tang and Toda, 2015).

1.7.3.2 Complex formation

Multiple lines of evidence point to the formation of a Klp5-Klp6 heterodimer, at least under normal physiological conditions in vivo, facilitated by the coiled-coil neck domains. Immunoprecipitation from asynchronous cultures revealed each could pull the other down, but at least Klp6 could not pull down itself (Klp5 was not tested in this instance) (Garcia et al., 2002b). Both Klp5 and Klp6 shuttle between the cytoplasm and nucleus during interphase in a Crm1/exportin-1-dependent manner that is dependent on their C-terminal NLSs, as the addition of leptomycin B (LMB) caused their accumulation in the nucleus in interphase (Fornerod et al., 1997, Unsworth et al., 2008). NLS mutation or deletion of Klp5 causes the loss of Klp6 spindle localization in mitosis, and vice versa, indicating that while each protein enters the nucleus separately, one is required for nuclear retention of the other (Unsworth et al., 2008). This suggest that their mitotic function, at least, relies on the presence of both kinesin-8 proteins, likely as a heterodimeric complex. Recent work has indicated there may be residual binding of one kinesin to the spindle in the absence of the other, but proper time-lapse analysis of the duration of the binding, and also the functionality of the binding is lacking (Gergely et al., 2016).

1.7.3.3 Deletion phenotypes

Initial experiments looking at the function of Klp5 and Klp6 studied their cellular deletion phenotypes, as individually and together, the genes are non-essential (West et al., 2001). The growth of single and double mutants in the presence of high concentrations of thiabendazole (TBZ) identified them as kinesins that could destabilise microtubules, as their absence lead to TBZ resistance, indicative of stable microtubules (West et al., 2001). Deletion strains are also resistant to cold shock, where microtubules persisted through the treatment and were quicker to regrow their interphase array (Garcia et al., 2002a). Visualisation of interphase microtubules in klp5Δ, klp6Δ or klp5Δ klp6Δ cells revealed their ability to curl at cell tips due to their increased length (West et al., 2001, Garcia et al., 2002b). These results, coupled with the similarity of these fission yeast kinesin-8 with the budding
yeast KIP3 confirmed their identity as microtubule destabilising motor proteins (Garcia et al., 2002b, Severin et al., 2001). Cells lacking Klp5 or Klp6, or both, are altered heavily in mitosis. Deletion strains show elongated metaphase spindles that do not maintain a constant length that is characteristic of wild-type mitosis (Garcia et al., 2002b, Syrovatkina et al., 2013), consistent with their ability to destabilize microtubules.

On these spindles, chromosomes do not congress to the spindle midzone but instead oscillate between the poles until anaphase onset (Garcia et al., 2002b, Mary et al., 2015). In contrast to the role of Kif18A in human cells, (Mayr et al., 2007, Stumpff et al., 2008), high temporal resolution of sister chromatid movements prior to anaphase found that congression defects in kinesin-8 deletion cells were not rescued by the reduction of their oscillatory movements by TBZ (Mary et al., 2015). This observation lead them to hypothesize that Klp5-Klp6 may center kinetochores by sliding them towards plus-ends, but this has not been validated. During this period, the metaphase-anaphase transition is delayed (West et al., 2002, Garcia et al., 2002a), due to activation of the SAC (Garcia et al., 2002a, Mary et al., 2015). Chromosome recapture assays using a cold-sensitive β-tubulin mutant, *nda3-KM311* (Toda et al., 1983, Hiraoka et al., 1984) that cause chromosomes to be lost from SPBs allows observation of their retrieval to the spindle by microtubules (Grishchuk and McIntosh, 2006). Recent work using this approach to understand the role of kinesin-8 in chromosome movements found that retrieval of kinetochores by depolymerising microtubules was hampered in cells lacking Klp5 and Klp6 (Gergely et al., 2016). Microtubules took longer to bring lost kinetochores back to the SPB and frequently paused or even pushed the kinetochores further away, behaviours that were not observed in wild-type cells (Gergely et al., 2016). These results are consistent with the role for kinesin-8 in proper kinetochore microtubules attachment and for the negative regulation of microtubule stability. However, the fact that these retrieving microtubules can still depolymerise suggests that spindle microtubules can be destabilized in other ways, either inherently or by some kinetochore factor? Think about this. Despite the characterization of the null phenotypes, little is known exactly how Klp5 and Klp6 contribute to stable kinetochore-microtubule attachments.
In kinesin-8 deletion cells, correct amphitelic attachments eventually establish and sister chromatids proceed to segregate from any location on the spindle (Garcia et al., 2002b). During anaphase, spindles continue to elongate, contrary to the maintenance of spindle length during this period seen in wild-type cells (Garcia et al., 2002b) and lagging chromosomes appear (Griffiths et al., 2008, Syrovatkina et al., 2013, Gergely et al., 2016). Despite the asymmetric positioning of metaphase sister chromatids on the spindle and the appearance of laggards, chromosome segregation is always equal in deletion cells, dependent on the presence of a functional SAC (Garcia et al., 2002b).

Additionally, overexpression of full length either or both kinesin-8 proteins was seen to be toxic, and visualization of DNA in cells overproducing Klp5 found that chromosomes could not undergo segregation on a proper spindle (Garcia et al., 2002b). However, the localisation of the highly expressed proteins was not examined, and the partners were not overexpressed together, so the reason for toxicity remains slightly unclear.

1.7.3.4 Role in SAC silencing

Klp5 and Klp6 have been implicated in silencing the activated SAC for a timely anaphase onset (Meadows et al., 2011). This was function was uncovered by an apparent synthetic lethality of klp5Δ and klp6Δ with bub3Δ, a component of the SAC implicated in silencing the signal, rather than activating it (Tange and Niwa, 2008, Vanoosthuyse et al., 2009, Windecker et al., 2009). This study used silencing assays, where cells are arrested in mitosis by the nda3-KM311 β-tubulin allele at low temperature in the presence of an analog-sensitive Aurora B mutant, ark1-as3 (Hauf et al., 2007) and fluorescently-tagged Cdc13 as a marker of anaphase onset (Vanoosthuyse and Hardwick, 2009). Addition of the analog to cold-arrested cells inhibited Aurora B activity to allow checkpoint silencing to proceed, as seen by a decrease in Cdc13 fluorescence after the APC/C becomes active. Kinesin-8 deletions caused a delay in silencing, which was found to be independent on the motor activity of the kinesins, although in this assay there are no spindle microtubules due to the β-tubulin mutation. Previous implication of PP1 in counteracting Aurora B phosphorylation for checkpoint silencing (Vanoosthuyse
and Hardwick, 2009) lead to the identification of PP1-binding motifs in the C-terminal tail domains; two motifs in Klp5 and one in Klp6 (Meadows et al., 2011). PP1-binding activity was indispensable for checkpoint silencing and a timely anaphase onset in unperturbed cells, but had no effect on spindle length (Meadows et al., 2011). Subsequent work from our group found that at least Klp5 could interact with Alp7, discovered through an Alp7 mutant that showed decreased Klp5-Klp6 at kinetochores and allowed an ndc80-NH12 more time to correct its kinetochore-microtubule attachment defects (Tang and Toda, 2015). This explains how Klp5-Klp6 can still bring PP1 to kinetochores in the absence of microtubules (Meadows et al., 2011).

1.7.3.5 Kinesin motility and behavior

Work on Klp5 and Klp6 in vitro has been limited by problems in expression of full-length heterodimeric constructs that yielded enough protein at an appropriate purity (Grissom et al., 2009, Erent et al., 2012). However, the use of truncations has provided some insight into their mechanism of function.

Microtubule gliding assays, using stabilized bovine brain microtubules, revealed that full-length recombinant Klp5-Klp6 have plus-end directed motility, dependent on the presence of ATP (Grissom et al., 2009). Moreover, motor-neck constructs of Klp6, when coupled to beads at a high concentration of protein, are capable of moving the beads towards the plus-ends of taxol-stabilised porcine brain microtubules (Erent et al., 2012). To date, there is no published work examining single molecule, or higher concentration, behavior of fluorescently-labelled Klp5-Klp6 on visible microtubules by high quality microscopy. Unpublished work from our lab by Yuzy Matsuo, in collaboration with Thomas Surrey, has performed such experiments using total internal reflection (TIRF) microscopy. Full-length Klp5-Klp6-msfGFP purified to a high level were seen to move processively towards the plus-ends of Cy5-labelleed taxol-stabilised porcine microtubules in the presence of ATP. Plus-end dwelling was also observed. Further, one group observed the movement of multivalent Klp5-Klp6-coated beads to move with the depolymerising end of elongated axonemes, suggestive of an ability for Klp5-Klp6 to track shrinking microtubule ends (Grissom et al., 2009). However, such events using purified
constructs on dynamic microtubules in our lab were never observed (Yuzy Matsuo, unpublished data).

The ATPase activity of full-length Klp5-Klp6 were seen to be dependent on the presence of both ATP and microtubules, but not free tubulin dimers (Grissom et al., 2009). Conversely, motor-neck constructs of both Klp5 and Klp6 individually displayed tubulin-activated ATPase activity, implying that full-length proteins are able to inhibit this activity (Erent et al., 2012). Additionally, these constructs were also found to bind to fission yeast microtubules more tightly than its tubulin. However, experiments were not performed with heterodimers of these constructs for proper comparison to the full-length heterodimer (Erent et al., 2012).

Mutations in the motor domain that affect ATPase activity have also been studied briefly in Klp5 and Klp6 (Rice et al., 1999) where mutations in the switch II region prevent kinesin translocation (Browning et al., 2003). Switch II mutants of Klp5 and Klp6 show decoration of anaphase spindles, indicative of loss of motility (Amy Unsworth’s thesis) (Meadows et al., 2011). Interestingly, the switch II mutants cannot negatively regulate spindle length, likely due their inability to efficiently accumulate at microtubule plus-ends and additionally show a delay in anaphase onset (Meadows et al., 2011).

1.7.3.6 Control of microtubule dynamics and behaviour

Further to the studies of recombinant kinesin-8 movement on purified microtubules, these groups also examined the behavior of microtubules in the presence of the motor proteins. Multiple experimental set ups using either taxol- or GMPCPP-stabilised bovine microtubules and full-length Klp5-Klp6 did not display any detectable shortening of microtubules (Grissom et al., 2009). The motor-neck constructs also did not have any significant effect on the shrinkage rate of GMPCPP-stabilised porcine microtubules when assayed in different ways, however this may be unsurprising given the use of the two constructs separately (Erent et al., 2012). Interestingly, the observation of dynamic unlabeled S. pombe microtubules found no significant effects on growth or shrinkage rates or the frequency of rescue or catastrophe when the microtubules of axonemes were
observed in the presence of individual or combined motor-neck constructs (Erent et al., 2012).

In contrast to these two bodies of work, recent studies from our lab has found that Klplp5-Klp6-msfGFP is capable of increasing the catastrophe frequency of labelled, dynamic porcine microtubules, compared to in the absence of motor proteins (Yuzy Matsuo, unpublished data). In these experiments, there was little change in growth rate and a modest decrease in the shrinkage rate of dynamic microtubules as the concentration of recombinant Klplp5-Klp6-msfGFP was increased (Yuzy Matsuo, unpublished data). Additionally, there was no change in the shrinkage rate of GMPCPP-stabilised microtubules when the motor complex was added, consistent with previous results (Erent et al., 2012). The inability of Klplp5-Klp6 to destabilize GMPCPP-microtubules highlights a difference between these fission yeast kinesin-8 and those of other species.

The nature of interphase cytoplasmic microtubules in S. pombe permit the visualization of microtubule events in vivo. Our group found that in the presence or absence of Klplp5/Klp6, cytoplasmic microtubule growth rate was unchanged and shrinkage rate remained within a similar range (Unsworth et al., 2008). The most prominent changes between wild-type and kinesin-8 deletions cells were the drop in both catastrophe frequency and rescue frequency (Unsworth et al., 2008), consistent with our recent results from in vitro experiments (Yuzy Matsuo). Detailed analysis of microtubule bundle tips at the cell cortex found that at this location catastrophe frequency is high and growth rate is low, due the interaction between the bundle and the cell tip (Tischer et al., 2009). At these bundle tips Klplp5-Klp6 is seen to increase in intensity as they remain on dwelling plus-ends and their deletion leads to a decrease in the frequency of catastrophe (Tischer et al., 2009).

Therefore, in contrast to their budding yeast and human counterparts, it seems that kinesin-8 in fission yeast influence microtubule dynamics by increasing catastrophe frequency to induce microtubule shrinkage. Although there may be some differences in activity between full-length and truncated constructs, biochemical studies of full-length kinesin-8 from other species see activity. This is contrast to the requirement of some kinesins for cargo proteins to relieve auto-inhibitory
activity for proper kinesin motility and function (Coy et al., 1999), but this does not seem to be the case for the kinesin-8 family.

1.8 Schizosaccharomyces pombe as a model organism

The fission yeast *Schizosaccharomyces pombe* is a member of the ascomycete division of fungi and is a unicellular eukaryote. As such, many genes are conserved from this fission yeast to higher eukaryotes and their encoded proteins perform largely similar functions. The fission yeast genome spans three chromosomes and the coding gene count currently stands at 5118 as it has been sequenced and continues to be reviewed (Wood et al., 2002)(Pombase). Gene locations are mapped and so are easy to delete, and as a haploid organism only one deletion event is required to obtain a phenotype, although some genes are essential and cannot simply be deleted. Additionally, the popularity of this model organism has driven the development of multiple tools for the study of this fission yeast. An extensive database detailing not only characteristics for the gene itself but compiling a list of population and cellular phenotypes as well as other forms of data and results exists through curation of published literature (PomBase - http://www.pombase.org/ ). Genetic disruption, deletion or gene tagging is facilitated by an online program for oligo generation (Bahler et al., 1998) and the ability of fission yeast undergo efficient homologous recombination.

Physiologically, fission yeast presents a strikingly similar cell cycle to higher eukaryotes with some variation in the timings and the proteins involved. One difference is that fission yeast undergo a closed mitosis (Ding et al., 1997), is in contrast to the open mitoses of higher eukaryotes, but many of the processes within mitosis are the same. One difference in mitotic events is anaphase, which is split into parts A and B. In contrast to higher eukaryotes, fission yeast exhibits a fast growth rate, where in rich media and a temperature of 30˚C, the generation time is about 2.5 hours, of which mitosis takes around 20 minutes. This factor permits ease of observation by microscopy and other experiments due to the quick generation time.
Chapter 2. Materials & methods

2.1 S. pombe cell culture and genetics

2.1.1 Growth and maintenance of strains

Standard media and methods were used for the growth and maintenance of fission yeast strains (Moreno et al., 1991). 30°C was used as permissive temperature. Strains were grown in YE5S media unless otherwise stated; all media used are shown in Table 1. For selection of genes marked with drug resistance, antibiotic was added to YE5S plates at concentrations shown in Table 2. For the selection of auxotrophic markers, EMM drop out plates were used.

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
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<tbody>
<tr>
<td>YE5S broth</td>
<td>0.5% Bacto yeast extract (BD), 3% (w/v) dextrose (supplier), 150 mg/l each uracil, adenine, histidine, lysine, leucine (all Sigma) Bacto agar (BD) added for plates.</td>
</tr>
<tr>
<td>YE5S+Phloxine B plates</td>
<td>YE5S + 7.5 µg/ml Phloxine B (Sigma), Bacto agar (BD)</td>
</tr>
<tr>
<td>EMM5S</td>
<td>14.7 mM potassium hydrogen phthalate, 15 mM Na₂HPO₄, 93.5 mM NH₄Cl, 2% (w/v) dextrose, salt stock, vitamin stock, mineral stock, 75 mg/l each uracil, adenine, histidine, lysine, leucine</td>
</tr>
<tr>
<td>EMM –ura/ade/his/lys/leu plates</td>
<td>EMM5S but lacking uracil, adenine, histidine, lysine or leucine. Bacto agar (BD) added for plates.</td>
</tr>
<tr>
<td>MEA4S plates</td>
<td>3% (w/v) Difco malt extract, 75 mg/l uracil, adenine, histidine, leucine, bacto agar</td>
</tr>
<tr>
<td>YFM</td>
<td>YE5S with 15% (v/v) glycerol</td>
</tr>
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Table 1 Growth media and components
<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Kanamycin</td>
<td>100 µg/ml G418 - Genetecin (Sigma)</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>300 µg/ml hygromycin B (Sigma)</td>
</tr>
<tr>
<td>ClonNAT</td>
<td>100 µg/ml nourseothricin (Werner Bio-agents)</td>
</tr>
<tr>
<td>Aureobasidin A</td>
<td>0.5 µg/ml aureobasidin (Takara Bio)</td>
</tr>
</tbody>
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Table 2 Drugs for selection and concentration

### 2.1.2 Transformation of S. pombe

1 x 10^8 cells were collected from log-phase cultures by centrifugation at 3,500 rpm for 5 minutes. Pellets were resuspended in 50 ml of water and centrifuged again. Cells were then washed in 1 ml of LiOAc/TE (100 mM lithium acetate pH 7.4, 10 mM Trish-HCl pH 7.4, 1 mM EDTA pH 8.0) and collected by centrifugation at 5,000 rpm for 1 minute before being resuspended in 100 µl of LiOAc/TE. 2.5 µl of salmon sperm carrier DNA (conc, supplier) was added to prepared cells in combination with a maximum of 15 µl of desired DNA and incubated at room temperature for 10 minutes. 260 µl of 40% (w/v) PEG 4000 in LiAOc/TE was then added to the mixture before incubating at 30°C for 45 minutes. After incubation, 43 µl of pre-warmed DMSO was added before a 42°C heat shock for 5 minutes. Cells were then collected by centrifugation at 3,000 rpm for 2 minutes, washed by water and then resuspended in 300 µl of water. 100 µl of transformation was plated onto YE5S in triplicate and incubated at 30°C for approximately 24 hours. After this, the resultant lawn of cells was replica plated to select for positive transformants using drug-containing plates.

### 2.1.3 Isolation of temperature-sensitive klp5 mutants in the dis1Δ background

A klp5’-5xFLAG-kan’ fragment was amplified from purified genomic DNA (gDNA) from a C-terminally tagged strain. gDNA was extracted from cell patches on plates using the MasterPure DNA Purification Kit (Epicentre) This fragment was used as a template for error-prone PCR using Vent polymerase (New England Biolabs) in the presence of ten times excess of deoxyguanosine triphosphate. The randomly mutagenised fragments were transformed into a dis1Δ strain and plated on to YE5S at 30°C for approximately 24 hours. Transformants were selected for by
replica plating onto YE5S + kan and incubating at 30°C for a further 4 days. Temperature sensitivity was tested by replica plating transformants onto YE5S + Phloxine B and incubating at both 30°C and 36°C for 24 hrs. Candidates were identified as those that grew as white, viable colonies at 30°C but formed dark pink, dying colonies at 36°C. Phloxine B stains dying cells dark as they are less able to pump out the pink dye, compared to viable active cells. Candidate mutants were then backcrossed to wild type to check that temperature-sensitivity segregated stably with kanamycin resistance during random spore analysis. Mutation sites were identified by in-house sequencing of a klp5<sup>ts</sup>-5FLAG-kan<sup>′</sup> fragment amplified from genomic DNA from each mutant (Crick STP).

### 2.1.4 Site-directed mutagenesis of klp5 and klp6

Two approaches were taken for site-directed mutagenesis of klp5 and klp6 at their endogenous loci. The first approach used gDNA as a template to amplify the klp-5FLAG-kan<sup>′</sup> fragment using Primestar polymerase (Takara). From this, a 5′ fragment and a 3′ fragment were generated, overlapping with each other by 15 bases, at the centre of which was the mutated codon. These were then stitched together in a fusion PCR reaction and the resultant mutant fragment was used for transformation.

The second approach involved using the Strataclone Blunt PCR Cloning kit (Agilent) to insert the template fragment into the supplied pSC-B-amp/kan cloning vector. The Q5 Site-Directed Mutagenesis kit (New England Biolabs) was used to introduce the desired SDM by mutagenic forward oligonucleotides and transformed. Transformants were sequenced to check for the substitution mutation before amplifying the mutant fragment for transformation into yeast.

### 2.2 Serial Dilution Assay – Spot Test

Cells were grown at 30°C overnight into mid-log phase (2-10 x 10<sup>6</sup> cells/ml). Dilutions were made to 2 x 10<sup>6</sup> cells in a volume of 1 ml for each strain and cells collected by centrifugation at 4,000 rpm for 2 minutes. Cells were resuspended in 200 µl of distilled water and diluted 10-fold in water before spotting onto appropriate
plates. Plates were then incubated at the required temperature(s) for 3 days before photographing for analysis of growth.

2.3 Live Cell Microscopy

2.3.1 Sample preparation for short-term time-lapse imaging at restrictive temperature

Mid-log phase cultures were shifted up to 36°C for 25 minutes in a shaking water bath then 1 ml was taken for sample preparation. Cells were pelleted at 4,000 rpm for 2 minutes then 400-800 µl of the supernatant media was removed. Cells were resuspended in the remaining volume, of which 200 µl was transferred to a lectin-coated (soy bean lectin, Sigma Aldrich) glass-bottomed culture dish (MatTek Corporation, Ashland, MA) and incubated for 3 minutes at room temperature to allow cells to stick. The 200 µl of cell suspension was then discarded before any free-floating unbound cells were removed by washing with 200 µl of warmed media. Bound cells were then covered by adding 2 ml of fresh warm media, to maintain nutrient supply during imaging. The dish was then incubated on the microscope at 36°C for a further 15 minutes to bring the total temperature shift prior to imaging to 40 minutes. Note that 40 minutes temperature shift was an optimal time to induce the temperature-sensitive phenotype but short enough to prevent the accumulation of brightly fluorescing dying cells. Cells were then imaged by time-lapse microscopy at 36°C.

2.3.2 Wide-field fluorescence microscopy

Images were acquired one of two wide-field inverted fluorescence Deltavision systems: either an Olympus IX70 microscope with an Olympus PlanApo 60x/NA 1.4, oil immersion objective or, an Olympus IX71 with a PlanApo 60x/NA 1.42 oil immersion objective. Ten fluorescent sections were taken through the Z-axis at an interval of 0.4 µm, followed by a bright field image taken from the middle of the sample. Images were captured and processed using the DeltaVision image acquisition software (softWoRx version; Applied Precision Ltd.) – Z-stacks were deconvolved and then combined to form a two-dimensional projected image following a maximum intensity algorithm. Images were analysed in FIJI and manipulated for figures using Adobe Photoshop CS5.1 and Adobe Illustrator CS5.1.


2.3.3 Image analysis

For the analysis of mitotic parameters, cells were synchronised \textit{in silico} by timing of mitotic entry. The frame prior to the first instance of SPB separation was classed as time zero and the end of mitosis was assigned to the frame before the spindle microtubules disassembled from between the two SPBs. Spindle length through mitosis was measured by drawing a straight line (or segmented for bent spindles) between SPBs. As metaphase could not be assigned based on spindle length, the last frame before anaphase onset – when the beginning of sister chromatid separation could be clearly observed - was used as a proxy for metaphase. Time zero up until and including the assigned metaphase time point was constituted time spent in pre-anaphase. The duration of chromosome segregation was classified as the time between the first instance of chromatid separation up until all chromosomes had reached their SPBs. Anaphase ‘A’ spindle lengths were extracted from this period of segregation. If a chromatid was still in the process of moving poleward when other chromatids had reached the same SPB, this was noted as a lagging chromosome.

2.4 Biochemical Analysis

2.4.1 Co-immunoprecipitation

2.4.1.1 Protein extraction

To test the interaction of Klp5^{Q582P}-5FLAG with Klp6-GFP, cultures were grown overnight at 30°C to mid-log phase before shifting to 36°C for 1 hour. Subsequent processing of samples was performed at 4°C or on ice. 100 ml of each culture was harvested then washed in 1 ml of STOP solution (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 0.007% NaN\textsubscript{3}). Cells were then washed in 1 ml HB buffer (homogenising buffer, composed of IP buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.05% NP-40 (Igepal CA630), 10% (v/v) glycerol) with 1 mM DTT, 10 mM PNPP, 1 mM PMSF and cOmplete protease inhibitor cocktail (Sigma)). Each sample was resuspended in 400 µl of HB buffer before acid-washed beads were added. Cells were then lysed by beating using a FastPrep120 at 4°C. Cell debris was pelleted by centrifugation at 13,000 rpm for 5 minutes and supernatant
taken as protein extract. Protein extracts were stored at -80°C until analysis. Protein concentration was determined using a Bradford assay (Bio-Rad).

2.4.1.2 Immunoprecipitation

A magnetic GFP-Trap system (ChromoTek GmbH, Martinsried, Germany) was used to pull down Klp6-GFP. To prepare GFP-Trap, beads were vortexed to remove aggregates then washed in IP buffer (described above) twice before resuspension in IP buffer. Protein extracts were made up to a total amount of 2.8 mg in 400 µl of HB buffer and incubated with 20 µl of prepared GFP-Trap for 1.5 hours at 4°C, rotating. After incubation, protein-bead complexes were washed with 1 ml of HB/wash buffer until solution became colourless. Complexes were then resuspended in 30 µl of wash buffer before boiling in Laemmli buffer (ingredients) for 5 minutes to separate proteins and beads. Whole cell extract (WCE) samples were prepared alongside. Beads were separated out magnetically and 16 µl of protein solution was subjected to SDS-PAGE, followed by immunoblotting. Klp5 was detected using mouse anti-FLAG M2 antibody (F-3165, Sigma) at 1:1,000 and Klp6 was detected by rabbit anti-GFP (AMS bio) at 1:1,000. Describe detection system.

2.5 Biochemical Studies of Kinesin Behaviour in vitro

2.5.1 Protein Expression and Purification

A pET-Duet plasmid (Novagen) containing codon-optimised \textit{klp5-HA-PreScission site-proteinA} and \textit{klp6-msfGFP} (Yuzy Matsuo, unpublished data) was used to express WT proteins and as a template for mutagenesis or further tagging. For the study of Klp5\textsubscript{WT/Q582P}-HA/Klp6-msfGFP, \textit{klp5-HA-PreScission site-proteinA} was mutated to \textit{klp5\textsuperscript{Q582P}} using an In-Fusion HD Cloning Kit (Clontech Laboratories Inc., Takara Bio, CA) as follows. A fragment containing the aforementioned components was amplified including flanking restriction sites - 5’ EcoRI and 3’ XhoI. Two overlapping mutant fragments were amplified from this fragment, using outward overlapping mutant primers at the Q582P nucleotide substitution site. A linearised backbone was generated by EcoRI/XhoI digestion of the original template and isolated by gel purification. The backbone was then incubated with the two mutant
fragments for plasmid assembly in the cloning reaction and subsequent transformation.

Klp5 and Klp6 constructs were co-expressed in BL21 DE3 codon-plus RIL *E. coli* cells and induced by 0.1 mM IPTG at 18°C for 16 hours. Cells were harvested in ice-cold LEW+ (Lysis, Equilibration, Wash buffer plus protease inhibitors and DNase) buffer (50 mM HEPES (pH 7.5), 300 mM KCl, 2 mM MgCl₂, 0.002% Brij-35, 1 mM DTT, 10% (v/v) glycerol, 0.2 mM ATP), plus a cocktail of EDTA-free protease inhibitors (Roche) and DNase I (Sigma). Cells were lysed by passing the suspension twice through a chilled high-pressure homogeniser (Emulsiflex C-5, Microfluidics). Samples were then passed through a sieve and clarified by centrifugation at 50,000 rpm for 40 minutes at 4°C to pellet debris. For purification of the kinesin complex, lysates were first incubated with IgG Sepharose-6 Fast-Flow resin (GE Healthcare) for 2 hours at 4°C. The resin was then transferred into a LEW+-equilibrated Econo-Pac column (Bio-Rad) and washed with 10 column volumes of LEW+ buffer to remove unbound protein. To cleave the Protein-A tag from Klp5-HA, samples were incubated at 4°C overnight in LEW buffer containing a PreScission protease (GE Healthcare). Cleavage was checked by SDS page and Coomassie Brilliant Blue staining before proteins were taken forward for further purification by gel filtration. A Superose-6 10/330 column at 4°C was equilibrated with LEW buffer and used to purify the Klp5-HA/Klp6-sfGFP complex (Klp5 ± mCherry), during which proteins eluted as a large majority peak, suggestive of a homogenous population of heterodimers. Purified proteins were then flash frozen in, and subsequently stored in liquid nitrogen until required.

**2.5.2 TIRF microscopy**

**2.5.2.1 Taxol-stabilised microtubule TIRF assay**

Flow chambers were assembled from a biotin-PEG functionalised coverslip attached to a PLL-PEG passivated microscope slide by double sided tape. TIRF assay buffer consisted of BRB80 supplemented with 85 mM KCl, 1 mM GTP, 10 mM β-mercaptoethanol, 0.1% Brij-35, 0.1% methylcellulose (Sigma-Aldrich) and an oxygen scavenger system [glucose, glucose oxidase (Serva) and catalase (Sigma-Aldrich)] and 1mM taxol and 1mM ATP. Cy5-labelled taxol-microtubules were used and the kinesins were added to a final concentration of 10 nM. For simultaneous
dual-colour time-lapse imaging of the Cy5 and GFP (and mCherry) channel, imaging was performed at 1-s intervals with 100 ms exposure time, using a ×100 objective lens at 30± 1°C on a custom TIRF microscope equipped with a Cascade II, cooled charge-coupled device camera (Photometrics), illuminating the sample with 488 nm and 640 nm (and 561 nm) lasers.

2.5.2.2 Image analysis

ImageJ software (NIH) was used to analyse TIRF microscopy data. Data acquired in separate fluorescence channels were firstly converted to 8-bit images then corrected for bleach using the ‘correct bleach’ macro. The two channels were then merged to create a composite and an image stabiliser macro applied to correct for drift of the field. Kymographs were generated by drawing a line down the microtubule of interest and reslicing the image, where the y-axis became time (1 sec/pixel) and the x-axis became distance (1 µm/pixel). To characterise GFP-tagged kinesin run behaviour on Cy5-labelled, taxol-stabilised microtubules, kymographs were generated for each microtubule and the bound kinesin complexes analysed. Processive runs were assigned to those showing smooth, constant and directed movement towards the plus end. Diffusive runs were characterised by stochastic back and forth continued movement on the microtubule. Note that diffusive runs exhibited an overall directionality for the plus end. In the event that a kinesin complex bound to the microtubule but did not show any movement over time, the run was classified as static. Only runs proceeding for 3 seconds or more were included for analysis.

2.6 Oligos used in this study

\textbf{klp5 sequencing:}

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CP020  R - 133  GTAGAAAAAGACGAGGAAACG
CP021  R + 237  CCAAGACACGTCCATCAAGT
CP022  R + 545  TAAACCAGGATCTTGCAATG
CP023  R + 925  AAAGTGCCAAAGTGTGGTC
CP024  R + 1275  GGCTTACATGTCGATCTACA
CP025  R + 1640  AATGATCTCTGCACGACGAG
CP026  R + 1951  TTAGGTTTCTCGCAAGACC
CP027  R + 2339  ATCTTCCAACATCGGGGAGA

klp5 SDM:
CP030  F'  Q582P  CAGGTTCTAGATGCTCCGAATAAAGTTGATGAG
CP031  R'  Q582P  CTCATCAACTTTATTCGAGGATCTAGGAACCTG
CP032  F'  D843G  GCTATTCACAACTTTGGTTTTTCTAAACCGAAG
CP038  R'  D843G  AGTCTTCGTTTAAACAAATGCTTGGAGGTCTTC
CP046  F'  T153A  TGTGTTAAACCACTCAATTAGTGGAACATG
CP047  R'  T153A  CATTGTCCAAATATGGCGTGATTTTACCACA
CP048  F'  K477R  AATGATCTATTATTGAAGTGAGATTTTTCCACA
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CP060  F'  Q788R  TTGATGCGACTGTGGCCGAGGAC
CP061  R'  Q788R  GAGTCTCGGCCACCGTGATGCTG

pET-Duet construction, klp5Q582P
YM443  R' Klp5 Q582P  GTAAATTCATCCACTTTTATTCGGGGGCAGATCCGT
YM444  F' Klp5 Q582P  AGTGGGATGATTTACCCCGG

Tail box mutants:
CP106  F'  Klp5-E575P Q5  ATCAATTTACCACTTTTATTCGGGGGCAGATCCGT
CP107  R'  Klp5 for Q5  TTTAGCAGCGACTTCATTC
CP102  F'  Klp6-E569P Q5  ATCTATTACCACTTTTATTCGGGGGCAGATCCGT
CP103  R'  Klp6 for Q5  TTTGCAATTTACCCGAGTCG

Table 3. List of oligonucleotides used in this study
2.7 Plasmids used in this study

pETDuet-1 Klp6-msfGFP-Klp5-HA-3C-Protein A
pETDuet-1 Klp6-msfGFP-Klp5(Q582P)-HA-3C-Protein A

2.8 Strains used in this study

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**Chapter 2 Materials and Methods**

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<td>Jonathan Millar</td>
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### Table 4. List of strains used in the experiments of this study

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<th>Strain Code</th>
<th>Description</th>
<th>Source</th>
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| CP582       | h' leu1 ura4 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} cen2::hph\textsuperscript{'}-lacO
his7\textsuperscript{-}:lacI-GFP-ura4\textsuperscript{-} sad1-dsRed-LEU2 aur\textsuperscript{-}mCherry-atb2 | This study      |
| CP180       | h' leu1 ura4 his2 nuf2-YFP-ura4\textsuperscript{+} sid4-mRFP-na\textsuperscript{'} aur\textsuperscript{-}mCherry-atb2 | Lab collection |
| CP324       | h' leu1 ura4 klp5::kan\textsuperscript{-} nuf2-YFP-ura4\textsuperscript{+} sid4-mRFP-na\textsuperscript{'} aur\textsuperscript{-}mCherry-atb2 | This study      |
| CP317       | h' leu1 ura4 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} nuf2-YFP-ura4\textsuperscript{+} sid4-mRFP-na\textsuperscript{'} aur\textsuperscript{-}mCherry-atb2 | This study      |
| CP544       | h' leu1? ura4? his? klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} cdc13-GFP sid4-mRFP-na\textsuperscript{'} aur\textsuperscript{-}mCherry-atb2 | This study      |
| CP557       | h' leu1 ura4 dis1::hph\textsuperscript{'} klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} cut11-GFP-ura4\textsuperscript{+} sid4-mRFP-na\textsuperscript{'} aur\textsuperscript{-}mCherry-atb2 | This study      |
| CP606       | h' leu1 ura4 ase1::nat                                                      | This study      |
| CP787       | h' leu1 ura4 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} ase1::nat               | This study      |
| CP306       | h' leu1 ura4 his2 klp5\textsuperscript{Q582P}-3FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'}                        | This study      |
| CP843       | h' leu1 ura4 cut7-21                                                      | Nurse lab       |
| CP838       | h' leu1 ura4 his2 cut7-21 dis1::hph\textsuperscript{'}                      | This study      |
| CP836       | h' leu1 ura4 his2 cut7-21 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-}                                   | This study      |
| CP840       | h' leu1 ura4 his2 cut7-21 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} | This study      |
| CP841       | h' leu1 ura4 his2 cut7-21 klp5\textsuperscript{Q582P}-5FLAG-kan\textsuperscript{-} dis1::hph\textsuperscript{'} | This study      |
Chapter 3. Screening for temperature-sensitive mutants of \textit{klp5}

Our aim was to begin to dissect the molecular functions of fission yeast kinesin-8, using a mutational approach instead of deletion or truncation mutants that have previously been studied. Although the members of the kinesin-8 family are non-essential genes in fission yeast, we exploited the synthetic lethality of \textit{klp5} with deletions of a subset of microtubule-associated proteins, including \textit{dis1}, \textit{alp14}, \textit{alp7} and \textit{dam1}. This chapter describes the random mutagenesis of \textit{klp5} in a \textit{dis1\Delta} background that produced multiple different temperature-sensitive \textit{klp5} alleles. Mutant strains were tested for growth defects, cell cycle progression, chromosome segregation behaviour and sensitivity to thiabendazole. The mutation sites were also analysed in regards to both sequence conservation and structural information or predictions. Using the \textit{in vivo} data acquired and \textit{in silico} analyses together provided the basis to select a mutation not only to elucidate novel domain functions of Klp5 protein but also to use as a conditional tool to study mitosis in the absence of key spindle factors Klp5/Klp6 and Dis1.

3.1 Rationale for the isolation of temperature-sensitive mutants of \textit{klp5}

The majority of previous studies on kinesin-8 in \textit{S. pombe} have focused largely on the use of deletions of \textit{klp5}, \textit{klp6} or both (West et al., 2001, West et al., 2002, Garcia et al., 2002a, Unsworth et al., 2008, Gergely et al., 2016, Syrovatkina et al., 2013, Mary et al., 2015). Others have used directed mutations in order to alter known domain functions, including those in the motor domains (Meadows et al., 2011), the NLS (Unsworth et al., 2008), potential CDK consensus sites (Unsworth and Toda, unpublished data) and PP1-binding motifs (Meadows et al., 2011, Tang and Toda, 2015). Truncation mutants have also been utilised in attempts to understand the interactions and functions of the distinct domains within the kinesin-8 heterodimer (West and McIntosh, 2008, Unsworth et al., 2008), but the validity of these results is questionable due to the methods by which the truncations were constructed and expressed. To our knowledge, no previous study has undertaken screening of kinesin-8 for mutations in a non-biased manner. Although some of the
kinesin-8 domains are well characterised, much of their regulation, structure and interactions are not. We sought to search for novel mutations in order to identify hitherto unknown motifs, understand characterised domain function and regulation or uncover novel interactions with other proteins. Both kinesin-8 genes are non-essential at a range of temperatures (West et al., 2001) therefore, mutations are likely to be tolerated in terms of cell viability and growth, similar to kinesin-8 deletions. Following this, screening for mutants in klp5 or klp6 would become labour-intensive at the primary stage, as phenotypes would have to be characterised by microscopy and other in-depth methods, rather than simply looking at colonies for growth problems.

The kinesin-8 gene deletions are known to be lethal with XMAP215/chTOG homologs dis1 and alp14 (Garcia et al., 2002a), the TACC adaptor alp7 (our unpublished data) and the DASH complex component dam1 (Sanchez-Perez et al., 2005). These genetic interactions indicate that the combined action of kinesin-8 and these MAPs is required for proper spindle function in mitosis. Therefore, klp5 and klp6 become essential in the absence of any of their collaborative MAPs. A genetic background in which kinesin-8 is essential makes it possible to screen klp5 or klp6 for temperature-sensitive mutations, where defective colony growth is an instant readout for a candidate mutant allele. Not only would using a deletion background facilitate a more efficient screening process, but it would shed light on the relationship between kinesin-8 and the collaborative microtubule-associated protein. Identifying a mutation in the kinesin that is only temperature-sensitive in the absence of a mitotic factor would imply a cooperative role for that region. Understanding the functional relationship between spindle factors is a key step towards a more global knowledge of mitosis.

3.2 Klp5/Klp6 are synthetically lethal with microtubule-associated proteins of the XMAP215/chTOG, TACC and DASH-complex families

To confirm the synthetic lethality between both kinesin-8 genes and the various MAPs, tetrad dissections were performed on crosses between the individual deletions. In line with previous observations
Chapter 3 Screening for temperature-sensitive mutants of klp5

(Garcia et al., 2002a, Sanchez-Perez et al., 2005), deletions of either klp5 or klp6 were always lethal with dis1Δ, alp14Δ/7Δ and dam1Δ (Figure 3.1). It is interesting to note that genetically, klp5 and klp6 show the same behaviour in these crosses. These results confirm the idea that the kinesin-8 functions overlap with those of this subset of MAPs, as cells cannot survive without their combined function.

3.3 Screening klp5 for temperature-sensitive mutations in the dis1Δ background

Although the deletions of various MAPs render klp5 or klp6 essential, not all deletion backgrounds are suitable for this mutagenesis screening. Deletions of alp7, alp14 or dam1 have been shown to be temperature-sensitive already (Sato et
al., 2004, Nakaseko et al., 2001, Sanchez-Perez et al., 2005), though viable at lower temperatures. Their intrinsic temperature sensitivity makes these deletions unsuitable for our screen. However, dis1Δ is a cold-sensitive deletion and displays normal growth at high temperature (Ohkura et al., 1988), making it an appropriate background to isolate mutants at high temperature. Consequently, the dis1Δ background was selected for the screening of kinesin-8.

![Diagram of klp5 and dis1Δ cells]

**Figure 3.2 Generation and identification of temperature-sensitive alleles of klp5**

A DNA fragment of *klp5* tagged with 5xFLAG-kanR was randomly mutated by error-prone PCR. Crosses within the represent mutations. Mutant fragments were then transformed into dis1Δ cells. Cells were plated onto and grown on YE5S for 24 hrs then replica-plated onto YE5S + kan to select for transformants. After 4 days, transformants were replica-plated to YE5S + phloxine B and incubated at the permissive (30°C) or restrictive (36°C) temperature for 24 hrs. Colonies were identified as candidates if viable at 30°C and inviable at 36°C.
The *klp5* gene was chosen for mutagenesis as it is the larger of the two kinesin-8 proteins. Figure 3.2 provides a scheme of the screening method used to isolate of temperature-sensitive mutations in the *klp5* gene in the absence of *dis1* (see Materials and Methods section 2.1.3 for full description). A permissive temperature of 30°C was chosen to prevent any cold-sensitive behaviour from the *dis1* deletion, while being low enough to allow colonies that are temperature-sensitive at 36°C to grow.

Between 3,000 and 4,000 colonies were screened across three independent experiments. After a primary screen of replica-plated transformant colonies for temperature-sensitivity, the candidates were backcrossed to a wild type strain and the progeny were checked for the segregation of temperature-sensitivity with kanamycin resistance. This process ensured that only candidates with intragenic mutations were carried through the screen, rather than those that showed temperature sensitivity as a result of a extragenic mutations. This process also helped to ensure that the mutations were stable and could be propagated through generations rather than being mutated back. After these steps, six suitable candidate *dis1Δ klp5*\(^{ts}\) mutants were selected for further analysis. From this point, a strain containing both a *klp5* mutation and *dis1* deletion is referred to as a double mutant, whereas a strain containing only the *klp5* mutation is referred to as a single mutant.

### 3.4 Confirmation of *dis1Δ klp5*\(^{ts}\) temperature sensitivities

The extent of temperature sensitivity of the six candidates isolated was assessed by serial dilution assays in which the plates were incubated at low, permissive and restrictive temperatures (Figure 3.3A). All double mutants displayed strong temperature-sensitivity at 36°C with very little to no growth, but appeared viable at 30°C. As expected from a non-essential gene, none of the single mutants showed temperature-sensitive growth defects a *dis1*\(^{ts}\) background. Interestingly, no *klp5* mutant was able to rescue the cold-sensitive
Chapter 3 Screening for temperature-sensitive mutants of *klp5*

Figure 3.3 Assay of temperature-sensitivity of *klp5*\textsuperscript{ts} mutants

Mutant candidates were assessed for temperature-sensitivity. (A) Cells were serially-diluted and spotted onto YE5S plates and incubated for 4 days at the indicated temperatures. (B) Liquid cultures were grown at 30°C overnight to mid-log phase before dilution and shifted to 36°C for 8 hours.
growth defect of dis1Δ at 22°C. In addition to assessing growth on plates, growth in liquid culture was also observed (Figure 3.3B). Cells cultured at 30°C were diluted to approximately 2 x 10^6 cells/ml and shifted to 36°C. Cell number was measured every 2 hours for a total of 8 hours, which equates to approximately four generations for wild type cells in rich media at this temperature. All six dis1Δ klp5ts mutants exhibit clear growth retardation by 4 hours, whereas WT, klp5Δ and dis1Δ control cells continue to grow. In line with the serial dilution assay, the candidate mutants exhibit a similar degree of temperature-sensitivity, which may reflect a related cellular phenotype across the mutants.

3.5 dis1Δ klp5ts cells have altered cell cycle progression

In order to understand why the double mutant cells cannot continue to divide at the restrictive temperature, the frequency of septation was measured in shifted cultures. Septum formation is initiated in late mitosis and matures after mitotic exit and passage through G1 into S-phase. Therefore, measuring the septation index, using

![Calcofluor](image)

**Figure 3.4 Double mutants have altered cell cycle progression and an incidence of septation defects**

Imaging of double mutant cells fixed and stained with calcofluor. Log-phase cultures were shifted from 30°C (0 hrs) to 36°C and samples taken at two and four hour time points for imaging. Panels show representative populations of cells, with examples of singly septated, multisepated or unseptated cells. Scale bar, 10 µm. Graph below shows percentage of cells with no septum, 1 septum or multiple septa at each time point. Number of cells counted for analysis indicated in white.
Calcofluor to stain the septa of dividing cells, can be used as a readout of cell cycle progression as the number of cells exiting, or trying to exit mitosis. Log-phase cultures grown at 30°C (0 hour time point) were shifted up to 36°C for a total of 4 hours (Figure 3.4). Samples were fixed then stained with calcofluor. Both control strains, WT and dis1Δ, throughout the experiment show relatively little variation in septation index, which never exceeds 25%, representative of viable cycling populations. In contrast the septation index of all double mutants increases over time at the restrictive temperature, some reaching over double that of wild type cells after 4 hours, indicating a change in the nature of cell cycle progression. It is possible that cells are spending longer in mitosis, resulting in an accumulation of cells exiting mitosis, or that septum formation takes longer or cannot be completed. While it is formally possible that the duration of septation is extended in the mutants, leading to an increase in the proportion of septated cells, it is unlikely that kinesin-8 play a role in septum formation or cell abscission. In double mutant cells expressing klp5<sup>CP49</sup> or klp5<sup>CP76</sup>, a substantial proportion of cells display multiple septa, an example of which is given in the top panel of Figure 3.4. Multiseptation is usually associated with problems in turning off the septation initiation network (SIN) for cell cleavage but may also be due to the inefficient termination of mitosis (Gould and Simanis, 1997). Although the incidence of multiseptation differs between individual mutants, it is interesting to note that after 4 hours at the restrictive temperature, the proportion of singly septated cells is quite similar among the mutants, within a small range of 30-40%. This similarity in the number of cells exiting mitosis may allude to a common defective phenotype amongst the six double mutants.
3.6 *dis1Δ klp5ts* cells show chromosome segregation defects

To clarify whether double mutant cells have a prolonged mitosis or have difficulty exiting mitosis, chromosome behaviour was monitored in an experiment identical to the one previously described. Cells were taken after 0, 2 and 4 hours at 36°C, fixed and then stained with the DNA-binding dye DAPI. Cells were categorised into groups based on the chromosome position and morphology phenotype (Figure 3.5). Wild-type and *dis1Δ* cells showed no defects in chromosome segregation or interphase DNA organisation: mononucleates show round nuclei with a darker nucleolus structure and binucleates show equal masses of decondensing DNA at either cell end. Even after two hours at restrictive temperature, all double mutants begin to show defects in DNA organisation, in both short and long cells. The most prominent phenotype in all the double mutants was a relatively tight mass of DNA localising towards one cell tip. As the cells are long and the chromosomes appear

![Figure 3.5](image_url)

**Figure 3.5** Chromosome defects progress over time in double mutants

Imaging of double mutant cells fixed and stained with DAPI. Log-phase cultures were shifted from 30°C (0 hrs) to 36°C and samples taken at two and four hrs after for imaging. Panels show two cells representative of each of the five phenotypic classes, with cell outlines marked on. Scale bar, 10 μm. Graph shows percentage of cells with normal DNA distribution (Normal), one asymmetrically positioned mass of DNA (One side), unequally distributed DNA (Unequal) and non-dividing cells with condensed DNA (Condensed) or no discernible DNA (No DNA) at each time point. Number of cells counted for analysis are indicated in white.
to be condensed, these cells can be classed as mitotic, with some even showing signs of septation. This phenomenon is reminiscent of the previously observed ‘non-disjoined’ mitotic phenotype (Ohkura et al., 1988), where replicated chromosomes do not segregate and appear to remain in a 6:0 distribution. In addition to this one-sided chromosome position, a smaller population of cells in each double mutant population display unequal masses of DNA in the vicinity of the cell tips. This may arise due to the movement of unsegregated chromosomes to form a 4:2 pattern (Ohkura et al., 1988), or after chromosome segregation has occurred although unequally. The two phenotypes seen in the smaller cells do not reflect interphase DNA arrangements, but rather very condensed DNA or no clear mass of DNA, aside from some mitochondria DNA staining (small dots). Due to the aforementioned high frequency of septation (Figure 3.4) and the fact that the two mitotic phenotypes observed are the result of improper chromosome segregation, it is likely that these two small cell phenotypes are the subsequent products of the defective mitoses. Septum formation in the absence of proper chromosome segregation is classed as a ‘cut’ phenotype and can occur with chromosomes remaining at the centre of the dividing cell, or away from the division site (Yanagida, 1998). Septation and division of a cell with a 6:0 chromosome pattern would produce one cell with condensed DNA and another totally lacking DNA; septation of a cell with the 4:2 distribution may result in asymmetrically condensed daughter cells. All four classified cell phenotypes share common defects in chromosome segregation, and as the majority of defects are that of mitotic cells, it can be concluded that this leads to the double mutants spending longer in mitosis. The extended duration of mitosis would lead to an increase in the number of septated cells observed, and the existence of the two small cell phenotypes suggest there to be no problems in the execution of septation. All temperature-sensitive dis1Δ klp5ts cells show severe chromosome segregation defects at the restrictive temperature.

### 3.7 klp5ts candidates have mutations across all domains

To begin to gain an understanding of how the mutations affect kinesin-8, the mutant klp5 alleles were sequenced. The sites of the amino acid changes are summarised in Figure 3.6. Substitution mutations were seen in nearly every domain of the Klp5 protein, except the N-terminal non-motor region (West et al., 2001).
Chapter 3 Screening for temperature-sensitive mutants of klp5

Five out of six of the candidates harboured multiple mutations, where only klp5<sup>CP85</sup> had a single substitution event. Two different candidates had surprisingly similar mutation distributions: CP1 and CP67 each had a substitution in the motor region and a second just after the neck domain, with an extra C-terminal mutation in CP67. As the motor domains are likely to be instrumental in the function of the kinesin-8 complex, it is not surprising that multiple alleles have mutations in this region. Only one allele, klp5<sup>CP3</sup>, contained a mutation in the neck domain. However, multiple alleles contain mutations clustered around a stretch immediately after the neck domain (CP1, CP49, CP67 and CP85) suggestive of an important function. Remarkably, two different alleles, CP3 and CP76, harboured mutations in a region of the tail that corresponds to the longest significant stretch of homology between Klp5 and Klp6 proteins, previously annotated as a ‘tail box’ (West et al., 2001). Again, the repetition of mutations in this domain may allude to the presence of a functional motif within kinesin-8. Three mutations arose in the final ~170 amino acids of the protein, again perhaps evidence of vital protein function in CP3, CP67.

Figure 3.6 Schematic summary of klp5<sup>ts</sup> mutants isolated

A pictorial representation of the Klp5 protein highlights the various structural or functional domains currently identified. Key residues for domain regions are noted on a simplified domain map. Below, the six candidate mutants show a variety of substitutions throughout the length of the protein, denoted by a cross at the residue position.
and CP76. Of these six candidates, five alleles contained more than one mutation. It is possible that a temperature-sensitive phenotype in the dis1Δ background could arise from the combined defects of multiple mutations, but this would be unfavourable for the current study as it would not shed light on individual domains and make the dissection of function more complex. For this reason, any further analysis was performed using klp5ts alleles containing only one mutation. With only one mutation, klp5CP85 is referred to as klp5L470P hereafter.

3.8 Dissection of key mutations in klp5ts alleles

To identify the causal mutations of the temperature-sensitivity of klp5, alleles containing individual substitutions were constructed by site-directed mutagenesis and checked for temperature sensitivity in the dis1Δ background. Figure 3.7 shows

![Figure 3.7 Identification of causal temperature-sensitive mutations in klp5](image)

Each point mutation was assessed for its contribution to the temperature-sensitivity of the original klp5ts allele isolated. Cells were serially-diluted and spotted onto YE5S plates and incubated for 4 days at the indicated temperatures.
the growth of cells with each individual mutation at the permissive and restrictive temperatures, with the original mutant cells as a control. CP49 was omitted from this investigation as the two mutations are relatively close together. In the case of CP1 and CP67, the motor domain mutations proved responsible for temperature sensitivity, suggesting that kinesin-8 motor activity is essential in the absence of Dis1. It should be noted that temperature sensitivity of double mutant cells containing Klp5T153A is not as severe as that of dis1Δ klpCP1 cells, perhaps due to an extremely small contribution from the K477R mutation. Also, the T463S mutation in klp5CP67 was not investigated as this area was already covered in klp5CP49. Interestingly, the neck mutation L426P in CP3 is the key mutation in producing temperature sensitivity in the dis1Δ background. As this region facilitates heterodimerisation with the Klp6 protein, L426P may have a knock-on effect on complex formation and hence nuclear retention in mitosis (Unsworth et al., 2008). For allele CP76, the most N-terminal tail mutation is responsible for the temperature-sensitive growth phenotype. In contrast, the A560V mutation just upstream of Q582 is not responsible for temperature sensitivity of CP3. This may be due to the difference in the structure of the new amino acid; the proline of Q582P is a ‘helix breaker’ while the effect of replacing an alanine with a valine at A560 may be a slightly more tolerable substitution. Curiously, the diverse set of mutants produce notably similar cellular phenotypes in terms of changes in cell cycle progression and chromosome behaviour (Figure 3.4 and Figure 3.5).

3.9 Function and sequence analysis of klp5ts proteins

With a role in the negative regulation of microtubules, deletion of klp5 or klp6 renders cells resistant to the microtubule-destabilising drug thiamine (TBZ) (West et al., 2001). Resistance to TBZ also indicates a fully functional SAC that allows cells to overcome problems with kinetochore-microtubule attachments induced by changes in plus-end dynamics. Conversely, deletion of the microtubule-polymerase dis1 can cause an increase in cellular sensitivity to TBZ, when the temperature approaches that of restrictive for dis1Δ (Matsuo et al., 2016), likely due to the activation of the SAC by imperfect kinetochore-microtubule attachments as a result of microtubule depolymerisation. However, in our experiments performed with a higher permissive temperature of 30°C, dis1Δ cells exhibit a very modest
increase in TBZ-resistance, compared to WT cells. With the responsible temperature-sensitive mutations identified, TBZ-resistance was assessed in both dis1Δ and dis1+ backgrounds at the restrictive temperature. Analyses of an in silico nature were also performed in order to understand the importance of the mutated residues through their sequence conservation and involvement in structural or motif contributions.
Figure 3.8 TBZ-sensitivity of final six *klp5* <sup>ts</sup> mutants
The final point mutations were assessed for TBZ-sensitivity in both dis1Δ and dis1<sup>+</sup> backgrounds. Cells were serially diluted and spotted onto plates containing the indicated concentration of TBZ and incubated for 4 days at 36°C. (A) Assay of the two motor mutants isolated. (B) Assay of the neck and tail mutants isolated.
3.9.1 Motor mutants V120A and T153A are functionally distinct

Both single motor mutants showed a similar resistance to TBZ as the klp5Δ control at the TBZ concentrations tested (Figure 3.8A), indicative of loss of kinesin-8 function that renders microtubules more stable. Though the two single motor mutants behaved similarly, when coupled with dis1Δ, the two different klp5 alleles cause cells to behave rather differently. The dis1Δ klp5V120A double mutant shows no growth in the presence of TBZ at 36˚C, whereas dis1Δ klp5T153A growth is restored to that of dis1Δ. It may be that addition of the microtubule poison is sufficient to compensate for the kinesin function affected by the T153A mutation at restrictive temperature, but not for that of V120A, even though their individual TBZ resistance is similar. Alignment of kinesin-8 protein sequences from multiple eukaryotes reveals a high degree of conservation for both residues (Figure 3.9).

The crystal structure of the motor domain of human kinesin-8, Kif18A, has previously been solved to high resolution (Peters et al., 2010), making it possible to infer the contribution of these residues to the structure. V120 lies in the α1-helix that contributes to the characteristic kinesin domain fold of a central β-sheet flanked on both sides by three α-helices (Sack et al., 1999). T153 follows the base interacting histidine of the α2-helix following the P-loop (Peters et al., 2010). These residues may make slightly different contributions to the conformation of the active Figure

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V120A T153A

Figure 3.9 Sequence conservation of Klp5 motor residues
Alignment of kinesin-8 from S. pombe, S. cerevisiae, D. melanogaster, M. musculus and two H. sapiens isoforms. Only region of interest shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with domains highlighted in white.
site and as such their mutation may reduce their affinity for ATP or its rate of hydrolysis.

3.9.2 L426P may affect Klp5 neck structure and function

The single neck mutation, *klp5*\(^{L426P}\), causes very little change in TBZ-resistance compared to wild type cells, suggestive of a near-functional kinesin-8 complex (Figure 3.8B). The residue L426 is mildly conserved and is at least maintained as a hydrophobic moiety in Klp6 (Figure 3.10A), likely due to its role in coiled-coil formation (West et al., 2001). We hypothesised earlier that the introduction of a proline into the neck domain may have a structural effect and thus lead to a deletion-like phenotype, as a result of inefficient heterodimerisation in the nucleus (Unsworth et al., 2008). However, the fact that the neck mutation does not result in significant TBZ resistance, like that of kinesin-8 deletion, implies that exclusion from the nucleus is not the effect of L426P. To understand how the mutation may affect the neck structure, the wild type and mutant sequences were evaluated for the probability of coiled-coil forming structures (Figure 3.10B) (Lupas et al., 1991). A mild change in the secondary structure is clear: while the wild type sequence shows a strong probability of coiled-coil formation in the neck region (solid black trace), Klp5\(^{L426P}\) shows a shortening of the predicted region (dashed blue trace). This minimal change in predicted change in the coil structure of the neck corroborates the TBZ assay result, inferring strong enough coiled-coil formation for nuclear retention in mitosis. Still, the neck mutation may have a more minor effect on complex formation or function that becomes essential in the absence of Dis1 function - double mutant cells become extremely temperature-sensitive and can barely grow on TBZ at restrictive temperature.
Figure 3.10 Sequence conservation and predicted structural analysis of Klp5\textsuperscript{L426P}

(A) Alignment of kinesin-8 from \textit{S. pombe}, \textit{S. cerevisiae}, \textit{D. melanogaster}, \textit{M. musculus} and two \textit{H. sapiens} isoforms. Only region of interest shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with domains highlighted in white. (B) The probability of coiled-coil formation was predicted using the online ‘COILED-COIL PREDICTION’ software from PRABI (Lupas et al., 1991). Parameters were set at window 28 and MTIDK matrix, with a weight of 2.5 at heptad positions a and d.
3.9.3 Early tail mutant residues are not conserved

Mutagenic screening of klp5 produced two different alleles, klp5<sup>CP49</sup> and klp5<sup>L470P</sup>, which contained mutations within a 30 amino acid stretch that is proximal to the coiled-coil-forming neck domain. Both single mutants are highly TBZ-resistant at restrictive temperature (Figure 3.8B) - a characteristic shared by the two motor mutants in this study and an indicator of loss of kinesin-8 function. Both residues, M462 and S469, of klp5<sup>CP49</sup> show extremely weak conservation, while the leucine of klp5<sup>L470P</sup> is retained in this position in Klp6, fly Klp67a and human Kif18B (Figure 3.11A). Overall, this region of interest has very low conservation and no structural annotation due to the flexibility of the tail region. A search for putative conserved domains within this region using the Blastp resource (NCBI) (Altschul et al., 1997) returned hits only from Klp5 orthologs within the fission yeast clade (S. octosporus, cryophilus and japonicus). Therefore, without further, more in-depth analysis, it is difficult to postulate the function of this region, if any outside of proper protein folding.

**Figure 3.11 Sequence conservation of the three Klp5 early tail mutation sites**
Alignment of kinesin-8 from *S. pombe*, *S. cerevisiae*, *D. melanogaster*, *M. musculus* and two *H. sapiens* isoforms. Only region of interest shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with domains highlighted in white.
3.9.4 Q582P disrupts predicted coiled-coil tail structure

The most C-terminal mutation isolated, *klp5*<sup>Q582P</sup>, shows increased resistance to TBZ compared to wild type cells. Interestingly, this level is distinct from that of both the almost wild type behaviour of and from the strong deletion-like resistance of the other four *klp5*<sup>ts</sup> mutants (Figure 3.8A and B), suggestive of a different degree of residual kinesin-8 function. There is a degree of conservation of a glutamine at this

![Alignment of kinesin-8 from S. pombe, S. cerevisiae, D. melanogaster, M. musculus and two H. sapiens isoforms. Only region of interest shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with domains highlighted in white.](image)

![The probability of coiled-coil formation was predicted using the online 'COILED-COIL PREDICTION' software from PRABI (Lupas et al., 1991). Parameters were set at window 28 and MTIDK matrix, with a weight of 2.5 at heptad positions a and d.](image)

*Figure 3.12 Q582P may cause a change in the tail structure of Klp5*  
(A) Alignment of kinesin-8 from *S. pombe*, *S. cerevisiae*, *D. melanogaster*, *M. musculus* and two *H. sapiens* isoforms. Only region of interest shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with domains highlighted in white.  
(B) The probability of coiled-coil formation was predicted using the online ‘COILED-COIL PREDICTION’ software from PRABI (Lupas et al., 1991). Parameters were set at window 28 and MTIDK matrix, with a weight of 2.5 at heptad positions a and d.
position, specifically in Kif18A from both humans and mice (Figure 3.12A). However, conservation of residues and their positions within this region seem to be limited to Klp5 and Klp6; Q582 lies 1 residue downstream of the previously identified ‘tail box’ (West et al., 2001). The coiled-coil prediction profiles of Klp5 presented in both this study and the previous from West and colleagues, primarily to look at the neck domain, show a smaller peak of formation probability in the sequence immediately prior to residue 600 (Figure 3.10). We postulated that Q582 may contribute to the integrity of this structure; its involvement was confirmed by prediction from the Klp5^Q582P sequence (Figure 3.12), where the introduction of proline (gold dashed trace) greatly reduces, but does not abolish, the coiled-coil forming structure found in the wild type protein (solid black trace). Perturbation of this conserved coiled region somehow has a minor effect on kinesin function as seen by the increased TBZ-resistance of single mutant cells. Again, as is seen for all but one Klp5 mutant, the addition of TBZ does not rescue the temperature-sensitivity of dis1^Δ klp5^Q582P cells. The fact that this region is conserved in Klp6 and may form part of a short coiled-coil structure is intriguing, as kinesin tails shows large stretches of disorder and both tail structure and function diverge across kinesin families and across species (Seeger et al., 2012). Of course, klp5 and klp6 are homologues within the same family and organism, but the fact that this region has been maintained through their evolution may allude to its importance.

3.10 Discussion

Screening for temperature-sensitive mutants of klp5 proved successful in the dis1^Δ background; six different alleles each lead to a comparable loss of mitotic control and chromosome segregation problems. This apparent universal terminal phenotype is slightly surprising due to the diversity of the mutation sites and their effect on kinesin function. The preliminary experiments and sequence/structure analyses presented above suggest that a variety of kinesin defects are lethal in the absence of Dis1 function, shedding light on both kinesin-8 function itself and its place in the context of the mitotic spindle.

The Klp5 mutants isolated show no growth defects at any temperature when Dis1 is functional, as concluded by the serial dilution assay. Nonetheless, similar to
kinesin-8 deletion cells, single Klp5 mutants may display a non-lethal phenotype when examined by microscopy. This notion is supported by the fact that five of the six mutants show resistance to TBZ, some approaching that of klp5Δ. Observation of single mutants at the restrictive temperature is required to further elucidate their specific defects. None of these mutants could suppress the cold-sensitivity of dis1Δ (Ohkura et al., 1988). As the mutants are sensitive to high temperature only, at 22°C kinesin-8 is fully functional and so double mutant cells will only exhibit the dis1Δ phenotype.

All six double mutants show a high septation index, representative of an enrichment of mitotic cells, corroborated by the observation of high frequency of chromosome segregation defects. Some double mutants, such as dis1Δ klp5Δ^CP49/klp5Δ^CP76, undergo multiple septation events more commonly than the others. It may be that these two double mutants spend the longest in mitosis before cell division, therefore the signal to septate persists. Indeed, the lowest abundance of normal chromosome behaviour is seen in these two double mutants. It is intriguing that the mitotic defects are so similar among the six different mutants considering that the mutation sites are spread across all domains of the kinesin. Perhaps it is not one function of the multifaceted kinesin-8 that is essential in the absence of Dis1, but all functions, and thus the knockdown of one aspect has dire consequences, or at least those isolated in our screen. This notion is reflected in the spread of mutations isolated, as if only a certain domain function was essential in dis1Δ, mutations likely would have clustered in that particular region. Non-essential functions of Klp5 would not have been targeted in our screen for temperature-sensitivity with dis1Δ, as the knockdown of these functions would have been tolerated by the cells.

The mitotic phenotypes seen in dis1Δ klp5Δ^ts cells at restrictive temperature are that of improper or the complete absence of chromosome segregation. In the majority of cases, all three pairs of condensed sister chromatids localise to one side of the cell in the majority of cases, with remarkable similarity to the first cold-sensitive phenotype observed for dis1Δ cells (Ohkura et al., 1988). However, there are some discrepancies in the literature about the nature of the non-disjoined phenotype. Previous work from our lab showed that spindles are short and sometimes snap in
dis1Δ cells or in an Ndc80 loop mutant that cannot interact with Dis1 (Hsu and Toda, 2011), whereas the initial work characterising the dis genes found that long spindles pushed the sister chromatids to one side of the cell (Ohkura et al., 1988). Differences in the restrictive temperatures used or the experimental set up in terms of both cell culturing and imaging methods may be responsible for the marked differences in phenotype. Without clarification of the dis1Δ cold-sensitive phenotype, the defects seen in dis1Δ klp5ts cannot be compared and interpreted in this context. Aside from this issue, it is clear that these cells that have not gone through anaphase, can septate and divide to produce the two small cell phenotypes, where one cell may have up to diploid copies of condensed DNA and the other contains none. APC/C cut mutants of the nuc2 (Hirano et al., 1988), cut4 (Yamashita et al., 1996) and cut9 (Samejima and Yanagida, 1994) subunits cause cells to septate aberrantly with a short metaphase spindle at one end of the cell. In contrast, some cut mutants undergo a spindle elongation event in the absence of chromosome segregation. These mutants include the phosphatase dis2 (Ohkura et al., 1989), which septates and divides like the APC/C mutants, and the DNA topoisomerase top2 (Uemura and Tanagida, 1986) in which non-disjoined chromosomes remains central, affecting proper septum formation. Further analyses are required to assign the cut phenotype seen in dis1Δ klp5ts cells into either one of these pre-existing categories or a novel group of cut mutants.

While all six double mutants seem to share a common terminal cut-style phenotype, differences exist amongst the single mutant growth phenotypes in the presence of TBZ, possible reflecting the differences in the location, nature of and implications of the mutated residues.

The two motor mutants isolate, klp5V120A and klp5T153A may affect ATPase activity or ATP binding affinity by changes in conformation of the active site. This in turn may affect microtubule-binding of the kinesin-8 complex. As rigor mutants have previously been isolated and studied in part, these two motor mutants were not taken further in this study.

Only one allele contained a mutation within the neck domain – klp5L426P. An in silico analysis of coiled-coil formation revealed a minor predicted change in the structure
of the neck region that has potential to affect dimerization with Klp6. This is likely not the effect of L426P, as an inability to form the kinesin-8 heterodimer would give the same TBZ-resistance as the nuclear-excluded klplp5Δ/klp6Δ phenotype. While there may be high enough levels of nuclear kinesin-8 to prevent TBZ-resistance, it is possible that some complexes containing Klp5L426P may not be quite strong enough to perform kinesin-motility or to be maintained as a dimer, hence reducing some mitotic function. Following this, it can be speculated that either strong complex formation is not absolutely required, or that a reduced population of functional dimerising kinesin-8 is sufficient to carry out its function. Although the L426P mutation may provide us with a conditional tool to reduce the population of motile or functional kinesin-8, the notion of heterodimerisation for motor protein function is not novel and provides no new insight on Klp5 domain structure. For these reasons, the Klp5L426P mutant was not pursued any further.

A group of mutations clustered in the neck-proximal region of the Klp5 tail domain: M462T, L470P and S496P, none of which are truly conserved residues. Of course, this may be a functional region specific to fission yeast kinesin-8 and the fact that three mutations arose here alludes to some importance. It is possible to postulate that this region so close to the neck is involved in motility-linked flexibility of the kinesin, conveying movements in the motor regions to the tail domains. However, with no structural information available and lack of conservation or any putative domain information, these three Klp5 mutants were not investigated any further.

The only other temperature-sensitive tail mutant isolated was klplp5Q582P. Although the residue itself and surrounding region is not conserved through all of the kinesin-8 orthologs aligned, by eye there is some detectable similarity between this region of Klp5 with that of Klp6. This stretch of amino acids has been previously identified as a ‘tail box’ (West et al., 2001) and we find that an overlapping region is predicted to take on a coil structure, potentially involved in the formation of a coiled-coil. Coiled-coils can form within a protein or between proteins usually as a mechanism of protein-protein interaction or sometimes for protein-DNA interfaces (Lupas, 1996). Kinesins oligomerise via their neck domains (Huang et al., 1994) to form a variety of coiled-coils structures, dependent on the stoichiometry of the final complex. Fewer reports exist about coiled-coil-forming domains outside of the neck.
or stalk. This Klp5 tail region could be involved in a secondary interaction with Klp6 for complex stability, or may interact with some other mitotic factors. Kinesins also undergo tail-motor interactions (Hackney et al., 2009), another possible role for this region of Klp5. The distinct level of TBZ resistance, conservation of the region in Klp6 and propensity of this stretch of sequence to potentially form a coiled-coil make \( \textit{klp5}^{Q582P} \) a very interesting novel allele to pursue in the study of kinesin-8 and its role within mitosis.
Chapter 4. Klp5 tail is required for overall kinesin-8 function

After the isolation, initial characterisation and selection of a randomly-generated kinesin-8 allele, we characterised the phenotype shown by this mutant. The experiments in this chapter are largely performed in the dis1+ background in order to focus on the kinesin-8 defects alone in an attempt to understand how the tail mutation affects the motor protein’s function. This chapter describes the assessment of the Klp5 mutant’s ability to interact with Klp6, to regulate particular aspects of mitotic events and their timing, and the involvement of the SAC, in terms of both activation and silencing. Kinesin activity was assessed in vitro to complement the cellular analyses. Further mutagenesis of both Klp5 and Klp6 and the subsequent observation of these mutants, highlight the functionality of the proposed tail motif, and its conservation in the two fission yeast kinesin-8 homologues.

4.1 Klp5<sup>CP76</sup> is expressed at normal levels

To ensure that any phenotypes seen were not due to low levels of Klp5 expression or expression of a truncated form of the protein, the levels of Klp5<sup>CP76</sup> protein were assessed by western blotting. Asynchronous cultures were shifted up to 36˚C for one hour before proteins were extracted. The levels of Klp5-5FLAG were observed in WT, dis1Δ and dis1Δ cells expressing Klp5<sup>CP76</sup>-5FLAG (Figure 4.1). In all cases, Klp5<sup>5FLAG</sup> is expressed at normal levels

![Western blot](image)

**Figure 4.1 Klp5<sup>CP76</sup> expression is normal in all genetic backgrounds**
Western blotting was performed to compare the expression levels of Klp5<sup>WT</sup>-5FLAG and Klp5<sup>CP76</sup>-5FLAG in the dis1Δ background. Cell extracts were obtained from asynchronous cultures after shifting to 36˚C for 1 hour. Total blot cropped to show only three lanes of interest – Klp5 bands and Cdc2 loading control.
the levels of either Klp5-5FLAG protein were similar and the products were all the same size, confirming that the growth defects seen in klp5<sup>CP76</sup> and dis1Δ klp5<sup>Q582P</sup> cells are in fact due to specific protein dysfunction and not a lack of expression or a truncated gene product.

### 4.2 Klp5<sup>Q582P</sup> retains the ability to interact with Klp6

Aligning the ‘tail box’ and surrounding region of Klp5 with other kinesin-8 proteins (see Figure 3.12A) revealed a stretch of homology limited to Klp6. To confirm this, only the Klp5 and Klp6 protein sequences were aligned (Figure 4.2A). It is clear that this region constitutes the largest stretch of homology within the two tails as the sequence surrounding the ‘tail box’ shows weak conservation with frequent interruptions. Looking at the predicted fit of residues to the heptad structure required for coil formation reveals three heptad repeats with a four-residue interruption after the second turn (Lupas et al., 1991). Though short and imperfect, the presence of a structured region in the tail that may contain a lot of disordered stretches is suggestive of function. One candidate function of this kind of structure is as a protein-protein interaction via coiled-coil formation, in a manner similar to that of the neck domain. Although TBZ-resistance of cells expressing Klp5<sup>Q582P</sup> is minimal and the mutation does not affect the neck domain (see Figure 3.8B), it is possible that this second coiled region has a minor contribution to heterodimerisation. To examine the possibility that Klp5<sup>Q582P</sup> impairs complex formation, its interaction with Klp6 was investigated at the restrictive temperature. Co-immunoprecipitation was performed using extracts from temperature-shifted, asynchronous cells co-expressing Klp6-GFP and either Klp5<sup>WT</sup>-5FLAG or Klp5<sup>Q582P</sup>-5FLAG (Figure 4.2B). Both WT and mutant Klp5 proteins were pulled down by Klp6-GFP, indicating that the tail mutation does not affect the ability to form a stable complex.
Chapter 4. Klp5 tail is required for overall kinesin-8 function

A

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Figure 4.2 Analyses of Klp5 'tailbox' homology and function regarding Klp6

(A) Alignment of full-length S. pombe kinesin-8s. Only a section of the tail region is shown. Calculated using T-coffee from Swiss Institute of Bioinformatics (Notredame et al., 2000). Mutated residues highlighted in bold black, with conserved 'tail box' highlighted in white. Coil forming heptads are marked on with bounding region of each turn marked by black lines. Heptad pattern generated by PRABI software (Lupas et al., 1991) using window 28, MTIDK matrix and 2.5 weighting for residues a and d.

(B) Co-immunoprecipitation was performed to confirm the interaction between Klp5<sup>WT</sup>/Klp5<sup>Q582P</sup> and Klp6 proteins in the dis1Δ background. Cell extracts were obtained from asynchronous cultures after shift to 36°C for 1 hour. WCE, whole cell extract. IP, immunoprecipitation.
4.3 Klp5$^{Q582P}$ has a weak phenotype at permissive temperature

Although cells expressing the Klp5$^{Q582P}$ protein do not show any temperature-sensitive growth defects when Dis1 is functional (see Figure 3.8B), the cells do exhibit a mild resistance to TBZ at the restrictive temperature (see Figure 3.8B), indicative of a change in kinesin-8 function. In order to understand the phenotype of this mutant, mitosis was observed in these cells by light microscopy to compare the behaviour to wild-type and kinesin-8 deletion cells. To do this, microtubules, SPBs and a pair of chromosomes were visualised by mCherry-Atb2, Sid4-mRFP and cen2-LacOp/LacI-GFP, respectively. It is possible to visualise the centromere of chromosome 2 by the insertion of a Lac operon array at the pericentric region, which can be bound by a GFP-tagged repressor molecule, LacI (Yamamoto and Hiraoka, 2003). Visualising one pair of centromeres simplifies the analysis of chromosomes as by doing so, the pairs cannot be confused. Time-lapse imaging was performed at 2 minute intervals: while this may be suboptimal for temporal resolution of mitosis, this imaging rate prevents drastic bleaching of the tubulin signal and hence allows more data to be obtained for longer during the course of the experiment. For the kinesin-8 deletion control, klp6$\Delta$ was used because the klp5 locus is closely linked to the centromere of chromosome 2 and therefore the desired strain could not be obtained by crossing. The klp5 point mutation was transformed into the klp5 locus to obtain the desired tagged strain. The following experiments show the observation of phenotype of klp5$^{Q582P}$ mutant cells firstly at the permissive temperature of the klp5$^{Q582P}$ dis1$\Delta$ cells (30˚C), and then at the restrictive temperature (36˚C).

To characterise the effects of the Q582P mutation on Klp5 function, the mutant was analysed at the permissive temperature. This approach also aids the understanding of the viability of dis1$\Delta$ klp5$^{Q582P}$ cells at 30˚C. Representative time-lapse imaging of WT, klp6$\Delta$ and klp5$^{Q582P}$ cells is shown in Figure 4.3. In all cases, even though only one pair of centromeres are visualised, the fact that spindles disassemble as in wild-type cells, the post-anaphase array appears and cells septate and divide suggests that the other two pairs of chromosomes are also segregating equally in these viable mutants.
Chapter 4. Klp5 tail is required for overall kinesin-8 function

Figure 4.3 Centromere and spindle behaviour in WT and kinesin-8 mutant cells

Time-lapse imaging of live cells expressing cen2-LacOp-LacI-GFP, Sad1-dsRed and mCherry-Atb2 in a WT, klp6Δ or klp8Q582P background at 30°C. Cells were observed every 2 minutes at 30°C. Images show chromosome 2 (cen2-GFP), microtubules and SPBs (mCherry-Atb2, Sad1-dsRed) and the merged image. Numbers, time in minutes. Scale bars, 10 µm.
4.3.1 *klp5*<sup>Q582P</sup>- spindle behaviour is mostly normal

An overall comparison of mitotic spindle behaviour was obtained by measuring spindle lengths over time (Figure 4.4A). At the permissive temperature, the profile of spindles in *klp5*<sup>Q582P</sup> cells is more similar to those of *WT* cells; the three phases are distinct compared to those of *klp6Δ* spindles. The appearance of clear changes in spindle dynamics during mitosis suggests a functional kinesin-8 complex. Maximum spindle lengths from these cells corroborates the functionality of Klp5 and Klp6 at the permissive temperature (Figure 4.4B). The mean maximum spindle length reached in *WT* cells was 10.50 ± 0.80 µm. While *klp6Δ* cells produced spindles with a maximum length of 11.47 ± 1.04 µm, the mean length for *klp5*<sup>Q582P</sup> cells was not significantly different from *WT*, at 10.67 ± 0.62 µm. From these results, it is clear that the Klp5 point mutant is able to perform its function in spindle length regulation at the permissive temperature.

4.3.2 *klp5*<sup>Q582P</sup> cells do not have an extended duration of mitosis

In addition to spindle length profiles, the duration of mitosis was also compared between the three strains at the permissive temperature (Figure 4.4C). Again, *WT* and *klp5*<sup>Q582P</sup> cells took the same amount of time to go through mitosis. The relatively large variability in these measurements may arise due to the moderately long intervals of two minutes between image acquisition.

4.3.3 *klp5*<sup>Q582P</sup> metaphase spindle length is similar to that of wild-type cells

Kinesin-8 has been shown to play a role in the negative regulation of spindle length specifically in early mitosis to size the metaphase spindle in multiple organisms (Syrovatkina et al., 2013, Mary et al., 2015, Stumpff et al., 2008). Therefore, metaphase spindle length was examined as a readout of kinesin-8 functionality at permissive temperature (Figure 4.5A). In this experiment, the instance of metaphase was defined by the frame before chromosomes started to segregate i.e. when chromosomes kinetochore-microtubule attachments are correct and chromosomes are aligned ready for anaphase. In this case, spindle length could not be used to distinguish metaphase, usually seen as a period of constant spindle length, as this varies in kinesin-8 mutants and was used here as the measured variable.
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Figure 4.4 Spindle length and time in mitosis is normal in klp5Q582P cells at permissive temperature
Quantification of mitotic parameters from time-lapse imaging as in Figure 4.6 at 30°C. (A) Quantification of spindle lengths against time in mitosis. (B) Maximum spindle lengths extracted from part (A). Lines represent mean and standard deviation. Statistical significance of difference was determined using a two-tailed t-test with Welch’s correction (****, p<0.0001; ns, p=0.2808, not significant). (C) Mean duration of mitosis, with standard deviation, based on the presence of a spindle. Statistical significance of difference was determined using a two-tailed Mann-Whitney test (****, p<0.0001; ns, p=0.2140, not significant). For all statistical analyses presented here, comparisons between the indicated strain and WT are represented in black and comparisons between the two kinesin-8 mutants are indicated in blue.
The metaphase spindle lengths in WT cells at 30°C (2.10 ± 0.34 µm) were in the normal range of ~2-3 µm (Nabeshima et al., 1998) while those for klp6Δ were much longer and more varied, with a mean of more than double that of WT (Garcia et al., 2002b, Syrovatkina et al., 2013). The mean metaphase spindle length for klp5Q582P cells was 2.31 ± 0.36 µm, almost the exact same as for WT cells, yet the statistical test used indicates a significant difference between the samples. This difference may arise from a larger range of values acquired for the klp5 mutant, and the large number of measurements taken. While the difference is reported to be significantly different, the similarity in mean and standard deviation of WT and klp5Q582P metaphase spindle length suggests that the mutant kinesin-8 complex is mostly functional in its role in metaphase spindle length regulation.

4.3.4 Chromosome congression is less efficient in klp5Q582P cells

Along with maintaining metaphase spindle length, kinesin-8 has been implicated in controlling chromosome congression for metaphase by regulating MT plus-end dynamics (Wargacki et al., 2010, Stumpff et al., 2012, Syrovatkina et al., 2013, Kim et al., 2014, Mary et al., 2015). Metaphase chromosome position was quantified as the absolute distance from the centre of the two cen2-GFP signals to the centre of the metaphase spindle (Figure 4.5C). Where cen2-GFP signals overlapped with the spindle centre, a zero value was recorded. Both the imaging set up and small size of the early spindle in S. pombe may contribute to large variation within the data from even WT cells. The relatively long two minute intervals used may not exactly capture true metaphase, and so the imaging frame that was analysed may be earlier, while the chromosomes are still oscillating. As the smallest average metaphase spindle was only 2 µm in length and in these cells the distance of the centromeres to the spindle centre would always be less than 1 µm, the error could be a large percent of the measurement. Nevertheless, differences between chromosome position in WT and kinesin-8 mutant cells were clear (Figure 4.5C). In WT cells, the centromeres clustered around the central 50% of the spindle (0.5 µm either side of the centre of a 2 µm-long spindle), with a quarter of the measurements being zero values as is expected for chromosome congression.
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Figure 4.5 Metaphase and anaphase parameters at the permissive temperature
Quantification of metaphase and anaphase parameters in cells expressing CEN2-LacOp LacI-GFP, Sad1-dsRed and mCherry-Atb2 in a WT, klp6Δ or klp5Q582P background at permissive temperature. (A) Representative images of metaphase cells taken from time-lapse imaging (see Figure 4.6). Scale bar, 10 µm. (B) Measurements of metaphase spindle lengths. Lines represent mean and standard deviation. Statistical significance of difference was determined using a two-tailed t-test with Welch’s correction (**, p=0.0038; ****, p<0.0001). (C) Quantification of chromosome position on metaphase spindles relative to spindle centre. Lines represent mean and standard deviation. (D) Mean and standard deviation of time spent in pre-anaphase, from the frame before SPB separation until the assigned metaphase spindle frame. For parts (C) and (D) Statistical significance of difference was determined using a two-tailed Mann-Whitney test (*, p=0.0179; ****, p<0.0001; ns, p=0.5651, not significant). (E) Frequency of lagging chromosome during segregation. Statistical significance of difference was determined using Fisher’s exact test (***, p=0.0008; ***, p=0.003; ****, p<0.0001). For all statistical analyses comparisons between the indicated strain and WT are represented in black and comparisons between the two kinesin-8 mutants are indicated in blue.
Cells lacking Klp6 displayed a vast spread of cen2-GFP positions with no obvious bias for any region of the spindle, with very few zero values. The mean centromere position for klp6Δ cells was 1.26 ± 0.85 µm away from the spindle centre, compared to 0.27 ± 0.23 mm for WT cells. Interestingly, the klp5Q582P mutant did affect chromosome position at the permissive temperature. On average, centromeres were positioned 0.42 ± 0.33 µm away from the spindle centre, approximately 1.5 times further away than in WT cells. With a significantly higher mean distance from the centre and a larger spread of positions, it appears that Klp5Q582P can affect chromosome positioning even at the permissive temperature.

4.3.5 klp5Q582P cells display a timely metaphase-anaphase transition

The lack of congression seen in klp5Q582P cells may be due to improper kinetochore-microtubule attachments that lead to the lack of tension generation by microtubules. cen2-GFP signals were never detected outside of the plane of the spindle and moved only in this dimension, suggestive of some degree of attachment to the spindle. To gain an idea of the extent of SAC activation in the klp5Q582P mutant cells, time spent in pre-anaphase, dictated by the extent of SAC activation, was quantified (Figure 4.5D). With a great effect on metaphase spindle length and chromosome position, klp6Δ cells clearly have an activated SAC, extending this period from the wild type 9.51 ± 1.56 minutes to 18.04 ± 5.59 minutes. Spending only 9.64 ± 1.75 minutes before pre-anaphase, the klp5Q582P mutant surprisingly did not cause any significant increase in this time. This suggests that the alignment defects in klp5Q582P cells at permissive temperature do not activate the SAC sufficiently to delay the onset of anaphase.

4.3.6 Lagging chromatids appear in klp5Q582P cells

Following the interesting result of impaired chromosome congression yet negligible activation of the SAC, klp5Q582P cells were assessed for the presence of lagging chromosomes. Kinesin-8 deletion mutants have been seen to have a chromosome still undergoing segregation while its sister has already reached its destination pole. The data presented in Figure 4.5E reflect previous findings, with approximately 63% of klp6Δ cells displaying a lagging centromere, while only one WT cell observed showed the phenotype. Intriguingly, 26% of klp5Q582P cells had lagging
chromosomes, a significantly different frequency to both WT and klp6Δ cells. As it appears that the SAC is not activated and spindle length is not increased in these kinesin-8 mutant cells at 30°C, the lagging chromosomes may be a direct result of the asymmetric position of centromeres on the spindle before anaphase. Altogether, the analyses of the klp5Q582P mutant at the permissive temperature of 30°C suggest the kinesin-8 complex formed is competent to regulate spindle length and kinetochore-microtubule attachment, but is impaired in its role in chromosome congression for the proper transition of metaphase into a coordinated anaphase.

4.4 klp5Q582P cells display pronounced mitotic defects at the restrictive temperature

Next, the experiments performed in section 4.3 were repeated at 36°C (the restrictive temperature used to isolate dis1Δ klp5Q582P cells) in order to understand the changes in kinesin-8 behaviour between temperatures, and to build upon the preliminary analyses of the mutant. Log-phase cultures of cells expressing cen2-LacOp LacI-GFP Sad1-dsRed mCherry-Atb2 were shifted up to 36°C for 40 minutes before time-lapse imaging.

4.4.1 Klp5Q582P drastically increases the length of the metaphase spindle

At 36°C, WT cells had an average metaphase spindle length of 2.17 ± 0.26 μm, while the deletion of klp6 caused this figure to more than double to 5.34 ± 1.69 μm (Figure 4.6A and B). Strikingly, at the restrictive temperature, the average metaphase spindle in klp5Q582P cells was more than 1.5 times longer than WT, at a length of 3.75 ± 0.71 μm, and showed more variation. While significantly different from the short spindles of a normal mitosis, these longer spindles are not as extreme as those in klp6Δ cells, suggesting that complexes containing Klp5Q582P can function partially in metaphase spindle length regulation. Also, comparing the same klp5Q582P strain at permissive and restrictive temperatures highlights the temperature-sensitivity of this kinesin-8 allele (Figure 4.6B), klp5Q582P red compared with blue).
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Figure 4.6 Metaphase parameters at the restrictive temperature
Quantification of metaphase parameters in cells expressing cen2-LacOp LacI-GFP, Sad1-dsRed and mCherry-Atb2 in a WT, klp6Δ or klp5Q582P background at the restrictive temperature. (A) Representative images of metaphase cells taken from time-lapse imaging (see Figure 4.10). Scale bar, 10 µm. (B) Measurements of metaphase spindle lengths. Lines represent mean and standard deviation. Data from previous experiment at 30˚C included for comparison in blue (see Figure 4.8B). (C) Quantification of chromosome position on metaphase spindles relative to spindle centre. Lines represent mean and standard deviation. (D) Mean and standard deviation of time spent in pre-anaphase, from the frame before SPB separation until the assigned metaphase spindle frame. For parts (B), (C) and (D) statistical significance of difference was determined using a two-tailed Mann-Whitney test (****, p<0.0001). For all statistical analyses, comparisons between the indicated strain and WT are represented in black and comparisons between the two kinesin-8 mutants are indicated in red.
4.4.2 Chromosome congression is severely impaired in klp5Q582P cells

The distribution of CEN2-GFP positions in WT cells was reproducible at high temperature (Figure 4.6C, also see Figure 4.5C), with the vast majority of chromosomes being found in the central 50% of the approximately 2 µm spindle and a mean distance of 0.22 ± 0.16 µm from the spindle centre. Congression measurements for klp6Δ cells were also reproducible, with a high mean displacement of chromosomes from the centre of the spindle (1.45 ± 0.96 µm away). In contrast, the temperature-sensitive Klp5Q582P mutant caused a more significant difference in the metaphase position of chromosomes at 36˚C than at 30˚C - sister chromatids were found further away from the centre of the longer spindles at restrictive temperature, with an average distance of 0.77 ± 0.49 µm.

4.4.3 Anaphase onset is delayed by Klp5Q582P

Longer metaphase spindles and more impaired chromosome congression may lead to a delay in anaphase onset in klp5Q582P cells at restrictive temperature. Indeed, the quantification of time spent in pre-anaphase revealed that klp5Q582P cells took, on average, 8.43 ± 1.47 minutes to go into anaphase, which is significantly longer compared to 6.50 ± 1.04 minutes for WT cells (Figure 4.6D). This extension of the time taken to transition from metaphase may be a result of SAC activation due to problems with kinetochore-microtubule attachment when kinesin-8 function is compromised.

4.4.4 The frequency of lagging chromatids increases at the restrictive temperature

Following the induction of temperature-sensitivity of the Klp5Q582P mutant protein, nearly 50% of mutant cells going through mitosis have lagging chromosomes (Figure 4.7). This frequency is more like that of klp6Δ cells (around 75%) in these conditions, than the relationship seen between these two strains at the permissive temperature (see Figure 4.5E). This suggests that although lagging chromosomes appear at 30˚C, increasing the temperature may further affect the protein function to compromise its role in timely chromosome segregation, perhaps as a result of impaired congression.
4.5 Anaphase onset is delayed in klp5<sup>Q582P</sup> cells due to activation of the SAC, and not silencing defects

4.5.1 The duration of kinetochore localisation of Mad2-GFP is extended

To confirm that the delay in anaphase onset seen in klp5<sup>Q582P</sup> cells at high temperature is in fact due an active SAC, the localisation of the checkpoint component Mad2 was observed. Mad2 is one of the most downstream proteins in the signalling cascade to be recruited to unattached kinetochores, where it undergoes a conformational change to form the MCC and inhibit the APC/C to prevent progression into anaphase. When kinetochore-microtubule attachments are satisfactory, the SAC is silenced by removal of the components, including Mad2, initiated by dephosphorylation of outer kinetochore components. Therefore the presence of Mad2 at the kinetochores represents an active checkpoint, and its disappearance signifies the silencing of the SAC. Mad2-GFP localisation was observed in cells expressing Sid4-mRFP and mCherry-Atb2 after these cultures
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Figure 4.8 Klp5<sup>Q582P</sup> leads to intermediate activation of the SAC

(A) Time-lapse imaging of live cells expressing Mad2-GFP, Sid4-mRFP and mCherry-Atb2 in a WT, klp5Δ or klp5<sup>Q582P</sup> background. Cells were observed every 2 minutes at 36°C. Arrowheads indicate last instance of kinetochore-Mad2 and the asterisk highlights persistent SPB localisation. Scale bar, 10 µm. (B) Percentage of cells displaying Mad2-GFP on kinetochores or the spindle plotted against time. Cells were aligned by their timing of mitotic entry. Sample sizes for WT, klp5Δ and klp5<sup>Q582P</sup> are 58, 50 and 62 cells, respectively. (C) Same data set as presented in part (B) but absolute time used to plot mean and standard deviation of Mad2-GFP signal duration. The statistical significance of differences was determined using a two-tailed Mann-Whitney test (****, p<0.0001). For the statistical analyses, comparisons between the indicated strain and WT are represented in black and comparisons between the two kinesin-8 mutants are purple.
were shifted to 36°C for 40 minutes before time-lapse imaging. In addition to localising to the kinetochores, Mad2 can also be seen on the spindle and SPBs; frequently it is possible to detect Mad2 at SPBs even after anaphase has occurred. Therefore, Mad2 removal from the kinetochores and spindle, but not from SPBs, was counted as its disappearance. Representative images of the Mad2-GFP time-lapses are shown in Figure 4.8A, where Mad2-GFP remains on the kinetochores and spindle in $klp5^{Q582P}$ cells for longer than in WT cells.

For quantification, cells were synchronised in silico by the timing of their entry into mitosis (again, time zero is defined as the frame before the first instance of SPB separation) and the percentage of cells retaining an active Mad2-GFP signal assessed at each time subsequent time point (Figure 4.8B). Compared to WT cells (black trace), the Mad2-GFP signal persists for longer in $klp5^{Q582P}$ cells (blue trace), indicative of an active spindle assembly checkpoint (SAC). While active in $klp5^{Q582P}$ cells, kinetochore-microtubule attachments are likely less defective than in $klp5\Delta$ cells, as seen by the substantial shift of the curve to the right (purple trace). The absolute times for Mad2-GFP presence on the spindle as measured in this experiment (Figure 4.8C) reiterate the differences between SAC activation in the three strains but also almost exactly recapitulate the duration of time spent in pre-anaphase as measured previously (see Figure 4.6D). Klp5$^{Q582P}$ extends Mad2-GFP localisation to unattached kinetochores from 6.74 ± 1.04 minutes in WT cells, which are promptly corrected and the SAC silenced in normal mitotic progression, to 8.94 ± 1.39 minutes. The problems with kinetochore-microtubule attachments induced by the absence of functional kinesin-8 are evident by the 10.8 ± 1.39 minutes taken to remove Mad2-GFP in checkpoint silencing.

Previously, Klp5 and Klp6 proteins have been shown to play a direct part in SAC silencing for anaphase onset (Meadows et al., 2011). Therefore, it is formally possible that the mitotic progression defects seen in $klp5^{Q582P}$ cells are due to problems with silencing the checkpoint.
4.5.2 Klp5\textsuperscript{Q582P} is not a checkpoint silencing mutant

Kinesin-8 was implicated in SAC silencing due to their synthetically lethal with \textit{bub3}\textsuperscript{Δ} (Meadows et al., 2011), a component of the checkpoint cascade that is thought to be required for efficient checkpoint silencing (Tange and Niwa, 2008, Vanoosthuyse et al., 2009). Their role in silencing was found to involve PP1-binding motifs in the C-terminal tails; PP1-binding mutants of both Klp5 and Klp6 (\textit{klp5}\textsuperscript{PP1} and \textit{klp6}\textsuperscript{PP1}, respectively) were found to be sick when Bub3 was deleted, compared to \textit{bub3}\textsuperscript{Δ} cells (Meadows et al., 2011). Similar to the kinesin-8 phenotype, PP1-binding mutants showed a delay in chromosome biorientation and anaphase onset, ascribed to prolonged phosphorylation of SAC targets, thus maintaining checkpoint activation. However, in contrast to \textit{klp5}\textsuperscript{Δ}/\textit{klp6}\textsuperscript{Δ}/\textit{klp5}\textsuperscript{Δ}\textit{klp6}\textsuperscript{Δ} cells, pre-anaphase spindle lengths were equal to that of wild-type in \textit{klp5}\textsuperscript{PP1} \textit{klp6}\textsuperscript{PP1} cells due to the functional motor activities of kinesin-8 (Meadows et al., 2011). The temperature-sensitive Q582P tail mutant exhibits elongated metaphase spindles (see Figure 4.6A), a phenotype that is not characteristic of \textit{klp5}\textsuperscript{PP1} mutant, suggesting the defect is different from that of PP1-binding. Interestingly, the elongated spindle phenotype of Klp5\textsuperscript{Q582P} is more like that of the \textit{klp5(SwII)} motor mutant, which produces slightly shorter spindles than those seen in full deletion mutants (Meadows et al., 2011).

To look for similarities and difference between these two mutant alleles, the behaviour of \textit{klp5}\textsuperscript{Q582P} was compared to \textit{klp5}\textsuperscript{PP1} in terms of growth at high temperature and behaviour in the absence of Bub3 (Figure 4.9A). All kinesin-8 mutants assayed grew similarly well in an otherwise wild-type background, but differently in the \textit{bub3}\textsuperscript{Δ} background. While neither PP1-binding mutant, or the double PP1-binding mutant, showed temperature-sensitivity in the absence of Bub3, \textit{klp5}\textsuperscript{Q582P} \textit{bub3}\textsuperscript{Δ} cells clearly struggled to grow at high temperature. The difference in growth suggests a distinction between the affected kinesin-8 activities in the two different Klp5 mutants, as one is temperature-sensitive in the absence of Bub3, and one is not. Intriguingly, the temperature sensitivity of \textit{klp5}\textsuperscript{Q582P-} \textit{bub3}\textsuperscript{Δ} is rescued by
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Figure 4.9 Klp5^Q582P is not a checkpoint silencing specific mutant
(A) Cells were serially-diluted and spotted onto YE5S plates and incubated for 4 days at the indicated temperatures. (B) Tetrad dissections of a cross between klp5Δ and bub3Δ. Dissected spores were germinated on YE5S at 30°C for 4 days before replica-plating for selection or to high temperature. Red rings indicate temperature-sensitive progeny.

the loss of PP1-binding by Klp6. Outside of its role in checkpoint silencing, Bub3 has been shown to be required for the transition of kinetochore attachments from mono-orientation to biorientation in perturbed mitoses (Windecker et al., 2009, Vanoosthuyse et al., 2009); its absence can lead to non-disjunction of chromosomes due to persistent mono-orientation (Vanoosthuyse et al., 2009, Akera et al., 2015). Thus, we speculate that in cells with compromised kinesin-8, where kinetochore-microtubule attachments are imperfect (as seen by the activation of the SAC (see
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Figure 4.6D and Figure 4.8)), Bub3 is depended on to aid attachment turnover to biorientation for anaphase onset. Reduction of PP1 at kinetochores Klp6$^{PP1}$ may promote more turnover of attachments and allow more time for correction, hence rescuing the sickness of klp5$^{Q582P}$ bub3$^{Δ}$ cells.

The fact that PP1-binding mutants of Klp5 and Klp6 appeared to grow normally in our serial dilution assay when Bub3 was absent was mildly surprising, as these cells had previously been reported to be sick (Meadows et al., 2011). Therefore, the viability of klp5$^{Δ}$ with bub3$^{Δ}$ was assessed by tetrad dissection (Figure 4.9B). Intriguingly, double mutant progeny did germinate (red rings) but were clearly temperature-sensitive, as signified by smaller colonies that stained darkly on plates containing Phloxin B. Communication with the authors showing their synthetic lethality revealed that a temperature of 33°C was used for germination of spores (Meadows et al., 2011), in contrast to our method which used 30°C. This is likely to account for the difference in viability of klp5$^{Δ}$ bub3$^{Δ}$ cells seen between our work and theirs. The fact that kinesin-8 PP1-binding mutants are not temperature-sensitive or sick in the absence of Bub3, yet kinesin-8 deletions are, suggests that another function of Klp5 and Klp6 is essential in the absence of Bub3, outside of checkpoint silencing.

Taken together, the prolonged Mad2-GFP signals at the kinetochore and the difference in lethality between klp5$^{Q582P}$ bub3$^{Δ}$ and klp5$^{PP1}$ bub3$^{Δ}$ cells suggest that the delay in anaphase onset seen in the temperature-sensitive kinesin-8 mutant is due to continued SAC signaling due to attachment issues and not solely an inability to silence the signal through compromised PP1 activity.

4.6 Klp5$^{Q582P}$ exhibits less processive behaviour in vitro

The mitotic phenotypes of cells expressing klp5$^{Q582P}$ observed so far point towards a loss of general kinesin-8 function, though the full-length gene product is expressed at normal levels. Various explanations could account for the loss-of-function phenotype, none of which are necessarily mutually exclusive. Mislocalisation of the kinesin-8 complex from microtubule plus-ends would mimic the state of these functional hotspots in cells deleted for kinesin-8. This may result
from changes in intrinsic kinesin behaviour, such as ATPase activity, as seen in Klp5 and Klp6 switch mutants (Meadows et al., 2011) or as a consequence of loss of a protein-protein interaction. Klp5 was shown to interact with Alp7 for its efficient accumulation at kinetochores in cells; disruption of this interaction somewhat increase metaphase spindle length and extends the time spent in pre-anaphase, akin to the deletion phenotype (Tang and Toda, 2015). It has also been demonstrated that human kinesin-8 rely on interaction partners for localisation – Kif18A is enriched at microtubule plus-ends by the kinetochore protein HURP (Ye et al., 2011) whereas Kif18B interacts with +TIP EB1 for its effective targeting (Stout et al., 2011). The location of a secondary microtubule binding site has been narrowed down to the last ~100 amino acids of the Kif18A tail is reportedly required for proper plus-end localisation (Weaver et al., 2011). Similarly, the tail domain of Kip3 has been proposed to contain microtubule-binding activity (Su et al., 2011). It may also be possible that localisation is not affected but some other vital function, be it intrinsic to kinesin function or reliant on extraneous factors, is compromised enough to produce a deletion phenotype. To understand whether the observed phenotypes are the consequences of changes of inherent kinesin-8 activity or are due to loss of association of interaction with extrinsic factors, the Klp5-Klp6 complex was subjected to in vitro study. Recombinant WT and mutant complexes were co-expressed in and purified from E. coli by affinity column and subsequent gel filtration. Kinesin behaviour was then assayed and compared by total internal reflection fluorescence (TIRF). For this section, template expression plasmids, protein purification and TIRF assays were made or carried out by a post-doctorate fellow, Yuzy Matsuo. Mutagenesis cloning, protein expression and TIRF analyses were performed by myself. To our knowledge, these are the first TIRF experiments performed using full-length co-expressed Klp5-Klp6 holocomplex purified to a high degree.

4.6.1 Constructs purify as heterodimers

For purification and the subsequent assays, Klp5 was C-terminally tagged with a hemagglutinin (HA) tag and Klp6 was C-terminally tagged with monomeric super-folder GFP (msfGFP) (Figure 4.10A). The kinesin-8 genes were co-expressed and after two steps of purification, both WT and mutant complexes eluted with high
clarity in a ratio of 1:1, implying heterodimerisation (Figure 4.10B). The profiles of gel filtration are almost exactly the same between Klp5\textsuperscript{WT}-Klp6 and Klp5\textsuperscript{Q582P}-Klp6, implying that the tail mutation has no effect on complex formation.

4.6.2 TIRF microscopy reveals substantial differences in run type between WT and Q582P constructs

To examine the motility of the recombinant kinesin-8 complexes, TIRF microscopy was performed to look at the movement of the fluorescently-tagged Klp5-Klp6 on stabilised microtubules. The experimental set up for the microscopy is schematised in Figure 4.11A. Cy5-labelled porcine brain tubulin was polymerised at 37°C then stabilised with 1 µM taxol. The biotin-Neutravadin system was employed to adhere stabilised microtubules to the coverslip, which was previously passivated to prevent other proteins binding. WT or Q582P mutant kinesin-8 complexes were then flowed into the buffer system containing an oxygen scavenging system and 1 mM ATP (see Materials & methods). Imaging was performed at 30°C, similar to the permissive temperature used for in vivo experiments. Attempts were made to use a higher temperature in the TIRF-microscope imaging chamber but at least for WT complexes, the rate of microtubule binding became too high to permit analysis of individual runs. It should be noted, as shown previously, Klp5\textsuperscript{Q582P} does produce marginally elongated metaphase spindles at permissive temperature (see Figure 4.4B), indicative of a slight loss of cellular kinesin function at 30°C. Klp5\textsuperscript{WT}-HA Klp6-
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Figure 4.11 Klp5\textsuperscript{Q582P} exhibits more diffusive run behaviour

(A) Schematic of TIRF assay for observation of Klp5-HA-Klp6-msfGFP on taxol-stabilised microtubules. (B) Kymographs dual colour TIRF microscopy was performed using 10 nM Klp5-Klp6 complex (green) and fluorescently-labelled taxol-stabilised microtubules (red) in the presence of 1 mM ATP at 30°C. (C) Categorisation of kinesin run events from data represented in part (B). A binding event was classified as a run if the complex remaining on the microtubule for more than 3 seconds (3 pixels in the Y dimension). N = 150 runs for each Klp5 construct. Lower part indicates representative runs for each category.
msfGFP has been observed to possess plus-end-directed motility on taxol-stabilised microtubules at 30°C (Yuzy Matsuo, unpublished data). In this assay, clear plus-end directed motility was seen for Klp5\textsuperscript{WT}-HA Klp6-msfGFP (Figure 4.11B, upper panels). Despite plus and minus ends of taxol-stabilised microtubules being indistinguishable by morphology or fluorescent labelling, the plus end was obvious as both WT and Q582P mutant complexes showed a clear overall preference for one of the microtubule ends, marking it as the plus-end. The WT complex frequently bound to microtubules and moved smoothly in a unidirectional manner, and could even be seen dwelling on the plus end (right hand side of kymograph). In striking contrast, Klp5\textsuperscript{Q582P}-HA Klp6-msfGFP clearly showed dramatically fewer binding events by eye (Figure 4.11B, lower panels). In addition to the infrequency of binding, the behaviour of the kinesin runs was very different from that of the WT complex (Figure 4.11C). A binding event was classified as a run if the GFP signal persisted on the microtubule for 3 seconds or longer (3 pixels in the y-axis). Runs were divided into three categories of behaviour (examples are given in the labelled image of Figure 4.11C). A static run involved the kinesin binding for the minimum time without any discernible movement along the microtubule in either direction, and so the GFP signal remained vertical through time. A processive run was assigned when the GFP signal moved clearly and smoothly in one direction, towards the plus end of the microtubule. Runs were defined as diffusive if the GFP signal showed jagged movement in both directions, the gradient of which did not match that of clearly processive runs. It should be noted that all diffusive runs exhibiting a clear overall directional motility moved towards the plus end, excluding the possibility of directionality switching as the cause of back and forth movement of signals. When the distribution of categories within each population of kinesin was quantified, the majority of runs were diffusive when the tail was mutated, whereas the most common behaviour from the WT complex was processive motility. The difference in overall kinesin-8 behaviour suggests the tail mutation has an effect on general function.

As described previously, kinesin-8 proteins are proposed to contain secondary microtubule binding motifs in the tail, to improve processivity and plus end localisation (Mayr et al., 2011, Stumpff et al., 2011), although no exact motif or sequence has been pinpointed for this function. These previous studies found that
recombinant tail-less constructs were impaired in proper plus-end accumulation and exhibited shorter runs due to more dissociation from the microtubule lattice, implicating secondary microtubule-binding in processivity. Therefore, the length, duration and velocity of each processive run was measured for the WT and Q582P complexes (Figure 4.12). As only 16 of the 150 mutant runs measured for the mutant complex were classified as processive, only the first 16 processive runs seen for the WT complex were included in this analysis. Both run time and run length were greatly reduced by the presence of the Q582P mutation (Figure 4.12A and B). WT runs actually showed more variation in addition to lasting for longer and covering a larger distance. As motility is normal, the major influence on run length is likely to be the starting position of the kinesin, which varies greatly, with frequent termination of measurement at the plus-end (see Figure 4.11B, upper panels). The shorter runs displayed by the mutant complexe are perhaps limited by the complexes' activity but will also infrequently be attenuated by reaching the microtubule plus end, and starting position likely has no effect on the measurements. Interestingly, while processive run distance was smaller and for a shorter period of time when the tail was mutated, the calculated velocity of processive runs was more similar than the other two parameters measured (Figure 4.12C). WT Klp5-Klp6 was relatively constant, ranging between 0.12 – 0.23 µm/s, comparable to that of recombinant full-length Kif18A at a similar temperature (Mayr et al., 2011). The mean velocity of mutant complexes was 0.13 ± 0.06 µm/s,
marginally lower than that of WT complexes at 0.15 ± 0.03 µm/s. Due to the small sample sizes for processive runs in the mutant, it is difficult to draw solid conclusions from these data. However, it does seem quite clear that mutant complexes, while capable of processive runs, cannot be retained on the microtubule lattice for long runs in terms of both distance and time. Also, processive runs are rare for mutant complexes, with the majority of runs being diffusive, implying problems even with initial lattice interactions. Further experiments would be required to validate these differences seen between WT and Q582P mutant recombinant Klps5-Klps6 complexes. Nevertheless, the fact that defective kinesin-8 behaviour is observed in the absence of any other MAPs or proteins, in our isolate in vitro system, suggests that mitotic phenotypes observed in klp5<sup>Q582P</sup> cells is primarily due to changes in intrinsic kinesin function, mediated by the tail mutation.

4.7 The kinesin-8 ‘tail box’ appears to be a functional motif

4.7.1 Mutagenesis of the conserved tail-box produces similar Klps5 and Klps6 tail mutants

The Q582P mutation, isolated by random mutagenesis, is located just one residue outside of the ‘tailbox’ (West et al., 2001) and is predicted to contribute to a coiled-coil forming structure (see section 3.9.4). While the introduction of a proline appears to change the probability of the region forming this secondary structure, disrupting the region more centrally may have a stronger effect on knocking down the function of this potential motif, if any. Also, Q582 is not conserved in Klps6, but the surrounding region is homologous. In order to understand if these tailboxes are functional kinesin-8 motifs and if the function/structure is conserved between Klps5 and Klps6, two new coiled-coil mutants were constructed. Various conserved residues were exchanged for proline and the mutant amino acid sequences were submitted for coiled-coil prediction, as described previously (Lupas et al., 1991). The two conserved residues that caused the largest reduction in the probability of coiled-coil formation, when mutated to proline, were glutamic acids (Figure 4.13A and B). For Klps5, E575P (left-hand panel, dashed purple trace) causes a much more dramatic loss of coiled-coil structure than the Q582P mutation is posited to (left-hand panel, dashed yellow trace), suggestive of a greater loss of function and
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Figure 4.13 Motif mutants are more extreme knockdown of function

(A) Clustal sequence alignments of kinesin-8 tail regions, with Klp5 and Klp6 mutants highlighted. (B) The probability of coiled-coil formation was predicted for Klp5 mutants (left) and Klp6 mutant (right) using the online ‘COILED-COIL PREDICTION’ software from PRABI (Lupas et al., 1991). Parameters were set at window 28 and MTIDK matrix, with a weight of 2.5 at heptad positions a and d. (C) Cells were serially-diluted and spotted onto YE5S plates and incubated for 4 days at the indicated temperatures in the presence of the indicated concentrations of TBZ.
likely a stronger phenotype. Although the peak of probability in the Klp6 tail region (right-hand panel, solid black trace) is considerably smaller than that of Klp5, the introduction of the proline completely abolishes it (right-hand panel, dashed pink line). Indeed, when all kinesin-8 mutants in hand were assayed for functionality by serial dilution assay in the presence of TBZ at high temperature, the new coiled-coil mutant cells behaved much more similarly to kinesin-8 deletion cells (Figure 4.13C). While Klp5Q582P renders cells moderately more resistant to TBZ than WT cells, Klp5E575P has the same effect as deletion of klp5. Interestingly, the klp6 mutant, Klp6E569P, causes cells to behave in exactly the same manner as Klp5E575P does, in addition to mimicking klp6Δ. It should also be noted that, both klp5Δ klp6Δ and klp5E575P klp6E569P do not show an additive phenotype compared to each single mutant. These results suggest that the two new proline mutations are more disruptive to tail structure and as such, have a stronger loss of function kinesin-8 phenotype.

4.7.2 Klp5E575P exhibits a more severe loss of mitotic control than Klp5Q582P

Next, the new tailbox mutants were examined more closely to ascertain whether their resistance to TBZ and hence loss of kinesin-8 functionality was caused by the same mitotic defects as seen in klp5Q582P cells. To this end, the selection of kinesin-8 mutants was studied by time-lapse imaging. In this experiment, microtubules, SPBs and the nuclear envelope (NE) were visualised by mCherry-Atb2, Sid4-mRFP and Cut11-GFP, respectively. Cut11 is an SPB docking protein required for insertion of the SPB into the nuclear envelope for mitosis and subsequent proper spindle formation (West et al., 1998). Cut11 localises to the nuclear pore throughout the cell cycle and becomes concentrated on mitotic SPBs from early prophase to sometime in anaphase (West et al., 1998). Therefore, in addition to the morphological information provided by nuclear envelope staining, it also provides a reference for mitotic timing in relation to anaphase. Log-phase cultures of WT, klp5Δ, klp6Δ, klp5Q582P and klp5E575P cells were shifted up to 36°C for a total of 40 minutes before live imaging at 36°C. As Klp5E575P and Klp6E569P exhibited the same TBZ resistance as each other and as the double tail mutant, and the main interest is the comparison between Klp5 tail mutants, klp6E569P and double mutant cells were omitted from this experiment.
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Time-lapse imaging of cells expressing Cut11-GFP, Sid4-mRFP and mCherry-Atb2 in a WT, klp5Δ, klp6Δ, klp5Q582P or klp5E575P background. Cultures were shifted to 36°C for 40 minutes before observation every 2 minutes at 36°C. (A) Representative cells from the point at which spindle length is equal to or greater than the diameter of the nucleus until the disappearance of Cut11-GFP from the SPB. (B) Left – proportion of cellular phenotypes, categorised as a spindle within a round nucleus (Normal), an extremely long spindle pushing the SPB(s) completely outside of the nuclear periphery (Protrusion) or a marginally longer spindle that causes SPBs to sit slightly but clearly outside of nuclear periphery (Lemon). Right – representative cells for each phenotype. Scale bars, 10 µm.

Figure 4.14 Tailbox mutants produce long spindles that distort the NE

Time-lapse imaging of cells expressing Cut11-GFP mCherry-Atb2 Sid4-mRFP at the restrictive temperature revealed pronounced differences between WT and kinesin-8 mutant cells (Figure 4.14A). Compared to the spherical nucleus in which the early short spindle is contained in WT cells, all kinesin-8 mutant cells show spindles that are longer than the diameter of the nuclear bulk and cause protrusion(s) of the spindle in the nuclear envelope. These nuclear envelope extrusions, or ‘tethers’, have been reported previously for kinesin-8 deletion strains.
(Gergely et al., 2016) but the frequency of this phenotype has not been quantified. For all cell types, nuclear envelope protrusion(s) were only observed when Cut11-GFP was present on the SPBs, meaning that these disruptive spindles are defective prior to anaphase, as Cut11 is removed from SPBs early in anaphase. This observation is in accordance with the findings presented in Figure 4.6B, where metaphase spindles increase in length from WT, to those regulated by a point mutant of Klp5 to those formed in the absence of kinesin-8. Interestingly, two types of nuclear distortion were obvious in mutant cells: one in which substantially longer spindles caused clear protrusions and a milder phenotype where the spindle that was only slightly longer than normal caused SPBs to sit just outside of the membrane, giving a 'lemon'-shaped NE. In all cases, the nuclear envelope was eventually resolved into two equal-sized nuclei. Quantification of the proportion of nuclear morphologies observed is presented in Figure 4.14B. Of the klp5Q582P cells exhibiting an abnormal spindle-NE morphology, around half of them had more severe protruding spindles, and half had the milder lemon phenotype. The fact that klp5Q582P cells exhibit a higher proportion of the less severe spindle phenotype reflects the milder TBZ resistance of these cells, compared to that of klp5Δ, and also the average metaphase spindle length being shorter, with a reasonable degree of variation (see Figure 4.6B). In sharp contrast, the frequency of the protrusion phenotype in klp5E575P cells was approximately twice that of klp5Q582P cells, with very few cells undergoing a morphologically normal mitosis. In this way, the new tailbox mutant behaves very similarly to cells lacking kinesin-8 function in mitosis. Furthermore, in this experiment, klp5Δ and klp6Δ cells were compared to ensure their interchangeability as kinesin-8 deletions, and it is clear that the frequency of aberrant spindle phenotypes is almost exactly the same.

Cut11 delocalises from the SPBs at the transition from anaphase A to anaphase B (West et al., 1998, Walde and King, 2014) and so can be used as a proxy for the timing of anaphase. Firstly, the spindle length in the last frame of Cut11-GFP at SPBs was measured to compare early anaphase spindle length in the kinesin-8 mutants (Figure 4.15A). A similar pattern of the severity of the kinesin-8 phenotype emerges from this data, as is seen in Figure 4.14B; spindle length increases with the level of kinesin perturbation. klp5Δ cells and klp6Δ cells presented the longest
Figure 4.15 Tailbox mutants increase spindle length and duration of early mitosis incrementally
Quantification of parameters in cells expressing Cut11-GFP Sid4-mRFP and mCherry-Atb2. (A) Spindle lengths at the last instance of Cut11-GFP foci on SPBs. Lines represent mean and standard deviation. (B) Mean and standard deviation of Cut11-GFP residence on SPBs from the beginning of mitosis to disappearance. (C) Maximum length of spindle protruding from cells exhibiting the phenotype, usually over multiple time points. Schematic shows spindle portion measured. For all parts, the statistical significance of difference was determined using a two-tailed Mann-Whitney test (**, p=0.0015; ****, p<0.0001, ns, not significant). Comparisons between the indicated strain and WT are represented in black and comparisons between the two kinesin-8 mutants are indicated in green.

early anaphase spindles with an average length of 5.71 ± 1.34 µm and 5.67 ± 1.25 µm, respectively. Klp5<sup>E575P</sup> also produced relatively longer spindles, with a mean of 4.76 ± 0.82 µm. Remarkably, this value is significantly different from that of both klp5<sup>D582P</sup> cells, where the average anaphase spindle length was 3.83 ± 0.59 µm, and from the kinesin-8 deletion cells. These data imply that the Klp5<sup>E575P</sup> coil mutant is more defective in kinesin-8 function than the original mutant isolated, but
retains enough of its abilities to perform some of its roles in comparison to the total loss of kinesin-8 in mitosis in deletion strains. Again, there is not a significant difference between early anaphase spindle lengths in each kinesin-8 deletion mutant, suggesting the same loss of function in each genetic background. The mean early anaphase spindle lengths for each genetic background generated in this data set are similar in magnitude to measurements of the metaphase spindle from the imaging of cen2-GFP Sad1-dsRed mCherry-Atb2 strains (see Figure 4.6B); values from the Cut11-GFP experiment are higher due to the later time point of measurement, as cells begin to undergo spindle elongation for anaphase B at the time of Cut11-GFP disappearance from SPBs.

In the same way that the timing of anaphase onset was used as a readout of kinesin-8 function (see Figure 4.6D and Figure 4.8B and C), the timing of anaphase marked by the disappearance of Cut11-GFP from SPBs was assessed in a similar manner (Figure 4.15B). Again, there appears to be three different degrees of loss of kinesin-8 function. On average, normal cells enter early anaphase after 9.38 ± 1.08 minutes in mitosis. In agreement with the extension of time spent in pre-anaphase seen previously, \textit{klp5}^{Q582P} cells take 10.24 ± 1.19 minutes to reach this stage. Furthermore, the Klp5\textit{E575P} mutant causes an even longer extension of this period, where cells take 11.84 ± 1.71 minutes to reach anaphase. While significantly longer than the time taken in both \textit{WT} and \textit{klp5}^{Q582P} cells, this duration is also significantly shorter than that of \textit{klp5}\textit{Δ} cells. These results reiterate the idea of varying degrees of kinesin-8 dysfunction across the three Klp5 mutants studied. Again, \textit{klp5}\textit{Δ} and \textit{klp6}\textit{Δ} cells display the same anaphase timing, with averages of 13.29 ± 2.51 minutes and 13.13 ± 3.22 minutes, respectively.

In addition to the two parameters quantified for all strains studied, the extent of the spindle protrusion into the nuclear envelope was assessed by measuring the protruded portion, as schematised in (Figure 4.15C). As spindles protruded from the bulk of the nucleus for multiple frames in the time-lapse imaging of the cells, the maximum value was taken from the multiple measurements and plotted. Unsurprisingly, no significant difference was found between the two sets of kinesin-8 deletion measurements as samples had an extremely similar average length and spread of data (Figure 4.15C). Therefore, for our study, we consider \textit{klp5}\textit{Δ} and
4.7.3 Central mutations of the tailbox of klp5 or klp6 are lethal with dis1Δ

The results described in this section so far suggest that the new coil mutations have a similar, but stronger, effect on the kinesin-8 tail than the original Q582P mutant isolated. To further clarify this notion, the lethality of these klp5 and klp6 mutants was checked in the dis1Δ background (Figure 4.16). No viable colonies were produced by progeny expressing both dis1Δ and either kinesin-8 mutant. The lethality of these alleles is a step up from the viability of dis1Δ klp5Q582P cells, which only show growth defects at high temperature, and is the same result as for full deletions of kinesin-8 in conjunction with dis1Δ.

**Figure 4.16 Central tailbox mutants are synthetic lethal with dis1Δ**

Tetrad dissections of crosses between dis1Δ and klp5E575P or klp6E569P. Dissected spores were germinated on YE5S at 30˚C for 4 days before replica-plating for selection. Yellow rings indicate inviable progeny.
4.8 Discussion

The analyses of kinesin-8 mutants conducted in this chapter clearly identify \( klp5^{Q582P} \) as a loss-of-function allele, when compared with kinesin-8 deletion behaviour. The Q582P mutation at the end of the tailbox renders Klp5 a temperature-sensitive protein that produces a clear phenotype, but is viable in terms of cell growth, both of which characteristics are also true for \( klp5^{Δ} \). The loss-of-function phenotype is not ascribable to problems in kinesin-8 heterodimerisation as seen by co-immunoprecipitation (Figure 4.2B), but somehow mildly phenocopies a deletion situation, as seen in the multiple observations of mitosis in live cells (Figure 4.6 to Figure 4.8, Figure 4.14 and Figure 4.15). Data obtained from \textit{in vitro} kinesin motility assays and the identification of further mutations in the tailbox support the notion that this may be a key motif that is integral to normal kinesin-8 function, in a previously unreported manner.

Characterisation of the Klp5\(^{Q582P}\) mutant began with the exclusion of the possibility that the tailbox mutation affects heterodimerisation with Klp6. At high temperature, Klp6 was able to pull down Klp5, wild-type or mutant, to a similar degree. This result suggests that the lethal temperature-sensitivity of this allele in the \( dis1^{Δ} \) background is not produced by impaired dimerisation that would cause a loss of proper nuclear retention of kinesin-8 in mitosis and hence mimic the synthetically-lethal deletion. Multiple attempts to obtain clear and reliable cellular localisation data for even wild-type kinesin-8 complexes proved extremely difficult, likely due to the motility of these proteins and the sensitivity of the camera used. Studying the localisation of wild-type and mutant Klp5 proteins would be extremely useful in understanding the effect of the tail mutation on inherent kinesin-8 behaviour and interactions \textit{in vivo}.

The behaviour of \( klp5^{Q582P} \) cells at the permissive temperature was assessed in order to understand the severity of the mutation and also to shed light on how \( dis1^{Δ} \ klp5^{Q582P} \) cells survive at 30°C. In all parameters measured in mitotic cells expressing spindle and chromosome markers, the only significant difference between \( WT \) and \( klp5^{Q582P} \) cells was found in chromosome congression and the nature of the subsequent chromosome segregation. The emergence of a
phenotype at permissive temperature in $klp5^{Q582P}$ cells is slightly surprising but not inexplicable; the introduction of a hydrophobic, helix-breaking proline in place of a polar, hydrophilic glutamine is likely capable of causing problems with protein function even at permissive temperature. The tail mutant caused the pair of cen2-GFP signals to be positioned further away from the spindle centre during metaphase, and with more variation in this position, compared to wild-type cells. Interestingly, these problems with chromosome congression do not appear to activate the SAC, as seen by a timely anaphase onset, similar to that for WT cells. This contrasts the greater loss of congression observed in $klp6\Delta$ cells, which is also accompanied by a prolonged delay in transitioning into anaphase. This mitotic behaviour was echoed in the experiments performed at high temperature. It may be that the milder congression defects in $klp5^{Q582P}$ cells, in comparison to $klp6\Delta$ cells, are not due to problems with kinetochore-microtubule attachment, which normally activate the SAC for their correction, and may be a result of other changes, such as the control of microtubule dynamics at plus ends.

Previous work suggests that the loss of congression in cells lacking kinesin-8 function is not due exclusively to the increased oscillation of chromosomes, as the addition of low levels of TBZ stabilised microtubules enough to reduce the amplitude of oscillations but not to rescue chromosome centering defects (Mary et al., 2015). This work also proposed that oscillations before anaphase are large due to the combined effects of altered microtubule dynamics and problems with kinetochore-microtubule attachment; Mad2 could be seen loaded onto the trailing kinetochore of an oscillating pair and was absent in the presence of TBZ when oscillations were reduced, implying that kinetochore-microtubule detachment contributed to the larger movements. Overall, their data imply kinesin-8 is required for the reduction of kinetochore oscillation by both the manipulation of microtubule dynamics and the fidelity of kinetochore-microtubule attachments. The latter of these roles may involve kinesin-8 proteins directly in a structural or interactive manner, or may be invoked by correct microtubule dynamics. The fact that $klp5^{Q582P}$ cells, with partially compromised Klp5 function at permissive temperature, have offset metaphase alignment in the absence of SAC activation sustain the idea that it is a loss of negative regulation of kinetochore-microtubule dynamics, and not attachment issues that is responsible for the mildly increased kinetochore
oscillations that give rise to random metaphase alignment positions. It is possible that sister chromatids in mutant cells are merotely attached to the spindle, giving rise to asymmetry in metaphase alignment, without SAC activation, and lagging chromosomes in anaphase. If this were the case, it would lend support to the notion that merotely attachments can generate tension across kinetochores, in addition to being attached, and hence are not detected by the SAC in fission yeast. The absence of merotely persisting into anaphase would imply that cells lacking proper kinesin-8 function are able to alter these aberrant attachments, excluding a role for kinesin-8 in the correction of merotely. However further experiments are needed to assign the nature of the kinetochore-microtubule attachments in kinesin-8-compromised cells.

With both increasing temperature (30°C to the restrictive 36°C for the klp5Q582P allele) and increasing loss of kinesin-8 function (klp5Q582P to klp6Δ), metaphase chromosome position spreads and the duration of SAC activation increases, in accompaniment with other phenotypes. This suggests that the Klp5Q582P protein becomes more inactive with increasing temperature, as cells behave more like the deletion. The increased SAC activation at the restrictive temperature, as observed by the increased time spent in pre-anaphase and prolonged Mad2 signals at kinetochores, highlights the competency of the Klp5Q582P protein at the permissive temperature to ensure proper kinetochore-microtubule attachments for timely anaphase onset. Perhaps the regulation of microtubule dynamics is the most sensitive function of kinesin-8 that is required for a concerted mitosis in terms of chromosome behaviour. At least, it can be said that bi-orientation occurs in the absence of congression, as occurs in kinesin-8 deletion cells.

At high temperature, multiple mitotic phenotypes are apparent in klp5Q582P cells. In addition to the problems in kinetochore attachment/chromosome congression and coordination of chromosome segregation, both of which defects become much more pronounced, metaphase spindle length increases. While this phenotype has been reported frequently for cells lacking klp5 or klp6 (Stumpff et al., 2008, Syrovatkina et al., 2013, Mary et al., 2015, Garcia et al., 2001), the molecular cause for this has not been explicitly addressed. Mutagenesis or deletion of a wide array of proteins that contribute to spindle formation and/or chromosome
segregation can lead to elongated spindles. Members of the kinesin-14 subfamily of motor proteins are known to produce inward sliding forces on spindles: homodimers localise to interpolar microtubules of the spindle and use their minus-end directed motility to slide the antiparallel microtubules over each other (Furuta and Toyoshima, 2008). Indeed, the knockdown of human kinesin-14 HSET rescues the long spindle phenotype of cells lacking the human kinesin-8 Kif18A (Weaver et al., 2011). Deletion of either of the kinesin-14 genes expressed in fission yeast, Pkl1 and Klp2, produces longer spindles in mitosis (Choi and McCollum, 2012, Yukawa et al., 2015), due to the loss of their antagonism against the outward force produced by the kinesin-5, Cut7 (Hagan and Yanagida, 1990, Yukawa et al., 2015). It is likely that aberrant outward sliding of interpolar microtubules is accompanied by microtubule polymerisation at the interdigitating plus ends to ensure sufficient kinesin-binding sites are available to continue sliding. Indeed, the microtubule cross-linker Ase1 is thought to stabilise outward force generation through its localisation to interdigitating microtubules at the midzone during anaphase, while microtubules are polymerising (Yamashita et al., 2005). Ase1 has also been found to stabilise long spindles in kinesin-8 deletion cells, as its deletion rescues the long-spindle phenotype (Syrovatkina et al., 2013). Recent work from our lab (Yukawa et al., 2017) has found that in the absence of the antagonistic spindle forces from kinesin-14 and kinesin-5, microtubule polymerisation by the XMAP215 homologue Alp14 is absolutely required. It is hypothesised that the force driving spindle elongation in the absence of the sliding of interpolar microtubules comes from growing kinetochore-microtubules that may push on opposing SPBs (Yukawa et al., 2017). Spindle anchoring proteins Msd1 and Wdr8 form a complex with Pkl1 and also produce a long spindle phenotype when deleted, implicating inward force generation by proper microtubule-SPB interactions as a factor in spindle length (Yukawa et al., 2015). This notion is reiterated by the fact that perturbation of other SPB-based spindle factors, including Mto1 (Zimmerman and Chang, 2005) and Sad1 (Hagan and Yanagida, 1995) can also lead to elongated spindles. Other contributors to inward force that are not members of the kinesin superfamily include the CCAN protein Mis6 and the outer kinetochore factor Mis12 (Goshima et al., 1999). Both of these static, chromosome-based proteins, amongst multiple others, manage to exert a force on the spindle to keep it short during metaphase, likely via kinetochore-microtubules, rather than interpolar microtubules. Similarly, Dam1, a
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constituent of the DASH complex that links microtubule plus ends and kinetochores, is also required to negatively control spindle length in early mitosis (Sanchez-Perez et al., 2005, Syrovatkina et al., 2013). To summarise, negative regulation of spindle length in fission yeast can be achieved through active kinesin-mediated inward sliding of interpolar microtubules, resistance of outward microtubule-sliding at SPBs and resistance to outward forces generated by kinetochore-microtubules. Where do Klp5 and Klp6 fit into this network of spindle regulation? Their localisation to kinetochores (Garcia et al., 2002b) and the clear defects in kinetochore-microtubule behaviour, as described above, permit the idea that they negatively regulate kinetochore-microtubules to regulate spindle length, and additionally generate inward force through proper kinetochore attachments. Conversely, Klp5 and Klp6 localise to the spindle midzone which is only clearly observable during anaphase B (West et al., 2002) and the spindle length phenotype can be rescued by ase1Δ (Syrovatkina et al., 2013). While rescue by the deletion of this midzone stabiliser makes a case for interpolar microtubules being those subjected to regulation by kinesin-8, the function of Ase1 transcends to kinetochore-microtubules as it is required to correct merotelic attachments in anaphase (Courtheoux et al., 2009), making the situation less clear. As the Klp5-Klp6 complex localises along the entire spindle during early mitosis when spindles are short (West et al., 2001), and it is difficult to distinguish different microtubule localisations on this small, linear S. pombe spindle, it is possible that Klp5 and Klp6 localise to the spindle midzone earlier than anaphase B. While this remains to be tested, it is not possible to accurately assign the population of microtubules that is subject to negative regulation by kinesin-8. It should be noted that regulation of kinetochore-microtubules or interpolar microtubules is not necessarily mutually exclusive. Nevertheless, abrogation of proper chromosome congression and the presence of lagging chromosomes at 30˚C is not lethal when coupled with dis1Δ as these cells are viable, implying that these roles for kinesin-8 is dispensable in the absence of Dis1 function. Therefore, we speculate that negative regulation of spindle length and proper kinetochore attachment for biorientation, not congression, by kinesin-8 is essential in the absence of Dis1 at the restrictive temperature.
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To further confirm the importance of the tailbox, mutations were made more central in the region in an attempt to completely knockout the function. The high degree of homology in this area permitted the mutations to be made in both klp5 and klp6, to check for conservation of function between the two homologues. As predicted, the two new tailbox mutants exhibited more severe phenotypes than the original mutant isolated, behaving much more like cells completely lacking kinesin-8 function. With increasing loss of tail function, spindle length increases, as does the time spent in early mitosis. Increased pre-anaphase spindle length has also been reported for kinesin-8 switch-II mutants that cannot hydrolyse ATP efficiently (Meadows et al., 2011). Switch-II mutants were seen to be localised along the entirety of the anaphase B spindle instead of their normal restriction to the midzone at this point in mitosis (Meadows et al., 2011). It is likely that the inability of these mutants to efficiently concentrate at plus ends leads to longer or more dynamic microtubules, in turn increasing spindle length, yet the population of microtubules affected (kinetochore or interpolar, or both) remains unknown. It is also formally possible that ATPase activity is directly required for the dampening of plus-end dynamics/catastrophe induction/depolymerisation by the Klp5-Klp6 complex. At least, studies of a motor-only construct of Kif18A showed that removal of tubulin dimers from microtubule ends does not rely on intrinsic plus-end-directed motility (Peters et al., 2010). Therefore, it is initially surprising that the tail mutations created in this study give a similar long spindle phenotype to motor domain mutants.

Multiple explanations as to why mutating the tailbox of Klp5 leads to a deletion-like phenotype. Full-length, fully expressed mutant Klp5 proteins are seen to be capable of interacting with Klp6. However, each mitotic parameter measured in the point mutants tend towards those of kinesin-8 deleted cells. Klp5<sup>Q582P</sup> produces a milder deletion-like phenotype, whereas a more central disruption to the tail box in klp5<sup>E575P</sup> or klp6<sup>E569P</sup> cells increases measurements further towards the values obtained for klp5<sup>∆</sup> or klp6<sup>∆</sup> cells. All statistical tests performed evaluating the significance of difference between the mean measurements for point mutants compared to deletions give a P value of less than 0.0001. The fact that the differences are significant to this degree implies that the point mutants retain some residual kinesin-8 function. While this notion is echoed in the viability of Klp5<sup>Q582P</sup> in
the \textit{dis1}\Delta background, it is not in the lethality of Klp5\textsuperscript{E575P} and Klp6\textsuperscript{E669P} in the absence of Dis1. Perhaps these mutants cause total abolition of the tail box structure and subsequent function, which is essential in the \textit{dis1}\Delta background. It is interesting to note the tail mutations affect every aspect of kinesin-8 function, as previously studied mutations in Klp5 of Klp6 appear to disrupt specific functions. Mutations in the PP1-binding motifs produce a delay in SAC-silencing but have no effect on spindle length (Meadows et al., 2011). Perturbing ATPase activity of the motor domains affects kinesin-8 localisation, which has knock on effects on both spindle length and time spent in pre-anaphase due to SAC-activation (Meadows et al., 2011), but not checkpoint silencing. It seems that the tail mutants isolated in this study exhibit characteristics like those of motor mutants, even though these two domains are completely separate in the protein.

Studying kinesin-8 behaviour in isolation through in vitro assays using recombinant proteins has shed a light on the nature of the Q582P tail mutant. In the absence of other mitotic proteins or interacting partners, the mutant Klp5-Klp6 complex exhibits much fewer processive runs on taxol-stabilised microtubules compared to the processive WT counterpart. The mutant appeared to favour more diffusive movements on microtubules, with these runs retaining an overall bias towards the microtubule plus end, indicative of residual function. It was also observable by eye that the mutant GFP-tagged complex appears to undergo fewer microtubule binding events than the WT complex. Together, these data suggest that the tail mutant could be impaired in proper microtubule-binding ability for processive activity. The mutant complex did display a low frequency of processive runs, of which the duration and distance covered were much shorter than those of processive runs made by the wild-type complex, yet the velocities were comparable. It is possible that, like Kif18A and Kip3, fission yeast kinesin-8 contain a secondary microtubule-binding region in the tail, disruption of which leads to reduced lattice interaction and fewer processive runs that are shorter in both duration and distance (Mayr et al., 2011, Stumpff et al., 2011). Kip3 relies on its C-terminal tail for improved run lengths and retention on microtubule plus-ends, but not for overall kinesin velocity (Su et al., 2011). It is unlikely that the tailbox is directly involved in in the hypothetical secondary microtubule-binding site, as its structure is predicted to be a coiled-coil, but its disruption may lead to changes in
tail structure that affect the proposed site. It also seems feasible that kinesin-8 may be subject to auto-inhibition of kinesin activity, like multiple other kinesins (for example kinesin-1, MACK), and that changes brought about by Q582P may act to strengthen an auto-inhibitory interaction. For example, two C-terminal coil regions in the human kinesin-1 heavy chain interact to bring the tail region closer to the motor domain for inhibition of microtubule-binding (Friedman and Vale, 1999). Klp5 is predicted to contain two C-terminal coiled-coil regions, one of which is expected to undergo a structural disruption when a proline is introduced at its end. It is also possible that the tailbox interacts with the motor domain itself to mediate an auto-inhibitory interaction, as is seen in the human kinesin-2, KIF17 (Hammond et al., 2010). A sequence with predicted coiled-coil structure, located between the neck and tail regions of KIF17, was seen to inhibit kinesin motor activity, separately from inhibition of microtubule-binding, which was found to rely on tail-motor interactions (Hammond et al., 2010). Tail-less constructs of Kif18A possess diminished microtubule-binding activity, due to the loss of a secondary microtubule-binding site, yet show increased velocity in kinesin motility or microtubule gliding assays, compared to full-length constructs (Mayr et al., 2011, Stumpff et al., 2011). Previously, no assumptions have been made about this finding where Kif18A truncations show increased activity, but experiments using full length and truncated Klp5-Klp6 found that only tail-less constructs possessed tubulin-stimulated ATPase activity (Erent et al., 2012), implicating a role for auto-inhibition in the regulation of fission yeast kinesin-8. Human kinesin-1 heavy chain appears to rely on interaction with cargo to release it from an inhibited state due to tail-motor interactions, where truncations show higher activity than the full-length constructs (Friedman and Vale, 1999). However, this requirement for cargo interaction has not been investigated for kinesin-8 in any species, as all full-length recombinant kinesin-8 proteins length have shown activity when assessed by in vitro assays. Therefore, we postulate that auto-inhibition and secondary microtubule binding are not mutually exclusive activities performed by the tail. This makes it difficult to interpret the data from the TIRF experiments described in this chapter, dissecting potential secondary microtubule-binding sites from a tail-motor interaction that affects activity, or even primary microtubule-binding activity. Given more time, it would be of great importance to compare the microtubule-binding activity of Klp5<sup>WT</sup> and Klp5<sup>Q582P</sup> constructs by way of a microtubule co-sedimentation assay, and also perhaps
purify a recombinant tail-only construct to validate a positive result. Structural studies, such as cryo-EM or circular dichroism, would shed light on any differences between the conformation of WT and mutant proteins, informing on the ability for tail-motor interactions to take place and any changes caused by mutating the tailbox. We surmise that the tailbox mutation affects secondary microtubule-binding directly, or strengthens an autoinhibitory interaction between the tail and motor regions, that in turn may disrupt primary microtubule-binding, ATPase activity, or both.

In conclusion, we find that the loss-of-function Q582P mutation has a negative effect on kinesin-8 activity by either affecting secondary microtubule-binding activity or by increasing an auto-inhibitory interaction, either of which produce a less processive Klp5-Klp6 complex. The consequences *in vivo* include less efficient accumulation at microtubule plus-ends where it would normally exert its negative influence on microtubule dynamics. Reduced levels of kinesin-8 at microtubule plus ends represents an intermediate population of functional complex, between full function in wild-type cells and complete lack of functionality in kinesin-8 deletion cells. For this reason, metaphase spindle length, chromosome alignment and kinetochore-microtubule interaction phenotypes are milder than observed in *klp5/klp6* deletion cells, but worse than in wild-type cells. Following this, we attribute the synthetic lethality of the *klp5*Q582P allele with *dis1Δ* to the fact that the tailbox mutant partially mimics *klp5* deletion in terms of the level of functional kinesin-8 on spindles.
Chapter 5. Coordination of microtubule dynamics by kinesin-8 and Dis1 is essential, in addition to kinetochore-microtubule attachments

Following the characterisation of \textit{klp5}^{Q582P} cells and protein behaviour, this chapter describes the use of the loss-of-function mutant as a tool to study the synthetic lethality between Dis1, a microtubule polymerase, and the kinesin-8 complex. \textit{dis1}\textDelta \textit{klp5}^{Q582P} cells were found to exhibit two distinct phenotypes in terms of spindle behaviour and chromosome segregation. Time-lapse imaging of defective mitoses reveals an interesting relationship between the microtubule polymerase and the microtubule-destabilising factors in spindle regulation. This chapter describes the imaging experiments performed to elucidate the functional interplay between Dis1 and kinesin-8 required to coordinate high-fidelity and viable mitoses.

5.1 Cells lacking Dis1 are extremely delayed in anaphase onset, distinct to that of kinesin-8 mutants

Dis1 is a fission yeast homologue of the XMAP215/chTOG family of MAPs (Garcia et al., 2001, Nakaseko et al., 2001, Ohkura et al., 2001). Dis1 is recruited to the outer kinetochore in mitosis (Nakaseko et al., 2001) via the internal loop of Ndc80, connecting kinetochores to microtubule plus-ends for stable attachment to microtubules and for spindle integrity (Hsu and Toda, 2011). Recently, the microtubule polymerase activity of Dis1 was confirmed \textit{in vitro}, in addition to the discovery that it interacts with the +TIP Mal3 in a non-canonical manner (Matsuo et al., 2016). While it is accepted that cells lacking Dis1 function, via deletion or mutagenesis, are cold-sensitive and show chromosome non-disjunction, the nature of the spindle phenotype in these cells at the restrictive temperature is conflicting. The work that originally isolated \textit{dis1} as a cold-sensitive mutation reported spindle elongation in the absence of sister chromatid segregation (Ohkura et al., 1988) while more recent work suggests that the spindles remain short with non-disjunction of chromosomes in both \textit{dis1}\textDelta cells and in a temperature-sensitive \textit{ndc80} mutant that
cannot recruit Dis1 (Hsu and Toda, 2011). Little work has characterised the behaviour of viable \textit{dis1}\(\Delta\) cells at 36°C, which is the restrictive temperature for the mutants isolated in this study. Therefore, the first step in understanding the lethality of \textit{dis1}\(\Delta\) \textit{klp5}\(^{Q582P}\) cells was to characterise mitosis in \textit{dis1}\(\Delta\) cells at 36°C. To this end, as described previously, a \textit{dis1}\(\Delta\) strain expressing cen2-GFP Sad1-dsRed and mCherry-Atb2 was used to visualise chromosome and spindle behaviour.

Again, log-phase cultures were shifted up to 36°C for 40 minutes before time-lapse

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5_1.png}
\caption{Centromere and spindle behaviour in wild-type, Dis1 and kinesin-8 mutants and the double mutant}
\end{figure}

Time-lapse imaging of live cells expressing cen2-LacOp LacI-GFP, Sad1-dsRed and mCherry-Atb2 in a WT, \textit{dis1}\(\Delta\), \textit{klp5}\(\Delta\), \textit{klp5}\(^{Q582P}\) or \textit{dis1}\(\Delta\) \textit{klp5}\(^{Q582P}\) background at 36°C. Cells were observed every 2 minutes at 36°C. Asterisks indicate the last frame of metaphases and arrowheads highlight lagging chromatids Scale bar, 10 \(\mu\)m.
imaging. Representative images of the cells observed are presented in Figure 5.1.

Compared to wild-type cells, dis1Δ cells appeared to pause in a metaphase-like state for a prolonged period of time (Figure 5.1). Measurement of spindle lengths over time revealed a wide range of variation in the delay of the phase 2 to phase 3 transition (Figure 5.2A), but phase 3 appeared to proceed as normal. In contrast to kinesin-8 mutants, cells lacking Dis1 were able to maintain a period of constant spindle length at high temperature (Figure 5.1 and Figure 5.2A). Interestingly, metaphase spindles were only slightly elongated with a mean length of 2.35 ± 0.38 µm, compared to 2.06 ± 0.36 µm in wild-type cells (Figure 5.4B), indicating functional metaphase spindle length regulation. Chromosome congression appears normal in dis1Δ cells with cen2-GFP signals found at an average of 0.18 ± 0.20 µm away from the centre of the spindle, similar to the 0.22 ± 0.19 µm for wild-type cells (Figure 5.4C), suggesting that Dis1 is not required for proper chromosome alignment. However, the absence of Dis1 extends the time spent in pre-anaphase from an average of 6.71 ± 1.67 minutes to 13.38 ± 5.55 minutes (Figure 5.4D), which is likely due to defects in Ndc80-mediated kinetochore-microtubule attachment (Hsu and Toda, 2011). Interestingly, klp6Δ cells are delayed to a similar extent, spending 11.44 ± 3.13 minutes in pre-anaphase. Although anaphase onset is delayed due to problems with kinetochore-microtubule interaction, the absence of Dis1 has no effect on attachments once anaphase A is initiated, as the frequency of lagging chromatids is negligible (1/46 cells) (Figure 5.4E).

Recent findings from our group showed that a Dis1 mutant, defective in binding to its recently identified partner, the +TIP Mal3, increased phase 3 spindle elongation rates compared to wild-type cells (Matsuo et al., 2016). However, no such difference was observed in dis1Δ cells at high temperature in this study (Figure 5.3). Anaphase spindle lengths increase similarly in wild-type and dis1Δ cells (Figure 5.3A), with average spindle lengths in phase 3 almost completely overlapping (Figure 5.3B). Indeed, when the average spindle elongation rate during this period was calculated for each cell, there was no significant difference between elongation rates for wild-type and dis1Δ cells, with means of 0.91 ± 0.16 µm/min and 0.85 ± 0.14 µm/min, respectively (Figure 5.3C). The difference between these results and those for the
Figure 5.2 Spindle length progression and phenotype distribution
Quantification of mitotic parameters at 36˚C from time-lapse imaging presented in Figure 5.1. (A) Measurement of spindle lengths against time in mitosis. (B) Distribution of phenotypes in dis1Δ klp5Q582P cells.
Dis1-LAPA Mal3-interacting mutant can be accounted for by the differences in temperature used for each experiment. In this study, a high permissive temperature is used, whereas the 23°C condition used for the Dis1-LAPA mutant analysis (Matsuo et al., 2016) approaches that of the restrictive temperature for cold-sensitive dis1∆ cells (20-22°C) (Ohkura et al., 1988, Hsu and Toda, 2011). Moreover, there are likely other phenotypic distinctions between the deletion and this specific mutant alleles.

These data suggest that at a high permissive temperature for dis1∆ cells, spindle length regulation is largely unperturbed, except for a small but significant increase in the length of the metaphase. The absence of drastic change in spindle length during mitosis in cells lacking dis1∆ may be slightly surprising after recombinant
Dis1 can clearly promote both the growth and shrinkage of microtubules *in vitro* (Matsuo et al., 2016). This subtle change may occur as a result of reduced tension generation at kinetochore-microtubule attachments to oppose outward spindle forces or de-localisation of other factors involved in spindle length regulation, such as Dam1 (Hsu and Toda, 2011, Kakui et al., 2013). However, kinetochore-microtubule attachment takes a long time to be established but eventually proceeds with no effect on congression and the subsequent anaphase. These findings are in sharp contrast to the previous results seen for *dis1Δ* cells at the low restrictive temperature, where cells were seen to have elongated spindles (Ohkura et al., 1988), or short unstable spindles (Hsu and Toda, 2011), and in either case, proper chromosome segregation could not occur.

### 5.2 Two distinct phenotypes exist in *dis1Δ klp5Q582P* cells

After characterising the phenotype of *dis1Δ* cells at high temperature, we could properly analyse the mitotic defects in cells lacking both kinesin-8 and Dis1 function. Again, mitosis was observed in cells expressing cen2-GFP Sad1-dsRed mCherry-Atb2 after shifting up to 36˚C for 40 minutes prior to imaging, in parallel with the imaging performed for *dis1Δ* analysis. The following data were obtained from the same imaging experiment represented in Figure 5.1.

Spindle behaviour was highly variable in the double mutant and did not resemble the profile of any single mutant (Figure 5.2A). Some cells produced extremely long spindles immediately that appeared to have no phase of constant length, whilst some showed evidence of spindle collapse and others remained short for a long period of time before going into anaphase B. Overall, the three phases of fission yeast mitosis were not discernible. It was apparent that two major phenotypes existed in mitotic *dis1Δ klp5Q582P* cells (Figure 5.2B). In some cells, cen2-GFP signals segregated to opposite poles in anaphase and subsequent exit from mitosis was clear, with some evidence of a post-anaphae array (PAA) (Type 1, 38.8% of cells, see Figure 5.1), while the majority of cells appeared to keep cen2-GFP signals paired together indefinitely (Type 2, 61.2% of cells). Subsequently, the length profiles of *dis1Δ klp5Q582P* spindle were split based on the chromosome segregation phenotype and the cells were analysed separately (Figure 5.4A and
B. As the presence and timing of the metaphase-anaphase transition was clearly defined in type 1 cells, the characteristics of \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) cells could be compared to that of single mutants.

### 5.3 Type 1 \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) cells can progress through anaphase, though spindle stability is compromised

While type 1 \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) cells were able to progress through to anaphase, these mitoses were not normal. Type 1 cells appear to undergo a variable period of spindle elongation with frequent spindle shortening or collapse, followed by the onset of anaphase B with variable timing (Figure 5.4A), showing some similarity to \( \text{dis1}^\Delta \) cells (Figure 5.2A). Phase 3 appeared to be the only clear period for each cell’s spindle elongation profile, with no period of constant spindle length, similar to kinesin-8 mutants (see Figure 5.2A). In both cases, overall spindle length behaviour appeared to be greatly affected. The behaviour of chromosome events visualised by \( \text{cen2-GFP} \) was the first analysis performed in order to understand the lethality of \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) cells.

#### 5.3.1 Dis1 may partially contribute to congression defects in cells lacking functional kinesin-8

The \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) double mutant showed an intermediate chromosome congression phenotype, compared to each single mutant (Figure 5.4C). The deletion of Dis1 appeared to partially restore \( \text{cen2-GFP} \) alignment, measured by the mean displacement of \( \text{cen2-GFP} \) signals, from \( 0.99 \pm 0.48 \) \( \mu \text{m} \) away in \( \text{klp5}^{Q582P} \) cells to \( 0.47 \pm 0.35 \) \( \mu \text{m} \) in \( \text{dis1}^\Delta \ \text{klp5}^{Q582P} \) cells. The rescue activity of \( \text{dis1}^\Delta \) implies that Dis1 somehow partially and redundantly contributes to chromosome congression defects in \( \text{klp5}^{Q582P} \) cells, however, expanding the sample size may instill more confidence in characterising the congression phenotype, as some measurements here appear extreme.
Chapter 5. Kinesin-8 and Dis1 coordinate MT dynamics & KT-MT attachments

A. \( \text{dis}1\Delta \text{klp5}^{G582P} \) n = 14

B. \( \text{dis}1\Delta \text{klp5}^{G582P} \) n = 24

C. Graph showing the distance of cen2-GFP from spindle centre (μm)

D. Time spent pre-anaphase (min)

E. % Cells with lagging chromatid

<table>
<thead>
<tr>
<th>(min)</th>
<th>WT</th>
<th>dis1Δ</th>
<th>klp6Δ</th>
<th>klp5G582p</th>
<th>dis1Δ klp5G582p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.71</td>
<td>13.38</td>
<td>11.44</td>
<td>9.10</td>
<td>24.62</td>
</tr>
<tr>
<td>Median</td>
<td>6</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>
5.3.2 Anaphase onset is extremely delayed in dis1∆ klp5Q582P cells

All single mutants showed a delay in anaphase onset, attributed to activation of the SAC due to problems with kinetochore-microtubule attachment (Figure 5.4E). While deletion of either Dis1 or Klp6 leads to a similar duration of time spent in pre-anaphase (13.38 ± 5.55 minutes and 11.44 ± 3.13 minutes, respectively), the loss of both protein functions was additive, extending the mean duration to 24.62 ± 11.27 minutes. As seen in Figure 5.2A, a small number of cells show extreme delays before phase 3 spindle elongation takes place, which may have heavily affected the mean time spent in pre-anaphase. Therefore, using the median as a measure of central tendency may better reflect the average time spent in pre-anaphase by the majority of type 1 cells, in comparison to single mutants. The table in Figure 5.4D shows the mean and median values of time spent in pre-anaphase for all strains, which are extremely similar for each cell type except the double mutant. As expected, the median time spent in pre-anaphase is lower than the mean for dis1∆ klp5Q582P cells and likely represents the behaviour of the population of type 1 cells better. Even so, the median time delay in anaphase onset in dis1∆ klp5Q582P cells is the most extreme and is over double that of kinesin-8 deletion cells. The fact that the time dis1∆ klp5Q582P cells spend pre-anaphase is almost the
sum of the times spent by each individual mutant suggests that Dis1 and kinesin-8 each contribute to proper kinetochore-microtubule attachment in distinct ways.

5.3.3 The absence of Dis1 function has no effect on the frequency of lagging chromosomes caused by defective kinesin-8

Although congression defects in dis1Δ klp5Q582P cells are not as severe in those completely lacking only kinesin-8 (Figure 5.4C), type 1 double mutant cells exhibit the highest frequency of lagging sister chromatids in anaphase (Figure 5.4E). Half of the type 1 cells analysed had lagging chromatids, which although higher than the frequency for klp5Q582P cells (39%) and even klp6Δ cells (47%), was not significantly different to either of these single kinesin-8 mutants. It should be noted that in type 1 dis1Δ klp5Q582P cells, all anaphase events finally resulted in equal segregation of 1 cen2-GFP signal to each pole and subsequent daughter cell and mis-segregation was never observed.

Altogether, these data reveal the similarities and difference between type 1 dis1Δ klp5Q582P cells and each single mutant. The absence of Dis1 in klp5Q582P cells has little effect on metaphase spindle length or the frequency of lagging chromosomes, but does appear to increase the efficiency of sister chromatid centering on the spindle despite anaphase onset being substantially delayed. It should be noted that while the difference between the displacement of cen2-GFP from the spindle centre in klp5Q582P and dis1Δ klp5Q582P cells was determined to be statistically significant, the data for the double mutant are all completely within the range of that acquired for the single mutant. There is also more variation in the data collected for dis1Δ klp5Q582P cells, whereas dis1Δ behaves exactly like wild-type cells. Therefore, it may be difficult to draw accurate conclusions about chromosome congression, but the facts that anaphase onset is dramatically delayed and the frequency of lagging chromosomes implies spindle-kinetochore attachment defects.

5.4 Type 2 dis1Δ klp5Q582P cells exhibit continuous spindle elongation but not like that of phase 3 spindles

While mitosis progresses in type 1 cells, albeit abnormally, it appears that the majority of dis1Δ klp5Q582P cells cannot segregate the replicated pair of sister
chromatids at least for chromosome 2. These type 2 cells appear to undergo only spindle elongation from the beginning of mitosis, then some spindles undergo periods of extreme length fluctuation (Figure 5.4B). The fluctuations and decrease in spindle length reflect the events where the spindle reaches cell length or longer and collapses or snaps in response to this. It may be that spindles progress into the anaphase portion of mitosis, with or without chromosome segregation. In order to gain more information regarding the nature of the spindles in mutant cells, the length and behaviour data extracted from the cells expressing cen2-GFP Sad1-dsRed mCherry-Atb2 was analysed and compared across strains to try to identify defective parts of mitosis.

5.4.1 Maximum spindle lengths are similar across all cell types

From the data presented in Figure 5.4A and B, all cell types were able to produce spindles that neared cell length, an event characteristic of anaphase B. Indeed, extracting the maximum spindle length for all cells tested revealed a similar range of values, with the maximum lengths clustering in the region of 10-12 µm (Figure 5.5A). Therefore, spindles in type 2 dis1Δ klp5-Q582P cells were capable of reaching lengths found during anaphase B in other cells.

5.4.2 Spindle elongation dynamics differ between single and double mutants

Anaphase B usually displays the fastest rate of spindle elongation, while elongation during spindle formation slows as cells advance towards metaphase. Spindle elongation velocities were extracted from the length profiles by calculating the first derivative of each curve followed by light smoothing of the curves. Wild-type cells exhibited the pattern described above in these experiments at 36˚C (Figure 5.5B). Spindle elongation slows as cell length is approached, before disassembly, reflected in the negative gradients of the final elongation events. Cells impaired in kinesin-8 function (klp6Δ or klp5-Q582P) exhibited more variation in elongation velocities than wild-type cells, with the initial period of growth lasting for longer, but still appeared to be slowing. Both types of dis1Δ klp5-Q582P cells have varied initial elongation rates; some even exhibit increasing elongation velocities up until 10 minutes into mitosis, which was not seen in the other three cell types (Figure 5.5B).
Figure 5.5 Spindles in type 2 cells have a slower maximum elongation rate
Quantification of spindle behaviour in cells expressing cen2-LacOp LacI-GFP, Sad1-dsRed and mCherry-Atb2 at 36°C. (A) Maximum spindle lengths. Lines represent mean and standard deviation. (B) The first derivative of spindle length data presented in Figure 5.2A and Figure 5.4A, smoothed using 2 neighbours and 2nd order polynomial to obtain velocities of spindle length changes. x-axes represent time in minutes. (C) Maximum spindle elongation extracted from part A. (D) Time point in mitosis at which the maximum elongation rates plotted in part C were reached. For all parts, black lines represent mean and standard deviation. For parts C and D, statistical significance of difference was determined using a two-tailed Mann-Whitney test (****, p<0.0001).
However, assessment of maximum spindle elongation velocities found that type 2 $dis1\Delta klp5^{Q582P}$ cells actually reached a lower maximum velocity than the two single kinesin-8 mutants (Figure 5.5C). Notably, both single kinesin-8 mutants, $klp6\Delta$ and $klp5^{Q582P}$, had similar maximum velocities to those of wild-type spindles. This implies that the constant elongation seen in kinesin-8 mutants is not due to an active elongation rate increase in the absence of kinesin-8 function, but likely due to simple loss of negative regulation. Maximum elongation rates for type 1 cells fell within the range of both wild-type and kinesin-8 mutant cells as well as that seen for type 2 $dis1\Delta klp5^{Q582P}$ cells, but was more similar to that of the former cells, due to their common ability to enter phase 3 after anaphase onset.

The timings at which maximum elongation velocity occurred confirmed the maximum measurements for wild-type, $klp6\Delta$ and $klp5^{Q582P}$ cells were taken during anaphase (Figure 5.5D), which began after an average of approximately 6, 11 and 9 minutes, respectively (see figure Figure 5.4D). Conversely, the elongation velocity in type 2 double mutant cells peaked earlier in mitosis (Figure 5.5C). Due to the severe delay in anaphase onset in type 1 double mutant cells maximum elongation wasn't reached for a markedly longer time, implying that these elongating spindles are those of phase 3.

Overall, the dynamics of spindle behaviour measured in these experiments suggest that while spindles in type 2 $dis1\Delta klp5^{Q582P}$ cells can reach the same lengths seen in a normal anaphase B, their elongation begins at a higher rate but generally cannot reach the rates seen in anaphase B in control cells. Perturbation of kinesin-8 activity appears to only relieve negative regulation of spindle elongation, negating the possibility that another factor overcompensates for spindle elongation in its absence. However, the fact that elongation velocities in kinesin-8-compromised cells do appear to slow before phase 3 suggests that there is still some form of negative regulation of spindle length. This may be an active process performed by other MAPs or may be an inherent property of the spindle structure as it approaches metaphase. The presence of increasing early elongation rates in both type 1 and type 2 $dis1\Delta klp5^{Q582P}$ cells imparts candidacy for that factor on Dis1, as somehow responsible for suppression of these events in single kinesin-8 mutants.
5.5 Type 2 cells show non-disjunction of sister chromatids

As type 2 dis1Δ klp5Q582P cells cannot segregate at least cen2-GFP signals in the above experiments, the behaviour of all kinetochores was also assessed to understand if any segregation events were occurring. To this end, all three pairs of kinetochores were visualised using Nuf2-YFP, a component of the Ndc80 outer kinetochore complex (Nabetani et al., 2001, Liu et al., 2005), in wild-type, klp5Δ and dis1Δ klp5Q582P cells. Log-phase cultures which were also expressing Sid4-mRFP and mCherry-Atb2 were shifted up to 36°C for 40 minutes before time-lapse imaging was performed at 36°C. Images were acquired every 2 minutes for 1 hour. The following sections describe and present data from the same experiments using Nuf2-YFP cells but are presented separately for clarity.

Upon observation of the behaviour of all six kinetochores on replicated chromatids, the same two phenotypes were seen in dis1Δ klp5Q582P cells, where 37.9% of cells were of the type 1 category, and the remaining 62.1% of cells had the type 2 phenotype (Figure 5.6A). Remarkably, these cells exhibited almost the exact same distribution of phenotypes as those expressing only cen2-GFP as a chromosomal marker (see Figure 5.2). The similarity in these figures suggests that the non-disjunction of cen2-GFP seen previously in type 2 dis1Δ klp5Q582P cells also extends to the other two pairs of sister chromatids. Further dissection of the chromosome segregation phenotypes revealed two sub-categories within each main type (Figure 5.6A and B). The majority of type 1 cells showed proper segregation of sister chromatids to each pole, albeit with lagging chromosomes that eventually reached their destination SPB ('normal') (Figure 5.6C). A small population of type 1 cells also underwent equal segregation of Nuf2-YFP signals but at least one sister chromatid could not reach its destined pole and was as such a distinct entity in the cell ('loss'). Although these are not technically mis-segregation events, the presence of non-SPB-tethered chromosomes is likely to affect the subsequent round of chromosome segregation (Hou et al., 2012). Interestingly, type 2 cells demonstrated two different outcomes of unsegregated sister chromatids: all three pairs towards one SPB ('6:0'), or two pairs at one SPB with the remaining pair at the opposite pole ('4:2'). These two phenotypes are reminiscent of the non-disjunction phenotypes that
Chapter 5. Kinesin-8 and Dis1 coordinate MT dynamics & KT-MT attachments

Figure 5.6 Type 2 cells exhibit non-disjunction of sister chromatids
Analysis of chromosome fate in cells expressing Nuf2-YFP Sid4-mRFP mCherry-Atb2 at 36˚C (A) Distribution of phenotypes in dis1Δ klp5Q582P cells. (B) Further separation of type 1 and type 2 phenotypes each into two distinct categories. (C) Representative images of phenotypes in dis1Δ klp5Q582P cells. (D) Distribution of mitotic fates of type 2 dis1Δ klp5Q582P cells. (E) Representative time-lapse images of mitotic fates of type 2 cells. Time difference between each frame shown is 8 minutes for all three cell types. Scale bars, 10 µm.
characterised mutations in the cold-sensitive dis1-3 genes at their low restrictive temperature, according to one group (Ohkura et al., 1988, Nabeshima et al., 1995). In addition to mimicking this reported dis phenotype in terms of chromosome behaviour, both categories of type 2 cells also showed broken or disassembled spindles between the two SPBs (Figure 5.6C), similar to the dis mutants’ phenotype at restrictive temperature. Type 1 dis1Δ klp5^{Q582P} cells usually proceeded to exit mitosis and form two new daughter cells that appeared to proceed normally through the next cell cycle. In stark contrast, type 2 cells remained either as one arrested mitotic cell, or proceeded to ‘cut’, where septation or division occurs in the absence of chromosome segregation and proper mitotic exit, as was seen for the majority of type 2 dis1Δ klp5^{Q582P} cells (Figure 5.6D and E). These data are in accordance with the high septation index and asymmetric DNA staining observed for the original dis1Δ klp5^{CP76} mutant isolated (see Figure 3.5 and section 3.6). As proposed earlier, either category of type 2 cells is likely to give rise to aneuploid cells as a result of the ‘cut’ phenomenon and subsequent cell scission.

Although type 1 dis1Δ klp5^{Q582P} cells appear to undergo anaphase, these results uncover a minority of cells that are poised to lose genetic material and likely give rise to lethal aneuploid cells after the current division or the subsequent round. Moreover, the majority of dis1Δ klp5^{Q582P} cells cannot segregate any pair of chromosomes and undergo spindle breakage prior to frequent cutting of cells in the presence of mitotic catastrophe. These results also confirm the inability of type 2 cells to undergo anaphase A or B in terms of chromosome segregation.

5.6 Both Dis1 and kinesin-8 are required to slow spindle elongation before and during anaphase A

Results so far suggest that dis1Δ klp5^{Q582P} cells are lethal due to premature exit from mitosis in the absence of chromosome segregation (type 2 cells), with additional lethality arising from a small population of cells that exhibit a type of chromosome mis-segregation (type 1 cells). It is clear that in addition to chromosome segregation problems, spindle morphology is altered in dis1Δ klp5^{Q582P} cells, both of which phenomena are not necessarily mutually exclusive in origin and consequence. To understand the reasons why cells lacking Dis1 and
Figure 5.7 Time-lapse imaging of all kinetochores reveals striking differences among mutants
Time-lapse imaging of live cells expressing Nuf2-GFP, Sid4-mRFP and mCherry-Atb2 in a WT, klp5Δ or dis1Δ klp5Q582P background at 36°C. Images were acquired every 2 minutes for a total of 1 hour. Asterisks signify the last frame of metaphase while arrowheads mark the end of anaphase A. Scale bars, 10 µm.
kinesin-8 function terminate in the above phenotypes, chromosome and spindle events were studied in more detail in dis1Δ klp5Q582P cells, using the aforementioned data acquired from Nuf2-YFP Sid4-mRFP mCherry-Atb2 cells. Representative time-lapse images are displayed in Figure 5.7. Visualisation of all kinetochores not only allowed the assessment of the fate of each pair of sister

![Figure 5.8](image)

**Figure 5.8** Altered microtubule length profiles are still able to produce maximum spindles of similar lengths

Analysis of cells expressing Nuf2-YFP Sid4-mRFP mCherry-Atb2 at 36°C. (A) Quantification of spindle lengths against time in mitosis. (B) Maximum spindle lengths extracted from part A. The statistical significance of difference was determined using a two-tailed Mann-Whitney test against klp5Δ data (****, p<0.0001; ns, p=0.3770, 0.6217 for T1 and T2, respectively). T1 and T2 were also compared (ns, p=0.2956).
chromatids, but allowed finer temporal resolution of chromosome events in mitosis. For these studies, \textit{dis1}$\Delta$ \textit{klp5}^{Q582P} cells were compared to only wild-type and \textit{klp5}$\Delta$ cells. As demonstrated previously, \textit{dis1}$\Delta$ cells behave in a manner akin to wild-type cells in almost every aspect of mitosis at 36°C, aside from the extension of metaphase due to kinetochore-microtubule attachment defects. Cells expressing \textit{klp5}^{Q582P} phenocopy \textit{klp5}$\Delta$ in all aspects of kinesin-8 function due to the likely reduced functional population of kinesin-8 on the spindle. Thus, \textit{dis1}$\Delta$ and \textit{klp5}^{Q582P} cells were not included in the following analyses.

As before, spindle lengths were measured over time in mitosis, but in these experiments, larger sample sizes were obtained for analysis than those presented in Sections 5.3 and 5.4. With nearly twice as many cells for analyses for each double mutant phenotype, the differences in spindle morphology throughout mitosis was striking between the two types of \textit{dis1}$\Delta$ \textit{klp5}^{Q582P} cells (Figure 5.8A). Again, most type 1 cells underwent a varied period of spindle elongation with some incidences of collapse during the middle of mitosis, yet were capable of rapid elongation towards the end of mitosis. In contrast, type 2 cells tended to extend spindles to extreme lengths before fluctuations and shortening with no period of anaphase.

Additionally, when maximum spindle lengths were extracted from the length over time profiles, once again all mutants produced much longer spindles than wild-type cells (Figure 5.8B). Cells lacking Klp5 function and both types of double mutant cells exhibited an average maximum spindle length of 11-12 µm, whereas in wild-type cells it was approximately 9 µm, significantly shorter than in \textit{klp5}$\Delta$ cells. Both types of \textit{dis1}$\Delta$ \textit{klp5}^{Q582P} cells produced similar maximum spindle lengths to those of \textit{klp5}$\Delta$ cells, and to each other. These data reiterate the ability of type 2 cells to reach anaphase B spindle lengths even though chromosome events do not reach this stage.

The variation in both spindle lengths, elongation rates and the timing of transition between different spindle stages, excluding type 2 cells, poses difficulties in assessing the stage at which these problems arise. While wild-type cells show relatively clear transitions in spindle elongation velocities and their timing, mutant
cells do not. To better understand if specific spindle morphology or behaviour contributed to chromosome segregation defects, spindle elongation was assessed in each stage of mitosis. Spindle lengths were split into three periods, where visualisation of all three pairs of kinetochores allowed accurate assignment of each period of mitosis. Pre-anaphase was taken as time zero up until the ascribed metaphase frame; anaphase A started with metaphase as its assigned time zero up and was completed when all segregating Nuf2-YFP signals reached their respective poles; anaphase B was taken as the period from segregated kinetochores associating with poles until the last frame before the spindle clearly underwent disassembly. At least in mutant cells, even within these shorter periods that show more uniformity in behaviour compared to mitosis overall, there exists a substantial amount of variation within both the spindle lengths and elongation rates. To reduce the noisy effect of this variation on average elongation rates taken from the data, maximum elongation rates were extracted and used for comparison between strains.

**Figure 5.9 Comparison of spindle elongation velocities reveals common slowing in early mitosis**

Analysis of spindle behaviour in cells expressing Nuf2-YFP Sid4-mRFP mCherry-Atb2 at 36°C The first derivative of the spindle length data presented in Figure 5.8 was smoothed using 2 neighbours and 2nd order polynomial to obtain velocities of spindle length changes. x-axes represent time in minutes.
5.6.1 Dis1 and kinesin-8 slow pre-anaphase spindle elongation

While all cell types appeared to undergo some form of general slowing of spindle elongation from the beginning of mitosis (Figure 5.13), both single and double mutants add length to their pre-anaphase spindles at a significantly quicker rate than in wild-type cells (Figure 5.10A). The removal of Klp5 causes the maximum spindle elongation rate to jump up dramatically, from 0.22 ± 0.04 µm/min to 0.37 ± 0.06 µm/min. Interestingly, type 1 cells that lack both kinesin-8 and Dis1 function are able to produce a similar elongation rate, 0.37 ± 0.07 µm/min, to klp5Δ cells. Furthermore, type 2 cells show the highest values for maximum spindle elongation rate at this point in mitosis, where the average rate was 0.47 ± 0.10 µm/min, over double the average seen in wild-type cells and significantly higher than klp5Δ and type 1 cells (Figure 5.10A). The marked increase in spindle elongation rate is, temporally, the first distinction between type 1 and type 2 dis1Δ klp5Q582P cells. While it is generally accepted that kinesin-8 in fission yeast is required to slow pre-anaphase spindle elongation, it appears that in the absence or reduction of kinesin-8 function, Dis1 is also required to negatively regulate the spindle during the pre-anaphase period of elongation. This may be mediated by the generation of stable kinetochore-microtubule attachments to generate inward force, or could also be through interaction partners at the outer kinetochore or microtubule plus-end.

Alongside spindle elongation rates, the duration of each period of mitosis was also measured (Figure 5.10B). Reflecting similar results acquired in the cen2-GFP background (see Figure 5.4E), wild-type cells spent approximately 6 minutes pre-anaphase, significantly shorter than the 10 minutes calculated for klp5Δ cells. Without any extremely delayed type 1 dis1Δ klp5Q582P cells in this data set, the mean time these cells spent in pre-anaphase was closer to 15 minutes, but was still 50% longer than the kinesin-8 single mutant. As type 2 cells remained in a pre-anaphase state, in terms of chromosome segregations, the duration of this period could not be measured.

The fact that the establishment of a proper metaphase is substantially delayed in cells that reach at least the minimum appropriate length (Figure 5.10C) faster than usual suggests that achievement of a certain absolute spindle length is not
necessary for metaphase. Instead, it is likely a balance of forces across the spindle on both interpolar-microtubules and kinetochore-microtubules that contribute to proper metaphase configuration of wild-type cells. These data support proposals set forward by recent work that implicates the importance of metaphase configuration in the context of kinetochore-microtubule attachments and the subsequent anaphase period (Syrovatkina et al., 2013). Additionally, many mutants with elongated metaphase spindles are still able to undergo chromosome segregation as viable cells, as is also seen in this study.
The data presented in this section highlight a role for Dis1 in negative spindle length regulation during pre-anaphase elongation, at least in type 2 cells, as its removal exacerbates the spindle elongation phenotype seen in \textit{klp5}\textsuperscript{\textDelta} cells. Although maximum elongation rates have been compared here, the general trend in pre-anaphase spindle behaviour is an overall slowing of elongation, albeit varying between the different mutant cells. Thus, although higher maximum rates are reached in all mutant cell types that lack negative spindle regulation from kinesin-8, a mechanism remains in place to dampen spindle elongation as cells progress through mitosis. Indeed, without such a mechanism, pre-anaphase spindles would approach cell length due to its imposition of a physical barrier, however this is not the case.

5.6.2 Anaphase A relies on both Dis1 and kinesin-8 to actively maintain a constant spindle length

Anaphase A, in wild-type cells, involves the orchestration of the segregation of sister chromatids from their central metaphase position towards opposite SPBs while the spindle is maintained at a relatively constant length throughout. For this to happen in wild-type cells, spindle elongation seen in the pre-anaphase period must slow to a speed where there is no substantial increase in spindle length for a brief period. Indeed, in wild-type cells, the maximum spindle elongation rates shift towards zero (Figure 5.11A). The maximum rates produced in \textit{klp5}\textsuperscript{\textDelta} cells remain significantly higher than wild type, 0.43 ± 0.14 \(\mu\)m/min compared to 0.21 ± 0.12 \(\mu\)m/min, in a similar relationship that is seen during the pre-anaphase period (see Figure 5.10A), confirming continued spindle elongation during anaphase A in cells lacking kinesin-8 function. Interestingly, in contrast to pre-anaphase where type 1 \textit{dis1}\textsuperscript{\textDelta} \textit{klp5}\textsuperscript{Q582P} cells exhibit similar maximum elongation rates to \textit{klp5}\textsuperscript{\textDelta} cells, type 1 cells displayed significantly high elongation rates in anaphase, with an average value of 0.53 ± 0.14 \(\mu\)m/min. This difference between the single and double mutant infers that the negative regulation of spindle length by Dis1 extends to the period of chromosome segregation, in addition to early mitosis.
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A

Anaphase A spindle elongation rate (μm/min)

WT  klp5Δ  dis1Δ klp5\(^{G522R}\)

B

Duration of anaphase A (min)

WT  klp5Δ  dis1Δ klp5\(^{G522R}\) T1

C

% Cells with lagging chromatid

WT  klp5Δ  dis1Δ klp5\(^{G522R}\) T1

D

Segregation time (mins)

wild-type  klp5Δ  dis1Δ klp5\(^{G522R}\) T1

Anaphase A spindle length increase (μm)
A logical reason for the spindle to remain at a controlled length from metaphase through to anaphase A is to maintain a distance short enough for the rapid arrival of sister chromatids at their respective poles, but at a long enough length to ensure the replicated material is separated enough from each other. Indeed, wild-type cells take an average of 2-3 minutes to complete anaphase A (Figure 5.11B) on short spindles that undergo relatively little growth (Figure 5.11A). The duration of anaphase A increases markedly from wild-type to klp5Δ cells, lasting an average of 6-7 minutes. Removal of Dis1 from the system significantly prolongs the duration of anaphase A to 9-10 minutes. This pattern of increase in time to segregate sister chromatids reflects the pattern of the increasing maximum spindle elongation rates as more MAPs are removed from the spindle.

As the duration of anaphase A was dramatically extended in mutant cells, the frequency of lagging chromosomes was re-examined, where all kinetochores were visible by Nuf2-YFP (Figure 5.11C). Intriguingly, in both klp5Δ and type 1 dis1Δ klp5ΔQ582P cells, nearly every anaphase A period observed contained one or more lagging chromatids (91.7% and 100% of observations, respectively). The high rate of spindle elongation during this period may be responsible for the delay in the completion of chromosome segregation as the SPBs continue to move apart.

To address the relationship between spindle length changes and the time taken for sister chromatids to segregate on these spindles, the total spindle length increase during anaphase A was plotted against the time spent in this period (Figure 5.11D). Overall, there appeared to be a correlation between the two variables in all cell types. For both klp5Δ and type 1 dis1Δ klp5ΔQ582P cells, the spearman’s rank
coefficients of 0.737 and 0.606, respectively, signify a strong positive correlation between the increase in anaphase A spindle length and the time taken for chromosomes to segregate. The correlation coefficient for the same relationship in wild-type cells is slightly lower at 0.454, likely due to the smaller range of time values due to the limitations imposed by 2 minute intervals of time-lapse imaging, but it is still a moderate positive correlation. Taken together, the above data suggest that both Dis1 and kinesin-8 are required to negatively regulate spindle length during anaphase A, for the rapid and timely completion of chromosome segregation to prevent the appearance of lagging chromatids.

The results presented in this section uncover multiple facets of mitosis that are regulated by both kinesin-8 and Dis1. In addition to their established function in negatively regulating metaphase spindle length (Syrovatkina et al., 2013), kinesin-8 is also required to suppress pre-anaphase elongation, in a manner distinct to that during metaphase-anaphase A. The difference may lie in the nature of negative regulation. Pre-anaphase spindles may rely on kinesin-8 to induce the shortening of microtubules to limit outward sliding. This is in contrast to the force-balance required in metaphase through to anaphase A for tension generation and maintenance of spindle length appropriate for timely and high-fidelity chromosome segregation. It is also possible that the difference in pre-anaphase and anaphase A regulation of spindle length can be found in the population of microtubules under regulation by kinesin-8, i.e. interpolar microtubules versus kinetochore-microtubules. Further, we propose that lagging chromosomes in klp5Δ cells arise due to a combination of off-centre metaphase alignment and an elongating spindle during anaphase A. Laggards in these cells are not likely to be merotelic during anaphase A as the induction of lagging chromosomes has been found to reduce spindle elongation rates (Courtheoux et al., 2009, Pidoux et al., 2000), whereas klp5Δ cells actually have increased elongation rates, compared to wild-type cells.

5.7 Anaphase B is shorter and slower in dis1Δ klp5Q582P cells
due to the extension of anaphase A

The last section of mitosis analysed was anaphase B, where poles are pushed outwards by spindle elongation to move genetic material as far apart as possible
and to properly position the nuclei in the subsequent daughter cells. In accordance with previous observations (see Figure 5.5C), wild-type and klp5Δ cells showed similarity in maximum spindle elongation rates during anaphase B (Figure 5.12A). Intriguingly, the maximum elongation rates seen in type 1 dis1Δ klp5Q582P cells was, on average, lower than the other two cell types, although sample size was markedly lower. In addition, dis1Δ klp5Q582P cells spent the shortest amount of time in anaphase B, an average of only around 3 minutes (Figure 5.12C). Actually, in

**Figure 5.12 Anaphase B is compromised in dis1Δ klp5Q582P cells**
Analysis of cells expressing Nuf2-YFP Sid4-mRFP mCherry-Atb2 at 36°C. For parts A and B, lines represent mean and standard deviation and the statistical significance of difference was determined using a two-tailed Mann-Whitney test. (A) Maximum spindle elongation rates in anaphase B (*, p=0.0160; ns, p=0.9982 not significant). (B) Duration of anaphase B (****, p<0.0001; *, p=0.0362). (C) Frequency of cells exhibiting bending spindles (left) with examples of these spindles (right). Scale bar, 10 µm.
this case, wild-type cells exhibited the longest duration of anaphase B, spending an average of about 6 minutes in this phase, whereas in klp5Δ cells, like double mutants, anaphase B was reduced to about 3.5 minutes. While it is formally possible that mutants are exiting mitosis quicker and cutting short anaphase B, the fact that the duration of anaphase A is so prolonged in these cells suggests that chromosome movement to poles contributes more to the process of chromosome segregation than outward spindle sliding does. The small sample size analysed for anaphase B in dis1Δ klp5Q582P cells also supports the idea that the majority of anaphase activity occurs during anaphase A, as the event of Nuf2-YFP signals reaching SPBs frequently coincided with the last frame of mitosis. It is interesting to note that anaphase B displays slower spindle elongation in type 1 dis1Δ klp5Q582P cells, even though some cells do manage to enter this period after anaphase A.

During these analyses, we observed the appearance of bending spindles at the end of mitosis in klp5Δ and dis1Δ klp5Q582P cells (Figure 5.12C). Spindle buckling or bending is a result of spindle length exceeding cell length, as is seen in budding yeast spindles when the kinesin-8 Kip3 is deleted (Rizk et al., 2014). The presence of bending spindles in the single and double mutant suggests that spindle elongation is somehow still not under proper negative control at this point in mitosis, even though the elongation rate is similar to or lower than in wild-type cells.

As both mutants have shorter durations of anaphase B, the extra-long, bending spindles are not due to cells simply spending longer to elongate their anaphase B spindles. On the other hand, the possibility exists that Dis1 is also required during anaphase B, however its localisation on the SPB-associated kinetochores makes it difficult to imagine this situation (Nakaseko et al., 2001). The observation of extremely long spindles in the absence of increased anaphase B elongation rate in both single and double mutants implies that kinesin-8 and Dis1 may not play direct roles in anaphase B themselves. Rather, it may be that the changes their absence bring about, during early mitosis and anaphase A, have a knock-on effect on other players in anaphase B, such as the microtubule cross-linker Ase1 or the kinesin-6 Klp9, that contribute to the bending of spindles.
5.8 Anaphase A and B are still distinct in cells lacking kinesin-8 function

As cells lacking kinesin-8 appear to be able to maintain high rates of elongation throughout all periods of mitosis, it is possible that there is no distinction between the separate phases in terms of spindle length regulation. To assess this possibility, spindle elongation rates for each portion of mitosis were compared within each cell type (Figure 5.13). In these analyses, wild-type cells clearly displayed the classical behaviour for each distinct phase in mitosis. A small but significant drop in the maximum spindle elongation rate, from an average of 0.22 ± 0.04 μm/min to 0.15 ± 0.11 μm/min, distinguishes metaphase and anaphase A spindles from those of pre-anaphase. Following that, a huge increase in elongation rate is implemented for anaphase B spindle extension, where maximum elongation rates reach an average of 0.58 ± 0.08 μm/min (Figure 5.13A), likely driven by the recruitment of Klp9 (Fu et al., 2009). In sharp contrast to this pattern of transitions, klp5Δ cells actually show a small increase in maximum elongation rate from pre-anaphase (0.37 ± 0.06 μm/min) to anaphase A (0.43 ± 0.14 μm/min) (Figure 5.13B). The fact that spindle elongation rate is not continuous between these two periods of mitosis implies that kinesin-8 is not only required to negatively regulate metaphase spindle length but to reduce pre-anaphase elongation as well. It follows that these roles performed by kinesin-8 before anaphase and during anaphase A are therefore distinct. It may be that kinesin-8 function in early mitosis is simply to limit elongation through interpolar microtubule destabilisation, while in metaphase and anaphase A, kinesin-8 directly antagonises the outward force produced by other MAPs and to balance forces in the spindle, as has been previously suggested (Syrovatkina et al., 2013). Additionally, a clear increase is observed between the maximum spindle elongation rates from anaphase A to anaphase B, where the average rate is 0.54 ± 0.19 μm/min, which as discussed previously, is similar to that of wild-type. These data not only contribute to the evidence that kinesin-8 do not function in anaphase B, but also reveal that anaphase B does not begin before anaphase A in cells lacking kinesin-8, contrary to previous suggestions (West et al., 2002), as elongation rate speeds up like wild-type when these cells enter anaphase B. From this, it can be proposed that the elongating spindles during chromosome segregation in cells
lacking kinesin-8 are still technically anaphase A, and that chromosome segregation does not finish during elongation as poles are still moving apart.

5.8.1 Anaphase B spindle elongation is impaired in \textit{dis1}\Delta \textit{klp5}^{Q582P} cells

Following the data presented above, where anaphase A spindle elongation in \textit{dis1}\Delta \textit{klp5}^{Q582P} cells is more rapid than in the corresponding period in wild-type cells, anaphase A spindle elongation is also significantly faster than the preceding period of early mitosis in these cells (Figure 5.13C). The rise in elongation rate between these two periods in these cells is more extreme than the corresponding jump seen.
in \textit{klp5}\Delta cells (see part B), confirming a role for Dis1 in the maintenance of anaphase A spindle length. This role may involve inward tension generation at kinetochores to oppose outward force. Surprisingly, even though spindles can reach longer maximum lengths than wild-type cells can (see Figure 5.8B), the velocity of anaphase B spindle elongation actually drops from anaphase A in \textit{dis1}\Delta \textit{klp5}^{Q582P} cells (Figure 5.13B). As this period of mitosis is markedly shorter in double mutants (see Figure 5.12B) it may be that these cells simply do not have time to reach maximum elongation velocity as the majority of spindle elongation and chromosome segregation has already occurred during the aberrant anaphase A. Again, the altered spindle morphology in \textit{dis1}\Delta \textit{klp5}^{Q582P} cells may occlude the proper function of other MAPs in anaphase B. It may be the Klp9 recruitment occurs and functions enough to elongate already long anaphase A spindles to bend within the confines of the cell, but there is not enough time to reach the same elongation velocities as seen in wild-type or \textit{klp5}\Delta cells.

Regarding their activity in anaphase B, we confirm that kinesin-8 does not contribute to spindle elongation directly, but the presence of bending spindles that exceed the length of the cells suggests that kinesin-8 play some part at this point in mitosis. The idea that kinesin-8 somehow regulates late spindle length is not unreasonable, in that the microtubule-depolymerising, but not anti-parallel sliding activity of Kip3 is required to prevent long, buckling spindles in budding yeast (Rizk et al., 2014). Intriguingly, we characterise Dis1 as essential for negative regulation of both pre-anaphase and anaphase spindle elongation rates in the absence of kinesin-8 function, as both phenotypes in \textit{klp5}\Delta cells are exacerbated in \textit{dis1}\Delta \textit{klp5}^{Q582P} cells. In some of these cells that can trigger a metaphase-anaphase transition, chromosome segregation is slow with lagging chromatids on elongating spindles, with occasional loss of some genetic material. The majority of \textit{dis1}\Delta \textit{klp5}^{Q582P} cells cannot undergo anaphase and show extreme spindle elongation from the start of mitosis until cell length, or longer, is reached. These data suggest that an upper limit for spindle length may exist, to ensure proper establishment of metaphase and the subsequent onset of a viable anaphase A, where type 1 cells are within this limit, yet type 2 cells exceed it. Additionally, type 1 cells show a delay in anaphase onset that is significantly longer than the delay seen in \textit{klp5}\Delta cells, implying that different aspects of kinetochore attachment are performed by
Dis1 and kinesin-8. Therefore, the synthetic lethality between Klp5/Klp6 and Dis1 arises from the loss of the functional commonality in negative regulation of spindle length in early mitosis for proper kinetochore-microtubule attachment and spindle tension in metaphase and a timely and faithful anaphase.

5.9 Premature spindle elongation in kinesin-8 mutants is not driven by the kinesin-6 Klp9

As discussed above, the long spindles in type 2 dis1Δ klp5Q582P cells can reach lengths like that of anaphase B spindles in viable cells. If long spindles in the double mutants were in fact anaphase B, this would signify progression of the spindle through mitosis in the absence of segregation. To clarify whether spindles were in fact behaving morphologically like anaphase B spindles, the localisation of the kinesin-6 family motor, Klp9, was studied. Klp9 is recruited from the nucleoplasm to the spindle midzone only during anaphase and is required to drive spindle elongation during anaphase B (Fu et al., 2009).

The recruitment of Klp9-YFP was followed in cells also expressing the H3 histone marker Hht1-CFP in addition to mCherry-Atb2, after cells were shifted up to 36°C for 40 minutes before time-lapse imaging. Representative images are presented in Figure 5.14A. In wild-type and klp5Δ cells, Klp9-YFP was clearly recruited to the spindle as chromatin underwent segregation, followed by spindle elongation. It was also seen to become restricted to the spindle midzone. While Klp9-YFP also localised to anaphase spindles in the minority of dis1Δ klp5Q582P cells that did undergo chromosome segregation (Figure 5.14B, 31%), the initial recruitment sites were not central, unlike in wild-type and klp5Δ cells. This pattern of localisation implies that the spindle midzone may not be central in these cells. In all mutants that underwent chromosome segregation, Klp9-YFP only moved from the nucleoplasm to the spindle midzone when spindles were substantially long. Anaphase spindle lengths at Klp9-YFP recruitment were much longer in the mutants compared to wild-
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Figure 5.14 Klp9 is not responsible for prematurely long spindles
(Figure on previous page). Analysis of cells expressing Klp9-YFP Hht1-CFP and mCherry-Atb2 at 36˚C. (A) Representative time-lapse images of recruitment of Klp9-YFP to anaphase spindles. The second frame shows the recruitment event with one time point before and one time-point after, where imaging intervals are 2 minutes. Both the merged channels (top) and YFP channel (bottom) are shown for clarity. T1, type 1. Scale bar, 10 µm. (B) Distribution of phenotypes in dis1∆ klp5Q582P cells. (C) Measurements of spindle length when Klp9-YFP is first recruited. Lines represent mean and standard deviation. The statistical significance of difference was determined using a two-tailed Mann-Whitney test compared to the result for klp5Δ cells (****, p<0.0001; ns, p=0.9615, not significant). (D) Quantification of the state of chromosome segregation at the time of Klp9-YFP recruitment to the spindle. (E) Timing of assigned metaphase compared with timing of Klp9-YFP recruitment within each cell type. The statistical significance of difference was determined using a two-tailed Mann-Whitney test (****, p<0.0001).

Type cells (Figure 5.14C). Although the length at which Klp9 was recruited appeared to be similar between klp5Δ and type 1 dis1Δ klp5Q582P cells, this may be an artefact of the small sample size for the double mutant as anaphase B was rare in these cells and anaphase A spindle prior to this elongate the fastest of all cell types. The fact that spindles are already long when Klp9 moves to the spindle midzone suggests that Klp9 is not the factor that mediates the aberrant spindle elongation. This notion is in accordance with recent findings that Klp9 is not seen to be recruited to the spindle before anaphase begins, even in cells compromised in bipolar spindle assembly (Yukawa et al., 2017, Rincon et al., 2017). These data reflect the previous finding that the fastest spindle elongations are similar among wild-type and kinesin-8 single mutants, as this is when Klp9 is recruited in late mitosis to drive rapid spindle elongation (Figure 5.5C). Assessment of the anaphase timing of Klp9-YFP localisation to the spindle revealed that in all cell types, Klp9-YFP could be recruited to spindles that were undergoing anaphase A, in addition to those in which chromosomes had already reached their destination poles (Figure 5.14D). The fact that Klp9 appears to be recruited earlier than anaphase B conflicts with the data presented in section 5.6, where anaphase B spindle elongation is faster than that of anaphase B, at least in wild-type and klp5Δ cells (see Figure 5.13). One possible explanation for this counter-intuitive observation may be that Klp9 is in fact recruited first and its sliding activity activated subsequently, only in anaphase B. Indeed, recent work found that the Klp9 plays a
role prior to anaphase B, where it is required to coordinate the onset of anaphase A, through distinct activities of chromosome passenger complex (CPC) localisation and microtubule sliding, respectively (Meadows et al., 2017).

Remarkably, in the majority population of type 2 dis1Δ klp5Q582P cells that enter mitosis (Figure 5.14B, 69%), Klp9-YFP was never observed at any location on the spindle. In these cells, spindles continued to elongate in the absence of chromosome segregation and Klp9-YFP remained in the nucleoplasm for a prolonged period of mitosis (Figure 5.15). As Klp9 does not appear to localise to these long spindles, normally only seen in anaphase B (see Figure 5.5A), these spindles in dis1Δ klp5Q582P cells are not those of anaphase B, suggesting that the spindle has not received signals to undergo changes yet. Therefore, the period of constant increase in spindle length seen at the beginning of mitosis in these type 2 cells is not due to Klp9 function and as such relies on early mitotic factors. In addition, the inability for these spindles to undergo chromosome segregation and the absence of anaphase spindles suggest that although mitosis is abnormal, mitotic chromosome and spindle events are still coupled in dis1Δ klp5Q582P cells.
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5.10 The establishment of bipolar kinetochore attachments is severely delayed in type 1 cells and absent from type 2 cells

5.10.1 Mad2 persists at kinetochores in cut dis1Δ klp5Q582P cells

The fact that type 2 dis1Δ klp5Q582P cells appear unable to undergo the transition from metaphase to anaphase, due to serious problems with spindle behaviour and kinetochore-microtubule attachments, suggests an arrest in a pre-anaphase state by activation of the SAC. However, these type 2 cells are frequently able to septate and even divide as part of the observed cut phenotype. To understand the nature of SAC activation in this situation, double mutant cells were assessed for the presence of Mad2-GFP during mitosis in cells also expressing Sid4-mRFP and mCherry-Atb2. Log-phase cultures were shifted up to 36°C for 40 minutes before

Figure 5.16 Mad2-GFP persists on unsegregated kinetochores through septation
Analysis of cells expressing Mad2-GFP Sid4-mRFP and mCherry-Atb2 at 36°C. (A) Distribution of phenotypes in dis1Δ klp5Q582P cells in terms of Mad2-GFP disappearance (-Mad2) or persistence (+Mad2). (B) Representative images of Mad2-GFP signals during cell division and septation. Images are presented in 12 minute intervals. Asterisks denote a septation event. Scale bar, 10 μm. (C) Quantification of the fate of type 2 cells.
time-lapse imaging at 36°C.

The distribution of type 1 (33.8%) and type 2 (66.2%) cells was similar to that found in previous experiments in this study, and reflected the mitotic phenotype (Figure 5.16A). Consistent with their ability to undergo anaphase, Mad2-GFP was observed disappearing from the spindle in all type 1 cells, (Figure 5.16B, type 1), but again Mad2-GFP localised to SPBs as previously noted (Ikui et al., 2002, Mayer et al., 2006). In contrast, Mad2-GFP foci as multiple distinct dots in all type 2 cells was extremely prolonged (Figure 5.16B, type 2), indicative of an activated checkpoint at kinetochores. Approximately half the population of type 2 cells underwent septation (Figure 5.16C), and remarkably, were able to retain Mad2-GFP on kinetochores during this process (Figure 5.16B, septated). The signals moved as pairs on the broken spindle, identifying them as actively kinetochore-localising signals rather than the residual SPB-localising events seen sometimes in late mitosis (Ikui et al., 2002, Mayer et al., 2006).

These data suggest that kinetochore-attachments in type 2 dis1Δ klp5<sup>Q582P</sup> cells cannot be corrected for anaphase onset and so remain unattached on an elongating spindle. Attachment defects likely arise due to the loss of the distinct activities of Dis1 and kinesin-8 at the kinetochore-microtubule interface, in conjunction with changes in kinetochore-microtubule dynamics. Additionally, the perseverance of active SAC signalling while the septation initiation network is clearly active may allude to an uncoupling of chromosome events with anaphase onset and cytokinesis signalling. In normal cells, the septation initiation is triggered when Cdc2 is inactivated by APC/C-mediated degradation of its mitotic cyclin B partner, Cdc13 (He et al., 1997). The APC/C becomes active when the SAC is silenced and Mad2 at kinetochores can no longer generate an inhibitory signal against the APC/C. Therefore, the presence of Mad2 at kinetochores in septating cells leads to the logical but paradoxical notion that the APC/C remains inactive even though septation has been initiated.
5.10.2 Cdc13/Cyclin B appears to persist through septation in type 2 cells

The presence of sustained SAC signaling at kinetochores in septated cells has also been observed previously in dis1 and nuc2 mutant cells at their restrictive temperatures (Ikui et al., 2002). The fact that septation can occur in cells apparently maintaining a SAC-mediated ‘metaphase’ arrest seems counter-intuitive, as Cdc2 activity - tied to Cdc13 levels - is seen to remain high in arrested cells (He et al., 1997), where the APC/C is kept inactive, and as such should continue to inhibit septation. A subset of APC/C mutants were also found to both septate in the absence of chromosome segregation (Hirano et al., 1988, Yamashita et al., 1996) and retain Mad2 at kinetochores in septated cells (Ikui et al., 2002). Work using synchronized cells found that these mutants had a reduced activity in targeting Cdc13 for degradation by the APC/C. Therefore, Cdc13 levels fluctuated enough to reduce Cdc2 activity and hence allow septation to proceed (Chang et al., 2001). However, in dis1Δ klp5Q582P cells, we predict that APC/C function itself is not compromised, as no link has been found between kinesin-8 or Dis1 and the APC/C.

Following the observation that the SAC appears to persist through septation of cells with the cut phenotype, we questioned whether Cdc13 levels also remained high in these cells, reflecting the activity of Cdc2. Log-phase cultures of dis1Δ klp5Q582P cells expressing Cdc13-GFP Sid4-mRFP and mCherry-Atb2 were shifted up to 36°C before time-lapse imaging was performed at 36°C. In these Cdc13-GFP cells, the type 2 phenotype once again constitutes the majority of dis1Δ klp5Q582P cells (Figure 5.17A). Of these type 2 cells, approximately 50% underwent septation. Examination of septated cells where the presence or absence of a GFP signal could be confidently assigned (51/83) found that all septated cells carried Cdc13-GFP through septation (Figure 5.17B). This result is in accordance with the previous observation that Mad2-GFP remains on unattached kinetochores through septation in type 2 cells.
In some cases, the Cdc13-GFP signal in just over 10% of septated cells seemed to dim or disappear after cells had begun to septate (Figure 5.17B). Type 1 cells were clearly able to degrade Cdc13-GFP before proceeding to divide (Figure 5.17C, left panels), as seen by the loss of signal from the spindle and SPBs. Type 2 cells, however, could septate in the presence of seemingly high levels of the fluorescently-tagged cyclin B (Figure 5.17C, middle and right panels). 90% of these

![Figure 5.17](image)

**Figure 5.17 Cdc13-GFP is appears to remain through septation**
Analysis of disΔ klp5<sup>Q582P</sup> cells expressing Cdc13-GFP, Sid4-mRFP and mCherry-Atb2 at 36°C. Only the GFP channel is shown here. (A) Distribution of disΔ klp5<sup>Q582P</sup> phenotypes. (B) Quantification of Cdc13-GFP behaviour in type 2 cells after septation. (C) Representative time-lapse images of Cdc13-GFP in disΔ klp5<sup>Q582P</sup> cells. Brightfield images (left-hand sides) depict cell fate in terms of septation while the GFP channel (right-hand sides) shows Cdc13-GFP behaviour at the corresponding time point. White numbers represent time in minutes from first imaging frame shown. Images were acquired every 2 minutes but only a selection are presented here.
cells appeared to maintain a strong Cdc13-GFP signal for the observable period after the onset of septation, where localisation to one or both SPBs and short microtubules of the broken spindle remained bright. Interestingly, for some cells, as is seen between 0 and 24 minutes in the representative type 2 cell (Figure 5.17C, right panels), a reduction and subsequent increase in the brightness of Cdc13-GFP was discernible. This did not always follow septum formation, but when this was the case, it could represent the synthesis of new Cdc13 and reflect the potential to enter G1 as a diploid. A small population of these septated type 2 cells showed dimming of the previously clear cyclin signal, but only after the septum was clearly discernible (Figure 5.17C, middle panels).

The analysis of levels of Cdc13-GFP through septation is limited in this dataset, as slight variation in GFP intensity across the imaging field and photobleaching prevent accurate quantification of the signal. Despite these drawbacks, the results presented here are still useful and relevant. The retention of Cdc13-GFP in septated cells coupled with the persistence of Mad2-GFP at kinetochores suggests that Cdc2 is still active and the APC/C is not. These inferences are supported by the maintenance of chromosomes in dis1∆ klp5Q582P cells in a pre-anaphase state, where Cdc2 activity is normally high and the APC/C is actively inhibited by Mad2 as part of the mitotic checkpoint complex (MCC), yet septation frequently occurs in these cells. The situation here is distinct from that of previous work in APC/C mutants, where the mutated subunits were hypomorphic in terms of Cdc13 destruction. The APC/C mutants arrested in a pre-anaphase state and cells septated with an asymmetrically placed nucleus, just as in the majority of type 2 dis1∆ klp5Q582P cells, indicative of sufficient loss of APC/C function to prevent progression to anaphase. However, the hypomorphic mutants retained enough function to allow the APC/C to degrade some Cdc13, to a level low enough to allow SIN activation. Our preliminary data reveal this is not the case in dis1∆ klp5Q582P cells, where chromosomes remain unattached to microtubules and Cdc13-GFP appears remain bright through septation, suggestive of paradoxical SIN activation in the presence of active Cdc2 and inactive APC/C. Further experiments to accurately quantify the fluorescence or protein levels of Cdc13 at high temporal resolution would be required to confirm this idea.
5.11 Asymmetric spindle morphology gives rise to aberrant daughter nucleus distribution in type 2 dis1Δ klp5Q582P cells

The results so far suggest that major lethality in cells lacking both Dis1 and kinesin-8 function is caused by the inability of cells to enter anaphase due to unrestrained spindle elongation and compromised kinetochore-microtubule attachments. Observation of kinetochores revealed that cells septate with unsegregated chromosomes, giving rise to diploid daughter compartments (see section 5.5). The subsequent daughters of type 2 dis1Δ klp5Q582P cells either contain a whole (6:0) or partial (4:2) diploid complement of DNA, and in the case of the former, a cell without any DNA. Although the observation of these DNA patterns in mutant daughter cells has been reported previously for Dis1 (Ohkura et al., 1988) and Dam1 (Sanchez-Perez et al., 2005), how these patterns arise has not been explicitly addressed.

To understand how the unequal distribution of DNA arises in type 2 dis1Δ klp5Q582P cells, Cut11-GFP was used to visualise the nuclear membrane (West et al., 1998) in addition to Sid4-mRFP and mCherry-Atb2 spindle markers. Log-phase cultures were shifted up to 36˚C for 40 minutes before time-lapse imaging was carried out at 36˚C.

Type 1 double mutant cells displayed spindles longer than the nucleus before and during anaphase (Figure 5.18A). Cut11-GFP was seen to disappear from SPBs before nuclear division was apparent, although the spindle continued to elongate during this process, consistent with previous observations of lagging sister chromatids. In sharp contrast, both categories of type 2 cells showed extremely long spindles, approaching cell length, where the tethered section of the spindle was always opposite to that of the bulk of the nucleus. Thus, it seems that the constant elongating spindles of type 2 cells are able to displace the nucleus from the centre of the cell as mitosis progresses. The ability of type 1 cells to partially restrain spindle elongation in early mitosis, relative to type 2 cells, combined with their ability to satisfy the SAC allows equal nuclear division to occur. Quantification of the frequency of spindle protrusion phenotypes in dis1Δ klp5Q582P cells reveals that basically all exhibit spindles longer than nuclear diameter, regardless of them
Figure 5.18 Protrusion causes displacement of the nucleus and disassembly of spindles
Analysis of cells expressing Cut11-GFP Sid4-mRFP and mCherry-Atb2 at 36°C. (A) Representative time-lapse images of dis1Δ klp5Q582P phenotypes. Images were acquired every 2 minutes but intervals of 8 minutes are shown here. (B) Left - proportion of cellular phenotype in all dis1Δ klp5Q582P cells, categorised as a spindle within a round nucleus (Normal), an extremely long spindle pushing the SPB(s) completely outside of the nuclear periphery (Protrusion) or a marginally longer spindle that causes SPBs to sit slightly but clearly outside of nuclear periphery (Lemon). Right – representative cells for each phenotype. Scale bars, 10 µm.
having the type 1 or type 2 phenotype (Figure 5.18B). What is not clear from these data is the distinction between cells terminating with a 4:2 DNA distribution and those ending in 6:0, except perhaps a small difference in spindle length, where the latter cells display the longest spindles. An earlier experiment in this study (see Figure 5.6) revealed that the Coupling these results with the earlier observation that Klp9, when recruited during majority of type 2 cells (approximately 85%) terminate with the 6:0 pattern of chromosome positioning. Therefore, the 4:2 pattern may occur due to randomness in kinetochore-microtubule dynamics in the absence of two key regulators.

In the 6:0 example cell, the nuclear envelope visualised by Cut11-GFP appears to contain the spindle up until a point (Figure 5.18A, second frame) before the signal disappears from the protruding portion of the spindle. After this event, the spindle disappears between the two SPBs to leave one diploid nucleus and one free-floating SPB. Subsequently new microtubules appear in the cytoplasm, which may reflect the beginnings of a post-anaphase array.

anaphase in type 1 dis1Δ klp5<sup>Q582P</sup> cells (see Figure 5.14), appears to localise asymmetrically to the spindle, it is not unreasonable to suppose that spindle microtubules themselves display asymmetry. The loss of force balance within the spindle in these cells may give rise to an off centre or distorted midzone of interpolar microtubules, as well as the varied distributions of kinetochore-microtubule plus-ends. In turn, variations in microtubule length between spindle halves could perpetuate further asymmetry as the capacity for the binding of MAPs differs, which would have further uneven consequences on microtubule and spindle regulation. In this way, long asymmetric spindles could displace the nucleus and genetic material.

5.12 Destabilisation of the spindle partially rescues the temperature-sensitivity of dis1Δ klp5<sup>Q582P</sup> cells

To further understand what is happening in the aberrant long spindles of dis1Δ klp5<sup>Q582P</sup> cells, the involvement of other MAPs in generating the lethal phenotypes was assessed. Ase1 is a microtubule cross-linker involved in spindle stabilisation at the midzone (Yamashita et al., 2005, Loiodice et al., 2005) and its deletion has
been shown to counteract the effects of kinesin-8 absence on metaphase spindle length and lagging chromosomes (Syrovatkina et al., 2013). It is proposed that Ase1 passively stabilises outward pushing forces on the spindle while kinesin-8 actively generate inward forces, and together work antagonistically to produce a balance of force. Therefore, it may be involved in stabilising the rapidly elongating spindles in dis1Δ klp5Q582P cells. While neither single or double mutants of ase1Δ and klp5Δ were temperature sensitive, the removal of Ase1 function from dis1Δ klp5Q582P cells partially rescued the temperature-sensitive growth defects (Figure 5.19A). The growth of dis1Δ klp5Q582P cells improved from a sensitivity to 32°C to growth like wild-type cells at 34°C, although triple mutants were still incapable of growth at 36°C. This result implies that Ase1 may be partly responsible for the long spindles seen in cells lacking Dis1 and kinesin-8 function through stabilisation at midzones. It may be that another factor is required to counter the effects of dis1Δ in negative spindle length regulation.

Another MAP involved in the generation of outward spindle forces is the kinesin-5 family member, Cut7 (Hagan and Yanagida, 1992). The plus-end directed motor activity and the microtubule cross-linking ability of Cut7 allows this kinesin to control the outward sliding of antiparallel microtubules in early mitosis. For this reason, Cut7 is regarded as an active outward force generator in spindle dynamics and, like Ase1, the absence of its function in cells deleted for kinesin-8 contributes to a reduction in spindle length (Syrovatkina et al., 2013). This finding suggests that Cut7 may play a role in the production of extremely long pre-anaphase spindles in dis1Δ klp5Q582P cells. As Cut7 is an essential gene, the temperature-sensitive cut7-21 allele was utilised, as this causes the appearance of monopolar spindles in the absence of proper kinesin-5 function (Yukawa et al., 2015). In contrast to the results seen for the deletion of Ase1, cut7-21 dis1Δ klp5Q582P cells showed a very modest rescue of temperature-sensitive growth defects at 32°C, compared to double mutants retaining functional Cut7, but remained unable to grow at 34°C or higher (Figure 5.19B). Further, cut7-21 dis1Δ and cut7-21 klp5Q582P remain as temperature-sensitive as cut7-21 alone. This implies that the problems caused by loss of outward sliding in early mitosis by cut7-21 exceed those induced by excessive spindle growth, certainly in klp5Q582P cells, and the subtler problems in dis1Δ cells. The meagre nature of the rescue of temperature-sensitive growth
defects in dis1Δ klp5Q582P cells suggests that Cut7 plays only a minor role in the production of aberrantly long spindles, even though kinesin-5 is a major driver of early spindle formation and elongation.

Together, the results of these genetic analyses reveal that active microtubule sliding by Cut7 and more importantly, the stabilisation of these interdigitating interpolar microtubules by Ase1 are both partially responsible for the mitotic spindle defects seen in cells lacking Dis1 and kinesin-8 function. As both these proteins...
function on structural interpolar microtubules, these results confirm the involvement of this population of microtubules in generating the long spindle phenotypes. However, whether their involvement is direct or indirect remains to be addressed. Recent work has found that in the absence of the antagonism of Cut7 and Pkl1, the XMAP215 homologue Alp14/TOG is responsible for spindle elongation in early mitosis (Yukawa et al., 2017). Alp14 may be a factor in aberrant spindle elongation in dis1Δ klp5Q582P cells, but its synthetic lethality with both Dis1 and Kl5/Klp6 prevents straightforward genetic analysis of triple mutants. Subjecting Alp14 to repressible expression would allow the assessment of growth at high temperature in dis1Δ klp5Q582P cells but may be complicated by the temperature sensitivity of alp14 mutation or deletion.

5.13 Discussion

This chapter details the experiments performed in order to understand why cells die in the absence of Dis1 and kinesin-8 function. We first examined cells lacking Dis1 at high temperature and found mitotic events to mirror those of wild-type in most aspects, aside from a prolonged metaphase-like state and a minor increase in metaphase spindle length (Figure 5.2 and Figure 5.3). Imaging of the double mutant revealed that dis1Δ klp5Q582P cells undergo excessive spindle elongation and the majority of them are unable to coordinate these changes with chromosome segregation. Approximately one third of double mutant cells can pass through the metaphase-anaphase transition point after a significant delay in a pre-anaphase state, though continued increasing spindle length contributes to the emergence of lagging chromosomes that sometimes result in mis-segregation (Figure 5.11). In early to mid-mitosis, both populations of dis1Δ klp5Q582P cells display spindles that continue to elongate through the normal points of regulation at faster rates than in wild-type cells or those lacking kinesin-8 function (Figure 5.2 and Figure 5.8). Our data suggest that Dis1, directly or indirectly, is responsible for some negative regulation of the spindle elongation that precedes anaphase B, at least in the absence of kinesin-8 at a temperature that is permissive for dis1Δ. Careful examination of spindle elongation between the different periods of mitosis showed that in all mutant cells, pre-anaphase and anaphase A spindle elongation rates were extremely high (Figure 5.10, Figure 5.11 and Figure 5.13). Additionally,
anaphase B did not begin prior to the completion of anaphase A, negating the possibility of premature activation of rapid anaphase B spindle elongation in early mitosis (Figure 5.13). The observation that Klp9 was only recruited to already long spindles towards the end of anaphase A in klp5Δ and type 1 cells, and never in type 2 cells, clarifies that this rapid outward slider is not responsible for increased spindle elongation rates (Figure 5.15). Rather, dis1Δ klp5Q582P cells that can enter anaphase B actually exhibit a clear slowing of spindle elongation after anaphase A takes on most of the responsibilities of chromosome segregation (Figure 5.12). Genetic analyses did however implicate the midzone microtubule-bundling factor Ase1 in the lethality of dis1Δ klp5Q582P cells, and also the outward force generating Cut7 kinesin, although to a lesser extent (Figure 5.19).

The absence of both Dis1 and kinesin-8 function not only perturbs microtubule dynamics and structure for spindle length regulation but also disrupts normal mitotic chromosome movements and events (Figure 5.4). The SAC is activated and maintained at kinetochores in double mutant cells, indicative of improper kinetochore-microtubule attachments (Figure 5.16). The delay in anaphase onset in type 1 dis1Δ klp5Q582P cells is greater than that of dis1Δ and klp5Δ single mutants, revealing a dichotomy in the roles of these two kinetochore-interacting MAPs in stable bipolar attachments (Figure 5.4 and Figure 5.10). Despite this, type 1 dis1Δ klp5Q582P cells are eventually able to establish attachments that satisfy the SAC and proceed into anaphase with lagging chromatids on lengthening spindles (Figure 5.11). In stark contrast type 2 cells, where the spindle elongation rates are the most elevated, bypass chromosome segregation of any form and proceed to septate, even though the SAC looks to be active (Figure 5.16) and the APC/C remains apparently inhibited (Figure 5.17). These type 2 cells, in our relatively short time-lapse imaging, generally give rise to septated cells in which one compartment contains a diploid nucleus of unsegregated chromosomes while the other is anucleate (Figure 5.18). The subsequent behaviour of these cells remains to be determined. Thus, Dis1 and kinesin-8 work together to orchestrate the negative regulation of early spindle lengths for proper metaphase and faithful anaphase, and work synergistically to coordinate bipolar kinetochore attachments for a viable mitosis.
The characterisation of dis1Δ cells at a permissive temperature of 36°C revealed delayed anaphase onset with slightly elongated metaphase spindles. In contrast to cells lacking kinesin-8, the pre-anaphase spindles stayed relatively constant in length during this period and centromeres, at least those of chromosome 2, remained central on the spindle up until eventual anaphase onset. Therefore, at least at permissive temperature, Dis1 has a minor role in the negative regulation of metaphase spindle length and is required for the timely onset of anaphase. Previous work has shown that Mad2 is recruited to cells that cannot localise Dis1 to the outer kinetochore (using the temperature-sensitive ndc80-21 mutant) (Hsu and Toda, 2011) and that the viability of a dis1 mutant relies on the presence of Mad2 (George and Walworth, 2015). Consistently, Dis1 is required for stable end-on kinetochore-microtubule attachment via its localisation to the outer loop of Ndc80, physically connecting the Ncd80 complex to the plus-ends of kinetochore microtubules (Hsu and Toda, 2011). This work also highlighted the instability of spindles in ndc80-21 cells at high temperature (Hsu and Toda, 2011), which coupled with the recently confirmed microtubule polymerase activity of the Dis1/XMAP215 family member (Matsuo et al., 2016), implicates a loss of microtubule stability in the phenotype. As chromosomes congress properly for metaphase in dis1Δ cells, it is likely the loss of physical bridging of kinetochores and microtubule plus-ends, rather than gross changes in microtubule dynamics that brings about the delay in anaphase onset. This is not to say that the regulation of microtubule plus-end growth is not involved in generating stable-kinetochore interactions; Dis1 may manipulate this in a limited way to generate and maintain bipolar attachments, but that is not responsible for large changes in microtubule length, when kinesin-8 is present. Following this, the previously observed spindle instability and collapse may arise from the loss of inward tension normally mediated by sister chromatid cohesion and translated to the spindle by proper kinetochore-microtubule attachments. In this way, Dis1 may negatively regulate metaphase spindle length in an indirect manner through stable bipolar attachments generated by physically connecting kinetochores to microtubules and coupling this to plus-end microtubule dynamics. Additionally, Dis1 has been found to be involved in the stable localisation of Dam1 to kinetochore-microtubule interface in mitosis (Hsu and Toda, 2011) and meiosis (Kakui et al., 2013). The latter function is required to efficiently retrieve lost kinetochores in meiotic chromosome recapture assays by
microtubule depolymerisation and relies on Dam1 localisation (Kakui et al., 2013). Therefore, the ability of Dis1 to actively reduce spindle length through destabilising microtubules cannot be dismissed and there is mounting evidence that in the absence of free tubulin, Dis1/XMAP215 can destabilise GMPCPP-microtubules, where the slowly hydrolysing analogue causes the microtubule lattice to mimic the GTP-cap of plus ends (Brouhard et al., 2008, Roostalu et al., 2015, Matsuo et al., 2016). As specific conditions dictate the switch between polymerising and destabilising activity of recombinant Dis1, the conditions in vivo, such as the presence or absence of kinesin-8 or spindle length, may also influence the behaviour of Dis1 in microtubule regulation.

Two distinct chromosome segregation phenotypes arose in dis1Δ klp5<sup>582Q</sup> cells – type 1 cells that were able to go through anaphase and exit mitosis, and type 2 cells that exhibited non-disjunction of sister chromatids. However, both cell types exhibited problems in negative spindle length regulation, although to different degrees.

Type 1 cells displayed some version of each of the three classical spindle phases of mitosis along with the overall chromosome behaviour. In early mitosis, spindle elongation rates and the resultant metaphase spindle lengths are higher than in wild-type, matching those of singly deleted klpΔ cells. In both cell types, and also in unperturbed mitoses, the general trend in spindle elongation is overall negative as cells approach the metaphase-anaphase transition. Together with the observation that metaphase spindles were rarely as long as cell length suggests that a mechanism to slow spindle elongation in early mitosis is still in place in these cells. This process may be a property inherent to the spindle, in that the presence of sister chromatid cohesion could generate some inward tension even in cells with sub-optimal kinetochore-microtubule attachments, or the dilution of positive regulators of spindle length as spindles continue to elongate. Alternatively, certain MAPs may be involved in the slowing of these rapidly elongating spindles. For example, the kinesin-14, Pkl1, is required to antagonise the outward sliding of spindle microtubules in early mitosis. Pkl1 uses minus-end directed motility to slide antiparallel microtubules over each other to limit spindle elongation (Yukawa et al., 2015, Pidoux et al., 1996). Pkl1 activity in single and double mutants in this study
may account for the tendency of spindles to shorten regardless of the increased elongation rate. Dam1 has also been shown to play a part in reducing spindle length (Sanchez-Perez et al., 2005). The Dam1/DASH-complex component may transduce inward pulling forces, generated by kinesin-8, from microtubule plus-ends in a manner that is similar to sister chromatid cohesion (Syrovatkina et al., 2013). Synthetic lethality between Dam1 and kinesin-8 supports a common role in negative regulation of spindle length, which would remain partially active in dis1Δ klp5Q582P cells. However, Dam1 levels at the kinetochore-microtubule interface appear reduced in cells lacking Dis1 (Hsu and Toda, 2011, Kakui et al., 2013), throwing the possibility of residual Dam1 function in negative spindle length regulation into question. Overexpression of these factors in dis1Δ klp5Q582P cells may provide insight into their involvement in regulating elongation, where a positive result would take the form of rescue of the spindle length phenotype. It is in this pre-anaphase period of rapid elongation where the first differences between type 1 and 2 cells arise.

Maximum elongation rates at this stage are significantly higher in type 2 cells, implying differential spindle behaviour within the same genetic background. This distinction may result from differences in the levels of functional kinesin-8 complexes at microtubule plus-ends, as follows. The results of chapter 4 indicate that klp5Q582P is a loss-of-function allele and that cells have reduced, but detectable kinesin-8 function. It may be that in type 2 cells, there is very little kinesin-8 at their functional location at microtubule plus-ends, due to their reduced processivity and hence motility and so cells present the most severe defects cause by the absence of Dis1 and kinesin-8. On the other hand, in type 1 cells, kinesin-8 levels at plus-ends may be low enough to produce a stronger phenotype than seen in dis1Δ cells. As Dis1 appears to negatively regulate microtubules/spindle length when kinesin-8 is compromised, the combination of a sub-optimal population of kinesin-8 at and the absence of Dis1 from plus-ends produce a phenotype similar in severity to klp5Δ cells. Although this theory may explain the spindle length phenotype, it does not explicitly justify the additive nature of the time dis1Δ klp5Q582P cells spend pre-anaphase, compared to each single mutant. It may be that the kinetochore attachment, compromised by dis1Δ, is more sensitive to changes in kinesin-8 levels than negative spindle length regulation is.
Chapter 5. Kinesin-8 and Dis1 coordinate MT dynamics & KT-MT attachments

The time spent in pre-anaphase is significantly increased in type 1 cells, reflecting the additive effect of dis1Δ on stable kinetochore attachment. Indeed, Mad2 localises to these kinetochores and maintains the pre-anaphase delay for longer than in dis1Δ and klp5Δ cells, indicative of distinct roles for Dis1 and kinesin-8 in kinetochore-microtubule attachment. While Dis1 has been shown to connect Ndc80 with microtubule plus-ends to couple attachment and plus-end dynamics (Hsu and Toda, 2011), no detailed mechanism has been reported for kinesin-8.

Klp5 and Klp6 have been found to interact with PP1 for timely silencing of the activated SAC likely through dephosphorylation of targets of Aurora B which in turn stabilises correct attachments (Meadows et al., 2011). Results from Chapter 4 of this study implicated that this process is distinct from their role in producing proper kinetochore-microtubule attachment. Interestingly, Kif18A has been found to interact with PP1 for its own dephosphorylation, which in turn allows the motor to accumulate at the plus-ends of kinetochore-microtubules for effective chromosome congression by dampening of microtubule dynamics (Hafner et al., 2014). The temporal nature of PP1-binding to Klp5 and Klp6 remains to be determined, as does their dephosphorylation by this phosphatase. Chromosome recapture assays, where the delocalisation of sister chromatids from the SPB is induced and their relocation to the spindle is analysed, found that kinesin-8 is required to ‘reel in’ the lost chromatids by shortening the microtubules that search for and capture them (Gergely et al., 2016). Reeling events in the absence of kinesin-8 showed pausing, as if kinetochores were not properly attached to the microtubule recapturing it and subsequent biorientation was delayed in these cells, confirming the role of Klp5 and Klp6 in bipolar attachment, but leaving the mechanism elusive. While kinesin-8 have been shown to localise to the kinetochore in metaphase-arrested cells (Garcia et al., 2002b), via Alp7 interaction (Tang and Toda, 2015), it may be that in the presence of the spindle kinesin-8 establish proper attachments through manipulation of plus-end dynamics and length rather than physically connecting chromosomes and the spindle. Indeed, destabilisation of kinetochore-microtubules would generate tension (Garcia et al., 2002b), and induction of microtubule shrinkage in the conversion of lateral kinetochore attachments to end-on attachments would be consistent with observations of centromere retrieval in
elegant experiments in budding yeast (Tanaka et al., 2007). Chromosomes in cells lacking kinesin-8 have been seen to undergo large oscillations in metaphase, where Mad2 was recruited prior to each directional movement, indicative of detachment each time the direction switched (Mary et al., 2015). One could envisage that polymerising microtubules in the absence of kinesin-8 contribute to large movements of chromosomes attached to plus-ends of microtubules emanating from one pole. Loss of chromosomes from the spindle plane was never observed, so rapid attachment to the other pole must occur, of which laterally would be the quickest due to surface area of the lattice compared to microtubule plus-end. As the conversion of lateral attachments to end-on may be impaired in kinesin-8 cells due to an inability of the microtubule to rapidly shrink back to where the kinetochore is attached, the waiting chromosome may be picked up by another kinetochore-microtubule and continue to oscillate until bipolar end-on attachments can finally be established. In the absence of Dis1, end-on attachment is further delayed or impossible as Ndc80 cannot make stable microtubule contacts and track the dynamics of microtubule plus ends. In this way, amongst others, kinesin-8 may indirectly contribute to proper-kinetochore microtubule attachment by regulating plus end dynamics, which is essential in the absence of Dis1, however this remains to be assessed experimentally. A relationship between kinesin-8 Kip3 and XMAP215/TOG Stu2 in budding yeast has been observed where Kip3 transports Stu2 from the kinetochore in a lateral attachment to the plus end for microtubule growth (Gandhi et al., 2011) however the additive phenotypes of Dis1 and kinesin-8 and the constitutive kinetochore interaction of Dis1 suggest the story is different in fission yeast.

After satisfaction of the SAC, sister chromatid cohesion is alleviated and sister chromatids move towards opposite poles in a concerted manner on spindles that remains at a relatively similar length to that of metaphase. However, in klp5Δ and type 1 dis1Δ klp5Q582P cells, these anaphase A spindles continue to elongate. Chromosomes segregate due to the depolymerisation of the attached kinetochore-microtubules, rather than the action of minus-end directed motors walking chromosomes towards poles (Grishchuk and McIntosh, 2006). Remarkably, chromosomes remain attached to these shortening microtubules due to the ring-forming Dam1/DASH complex that acts as a collar to couple kinetochore
movement to microtubule shrinkage (Grishchuk et al., 2008). At least in budding yeast, multiple experiments show that this kinetochore tracking of plus-ends is mediated by the interaction of Dam1 with Ndc80 (Lampert et al., 2010, Kim et al., 2017). Recent work by our lab proposed that kinesin-8 play a part in the dynamics of poleward chromosome segregation, where reduction of Klp5-Klp6 at kinetochores by an alp7-LA6 mutant lead to slower segregation (Tang and Toda, 2015). This may reflect a requirement for kinesin-8 in sustaining the shrinkage of kinetochore-microtubules, in addition to triggering the initial event by inducing catastrophe. However, even though the coating of beads with recombinant truncated Klp6 construct appears to couple its movement to depolymerising microtubules in vitro (Grissonom et al., 2009), observations from our group of fluorescently-tagged recombinant full-length Klp5-Klp6 on dynamic microtubules saw no signals on depolymerising microtubule plus ends (Yuzy Matsuo and Takashi Toda, unpublished data). Additionally, these unpublished experiments saw no change in shrinkage rate between the presence and absence of Klp5-Klp6 on dynamic microtubules. However, the Klp5-Klp6 complex was capable of increasing catastrophe frequency, confirming its function in destabilising microtubules. The fact that chromosomes are still able to segregate to poles in the absence of kinesin-8 suggests that the residual microtubule depolymerisation, is sufficient to pull chromosomes to poles. What remains unclear is how the initial event is triggered, if not by induction of catastrophe, in cells lacking kinesin-8. Temporally higher resolution imaging, perhaps in conjunction with automated tracking of chromosome movements may provide more insight in the changes of segregation speeds.

Although the data presented in this study do not aid clarification of the role of kinesin-8 and Dis1 in anaphase movement of chromosomes, aside from this event proceeding in their absence, these data show a clear role for both of these factors in regulating anaphase A spindle length. While kinetochore-microtubules undergo depolymerisation and likely do not contribute to spindle length during this period, interpolar microtubules must be regulated to maintain a constant spindle length and be poised for the subsequent outward sliding in anaphase B. Photo-bleaching of phase 2 spindles in both fission yeast (Mallavarapu et al., 1999) and budding yeast (Higuchi and Uhlmann, 2005) uncovered the high dynamicity of these microtubules,
consistent with kinetochore-microtubules undergoing search and capture prior to anaphase onset. Once anaphase was triggered, a switch to stability in this population was observed. Stability in this case refers not to microtubules remaining the same length, but rather stably undergoing shrinkage for chromosome segregation. However, it is experimentally difficult to ascertain the behaviour of interpolar microtubules before and during anaphase A, as these overlap with kinetochore-microtubules and are likely less dynamic, preventing the assessment of their fluorescence recovery. Relatively little is known about the regulation of spindle length from metaphase through to the completion of anaphase A, with few mutants exhibiting an elongation phenotype as described here. Dynamic kinetochore-microtubules may play a part in overall spindle length but as they switch to a more stable, depolymerising state, are unlikely to be sites of active regulation in maintaining spindle length. This is likely to be especially true during chromosome segregation where kinetochore-microtubules no longer have a direction connection to the other pole through sister chromatids. Indeed, visual inspection of chromosome movements in type 1 dis1Δ klp5<sup>Q582P</sup> cells found that segregating sisters tended to move unidirectionally towards poles, suggesting that kinetochore-microtubules are only depolymerising, reducing the possibility that polymerisation of them could contribute to spindle elongation.

In this study, we observe a difference in elongation rates between pre-anaphase and anaphase A spindles in both klp5Δ and dis1Δ klp5<sup>Q582P</sup> cells, suggesting that the phenotype of increasing spindle length is slightly different in each phase. This is logical in that these two phases are normally extremely distinct from each other, where the former involves spindle elongation and the latter usually halts such processes. During spindle formation and elongation up until metaphase, where bipolar attachment of paired sister chromatids can generate inward tension, we propose that kinesin-8 negatively regulate microtubules to produce overall elongation of early spindles in potentially three ways, none of which are mutually exclusive. Firstly, Klp5-Klp6 is clearly restricted to the spindle midzone in anaphase B (West et al., 2002) but the possibility of this occurring in earlier mitosis cannot be disregarded. Therefore, kinesin-8 may induce catastrophe on or dampen interpolar microtubule-plus-ends to limiting both substrate and binding sites for outward forces like Cut7 and so slow spindle elongation. Secondly, a similar activity may
Chapter 5. Kinesin-8 and Dis1 coordinate MT dynamics & KT-MT attachments

occur on kinetochore-microtubule plus-ends that mediate outward pushing forces via sister chromatid cohesion against SPBs. Thirdly, the role of kinesin-8 in proper attachments, help to generate tension through stable attachments which also limits spindle elongation. The loss of Dis1 in addition to loss of the above activities in dis1Δ klp5Q582P cells further exacerbates the third point, causing greater increase in spindle length and delaying the establishment of stable bipolar kinetochore-microtubule attachments. Type 1 cells, with perhaps residual functional Klp5Q582P-Klp6 at plus-ends may be able to overcome these issues. In contrast, type 2 cells, where there are likely insufficient levels of the complex to exert its function, which may be cooperatively, attachments cannot be established, and even if they could the spindle is likely too long to promote efficient search and capture from both poles. Spindle length, although anaphase can be triggered at various spindle lengths, may not directly play a role in coordinating chromosome attachments, is thought to contribute to the process by limiting the distance plus-ends have to travel in order to find and capture kinetochores. As single dis1Δ mutants do not produce a strong spindle length phenotype at point during mitosis, the loss of kinesin-8 clearly sensitisises the spindle to further loss of inward force generators.

In anaphase A, both klp5Δ and dis1Δ klp5Q582P cells exhibit an increase in spindle elongation rate compared to that of pre-anaphase. As cohesin is cleaved and sister chromatids no longer connect spindle halves, there is likely a natural reduction in the levels of inward force as spindle halves are no longer connected. Therefore, anaphase A may rely primarily on the length regulation of interpolar microtubules by kinesin-8 to limit spindle growth. Subsequently, in both the mutant cell types aberrantly high spindle elongation rates may increase from those of early mitosis as a result of anaphase A being more reliant on interpolar-microtubule regulation in the absence of inward tension generation by chromosomes. It is difficult to resolve the role of Dis1 in this process as it segregates with chromosomes (Nakaseko et al., 2001). However, careful inspection of published images (Decottignies et al., 2001, Nakaseko et al., 2001) and unpublished image from our lab (Kuo-Shun Hsu’s thesis) reveals some fluorescence remains on the spindle when chromosomes segregate in cells expressing tagged Dis1 constructs. High resolution microscopy could confirm or dismiss this possibility. It should be noted that the depolymerase activity of Dis1 has only been reported for GMPCPP-microtubules in vitro under
certain conditions, including the absence of free tubulin. This is in contrast to anaphase A, where the shrinkage of kinetochore-microtubules releases tubulin. One explanation that does not rely on hitherto unknown roles of Dis1 is that if cells simply enter anaphase A with a spindle that is already long and undergoing rapid elongation, the alleviation of kinetochore tension may simply amplify this, as is seen in type 1 dis1Δ klp5Q582P cells. Nevertheless, we find that the roles of Dis1 and kinesin-8 and in negative spindle length regulation extend into anaphase A. It is possible that Klp5-Klp6 may regulate spindle length via kinetochore-microtubules up until metaphase and translocate to interpolar microtubules at the onset of anaphase, the transition of which may be mediated by something such as phosphorylation.

Anaphase B is the only period of mitosis in which spindle elongation rates are not increased in type 1 dis1Δ klp5Q582P cells. Remarkably, spindles appear to be growing at a slower rate than in wild-type or klp5Δ cells, even though all cell types are able to recruit Klp9 to mediate this process. Anaphase B is also seen to last for a much shorter time in double mutant cells, as anaphase A performs the majority of chromosome segregation on already long spindles. Currently, it cannot be formally disregarded that Dis1 and kinesin-8 play an active role in anaphase B elongation, indeed Klp5-Klp6 is seen to localise to the midzone at this point (West et al., 2002) and the budding yeast XMAP215 homologue Stu2 has been implicated in promoting spindle elongation in anaphase (Severin et al., 2001). It may simply be that elongation rate in anaphase B is limited in dis1Δ klp5Q582P cells as the space for length increase by Klp9 is less than that in other cell types as anaphase A spindles are much longer already. Therefore, steps of length increase would be shorter as spindle length approaches that of the cell, but not impossible, as spindles are seen to bend in klp5Δ and dis1Δ klp5Q582P cells. Consistent with this observation, Kip3 has been reported to function in spindle disassembly as budding yeast cells lacking the kinesin-8 homologue show long bent and buckled spindles (Rizk et al., 2014). Klp5-Klp6 may be required in late mitosis to destabilise microtubules for proper spindle morphology before mitotic exit.

Intriguingly, we find that type 2 dis1Δ klp5Q582P cells that are unable to undergo anaphase due to an inability to attach chromosomes to greatly elongated spindles
can eventually septate in while the SAC is activated. Spindles greatly distort the nuclear envelope and eventually cause it to break around the protruding portion of the spindle. After these events spindles were frequently seen to snap or disassemble between the two SPBs to leave one SPB alone now in the cytoplasm and one in the remnants of the nucleus, at least in cells with the 6:0 kinetochore pattern. Spindles may snap due to the decrease in local concentration of stabilising factors after the pro-spindle environment of the nucleus is lost. Coarse observation of Cdc13 found it to persist through septation in conjunction with Mad2 at unattached kinetochores. Septated cells usually displayed one diploid nucleus and an anucleate compartment that contained the other free SPB. The preference of chromosomes for the vicinity of one SPB in this process is interesting, considering that the spindle is thought to be symmetrical in terms of microtubules and their associated spindle factors. However, asymmetry may arise from the identity or age of the SPBs, where mother SPBs that are ‘experienced’ in mitosis may be better at capturing chromosomes at the onset of mitosis when they are initially released from their interphase configuration and this is maintained with little challenge. On the other hand, one-sided chromosome placement in these cells may result from an asymmetry in the spindle and the microtubules themselves, that is establish in the beginning of mitosis and becomes self-perpetuating as longer microtubules produce more binding sites for stabilising factors and so on. In some septating cells, the post-anaphase array is seen suggestive of some form of mitotic exit following activation of the SIN. It is difficult to reconcile the ability of these cells to maintain pre-anaphase signalling and potentially CDK activity yet septate and somehow exit mitosis. Higher temporal resolution may find a dip in Cdc13 levels sufficient to allow septation, but how this may be mediated remains a mystery in the absence of APC/C activity, unless the ubiquitin ligase activity can be activated while Mad2 localises to unattached kinetochores. It is interesting to note that in some cases, the diploid and anucleate compartments, either within the same septated cell or as two separate cells after division were seen to undergo a brightening of the Cdc13 signal and appear to show microtubule nucleation activity in the cytoplasm. This leads to the idea that these cells may be enter the subsequent G1 and S-phases of the cell cycle, even though Securin/Cut2 may still be present and cohesin still intact. DAPI staining of DNA appeared to show that chromosomes remain condensed but further investigation is required to understand the nature of these cells in terms of
DNA compaction, CDK activity levels and ability to perform cellular functions as a small diploid cell.
Chapter 6. Concluding remarks

The aim of this study was to learn more about the way kinesin-8, Klp5-Klp6 function in mitosis and how this role overlaps with that of an XMAP215/TOG microtubule polymerase, Dis1. To this end, temperature-sensitive mutants of klp5 were isolated in the absence of dis1. The major phenotype of all of these mutants was the non-disjunction of sister chromatids leading to a cut phenotype.

Subsequent analyses of one mutant in particular – klp5Q582P – revealed a role for the tail of Klp5 in intrinsic kinesin-8 function as seen by in vitro motility assay. klp5Q582P yielded moderate TBZ-resistance, increased metaphase spindle length, mild problems with chromosome congression, SAC activation and lagging chromosomes in anaphase. The nature of these phenotypes reflects those of klp5Δ cells but are less severe in strength, indicative of partial loss of kinesin-8 function, that was nearly completely restricted to high temperature. Further analysis of kinesin-8 and microtubule behaviour in vitro would confirm these findings and provide insight into the molecular mechanism of microtubule control by kinesin-8, which is much needed in a subclass that varies in function and molecular mechanism between species. Moreover, it would be extremely interesting and informative to observe the behaviour of dynamic microtubules in the presence of both kinesin-8 and Dis1 to see their interplay. Structural or biophysical studies would complement these experiments to look for any conformational changes in the mutant compared to the wild-type, which may allude to the nature of the auto-inhibitory mechanism. Direct comparison of microtubule-binding by cosedimentation assay would also support our study.

With the conditional klp5 mutant in hand, it was possible to study mitosis in the absence of both kinesin-8 and Dis1. Two phenotypes arose in dis1Δ klp5Q582P double mutant cells, where one population could transition to and complete anaphase (type 1) and one remained stuck pre-anaphase (type 2). The severity of mitotic defects in double mutant cells was additive in nearly every aspect. Both types of cells exhibited increased rates of spindle elongation in early mitosis: type 1 cells were able to overcome this eventually and produce bipolar kinetochore-
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microtubule attachments but type 2 cells could not and went on to cut with long spindles and asymmetrically placed diploid nuclei.

The experiments performed in this study reveal two major novel findings. The first is that fission yeast kinesin-8, like many other kinesins, including kinesin-8, relies on tail function for motility. Recombinant kinesin-8 composed of Klp5\textsuperscript{Q582P}-Klp6 exhibited a marked decrease in the number of processive runs on stabilised microtubules and behaved more diffusively. Additionally, the number of microtubule-binding events and amount of kinesin-8 on plus-ends, by visual inspection, appeared reduced, compared to the wild-type complex. These data point to a role for the tail of Klp5 to influence motility of the overall kinesin-8 complex. Proper tail function is required \textit{in vivo} for all functions of the Klp5-Klp6 function. It should be noted that Klp5\textsuperscript{Q582P} is not a rigor mutant, as these cannot hydrolyse ATP and so remain tightly bound to microtubules. This mutant must possess some ATPase activity to allow its dissociation from microtubules, unless, as is seen for other kinesins, the tail can directly prevent the motor region from interaction with microtubules.

The second key revelation from our data is the synergy between Dis1 and kinesin-8 for the negative regulation of microtubule dynamics in both early mitosis for metaphase spindle length and also in anaphase A, the latter of which has not been described before for either factor. This hitherto uncharacterised function of Dis1 may underpin the requirement of two XMAP215/TOG family proteins in a eukaryote that does not encode a kinesin-13. It would be interesting to assess the behaviour of cells lacking Alp14 and Klp5 function to see if this role for XMAP215/TOG is conserved between family members. Type 1 double mutant cells exhibit long metaphase spindles due to increased spindle elongation rates during early mitosis. Either residual kinesin-8 function or another process is able to prevent these spindles reaching cell length and slows their elongation slightly before anaphase A. On these long metaphase spindles, bipolar attachment of kinetochores is heavily delayed, more so than each single mutant, implying that Dis1 and kinesin-8 make distinct contributions to proper attachments. Imaging of chromosomes at a high frame rate in single and double mutants in kinetochore recapture assays would reveal the nature of attachments and how some cells can establish amphitely. High
frame rate imaging would also reveal more about chromosome oscillations to improve our understanding of congression in double mutants.

Type 2 \(dis1\Delta klp5^{Q582P}\) cells appeared to bypass any residual spindle length shortening and continue to elongate their spindles until the spindle broke due to limitations by cell size. This caused chromosomes to move asymmetrically to one side of the cell with SAC signalling, indicative of improper attachment to both poles. It may be useful to address the identity of the SPBs that non-disjoined chromosomes prefer to side with in these cells, if any. It may be that one SPB has a higher affinity for capture of kinetochores in early mitosis that is not overcome during this period in cells lacking Dis1 and kinesin-8 function.

In contrast to the long spindles in type 1 cells, these extreme lengths are too long for proper chromosome segregation. It is interesting to ponder if this inability to segregate chromosomes on spindles this long is due directly to changes in the ability of kinetochore-microtubules to capture chromosomes or if it is a pre-emptive move against anaphase on an exceedingly long spindle in which lost single sisters may be more detrimental to the cell than non-disjunction. Notably, there were some \(dis1\Delta klp5^{Q582P}\) cells that appeared to attempt to reform spindles after they snapped but the frequency was low and the fate of these cells was not captured within the imaging time. Observation of this population of cells may reveal that the latter hypothesis is true. Further, a search for mutants that could rescue the phenotype, either by overexpression or deletion, would shed light on exactly how these spindles can achieve such great lengths or if there is delocalisation of other MAPs that contribute to spindle length regulation or kinetochore-microtubule attachment in these circumstances.

Additionally, we find that lagging chromosomes in \(klp5\Delta\) and type 1 \(dis1\Delta klp5^{Q582P}\) cells are not due to problems in coupling anaphase kinetochore-microtubule attachments to depolymerising microtubules but is a result of continuous spindle elongation during anaphase A. Following this, it appears that cells are still able to trigger anaphase onset in the absence of both Dis1 and kinesin-8 function. This is not due to residual Klp5 function as \(klp5\Delta\) cells show the same phenotype and ability. Hence in the absence of two negative regulators of microtubules,
kinetochores and microtubules can still depolymerize, suggesting that either another factor is responsible for this or that this property is intrinsic to anaphase kinetochores.

While this study uncovers an autoregulation role for the Klp5 tail in the kinesin-8 complex and highlights the strict requirement for both kinetochore attachment and microtubule length regulation by Dis1 and kinesin-8 for viability, it sheds light on wider aspects of mitosis but also stimulates more questions, which is not a bad thing. Clearly, these MAPs are extremely important in the coordination of mitotic events for viable cell division in S. pombe and some aspects are likely conserved in human. However, it is becoming clearer that orthologs do not necessarily retain exact molecular mechanisms of function across species. The tight control of chromosome segregation is essential for the proliferation of normal healthy cells. Aneuploidy, which can arise from improper chromosome segregation is a hallmark of cancer and can give rise to other transformative events. Indeed, high levels of XMAP215/TOG and kinesin-8 expression are observed in some human malignancies, re-iterating their importance in high fidelity cell division. The fact that disruption of the fission yeast orthologs presents such a strong phenotype opens the door for potential translational experiments in this unicellular eukaryote, such as expression of human proteins in fission yeast and screening for inhibitors or ways to return cells to normal. Even if this is not possible, basic research on eukaryotic chromosome segregation lays a foundation for disease research and establishes a wealth of knowledge as a good reference point for new ventures in the investigation of how cells divide normally and abnormally.


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