Spindle assembly checkpoint-independent functions of Bub1 and Bub3 in fission yeast mitosis

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

A	Adenine (in media)
A	Alanine (in proteins)
A. nidulans	*Aspergillus nidulans*
APC/C	Anaphase promoting complex/Cyclosome
Ark1	Aurora related kinase 1
Bub	Budding uninhibited by benzimidazole
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CENP-E	Centromere associated protein E
ChIP	Chromatin immunoprecipitation
CPC	Chromosomal passenger complex
CPT	Camptothecin
cut	Cell untimely torn
D	Aspartate
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EMM	Edinburgh minimal medium
F	Phenylalanine
5-FOA	5-Fluoroorotic acid
G	Glycine
GFP	Green fluorescent protein
GLEBS	Gle2-binding sequence
H	Histidine
h	Hour
HP1	Heterochromatin protein 1
HU	Hydroxyurea
Ipl1	Increase in ploidy 1
K	Lysine
Klp	Kinesin-like protein
klpΔ	*klp5Δ*, *klp6Δ* and *klp5Δ klp6Δ*
L	Leucine (in proteins and media)
L	Liter
Mad	Mitotic arrest deficient

MBC	Carbendazim, methyl-2-benizimidazole carbamate
µg	Microgram
min	Minute
mL	Milliliter
µL	Microliter
MM	Minimal medium
Mps1	Monopolar spindle 1
N	NH_4Cl (in media)
N	Asparagine (in proteins)
P	Proline
PCR	Polymerase chain reaction
PEM	A buffer consisting of PIPES, EGTA and magnesium sulphate
PFA	Paraformaldehyde
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PP1	Protein phosphatase 1
Q	Glutamine
R	Arginine
RNAi	RNA interference
rpm	Rotations per minute
S	Serine
SAC	Spindle assembly checkpoint
Scc	Sister chromatid cohesion
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Synthetic dextrose
SDS	Sodium dodecyl sulfate
Sgo	Shugoshin
SIN	Septation initiation network
SPB	Spindle pole body
S. pombe	*Schizosaccharomyces pombe*
T	Threonine
TBZ	Thiabendazole
TPR	Tetratricopeptide repeat
Tris	Tris(hydroxymethyl)aminomethane
W	Tryptophane
wt	Wild type
Y	Tyrosine
YE	Yeast extract
YEA	Yeast extract supplemented with adenine

SUMMARY

To prevent errors in chromosome segregation, chromosomes have to achieve bi-orientation, i.e. the kinetochores of both sister chromatids have to attach to microtubules emanating from opposite spindle poles. The spindle assembly checkpoint (SAC) prevents anaphase onset as long as chromosomes are not correctly bi-oriented. In the fission yeast *Schizosaccharomyces pombe* and most other eukaryotic organisms studied, the core proteins of the SAC comprise Bub1, Bub3, Mad1, Mad2, Mad3 and Mph1 (Mps1 in other organisms). These proteins localize to kinetochores in early mitosis and are specifically enriched at malattached kinetochores, which is considered to be required for SAC signaling.

The SAC kinase Bub1 has also checkpoint-independent functions. Bub1 is known to recruit Sgo2 (shugoshin) to centromeres, which is required for accurate chromosome segregation. In addition, my genetic data indicated that Bub1 and its binding partner Bub3 share a function that is independent of Sgo2 and the SAC, but is presumably connected to regulation of microtubule dynamics.

Bub3 was previously believed to be one of the core proteins of the SAC, but we and others surprisingly found that it is not essential for SAC activity in fission yeast. Furthermore, Sgo2 localization is not completely abolished in *bub3Δ* cells, in contrast to *bub1Δ* cells. However, cells lacking Bub3 are sensitive to microtubule-destabilizing drugs and delay in unperturbed mitosis, indicating that Bub3 has a function in mitosis. I found that after release from conditions that cause disruption of spindles in early mitosis, *bub3Δ* cells show pronounced chromosome missegregation. In cells recovering from spindle disruption, unclustered chromosomes are usually captured by astral microtubules and retrieved to the spindle pole bodies (SPBs), before they get bi-oriented on the spindle. In the absence of Bub3 and in a Bub1 mutant lacking the region required for interaction with Bub3, chromosomes were retrieved but remained mono-oriented close to the SPB for a prolonged period. This defect in bi-orienting chromosomes on the spindle was not observed in cells lacking Sgo2, indicating that Bub1 and Bub3 share a function in bi-orienting chromosomes which is independent of Sgo2. Based on my results, I propose that Bub1 and Bub3 have an influence on microtubule dynamics at the kinetochore and are therefore required for establishing stable kinetochore microtubule attachment. This function is largely dispensable in normal unperturbed mitosis, but becomes essential when chromosome segregation is challenged under certain growth conditions or in a specific genetic background.

Even though Bub3 is not essential for SAC activity in fission yeast, it is nevertheless required for kinetochore localization of Mad1, Mad2, Bub1 and Mad3, indicating that – in contrast to the current model - enrichment of these proteins at unattached kinetochores is not essential for SAC signaling. This raises the questions of how the SAC signal is generated at the kinetochore, which I am currently still investigating and for which I present initial results here. As the checkpoint kinase Mph1 seems to localize even in the absence of Bub3, it is likely to be the key player at the kinetochore.

ZUSAMMENFASSUNG

Um Fehler bei der Chromosomensegregation zu vermeiden, müssen Chromosomen bi-orientiert werden, d. h. die Kinetochore beider Schwesterchromatiden müssen mit Mikrotubuli verknüpft werden, die von den entgegengesetzten Spindelpolen ausgehen. So lange die Chromosomen noch nicht korrekt bi-orientiert sind, wird das Einsetzen der Anaphase durch den 'Spindle Assembly Checkpoint' (SAC) verhindert. Bei der Spalthefe *Schizosaccharomyces pombe* und anderen untersuchten, eukaryotischen Organismen sind Bub1, Bub3, Mad1, Mad2, Mad3 and Mph1 (Mps1 in anderen Organismen) die zentralen Proteine des SACs. Diese Proteine lokalisieren in der frühen Mitose am Kinetochor und sind speziell an falsch an Mikrotubuli angebunden Kinetochoren angereichert, was als Voraussetzung für die Entstehung des SAC-Signals gesehen wird.

Die SAC-Kinase Bub1 hat zusätzlich Checkpoint-unabhängige Funktionen. Bub1 rekrutiert bekanntermaßen Sgo2 (shugoshin) zu den Zentromeren, was für akkurate Chromosomensegregation notwendig ist. Außerdem deuten meine Ergebnisse darauf hin, dass sich Bub1 mit seinem Interaktionspartner Bub3 eine Aufgabe teilt, die unabhängig von Sgo2 und dem SAC ist, aber vermutlich mit der Regulation der Mikrotubulidynamik zu tun hat.

Man glaubte bislang, dass Bub3 eine Hauptkomponente des SACs ist. Überraschenderweise fanden wir und andere Forschungsgruppen jedoch heraus, dass Bub3 in der Spalthefe nicht essentiell für SAC-Aktivität ist. Zudem war die Lokalisation von Sgo2 in *bub3Δ*-Zellen, im Gegensatz zu *bub1Δ*-Zellen, nicht komplett verhindert. Dennoch sind Zellen ohne Bub3 empfindlich gegenüber Mikrotubuli-destabilisierenden Substanzen und verbleiben länger in der Mitose, was darauf hindeutet, dass Bub3 andere mitotische Funktionen aufweist. Ich konnte zeigen, dass Missegregation von Chromosomen vermehrt auftritt, nachdem *bub3Δ*-Zellen aus Bedingungen entlassen wurden, die Spindelbildung in der frühen Mitose verhindern. In Zellen, die sich von solchen Bedingungen erholen, werden freie Chromosomen von astralen Mikrotubuli aufgegriffen und zurück zum Spindelpol transportiert, bevor sie auf der Spindel bi-orientiert werden. In Abwesenheit von Bub3 und in Bub1-Mutanten, die nicht mit Bub3 interagieren können, werden Chromosomen zwar zum Spindelpol zurückgebracht, aber dort verbleiben sie über längere Zeit in einem mono-orientierten Zustand. Dieser Defekt in der Bi-orientierung der Chromosomen auf der Spindel konnte in Zellen ohne Sgo2 nicht beobachtet werden, was darauf hindeutet, dass Bub1 und Bub3 unabhängig von Sgo2 eine Funktion in der Bi-orientierung der Chromosomen aufweisen. Basierend auf meinen Ergebnissen stelle ich die Vermutung auf, dass Bub1 and Bub3 die Mikrotubulidynamik am Kinetochor beeinflussen und daher für die Entstehung stabiler Kinetochore-Mikrotubuli-Verknüpfungen benötigt werden. Diese Funktion ist unter normalen Umständen in der Mitose nicht essentiell. Sie ist jedoch unabkömmlich wenn die Chromosomensegregation unter bestimmten Wachstumsbedingungen oder in einem spezifischen genetischen Hintergrund beeinträchtigt ist.

Obwohl Bub3 in der Spalthefe nicht essentiell für SAC-Aktivität ist, wird es trotzdem für die Kinetochor-Lokalisation von Mad1, Mad2, Bub1 und Mad3 benötigt, was darauf hindeutet, dass die

Anreicherung dieser Proteine am Kinetochor – im Widerspruch zur derzeitig akzeptierten Modellvorstellung – nicht essentiell für das SAC-Signal ist. Das wirft die Frage auf, wie das SAC-Signal am Kinetochor entsteht. Diesen Aspekt untersuche ich im Moment und präsentiere erste Ergebnisse in dieser Arbeit. Da die Checkpoint-Kinase Mph1 auch in Abwesenheit von Bub3 noch am Kinetochor zu lokalisieren scheint, ist es wahrscheinlich, dass sie die Hauptaufgabe in der Bildung des SAC-Signals am Kinetochor übernimmt.

1 Introduction

1.1 The cell cycle

During proliferative growth, cells pass through specific stages in a defined order, which – after cell division – are repeated by the daughter cells. This repetitive series of events is generally referred to as the 'cell cycle'. The cell cycle is divided into four phases: a gap phase (G_1), followed by DNA replication (S phase), a second gap phase (G_2) and M phase, which comprises nuclear division (mitosis) and subsequent cell division (cytokinesis). The period between M phases, comprising G_1, S, and G_2 phase, is termed interphase. Exceptions of the strict order of events exist. For example, during a brief period in *Drosophila melanogaster* embryonic development, nuclear divisions occur without subsequent cell division, forming a syncytium. 13 rounds of DNA replication are followed by nuclear division without cytokinesis and intervening gap phases (Morgan, 2007).

Cells of multicellular organisms can stop proliferating and after G_1 phase enter the so-called G_0 phase, a quiescent state. This can happen when cells are damaged and become senescent, or when cells are fully differentiated (Morgan, 2007).

In the G_1 phase, cells mainly take up nutrients and grow in size. Subsequently, the genetic material is replicated in S phase. To ensure accurate chromosome segregation later in mitosis, it is crucial that as soon as DNA is replicated, the two copies of each chromosome, called sister chromatids, remain in close association with each other, a task that is primarily carried out by a protein complex called cohesin. The G_2 phase spans the period from S phase to M phase and provides additional time for cell growth. In mitosis, the genetic material is distributed, and two daughter nuclei are formed (see chapter 1.3). Finally, cytokinesis separates the cytoplasm to form two daughter cells (Morgan, 2007).

The order and the timing of cell cycle events is regulated by a number of proteins, of which cyclin-dependent kinases (Cdk) and their activators (cyclins) are the key players. There are several different types of cyclins, which are expressed at different stages of the cell cycle, and - as their name suggests - their expression fluctuates. As a consequence, Cdk activity oscillates, even though the Cdk protein levels itself stay constant (Morgan, 2007). Cdk activity is low when cells enter G_1, and soon after it rises, cells progress through S phase, G_2 phase and M phase. At the very end of M phase, Cdk activity drops again (Morgan, 2007).

When cells proliferate, they have to ensure that the descendents receive all the components they need for survival. Thus, a cell must not start to divide before it has duplicated its set of chromosomes, its organelles and cytoplasmic components. To ensure that cells do not enter the following phase in the cell cycle unless prior events have been completed successfully, the cell cycle has several checkpoints, transition points at which progression of the cell cycle can be delayed by negative signals (Hartwell and Weinert, 1989; Li and Murray, 1991; Musacchio and Salmon, 2007). Per definition, checkpoint components are not essential in an unperturbed cell cycle, but become essential when damage is inflicted on cells either by the environment or by internal mishaps.

1.2 Fission yeast as a model organism

Work with the fission yeast *Schizosaccharomyces pombe (S. pombe)* as a model organism started when Urs Leupold initiated genetic studies in the middle of the last century (Egel, 2000; Mitchison, 1990; Yanagida, 2002). The strains used nowadays are all descendents from his original lab strains. One of the main advantages of fission yeast as a model organism is the short generation time of about 2.5 hours at 30 °C in full medium. Furthermore, it is a genetically tractable organism, whose genome sequence has been published in 2002 (Wood et al., 2002). *S. pombe* has three chromosomes and a relatively small genome, with about 5,000 predicted genes, of which about half contain introns. The three chromosomes condense visibly in mitosis and can be distinguished as individual chromosomes when cells arrest in mitosis, which facilitates analysis of chromosome segregation by microscopy (Russell and Nurse, 1986). Visible chromosome condensation is one of the features fission yeast does not share with the other main yeast model organism, the budding yeast *Saccharomyces cerevisiae (S. cerevisiae)*. The two yeasts differ in many ways, which is not surprising considering that according to gene sequence comparisons they have diverged from each other as early as 400 Million years ago (Yanagida, 2002). Whereas *S. cerevisiae* has experienced a whole genome duplication event in its evolution, only a region of about 50 kb in the sub-telomeric regions of chromosomes I and II seems to have duplicated in *S. pombe* (Wolfe and Shields, 1997; Wood et al., 2002). Fission yeast resembles metazoans in many ways. It has a mitochondrial genome that is similar to the mammalian one, and in contrast to budding yeast, mitochondria are essential. The three chromosomes of fission yeast are similar to those of metazoans in that they have large regional, heterochromatic centromeres and diffuse replicative origins (Forsburg, 2003). Accordingly, proteins that are found in *S. pombe* and metazoans, but not in *S. cerevisiae*, include heterochromatin factors (e.g. Swi6/HP1) and centromere-associated proteins as well as components of the RNAi machinery, which is non-functional in *S. cerevisiae* (Forsburg, 2003).

Fission yeast cells have a rod-shaped structure and grow only at their cell ends, maintaining a constant width (Mitchison, 1990). As their names indicate, budding yeast cells proliferate by forming a bud, which grows and eventually is separated from its mother cell, whereas fission yeast cells undergo a symmetric cell division, commencing with the formation of a septum in the cell center. In contrast to budding yeast, fission yeast normally proliferates in a haploid state. Cells only mate when they are starved, and the resulting diploid cell subsequently undergoes meiosis, forming an ordered tetrad with four haploid spores, which germinate when growth conditions improve. Similar to budding yeast, fission yeast has two different mating types (h+ and h-), and only cells of opposite mating types can mate. In wild type cells, the mating type switches, so that there are always mating partners available within a colony. In the lab, h+ and h- strains that have lost the ability for mating type switching are used in addition to strains that are still able to switch (h90). Fission yeast can proliferate as diploids under certain selective conditions in the lab, but when the selective pressure is released, cells undergo meiosis and form so-called azygotic asci, as opposed to zygotic asci, which are formed under normal conditions, directly after zygote formation.

Whereas budding yeast has a long G_1 phase but no distinct G_2 phase, the latter constitutes the longest phase in fission yeast under ideal growth conditions (Forsburg, 2003). The G_2 phase takes 70% of the total generation time, whereas the other phases each make up approximately 10% of the cell cycle (Figure 1.1). A special feature of the fission yeast cell cycle is that cells pass through G_1 and S phase before cytokinesis has been completed, and as a consequence single cells usually possess a duplicated genome. If cells are starved for nitrogen, they arrest in G_1 (but nevertheless complete cytokinesis), which is a prerequisite for mating, whereas if they are starved for glucose, they arrest in G_2 (Costello et al., 1986).

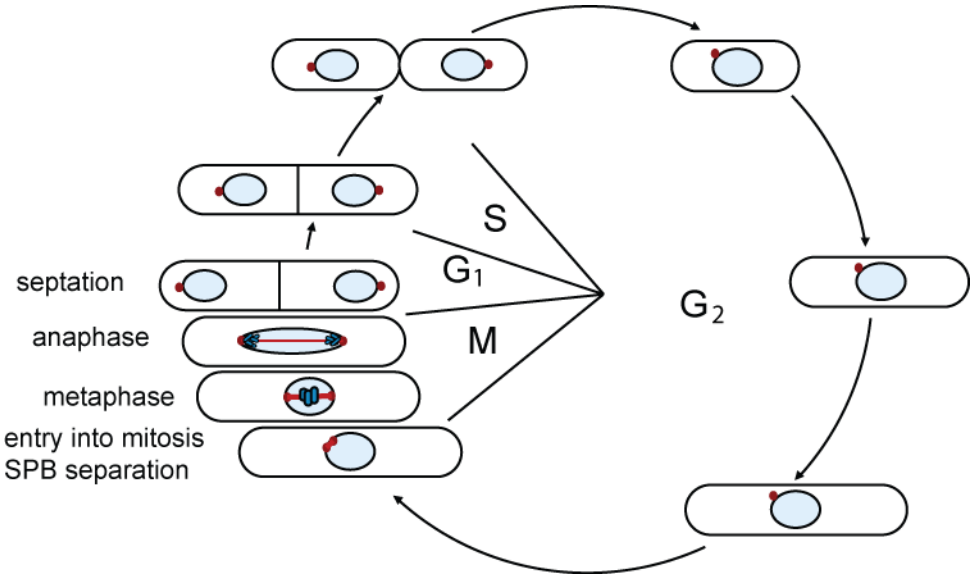

Figure 1.1 The fission yeast cell cycle
Under ideal growth conditions, fission yeast cells spend about 70% of the total generation time (approximately 2.5 hours) in G_2 phase. The remaining 30% are distributed more or less equally to the remaining phases of the cell cycle. Fission yeast, similar to budding yeast, undergoes a closed mitosis, i.e. without nuclear envelope break-down. Fission yeast cells proliferate by symmetrical division. Septation is initiated when the mitotic spindle disassembles, but cytokinesis is usually not completed before cells have passed through G_1 and S phase. In interphase, the spindle pole body (SPB) is associated with the cytoplasmic surface of the nucleus. As the cell progresses into mitosis, the SPB duplicates and enters the nuclear envelope.

1.3 Mitosis

Before cytokinesis (division of the cytoplasm) occurs, the genetic material has to be separated into two identical chromosome sets, and two nuclei have to be reformed. This process is called mitosis. Mitosis in vertebrates is generally divided into five stages, prophase, prometaphase, metaphase, anaphase and telophase (Pines and Rieder, 2001). In prophase, chromosomes start to condense, the microtubule-organizing centers responsible for spindle formation, called centrosomes, separate, and spindle formation is initiated. At the beginning of prometaphase, the nuclear envelope breaks down. The chromosomes condense further, attach to spindle microtubules via

their kinetochores, large protein complexes that assemble on centromeres, and start to congress to the spindle midzone. Correct attachment of kinetochores to the spindle microtubules is crucial for accurate chromosome segregation. The chromosomes have to bi-orient on the spindle, so that the kinetochores of sister chromatids are attached to microtubules emanating from opposite spindle poles (bipolar attachment) (Figure 1.2). In metaphase, the chromosomes are aligned at the equatorial plane of the spindle (metaphase plate). Subsequently, in anaphase, the sister chromatids of the chromosomes are separated and segregate to the two spindle poles. Anaphase events are triggered by activation of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase comprising at least 11 conserved core subunits, plus about one to three species-specific subunits (Kops et al., 2010). According to the generally accepted model for chromosome segregation, sister chromatid separation occurs when the cohesin complexes linking the two DNA strands are cleaved by the protease separase (Cut1 in *S. pombe*, Esp1 in *S. cerevisiae*), which becomes active when its binding partner securin (Cut2 in in *S. pombe*, Pds1 in *S. cerevisiae*) is ubiquitinated by the APC/C and subsequently degraded by the 26S proteasome (Peters, 2006). The other major target of the APC/C in mitosis is cyclin B (Cdc13 in *S. pombe*). Its degradation leads to a reduction in Cdk1 activity, a prerequisite for late mitotic events and cytokinesis (Peters, 2006). Anaphase can be sub-divided into anaphase A and B. In anaphase A, separation of sister chromatids is mainly driven by their movement toward the spindle poles and only marginally by elongation of the spindle. In contrast, during anaphase B the whole spindle elongates, and this is the motor for delivering the chromosomes to the opposite poles. In telophase, chromosomes decondense, the mitotic spindle breaks down and the nuclear envelopes reform around the two daughter nuclei.

Not in all eukaryotic organisms mitosis follows precisely the described stages. In yeasts, the nuclear envelope does not break down (closed mitosis), and a clear prophase cannot be discerned. Therefore, all early mitotic stages in yeast, including chromosome condensation, spindle formation and chromosome alignment, are often referred to as prometaphase.

In fission yeast, a single spindle pole body (SPB), the functional equivalent of the human centrosome, lies on the cytoplasmic surface of the nucleus during interphase. When the cell progresses into mitosis, the SPB enters the nuclear envelope, duplicates and co-ordinates the mitotic spindle until it returns to the cytoplasm in anaphase (Ding et al., 1997). In fission yeast G_2 phase, the centromeres of chromosomes cluster at the nuclear periphery, close to the SPB (Funabiki et al., 1993). Therefore, centromeres are closely associated with the SPBs when the cell enters mitosis, which presumably facilitates capture of kinetochores by newly nucleated spindle microtubules.

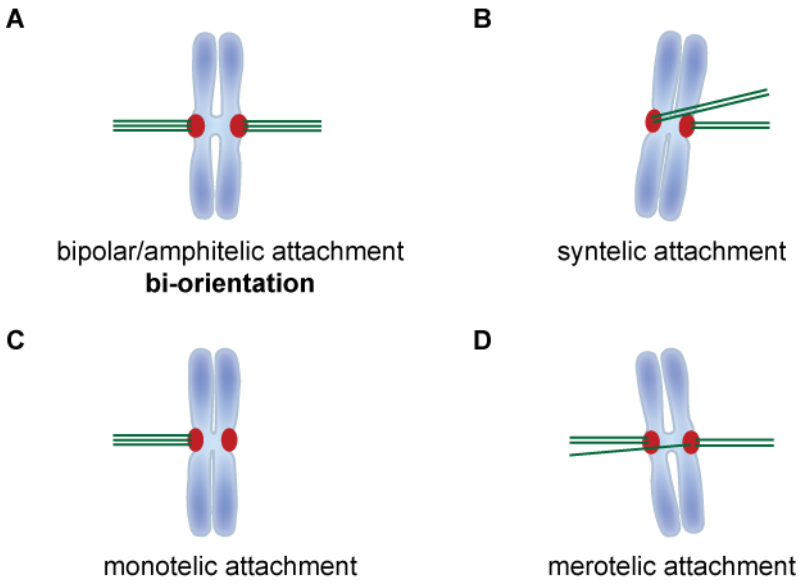

Figure 1.2 Chromosome attachment
Kinetochores of bi-oriented chromosomes are correctly attached to microtubules emanating from opposite spindle poles, so-called amphitelic or bipolar attachment (**A**). Common attachment errors are syntelic attachment (**B**), where both kinetochores are attached to microtubules emanating from the same spindle pole, monotelic attachment (**C**), where only one kinetochore is attached and merotelic attachment (**D**), where one kinetochore is attached to microtubules emanating from opposite spindle poles.

1.3.1 The mitotic spindle in fission yeast

In interphase, microtubules form bundles along the longitudinal axis at the periphery of the fission yeast cell. Their minus ends are nucleated at microtubule-organizing centers close to the nucleus in the cell center, and their plus ends reach toward the cell tips. Microtubules, especially their plus ends, are highly dynamic and often switch between stages of shrinking and growing. There are no nuclear microtubules in interphase, but when the cell enters mitosis, cytoplasmic microtubules depolymerize, and the spindle is formed inside the nucleus. The only cytoplasmic microtubules that can be observed in mitosis are astral microtubules, which appear in anaphase. They are associated with the SPBs on the cytoplasmic side of the nuclear envelope and are oblique to the axis of the spindle (Ding et al., 1993; Hagan and Hyams, 1988).

Fission yeast spindle elongation in mitosis can be divided into three phases (Nabeshima et al., 1998). Phase 1 describes the period of spindle formation, from spindle pole body separation until the spindle reaches a length of approximately 1.5 µm (Mallavarapu et al., 1999). The spindle elongates only very slowly to approximately 2.5 µm to 3 µm in phase 2, which comprises late prometaphase, metaphase and anaphase A. In phase 3, which equates to anaphase B, the spindle elongates rapidly to approximately 12-15 µm (Mallavarapu et al., 1999; Nabeshima et al., 1998).

Careful studies using electron microscopy and three-dimensional reconstruction revealed that there are approximately 20 microtubules in the vicinity of each SPB in early mitosis (Ding et al., 1993). Some of these microtubules are nucleated at the opposite spindle pole and span the entire spindle

(Ding et al., 1993; Tanaka and Kanbe, 1986). Each kinetochore is attached to 2-4 microtubules, adding up to 6-12 kinetochore microtubules in total per half spindle (Ding et al., 1993). Other microtubules reach toward the middle of the spindle but are not connected to kinetochores. Once the cell enters anaphase B, the microtubules that span from pole to pole disappear, and the spindle elongates by sliding apart of antiparallel interdigitating microtubules, which form a stable bundle (Ding et al., 1993; Mallavarapu et al., 1999). During this time, the microtubules elongate at their plus ends in the midzone of the spindle, but neither polymerize nor depolymerize at their minus ends. Thus, in contrast to mammalian cells, there is no poleward flux of microtubules during anaphase in fission yeast (Mallavarapu et al., 1999). In telophase, the mitotic spindle disassembles and the post-anaphase array is formed at the cell center, the site where septation is initiated.

1.4 The spindle assembly checkpoint

The spindle assembly checkpoint (SAC) is a mechanism that ensures a delay in anaphase onset when chromosomes are not correctly attached to the mitotic spindle. It is also often referred to as mitotic, spindle, kinetochore or metaphase checkpoint. The discovery of the first SAC components dates back to the year 1991, when SAC genes were identified in two independent budding yeast screens for mutants that failed to delay the cell cycle in the presence of microtubule-destabilizing drugs (Hoyt et al., 1991; Li and Murray, 1991). The Roberts lab identified *BUB* (*b*udding-*u*ninhibited by *b*enzimidazole) genes (Hoyt et al., 1991), while the Murray lab identified *MAD* (*m*itotic *a*rrest-*d*eficient) genes (Li and Murray, 1991). The SAC components Mad1, Mad2, Mad3 (BubR1 in vertebrates and flies), Bub1 and Bub3 are conserved in all eukaryotes (reviewed in Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007; Taylor et al., 2004). Additional SAC components, including the kinases Mps1 (Mph1 in fission yeast) and Aurora B (Ipl1 in budding yeast, Ark1 in fission yeast), were identified in subsequent studies (see chapter 1.4.2 for references for Mps1; Ruchaud et al., 2007).

The ultimate downstream target of the SAC is Cdc20 (Slp1 in fission yeast), a co-factor and activator of the anaphase-promoting complex/cyclosome (APC/C). By preventing APC/C activation, the SAC prohibits ubiquitination and thereby also degradation of the major mitotic APC/C substrates, securin (Cut2) and cyclin B (Cdc13). As a result, progression into anaphase is delayed and sister chromatids remain cohesed.

Since initial capture of kinetochores by spindle microtubules is partly a stochastic process (Desai and Mitchison, 1997; Kirschner and Mitchison, 1986; Rieder and Salmon, 1998), attachment errors are inevitable (Figure 1.2). In monotelic attachment, one of the sister kinetochores is completely unattached, whereas in syntelic attachment, the kinetochores of both sister chromatids attach to microtubules emanating from the same spindle pole; merotelic attachment describes the connection of one sister chromatid to both spindle poles. Note that the latter attachment defect does not occur in budding yeast, because its kinetochores are attached to only one microtubule (Peterson and Ris, 1976). Attachment errors have to be corrected before sister chromatid cohesion is lost in anaphase. The SAC senses the errors and causes a delay, which allows time for

correction of misattachment. Ultimately, chromosomes have to become bi-oriented, i.e. achieve tension-generating attachment, with connection of sister kinetochores to microtubules emanating from opposite poles, which is also called bipolar or amphitelic attachment (Figure 1.2).

How exactly the SAC senses attachment errors is not known. Laser ablation experiments revealed that kinetochores are the catalytic site where the 'wait anaphase' signal is created (Rieder et al., 1995). Consistently, some mutants of constitutive kinetochore components are not only impaired in establishing correct microtubule attachment but are also unable to activate the SAC, resulting in precocious sister chromatid separation and missegregation of chromosomes (McAinsh et al., 2003; Meraldi, 2004). SAC components and Cdc20 localize to kinetochores in early mitosis and get enriched when chromosomes are misattached, which has been assumed to be a prerequisite for SAC signaling.

A detailed model of how a SAC signal is generated has been described for Mad1 and Mad2 (see below, Figure 1.3), but much less is known about the role of the other SAC components in signaling. A major SAC effector complex is the MCC (*m*itotic *c*heckpoint *c*omplex), consisting of Cdc20, Mad2, Mad3/BubR1 and Bub3 (with fission yeast forming an exception, see chapter 1.4.4.5). The MCC binds the APC/C and inhibits its E3 activity (Musacchio and Salmon, 2007).

1.4.1 The 'Mad2-template model'

Mad1 forms a stable complex with Mad2 and is required for localization of Mad2 to kinetochores (Chen et al., 1999; Chen et al., 1998; Ikui et al., 2002; Sironi et al., 2001). The Mad2-interacting region in Mad1 lies within its C-terminus, whereas the N-terminal part is required for kinetochore localization (Chen et al., 1999; Chung and Chen, 2002; Luo et al., 2002; Sironi et al., 2002) (Figure 1.4). When Mad2 binds to Mad1 it undergoes a conformational change from an open (O-Mad2) to its closed (C-Mad2) form (Luo et al., 2002; Sironi et al., 2002)(Figure 1.3). In addition, C-Mad2 and O-Mad2 can dimerize (De Antoni et al., 2005; Mapelli et al., 2007). The Mad1-C-Mad2 complex, consisting of two Mad1 and two Mad2 molecules, binds stably to kinetochores, forming a receptor for a pool of free O-Mad2, which has a high turnover rate at the kinetochore (De Antoni et al., 2005; Howell et al., 2004; Shah et al., 2004; Sironi et al., 2002; Vink et al., 2006)(Figure 1.3). Most of Mad1 in the cell is bound to Mad2, but there is an excess of Mad1-free O-Mad2 (Chung and Chen, 2002). Furthermore, Mad2 can form a complex with the APC/C activator Cdc20, which has a similar Mad2-binding motif as Mad1. Consistently, Mad2 undergoes a similar conformational change when it binds to Cdc20 as it does upon Mad1-binding, and the binding of Cdc20 and Mad1 to Mad2 is mutually exclusive (Luo et al., 2000; Luo et al., 2002; Sironi et al., 2002; Sironi et al., 2001). Mad2 dimerization occurs only between O-Mad2 and C-Mad2, which is either bound to Mad1 or Cdc20, but not between two molecules of the same conformation (De Antoni et al., 2005). The 'Mad2-template model' proposes that free O-Mad2 binds to the Mad1-C-Mad2 receptor, using C-Mad2 as a template for changing its conformation to C-Mad2 after binding of Cdc20 (De Antoni et al., 2005). Cdc20-C-Mad2 complexes can in turn function as a template for other free O-Mad2 molecules, triggering formation of more Cdc20-C-Mad2 complexes, in a positive-feedback loop (reviewed in Musacchio and Salmon, 2007; Nasmyth, 2005).

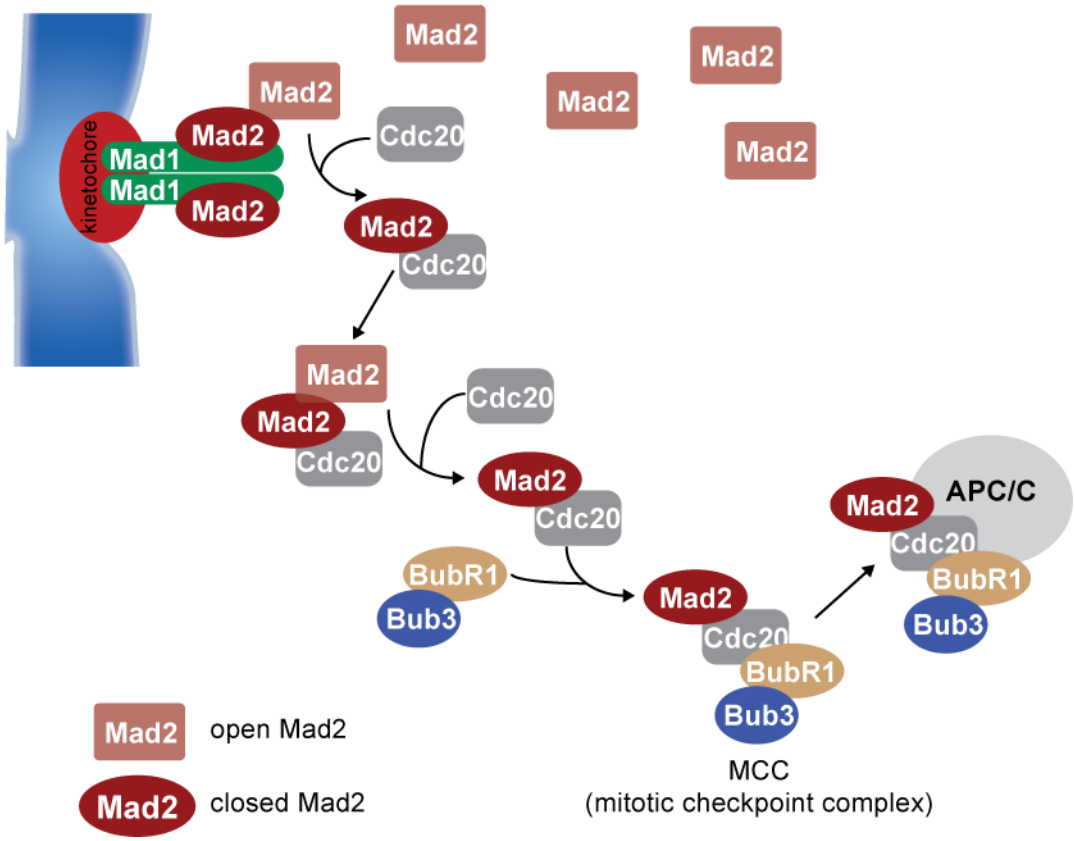

Figure 1.3 Model for SAC signaling based on the 'Mad2-template model'

The 'Mad2-template model' describes the formation of an inhibitory Mad2-Cdc20 complex. A Mad1-Mad2 tetramer is stably associated with unattached kinetochores. Open-Mad2 (O-Mad2) binds transiently to closed-Mad (C-Mad2) in the Mad1-Mad2 complex and undergoes a conformational change to C-Mad2 upon binding of free Cdc20 (Slp1 in fission yeast). The newly formed Cdc20-C-Mad2 complex in turn can function as a template for the formation of additional Cdc20-C-Mad2 complexes. These can bind to Bub3 and BubR1 (Mad3 in yeasts) to form the mitotic checkpoint complex (MCC). This complex is a potent inhibitor of the anaphase promoting complex/cyclosome (APC/C). In *S. pombe*, the MCC seems to lack Bub3.

1.4.2 The spindle assembly checkpoint kinase Mps1 (Mph1)

The kinase Mps1 (Mph1 in fission yeast) is a conserved component of the spindle assembly checkpoint in *S. pombe*, *S. cerevisiae*, *Drosophila*, *Xenopus*, mice and humans, but is absent in nematodes (Abrieu et al., 2001; Essex et al., 2009; Fisk and Winey, 2001; Fisk and Winey, 2004; He et al., 1998; Stucke et al., 2002; Weiss and Winey, 1996) (Figure 1.4). It was first described as an essential kinase required for spindle pole body duplication in budding yeast (Lauzé et al., 1995; Winey et al., 1991). The fission yeast homolog Mph1 (*M*ps1-like *p*ombe *h*omolog) in contrast is not essential, probably because it is not required for SPB duplication (He et al., 1998). Whether Mps1 is required for centrosome duplication in human cells is debated (Fisk et al., 2003; Liu et al., 2003; Stucke et al., 2002).

Mps1/Mph1, like other spindle assembly checkpoint proteins, localizes to kinetochores in mitosis (Abrieu et al., 2001; Fisk and Winey, 2001; Martin-Lluesma et al., 2002; Stucke et al., 2002). It has

been shown in human cells that its kinetochore localization is mediated by its N-terminal region and depends on the Ndc80 complex of the outer kinetochore (Liu et al., 2003; Maciejowski et al., 2010; Martin-Lluesma et al., 2002; Meraldi, 2004; Stucke et al., 2004). Mps1/Mph1 localization is in turn required for recruitment of other SAC components to the kinetochore (Abrieu et al., 2001; Martin-Lluesma et al., 2002; Millband and Hardwick, 2002; Vanoosthuyse et al., 2004; Vigneron et al., 2004; references in Lan and Cleveland, 2010; Stephanie Heinrich, unpublished data), indicating that Mps1/Mph1 acts upstream in the SAC. As it has a high turnover rate at the kinetochore (Howell et al., 2004), it has been suggested that its function in localizing other SAC components to the kinetochore is based on a regulatory role rather than a structural role (Musacchio and Salmon, 2007).

In fission yeast, *mph1* was identified, together with *mad2*, in a screen for genes that show a mitotic arrest when overexpressed (He et al., 1998). Whereas the delay caused by overexpression of *mph1/mps1* is dependent on Mad2, which is another indication that Mps1/Mph1 acts upstream in the SAC, the delay caused by high levels of Mad2 is not abolished in the absence of Mps1/Mph1 (Abrieu et al., 2001; Hardwick et al., 1996; He et al., 1998). Therefore, the putative upstream signal created by the Mps1/Mph1 kinase can be circumvented when Mad2 is overabundant.

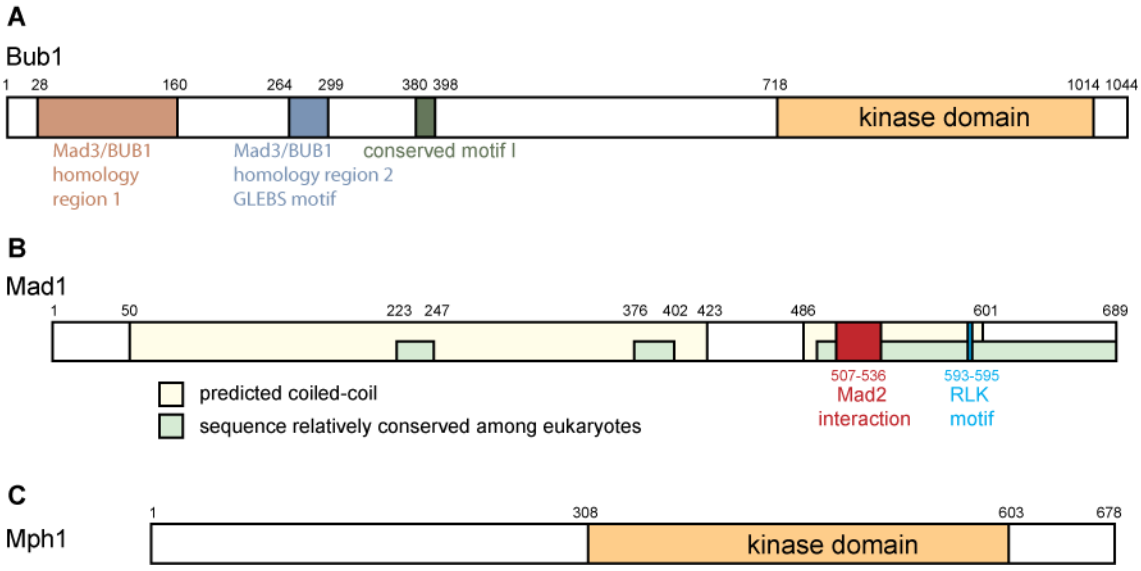

Figure 1.4 Motifs and domains of the fission yeast SAC proteins Bub1, Mad1 and Mph1
(**A**) Bub1 has two Mad3/BUB1 homology regions, which are, as the name indicates, similar to regions in Mad3/BubR1. The first of these regions has been reported to be required for kinetochore localization and the spindle assembly checkpoint. The second region is also called GLEBS motif and has been shown to mediate interaction with Bub3. Bub1 has a C-terminal kinase domain. (**B**) Mad1 has long predicted coiled-coil regions. In the C-terminal half of the protein lie two very conserved regions, the region which is required for interaction with Mad2 and an invariant RLK motif, which has been reported to be required for co-immunoprecipitation with Bub1 and Bub3. (**C**) Mph1, the fission yeast homolog of Mps1, has a C-terminal kinase domain. The N-termini of Mph1/Mps1 proteins are less conserved.

Studies from budding yeast and human cells revealed that Mps1 is additionally required for bi-orientation of chromosomes in mitosis (Jelluma et al., 2008; Jones et al., 2005; Maure, 2007; Shimogawa et al., 2006). In human cells, it directly phosphorylates the chromosomal passenger protein Borealin/Dasra (Jelluma et al., 2008), thereby enhancing activity of the kinase Aurora B, which is required for correcting erroneous kinetochore microtubule attachments (Ruchaud et al., 2007). In budding yeast, an influence on Aurora kinase activity has not been found (Maure, 2007).

1.4.3 Activation of the spindle assembly checkpoint

The SAC has to monitor the attachment state of kinetochores. Not only lack of attachment, but also other defects in chromosome bi-orientation have to be sensed. In syntelic attachment, for example, both kinetochores are attached to microtubules (Figure 1.2). Nonetheless, the SAC has to remain active, because the chromosomes are mono-oriented. In many organisms, slight separation of the centromeres can be observed when chromosomes achieve bi-orientation, indicating that the microtubules emanating from opposite spindle poles exert pulling forces on the sister chromatids that are counteracted by cohesin (Zhou et al., 2002). Thus, tension is generated at kinetochores and centromeres. Thereby, an increase in distance occurs not only between kinetochores (interkinetochore stretch), but also within kinetochores (intrakinetochore stretch) (Maresca and Salmon, 2009). The first experiment showing the importance of tension for progression into anaphase was performed in spermatocytes of praying mantids (Li and Nicklas, 1995). Spermatocytes with unpaired X-chromosomes delayed anaphase onset for several hours. Pulling on such a chromosome with a microneedle abolished the delay, indicating that it was the absence of tension that had caused the delay. It is still a matter of debate whether the SAC senses the lack of tension directly or whether the lack of tension leads to destabilization of kinetochore microtubule attachment, thereby generating unattached kinetochores, which are then detected by the spindle assembly checkpoint (reviewed in Amon, 1999; Khodjakov and Rieder, 2009; Millband et al., 2002; Musacchio and Hardwick, 2002; Nezi and Musacchio, 2009; Pinsky and Biggins, 2005; Vader et al., 2008; Zhou et al., 2002). Erroneous attachments certainly have to be released to allow formation of correct attachments. A key role in error correction is played by the Aurora B kinase (Ark1 in *S. pombe*, Ipl1 in *S. cerevisiae*). The kinase was found to destabilize erroneous kinetochore microtubule attachments by phosphorylation of kinetochore substrates (reviewed in Liu and Lampson, 2009; Ruchaud et al., 2007; Vader et al., 2008). The phosphorylation of Aurora B substrates was shown to depend on the physical distance from the kinase, which localizes to the inner centromere (Liu et al., 2009). Thus, the destabilization of kinetochore microtubule attachments seems to be regulated by spatial separation of Aurora B from its substrates. As soon as a chromosome achieves bi-orientation, the tension at the kinetochore increases the distance between the kinase and its substrates, therefore allowing stable kinetochore microtubule attachment. Aurora B was found to be required for delaying anaphase onset in situations where tension-generating attachment is abolished (Pinsky and Biggins, 2005). Budding yeast Ipl1 is exclusively required for SAC activity in the absence of tension, and not when kinetochores are unattached (Biggins, 2001), supporting the hypothesis that creation of a SAC signal in lack of

tension situations is an indirect process and requires the formation of unattached kinetochores. However, in *Xenopus* and fission yeast, Aurora B/Ark1 is additionally required for delaying anaphase in response to unattached kinetochores (Kallio et al., 2002; Petersen and Hagan, 2003; Vanoosthuyse and Hardwick, 2009; Nicole Hustedt, unpublished data), and also human Aurora B seems to be involved, at least to a certain extent, in SAC signaling in no-attachment situations (Ditchfield et al., 2003; Hauf, 2003; Morrow et al., 2005), indicating that the kinase also has a direct role in the SAC. In budding yeast, Mad3 phosphorylation by Ipl1 is required for SAC activity in the absence of tension, thus also in this organism Ipl1 has a direct role in SAC signaling (King et al., 2007). Similar to Ipl1, shugoshin proteins, Sgo2 in fission yeast and Sgo1 in budding yeast, have been reported to be exclusively required for delaying anaphase onset in low tension situations, but not in situations where kinetochores are unattached (Indjeian et al., 2005; Kawashima et al., 2007; Vanoosthuyse et al., 2007) (see chapter 1.4.4.4).

It has been proposed that the SAC has two branches, one being responsible for detecting the lack of attachment and the other one for detecting the lack of tension (Skoufias et al., 2001). This hypothesis is mainly based on the observation that human Mad2 binds to unattached kinetochores, but not to those not under tension, whereas Bub1 and BubR1 bind tension-less as well as unattached kinetochores (Shannon et al., 2002; Skoufias et al., 2001; Taylor et al., 2001; Waters et al., 1998). In fission yeast, it has been observed that Bub1 can localize to kinetochores to which Mad2 does not localize, but there are no kinetochores that harbour Mad2 but not Bub1, which is also consistent with the observation that Bub1 is required for kinetochore localization of Mad2 (Garcia et al., 2002a; Gillett et al., 2004; Johnson et al., 2004; Meraldi, 2004; Sharp-Baker and Chen, 2001; Windecker et al., 2009; this thesis). Some fission yeast mutants, which presumably have reduced tension at the kinetochore, require only specific sets of SAC components for survival and delay anaphase onset in the absence of *mad2*, whereas deletion of *bub1* shortens the delay (Asakawa et al., 2006; Asakawa and Toda, 2006; Asakawa et al., 2005; Meadows and Millar, 2008). However, the existence of the separate branches has not been proven so far.

1.4.4 Bub1 and Bub3

1.4.4.1 Bub1

Bub1 is a conserved protein that has been shown to be required for SAC signaling in many organisms, including budding yeast (Hoyt et al., 1991; Roberts et al., 1994), fission yeast (Bernard et al., 1998) and *Xenopus* (Sharp-Baker and Chen, 2001). Early studies in human cells suggested that Bub1 might not be required for checkpoint activity (Johnson et al., 2004; Tang et al., 2004b). Later it turned out that this discrepancy was due to a low efficiency of the Bub1 RNAi in these studies and that very low levels of Bub1 are sufficient for a spindle assembly checkpoint arrest, whereas depleting Bub1 further leads to complete checkpoint abrogation (Meraldi, 2004; Meraldi and Sorger, 2005).

Similar to the other checkpoint proteins, Bub1 is recruited to kinetochores in mitosis. Using *Xenopus* egg extracts, Wong and Fang (2006) showed that assembly of SAC components on kinetochores follows a strict order, with Bub1 being one of the first proteins to be recruited.

Therefore, Bub1 is considered to act rather upstream in SAC signaling, which is supported by the fact that localization of most of the other checkpoint components is dependent on Bub1 (Basu et al., 1998; Gillett et al., 2004; Johnson et al., 2004; Logarinho et al., 2008; Meraldi, 2004; Meraldi and Sorger, 2005; Millband and Hardwick, 2002; Morrow et al., 2005; Sharp-Baker and Chen, 2001; Vanoosthuyse et al., 2004; Windecker et al., 2009).

Crucial for targeting Bub1 to kinetochores is its N-terminal Mad3/BUB1 homology region 1 (Klebig et al., 2009; Vanoosthuyse et al., 2004) (Figure 1.4). The human kinetochore protein Blinkin (*b*ub-*lin*king *kin*etochore protein) directly interacts with Bub1 in this region. The Mad3/BUB1 homology region adopts a tetratricopeptide-like fold with a tandem array of three tetratricopeptide repeat (TPR) motifs (Bolanos-Garcia et al., 2009; Kiyomitsu et al., 2007). A deletion mutant of this region in fission yeast (*bub1-Δ28-160*) does not have a functional checkpoint and fails to efficiently recruit Bub3 and Mad3 to kinetochores (Vanoosthuyse et al., 2007; Vanoosthuyse et al., 2004). Similarly, deletion of this region in human Bub1 leads to a reduction in checkpoint activity (Klebig et al., 2009).

Bub1 has a second, structurally probably unrelated, Mad3/BUB1 homology region (Figure 1.4), which mediates interaction with Bub3 (Klebig et al., 2009; Larsen et al., 2007; Taylor et al., 1998; Vanoosthuyse et al., 2009; Wang, 2001). A similar sequence motif occurs in the nuclear pore complex protein Nup116, where it mediates interaction with Gle2 (Rae1 in human and fission yeast) (Bailer et al., 1998). Therefore, this region is often referred to as a GLEBS motif, short for *Gle*2-*b*inding *s*equence (Bailer et al., 1998; Wang, 2001). A GLEBS motif is also found in the SAC protein Mad3 (BubR1 in vertebrates and flies), which has been shown to be also a binding partner of Bub3 (Hardwick et al., 2000; Larsen et al., 2007; Millband and Hardwick, 2002; Taylor et al., 1998; Wang et al., 2001)(see also chapter 1.4.4.5). The *bub1* allele isolated in one of the two initial screens for spindle assembly checkpoint components in budding yeast (*bub1-1*) (Hoyt et al., 1991) has a point mutation in the GLEBS motif, in which the conserved residue glutamate 333 is replaced by a lysine (Warren et al., 2002). This finding as well as data from human cells indicate that the GLEBS motif, and therefore probably also interaction with Bub3, is required for a functional SAC (Klebig et al., 2009).

Similar to Mad3/BUB1 homology region 1, the GLEBS motif is required for efficient recruitment of Bub1 to kinetochores, which was shown for human cells (Klebig et al., 2009) and fission yeast (Windecker et al., 2009). Consistently, in fission yeast, not only Bub3 requires Bub1 for localization to kinetochores, but also Bub1 requires Bub3 (Vanoosthuyse et al., 2004; Windecker et al., 2009). Similar results have been observed for human BubR1, which is known to depend on Bub3 for kinetochore localization (Logarinho et al., 2008; Meraldi, 2004). Consistently, deletion of the BubR1 GLEBS motif abolishes kinetochore localization of the protein (Taylor et al., 1998).

Studies in budding yeast indicate that Bub1 and Bub3 form a complex with Mad1 in a cell cycle-regulated manner (Brady and Hardwick, 2000). In contrast, Mad2 co-immunoprecipitates with Mad1 throughout the cell cycle (Brady and Hardwick, 2000). Co-immunoprecipitation of Bub1 and Bub3 with Mad1 increases substantially in mitotic arrest, caused by treatment with the microtubule-destabilizing drug nocodazole or by *MPS1* overexpression, but is abolished in the absence of Mps1

or Mad2, indicating that a functional spindle assembly checkpoint is required (Brady and Hardwick, 2000).

Furthermore, whereas the kinase function of Bub1 is dispensable for this association (Brady and Hardwick, 2000), the region between amino acids 368 and 608 of budding yeast Bub1 is required (Warren et al., 2002). This region encompasses a conserved motif (conserved motif I), which was described by Klebig et al. (2009)(Figure 1.4). Deletion of this motif in human cells abolishes SAC activity and leads to loss of Mad1 from kinetochores, indicating that it could be directly involved in Mad1 binding. However, the authors could not observe a direct interaction between Mad1 and Bub1 or Bub1 plus Bub3. Similarly, co-immunoprecipitation of Mad1 and Bub1 in mitosis could not be detected in fission yeast (Nicole Hustedt, unpublished data). The interaction is possibly only very weak or completely absent in these organisms.

On the Mad1 side, a conserved motif of three amino acids (RLK) in the C-terminal region is required for co-immunoprecipitation with Bub1 and Bub3 in budding yeast (Brady and Hardwick, 2000)(Figure 1.4). How Mad1 and Bub1 interact and whether the interaction is mediated via other proteins, e.g. kinetochore components, remains an open question. Furthermore, it remains to be unequivocally resolved whether Mad1 and Bub1 interact in fission yeast, and if they do not, which role the conserved motif I plays in the checkpoint.

1.4.4.2 Bub1 kinase activity

Bub1 carries a kinase domain at its C-terminus (Figure 1.4). Not many Bub1 kinase substrates have been described. Early experiments with budding yeast Bub1 revealed that it can phosphorylate itself and Bub3 *in vitro* (Roberts et al., 1994). The physiological relevance of this has not been investigated. The authors proposed that Bub3 binding activates the kinase function of Bub1. *In vitro* data using human and mouse proteins could not confirm activation of kinase activity by Bub3 binding (Martinez-Exposito et al., 1999; Seeley et al., 1999). Furthermore, mammalian Bub3 was not found to be a substrate *in vitro* (Martinez-Exposito et al., 1999; Seeley et al., 1999). Instead, Seeley et al. (1999) identified human Mad1 as a substrate of Bub1 *in vitro*.

Tang et al. (2004a) showed that human Bub1, which had been co-purified with Bub3, can directly phosphorylate Cdc20 *in vitro*, at sites that were also found to be phosphorylated *in vivo*. Phosphorylation of Cdc20 by Bub1-Bub3 led to inhibition of the APC/C_{Cdc20} *in vitro*, and a Bub1 fragment lacking the kinase domain did not inhibit APC/C_{Cdc20}. However, there is a lot of controversy in the literature regarding the requirement for kinase activity of Bub1 in the SAC. Other studies from mammalian cells show that loss of Bub1 kinase function has no or only a partial effect on SAC activity (Klebig et al., 2009; McGuinness et al., 2009; Perera and Taylor, 2010). The phosphorylation of Cdc20 by Bub1 seems to be not sufficient for checkpoint activation but rather an amplification mechanism, given that cells expressing a non-phosphorylatable mutant of Cdc20 still had partial SAC activity (Tang et al., 2004a), whereas point mutations on yeast Cdc20 that abolish interaction with Mad2 abrogated the SAC completely (Hwang et al., 1998; Kim et al., 1998).

Early results from budding yeast pointed in the direction that the Bub1 kinase activity is required for SAC activity: the kinase dead mutant *bub1-K733R* was partially checkpoint deficient (Warren et al.,

2002). However, it turned out that this mutant protein is expressed at a much lower level than wild type *bub1* (Roberts et al., 1994; Warren et al., 2002), and a mutant lacking the entire kinase domain was able to arrest in mitosis in the presence of nocodazole (Fernius and Hardwick, 2007; Warren et al., 2002), indicating that the kinase function of Bub1 is dispensable for checkpoint activity in budding yeast.

In *Xenopus* egg extracts, a kinase dead mutant of Bub1 failed to arrest in the presence of low concentrations of nocodazole, but arrested normally in the presence of higher concentrations (Sharp-Baker and Chen, 2001), indicating that the kinase activity becomes essential for SAC activity only when there are subtle attachment defects (Chen, 2004).

Fission yeast Bub1 kinase activity also seems to play a minor role in SAC signaling, as a kinase dead mutant and a mutant lacking the entire kinase domain did not or only partially inhibit cell cycle progression when treated with the microtubule-destabilizing drug MBC (*m*ethyl-2-*b*enzimidazole *c*arbamate) or in a cold-sensitive β-tubulin mutant (*nda3-KM311*) at restrictive temperature (Kawashima et al., 2009; Vanoosthuyse et al., 2004; Yamaguchi et al., 2003). However, in the temperature-sensitive cohesin mutant *psc3-1T,* the kinase function of Bub1 is required to delay anaphase onset at the restrictive temperature, indicating that it plays a role in no tension-sensing SAC activity (Kawashima et al., 2009).

Overall, the data from different organisms indicate that the kinase activity of Bub1 has only a minor function in the spindle assembly checkpoint, which is possibly restricted to amplification of the signal in the presence of weak attachment defects or to activation of the SAC in the absence of tension at the kinetochores.

Only recently, a crucial target of the Bub1 kinase has been identified. Histone H2A is phosphorylated by Bub1, and this is essential for localizing shugoshin proteins to centromeres (see chapter 1.4.4.4) (Kawashima et al., 2009).

1.4.4.3 Bub1 has functions outside the SAC

It has been proposed already a number of years ago that Bub1 has roles in mitosis outside the SAC. In budding yeast, it has been shown that deletion of *BUB1* leads to a higher rate of chromosome loss than deletion of the checkpoint genes *MAD1*, *MAD2* and *MAD3* (Warren et al., 2002). Similarly, *bub1*-deleted fission yeast cells show an elevated rate of chromosome loss, higher than the chromosome loss rate in mutants of other spindle assembly checkpoint genes (Tange and Niwa, 2008). In addition, overexpression of *BUB1* in budding yeast leads to an increase in chromosome loss (Warren et al., 2002) and a slight increase in sensitivity toward the microtubule-destabilizing drug benomyl (Roberts et al., 1994). In human cells, Bub1 is known to be required for chromosome congression. That this is not only due to loss of checkpoint activity became clear when anaphase onset was blocked by treatment with the proteasome inhibitor MG132 (Johnson et al., 2004; Logarinho et al., 2008; Meraldi and Sorger, 2005). Bub1-depleted cells failed to align their chromosomes efficiently and showed bi-orientation defects. Furthermore, partial depletion of Bub1 in human cells causes a delay in mitosis (Johnson et al., 2004; Logarinho et al., 2008; Tang et al., 2004b). This has been shown to be due to chromosome attachment

defects (Logarinho et al., 2008). Only when Bub1 levels are reduced further, the spindle assembly checkpoint function of Bub1 is lost in addition to its function in chromosome congression (Meraldi and Sorger, 2005).

Another indication that Bub1 has functions outside the SAC came from studies of fission yeast meiosis, where the lack of Bub1 resulted in precocious sister chromatid separation and non-disjunction of homologous chromosomes in meiosis I as well as random segregation of sister chromatids in meiosis II (Bernard et al., 2001b; Vaur et al., 2005). This was in sharp contrast to *bub3Δ*, *mph1Δ*, *mad1Δ*, *mad2Δ* and *mad3Δ* mutants, which had no or only slight defects in chromosome segregation in meiosis (Vaur et al., 2005). It was later found that Bub1, and specifically its kinase activity, is required for localization and function of shugoshin proteins (see chapter 1.4.4.4). This has been confirmed by now in many organisms, including budding yeast (Fernius and Hardwick, 2007; Riedel et al., 2006), fission yeast (Kitajima et al., 2004; Vaur et al., 2005), mammalian cells (Kitajima et al., 2005; Klebig et al., 2009; Perera and Taylor, 2010; Tang et al., 2004b) and *Xenopus* (Boyarchuk et al., 2007). It became clear that the meiotic chromosome segregation defects observed in *bub1*-deleted fission yeast cells are mainly due to loss of shugoshin proteins from centromeres (Kawashima et al., 2009; Kitajima et al., 2004; Vaur et al., 2005).

In fission yeast, *bub1* was originally identified in a screen for genes that become essential in cells lacking the heterochromatin protein Swi6 (*S. pombe* HP1 homolog) (Bernard et al., 1998). Swi6 localizes to heterochromatic regions, including centromeres, telomeres and the mating type locus (Cam et al., 2005). It is mainly required for centromere function, and cells lacking Swi6 have an increased rate of chromosome missegregation and chromosomes sometimes lag on anaphase spindles, indicative of merotelic attachment (Ekwall et al., 1995), both phenotypes that they share with *bub1Δ* cells (Bernard et al., 1998). Subsequent studies revealed that both Swi6 and Bub1 are involved in localizing the essential kinase Ark1 (the fission yeast Aurora B homolog) to the centromere, which could explain the synthetic sick interaction of *swi6Δ* with *bub1Δ* (Kawashima et al., 2007; Vanoosthuyse et al., 2007) (see chapter 1.4.4.4).

Apart from its role in chromosome congression, in shugoshin localization and in the spindle assembly checkpoint, Bub1 has been implicated in senescence and apoptosis, but not much is known about underlying mechanisms and there are controversial reports. Early studies on Bub1 in *Drosophila* revealed that Bub1 mutants have elevated rates of apoptosis (Basu et al., 1999). Gjoerup et al. (2007) report that in human cells, reduced Bub1 levels lead to premature senescence and that this depends on p53. In contrast, another study in human cells describes a delayed senescence when Bub1 levels are decreased (Musio et al., 2003), whereas Niikura et al. (2007) report that cells with reduced Bub1 levels undergo caspase-independent mitotic death, which is independent of p53. In mice, reduced Bub1 levels suppress cell death in response to chromosome missegregation and might facilitate tumorigenesis (Jeganathan et al., 2007; Taylor and McKeon, 1997).

1.4.4.4 Bub1 acts upstream of shugoshin

1.4.4.4.1 Functions of Shugoshins

Shugoshin proteins were first identified because of their crucial role in meiosis (Davis, 1971; Kitajima et al., 2004). In meiosis, two consecutive rounds of chromosome segregation, meiosis I and meiosis II, follow a single round of DNA replication. In meiosis I, homologous chromosomes pair up, become connected by chiasmata and achieve bi-orientation. In contrast to mitosis, the sister chromatids of the individual chromosomes are now mono-oriented. The homologous chromosomes are pulled to opposite poles in anaphase I. The second meiotic division resembles mitosis in that the sister chromatids get bi-oriented and segregate to opposite poles in anaphase II. Due to the formation of chiasmata in meiosis I, cohesion, mediated by the cohesin complex, occurs not only between sister chromatids of one chromosome, but also between sisters of the homologous chromosomes. Cohesin has to be removed from the chromosome arms to allow separation of homologous chromosomes in anaphase I, whereas sister chromatid cohesion at the centromeres has to be maintained, so that sister chromatids are not separated until anaphase II.

Genetic screens in fission yeast and budding yeast revealed proteins that protect centromeric cohesion of sister chromatids in meiosis I (Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). In analogy to their function, they were dubbed shugoshin, which means 'guardian spirit' in Japanese. Homologs in many other eukaryotic organisms were found, including the *Drosophila mei-S332* gene, which had been described previously as being required to prevent precocious separation of sister chromatids in the first meiotic division (Davis, 1971; Kerrebrock et al., 1992). In metazoans, where most of the cohesin on chromosome arms is lost during prometaphase ('prophase pathway'), shugoshin proteins are also responsible for maintaining centromeric cohesin in mitosis (Lee et al., 2005; Watanabe, 2005).

Budding yeast and *Drosophila* have only one shugoshin protein, whereas fission yeast, mammals and plants have two (Watanabe, 2005). The fission yeast shugoshin proteins are Sgo1, which is meiosis I-specific, and Sgo2, which is expressed in mitosis as well as meiosis (Kitajima et al., 2004; Rabitsch et al., 2004). Similar to the deletion of the single shugoshin gene in budding yeast (*SGO1*), deletion of fission yeast *sgo1* leads to random segregation of sister chromatids in meiosis II due to loss of centromeric cohesin in meiosis I.

In contrast, deletion of fission yeast *sgo2* does not lead to a loss in cohesion, neither in meiosis nor in mitosis (Kawashima et al., 2007; Kitajima et al., 2004; Rabitsch et al., 2004; Vaur et al., 2005). Instead, Sgo2 is required for mono-orientation of sister chromatids and bi-orientation of homologous chromosomes in meiosis I (Kitajima et al., 2004; Rabitsch et al., 2004; Vaur et al., 2005) and for proper chromosome segregation in mitosis (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004; Vanoosthuyse et al., 2007). Cells lacking Sgo2 show sensitivity toward microtubule-destabilizing drugs and have an elevated rate of chromosome missegregation (Kitajima et al., 2004). The rate of missegregation is much increased after release from a mitotic arrest that was caused by microtubule depolymerization (Kawashima et al., 2007; Vanoosthuyse et al., 2007; Windecker et al., 2009; this thesis). In addition, lagging chromosomes can be observed in mitosis as well as in anaphase of the first meiotic division, indicating that Sgo2 is required for

preventing merotelic attachment (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004; Rabitsch et al., 2004). It has been described that *sgo2Δ* cells are able to arrest in mitosis in the presence of unattached kinetochores (Kawashima et al., 2007; Vanoosthuyse et al., 2007), whereas they fail to delay anaphase onset in the absence of tension at kinetochores (Kawashima et al., 2007)(see discussion, chapter 3.5), similar to budding yeast *sgo1* mutants (Indjeian et al., 2005).

1.4.4.4.2 Shugoshin localization depends on Bub1

Sgo1 localizes to pericentromeric heterochromatin in the first meiotic division and disappears after onset of anaphase I (Kitajima et al., 2004). It was known already since the discovery of Sgo1 in fission yeast that Bub1 is required for shugoshin localization (Kitajima et al., 2004), but only recently the relevant substrate was discovered: Bub1 targets shugoshin proteins to chromatin by phosphorylating histone H2A (Kawashima et al., 2009). Localization of Sgo1 to pericentromeric heterochromatin also depends on Swi6, but there is some residual localization in *swi6Δ* cells, whereas abolishing H2A phosphorylation seems to disrupt recruitment of Sgo1 completely (Kawashima et al., 2009) (Figure 1.5). Kawashima et al. (2009) found that in a Bub1 mutant lacking the N-terminal part of the protein (*bub1-ΔN)*, which itself does not localize to kinetochores, H2A can still be phosphorylated. However, consistent with loss of kinetochore localization of Bub1-ΔN, H2A phosphorylation is not anymore restricted to the pericentromeric heterochromatin, but occurs all along the chromosomes. As mentioned in 1.4.4.3, Swi6 is known to localize to heterochromatic regions, including centromeres, the mating type locus and telomeres (Cam et al., 2005). Consistently, Sgo1 also localizes to these regions in *bub1-ΔN* cells, indicating that Bub1 localization to kinetochores is not required for efficient phosphorylation of histone H2A *per se* but for specifically enriching H2A phosphorylation at the centromere and thereby, in conjunction with Swi6, restricting Sgo1 localization to the pericentromeric heterochromatin (Kawashima et al., 2009) (Figure 1.5).

Sgo2 localizes to outer subtelomeric, euchromatic regions in interphase and to pericentromeric heterochromatin in mitosis and meiosis (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004). Bub1 kinase mutants and a non-phosphorylatable H2A mutant largely abolish localization of Sgo2 not only in mitosis but also in interphase, indicating that phosphorylation of H2A by Bub1 is also required for interphasic localization of Sgo2 (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004) (Figure 1.5). However, it is unclear, what function Sgo2 fulfills at the subtelomeric region and how exactly its interphasic localization is determined, considering that histone H2A phosphorylation is not increased in the outer subtelomeres compared to other euchromatic regions (Kawashima et al., 2009). For Sgo2, unlike Sgo1, no localization-dependency on Swi6 was reported, but association with pericentromeric heterochromatin in mitosis was shown to depend on the chromosomal passenger protein Bir1 (Kawashima et al., 2007) (Figure 1.5). The localization of the two proteins is mutually dependent, as Sgo2 is required for efficient mitotic centromere localization of the CPC (*c*hromosomal *p*assenger *c*omplex) components Ark1, Pic1 and Bir1 (Aurora B/Ipl1, INCENP/Sli15 and Survivin in other organisms) (Kawashima et al., 2007; Vanoosthuyse et al., 2007). Among many other functions, Ark1 is

required for mono-orientation of sister chromatids and bi-orientation of homologous chromosomes in meiosis I, as well as bi-orientation of sister chromatids in mitosis (Hauf et al., 2007), indicating that chromosome segregation defects observed in *sgo2Δ* could be due to loss of the chromosomal passenger complex from centromeres (Kawashima et al., 2007). Localization of the essential kinase Ark1 not only depends on Sgo2 but, as mentioned above (1.4.4.3), also depends on Swi6 (Kawashima et al., 2007). This dependency is presumably at least in part indirect. Swi6 is involved in recruiting cohesin to centromeres, and cohesin in turn influences Ark1 localization (Bernard et al., 2001a; Kawashima et al., 2007; Morishita et al., 2001; Nonaka et al., 2002) (Figure 1.5). This could explain why *swi6Δ sgo2Δ* cells show a strong increase in lagging chromosomes in anaphase and an increased rate of chromosomes missegregation compared to the single mutants (Kawashima et al., 2007; Kitajima et al., 2004). Furthermore, the described localization dependencies place Bub1 upstream of Sgo2, and Sgo2 and Swi6 in turn upstream of the CPC, which is consistent with the synthetic sickness of *swi6Δ* with *bub1Δ* (Bernard et al., 2001b), with a *bub1* kinase-dead mutant and with *sgo2Δ* (Kitajima et al., 2004).

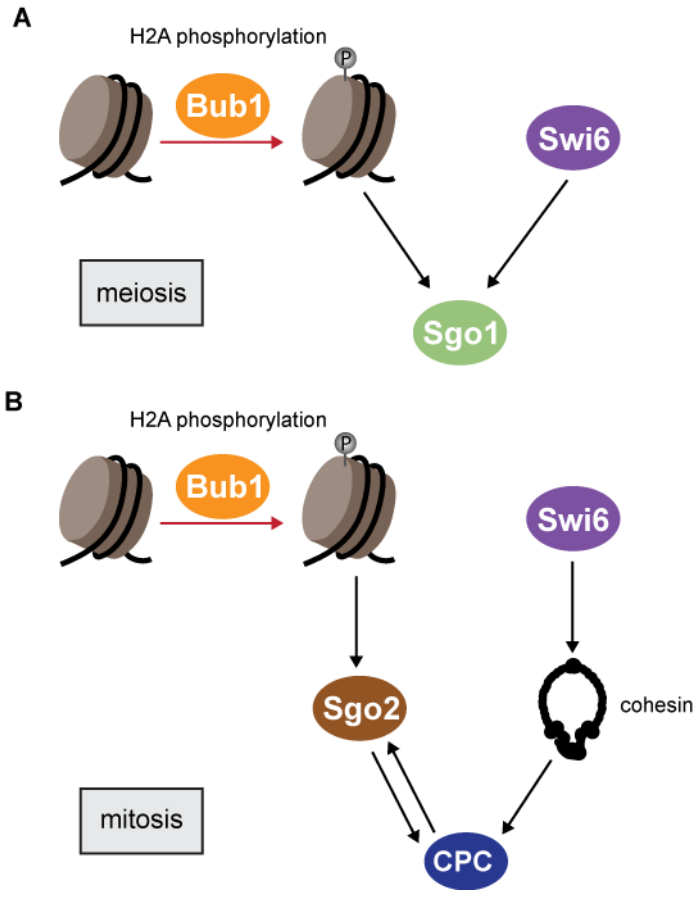

Figure 1.5 Shugoshin localization dependencies in fission yeast
Localization dependencies of Sgo1 in meiosis (**A**) and of Sgo2 in mitosis (**B**). Sgo1 localization depends on histone H2A phosphorylation by Bub1, represented by a red arrow, and on the heterochromatin protein Swi6. Localization of Sgo2 similarly depends on histone H2A phosphorylation by Bub1 and on the chromosomal passenger complex (CPC), which in turn also requires Sgo2 for efficient localization. Localization of the CPC also depends on Swi6, most likely mediated via cohesin. See text for details.

In accordance with Bub1 acting upstream of Sgo1, *bub1*-deleted cells show random segregation of sister chromatids in meiosis II (Vaur et al., 2005). Cells lacking Bub1 also show a remarkable increase in non-disjunction of homologous chromosomes in meiosis I (Bernard et al., 2001b), which can be explained by loss of Sgo2 from centromeres (Kitajima et al., 2004; Vaur et al., 2005). In addition, similar to *sgo2Δ* cells, *bub1Δ* cells have an elevated rate of equational segregation of sister chromatids in meiosis I (Kitajima et al., 2004; Vaur et al., 2005). Both segregation defects, non-disjunction of homologs and equational segregation of sisters, are a consequence of defects in bi-orientation of homologous chromosomes and are presumably the result of impaired Ark1 function at the kinetochores. However, not all meiotic defects observed in the *bub1Δ* mutant can be explained by loss of Sgo1 and Sgo2 functions. Cells lacking Bub1 show a higher rate of equational segregation of sister chromatids in meiosis I than the *sgo1Δ sgo2Δ* double mutant (Kawashima et al., 2007). It is possible that the stronger defect results from loss of spindle assembly checkpoint activity. However, this has not been addressed in the published studies.

1.4.4.5 Bub3

Bub3 was first described in the publication presenting the initial budding yeast screen for *bub* (*b*udding-*u*ninhibited *b*y *b*enzimidazole) mutants (Hoyt et al., 1991). Unlike Bub1, it was not identified as a mutant in the screen itself, but isolated as an extra copy suppressor of the *bub1-1* allele (Hoyt et al., 1991). It was soon discovered that Bub3 can form a complex with Bub1 and with BubR1/Mad3 (Hardwick et al., 2000; Larsen et al., 2007; Martinez-Exposito et al., 1999; Millband and Hardwick, 2002; Roberts et al., 1994; Seeley et al., 1999; Sharp-Baker and Chen, 2001; Taylor et al., 1998; Vanoosthuyse et al., 2004; Wang et al., 2001). In budding yeast and mammalian cells, these interactions have been shown to be direct and mediated via the GLEBS motif present in Bub1 and BubR1/Mad3 (Hardwick et al., 2000; Larsen et al., 2007; Taylor et al., 1998; Wang et al., 2001). However, even though fission yeast Mad3 co-immunoprecipitates with Bub3 (Millband and Hardwick, 2002), it lacks a canonical GLEBS motif (Millband and Hardwick, 2002; Musacchio and Salmon, 2007). Thus, whether there is a direct interaction between Bub3 and Mad3 in fission yeast remains an open question. Bub3 is a WD40 protein[1] which forms a seven-bladed β-propeller (Larsen and Harrison, 2004; Wilson et al., 2005). In the crystal structures of budding yeast proteins, the GLEBS motif of Bub1 and the GLEBS motif of Mad3 bind to the top surface of Bub3 in a mutually exclusive manner (Larsen et al., 2007).

In fission yeast, Bub3 plays an important role for localizing spindle assembly checkpoint proteins to kinetochores. It is required for kinetochore localization of Bub1 and Mad3 as well as of Mad1 and Mad2 (Millband and Hardwick, 2002; Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2004; Windecker et al., 2009; this thesis). Similarly, localization of the budding yeast SAC components

[1] WD40 proteins are a large family found in all eukaryotes. They contain repeated motifs of approximately 40 amino acids with characteristic conserved tryptophan (W) and aspartic acid (D) dipeptides. The repeats form β-propeller structures with usually seven or eight blades. The propeller forms a frustum, of which the plane with the smaller diameter is defined as the top surface. WD-repeat proteins act as a platform for binding partners and are involved in a variety of cellular processes. Smith, T.F., Gaitatzes, C., Saxena, K., and Neer, E.J. (1999). The WD repeat: a common architecture for diverse functions. In Trends in Biochemical Sciences, pp. 181-185.

Mad1, Mad2 and Bub1 depend on Bub3 (Gillett et al., 2004). However, Meraldi (2004) reports that Mad1 and Bub1 localization to kinetochores is not impaired in Bub3-depleted HeLa cells and that Mad2 is only slightly affected, whereas, similar to fission yeast, BubR1/Mad3 localization to kinetochores is abolished. This is consistent with data from Logarinho et al. (2008), who saw only a slight reduction of Bub1 localization in the absence of Bub3, whereas BubR1 localization was completely abolished. In contrast, Taylor et al. (1998) report that in human cells, a Bub1 mutant lacking the GLEBS motif does not bind to kinetochores anymore, indicating that the Bub1-Bub3 interaction is important for efficient kinetochore localization of Bub1.

In agreement with its role in localizing spindle assembly checkpoint proteins to kinetochores, Bub3 was found to be required for checkpoint activity. This has been shown not only for budding yeast (Farr and Hoyt, 1998; Hardwick et al., 1996; Hoyt et al., 1991; Wang and Burke, 1995), but also in mice (Kalitsis et al., 2000), *Xenopus* (Campbell and Hardwick, 2003), *Drosophila* (Lopes, 2005), human cells (Logarinho et al., 2008) and *C. elegans* (Essex et al., 2009). Bub3 is a stable component of the mitotic checkpoint complex (MCC) (Fraschini et al., 2001; Hardwick et al., 2000; Sudakin, 2001). Its association with Cdc20 is dependent on Mad1 and Mad2 (Fraschini et al., 2001), and mutation of the two conserved WD motifs of budding yeast Bub3 disrupts co-immunoprecipitation of Mad2, Mad3 and Cdc20 (Fraschini et al., 2001). Strikingly, Bub3 in fission yeast seems to form an exception. Contrary to what was believed before (Millband and Hardwick, 2002; Vanoosthuyse et al., 2004), recent studies revealed that fission yeast Bub3 is not essential to delay anaphase onset in the presence of chromosome attachment errors (Tange and Niwa, 2008; Vanoosthuyse et al., 2009; Windecker et al., 2009; this thesis). Consistently, fission yeast Bub3 is neither present in the MCC nor involved in MCC formation and does not associate with the APC/C (Sczaniecka et al., 2008; Vanoosthuyse et al., 2009).

Like Bub1, Bub3 seems to have functions in chromosome segregation other than the spindle assembly checkpoint. In budding yeast, deletion of *BUB3* leads to a similarly high rate of chromosome loss as deletion of *BUB1*, and this is much higher than for other SAC component deletion mutants (Warren et al., 2002). In addition, the double mutant *bub1Δ bub3Δ*, as well as the *bub1-E333K* mutant, which abolishes interaction between Bub1 and Bub3, have a similar rate of chromosome loss. Taken together, these results indicate that budding yeast Bub1 and Bub3 share a role in chromosome segregation outside the spindle assembly checkpoint (Warren et al., 2002). In addition, overexpression of both *BUB1* and *BUB3* results in a severe growth defect. This is not due to kinase activity of Bub1, since overexpression of a kinase dead mutant had a similar effect (Roberts et al., 1994). In accordance with data from budding yeast, Bub3-depleted human cells show similar chromosome congression and kinetochore microtubule attachment defects as Bub1-depleted cells and these are independent from loss of SAC activity (Logarinho et al., 2008). In these cells, the kinetochores of unaligned chromosomes are often attached to microtubules in a side-on configuration. BubR1-depleted cells also show chromosome alignment defects, but they are distinct from those observed in Bub1 and Bub3 RNAi. Chromosome alignment defects in BubR1-depleted cells are suppressed by additional inhibition of the Aurora B kinase, whereas in Bub1- and Bub3-depleted cells, misalignment is exacerbated when Aurora B is inhibited (Lampson

and Kapoor, 2005; Logarinho et al., 2008; Meraldi and Sorger, 2005). In fission yeast, on the other hand, *bub3*-deleted cells have a lower chromosome loss rate than other checkpoint deletion mutants (Tange and Niwa, 2008). This difference can be attributed to the functional SAC in *bub3Δ* cells, since abrogating the checkpoint by deleting *mad2* or *mad3* in addition to *bub3* leads to a prominent increase in chromosome loss, to a level similar to that in *bub1Δ* cells and higher than that in *mad3Δ* and *mad2Δ* single mutants (Tange and Niwa, 2008).

Tange and Niwa (2008) report that in *bub3Δ* cells, chromosome oscillations between the spindle poles are increased compared to wild type in phase 2 of spindle elongation. They also state that the interkinetochore distance is increased, but it is not clear whether this is due to higher pulling forces at the kinetochore or to weakened cohesion at the centromeres. In a recent publication, it was suggested that fission yeast Bub3 is required for efficient silencing of the spindle assembly checkpoint (Vanoosthuyse et al., 2009). The authors consider it likely that Bub3-mediated enrichment of checkpoint proteins at the kinetochore is required for switching off the checkpoint signal.

The mRNA export factor Rae1 has extensive sequence homology with Bub3 (Brown et al., 1995; Martinez-Exposito et al., 1999; Reddy et al., 2008). Mouse studies revealed that it interacts directly with the Bub1 GLEBS motif (Wang et al., 2001) and that it can perform spindle assembly checkpoint functions (Babu, 2003). Rae1 haplo-insufficient mice have defects in the spindle assembly checkpoint and show chromosome missegregation (Babu, 2003). Overexpression of Rae1 can suppress haplo-insufficiency of Bub3 (Babu, 2003). Moreover, Jeganathan et al. (2005) found that mammalian Rae1 together with its binding partner Nup98 (Nup189 in fission yeast), which also carries a GLEBS motif, inhibits APC^{Cdh1}-mediated ubiquitination of securin. Since fission yeast Bub3 turned out to be dispensable for spindle checkpoint activity, it was possible that Rae1 can fill in for Bub3 when *bub3* is deleted. A temperature-sensitive mutant of the essential *rae1* gene has been described (*rae1-167*), which upon shift to restrictive temperature shows accumulation of mRNAs in the nucleus and cell cycle arrest at the G_2/M boundary (Yoon et al., 1997). In contrast to mice, even though the *rae1-167* mutant shows an elevated rate of chromosome loss, no function in the spindle assembly checkpoint could be ascertained for Rae1 in fission yeast (Tange and Niwa, 2008; Vanoosthuyse et al., 2004). The *rae1-167* mutant and also the double mutant *rae1-167 bub3Δ* were able to delay exit from mitosis in the presence of a microtubule-depolymerizing drug (Tange and Niwa, 2008). Furthermore, overexpression of *rae1* did not efficiently suppress sensitivity of *bub3Δ* cells toward the microtubule-destabilizing drug thiabendazole (TBZ), and no physical interaction of Rae1 with Bub1 or Mad3 could be detected (Vanoosthuyse et al., 2004).

1.5 The fission yeast kinesin-8 family members Klp5 and Klp6

Kinesins are a superfamily of molecular motor proteins, which use ATP-hydrolysis to move along microtubules or to regulate microtubule dynamics (Woehlke and Schliwa, 2000). They usually form homodimers and move along microtubules in a processive manner, using a 'hand-over-hand' mechanism of the globular motor domains (Howard et al., 1989). Kinesins are classified into 14 subfamilies plus orphans, which cannot be assigned to any of the others families (Lawrence, 2004; Miki et al., 2005). Most kinesins are plus end-directed motors, only those of the kinesin-14 family are minus end-directed (Cross, 2010). Members of the kinesin-13 and kinesin-8 families are able to destabilize microtubules (Wordeman, 2005). In *S. pombe*, members of the kinesin-13 family have not been found, but kinesin-8 proteins are present. The first identified members of the kinesin-8 family were budding yeast Kip3 (Cottingham and Hoyt, 1997; DeZwaan et al., 1997) and *Drosophila* Klp67A (Pereira et al., 1997). Mutants of *KIP3* showed defects in nuclear positioning and increased microtubule stability (Cottingham and Hoyt, 1997; DeZwaan et al., 1997). Using speckle microscopy, Gupta et al. (2006) showed that Kip3 moves toward the plus ends of microtubules. This was confirmed by *in vitro* studies, which also revealed that Kip3 is a plus end-specific motor as well as a microtubule depolymerase (Gupta et al., 2006; Varga et al., 2006). Similar results were obtained with the human homolog Kif18A (Mayr et al., 2007). Both Kip3 and Kif18A depolymerize longer microtubules more efficiently than shorter ones (Mayr et al., 2007; Varga et al., 2006). In a recent publication, the depolymerization mechanism of Kip3 was described in more detail. Varga et al. (2009) observed *in vitro* that Kip3 pauses at plus ends, and their experiments suggest that it dissociates together with one or two tubulin dimers when it is reached and displaced by an incoming Kip3 molecule. This makes deployerization dependent on the continuous delivery of Kip3 molecules to the microtubule plus end.

The fission yeast genome contains two kinesin-8 genes, *klp5* and *klp6*. Deleting either of them does not lead to a pronounced growth defect, but it results in resistance toward microtubule-depolymerizing drugs, elongated mitotic spindles and increased microtubule length in interphase (Garcia et al., 2002b; West et al., 2002; West et al., 2001). This is consistent with the notion that Klp5 and Klp6, like the other kinesin-8 family members described, act as microtubule depolymerases. The double deletion mutant does not result in a more severe phenotype, and Klp5 and Klp6 colocalize throughout the cell cycle (West et al., 2001), indicating that the two proteins function together and may form a heterodimer. This is supported by data from Grissom et al. (2009), who saw co-sedimentation of Klp5 and Klp6 in a sucrose gradient and co-elution in gel filtration and ion-exchange chromatography. Furthermore, the two kinesins co-immunoprecipitate, whereas co-immunoprecipitation between two differently tagged Klp6 molecules in diploid cells could not be detected (Garcia et al., 2002b). Similarly, in a yeast two-hybrid assay, Klp6 interacted with Klp5 but not with itself (Li and Chang, 2003). However, in contrast to Klp6, Klp5 interacted also with itself in this assay. The two kinesins might have additional, mutually independent functions, which is supported by their divergent deletion phenotypes in meiosis (West et al., 2001). Klp5 and Klp6 localize to the spindle and kinetochores in mitosis, to the midspindle in anaphase B and to cytoplasmic microtubules in interphase (Garcia et al., 2002b; West et al., 2002; West et al., 2001;

West and McIntosh, 2008). ChIP assays revealed that Klp5 binds to the outer repeats of centromeres, but when cells were treated with the microtubule-destabilizing drug TBZ, Klp5 was lost from centromeres, indicating that kinetochore localization is microtubule-dependent (Garcia et al., 2002b). This is consistent with data from human cells, where Kif18A also localizes to kinetochores in a microtubule-dependent manner (Mayr et al., 2007; Stumpff et al., 2008). While Klp5 and Klp6 seem to be functional only as a heterodimer, their localization to interphase microtubules has been reported to be independent of the respective binding partner (Unsworth et al., 2008; West et al., 2001). Recently, it has been shown that their mitotic localization is mutually dependent (Unsworth et al., 2008). However, blocking nuclear export rescued localization, indicating that the proteins require each other for retention in the nucleus, rather than localization within the nucleus (Unsworth et al., 2008).

Kif18A-depleted human cells have defects in chromosome congression (Huang et al., 2009; Mayr et al., 2007; Stumpff et al., 2008; Zhu et al., 2005). However, some of these alignment defects have been attributed to a reduction of CENP-E levels in Kif18A RNAi cells (Huang et al., 2009). CENP-E is a kinesin-7 that has plus end-directed motor activity and is required for chromosome congression (Wood et al., 1997). There are no CENP-E homologs in fission yeast and budding yeast. In normal mitosis, chromosomes show oscillatory movements on the mitotic spindle in prometaphase and metaphase. These movements are increased in Kif18A-depleted cells, in terms of amplitude and velocity, and the direction of movement changes less frequently (Jaqaman et al., 2010; Stumpff et al., 2008). Overexpression of Kif18A reduces chromosome oscillation. Consistently, velocity of chromosome movements in anaphase is increased when Kif18A is depleted and decreased when Kif18A is overexpressed, indicating that Kif18A dampens chromosome movements (Stumpff et al., 2008). Fission yeast Klp5/6 also seems to be a major regulator of chromosome movements in mitosis (West et al., 2002). In *klpΔ* (*klp5Δ*, *klp6Δ* or *klp5Δ klp6Δ*) cells, the chromosomes do not align in the middle of the spindle, but can be positioned all along the elongated spindle at the time of anaphase onset. Nonetheless, in most cases they segregate correctly, so that *klpΔ* cells have only a slight increase in chromosome missegregation (Garcia et al., 2002b; West et al., 2002). How exactly Klp5 and Klp6 regulate chromosome movement is not clear. The deletion phenotypes (see above) clearly point in the direction that they act as microtubule depolymerases, but this could not be confirmed *in vitro* (Grissom et al., 2009). It has been shown *in vivo* that deletion of *klp5* and *klp6* results in a reduction of the catastrophe frequency of cytoplasmic microtubules (Tischer et al., 2009; Unsworth et al., 2008). However, also the rescue frequency was reduced in *klp5Δ klp6Δ* mutants (Unsworth et al., 2008). Kinesin-8 deletion mutants show a mitotic delay. Deletion of *mad2* abolishes the delay and leads to an increase in the rate of chromosome missegregation, suggesting that the spindle assembly checkpoint is activated in *klpΔ* mutants and prevents chromosome loss (Garcia et al., 2002a). However, the spindle assembly checkpoint is not essential for survival of *klpΔ* cells, as neither *klpΔ mph1Δ* nor *klpΔ mad2Δ* double mutants show an obvious growth defect (West et al., 2002). In contrast, deleting *bub1* in addition to kinesin-8 genes leads to a severe growth defect (West et al., 2002; this thesis), indicating that these proteins share a function. Alternatively, Bub1 could have a

checkpoint function that it does not share with Mad2, but is required for survival of *klpΔ* cells (see chapter 1.4.3).

1.5.1 Synthetic lethal interactors of *klp5*

It has been described for several genes that they have a synthetic growth defect in conjunction with *klpΔ*. Apart from *bub1Δ*, this has been reported for mutants of α- and γ-tubulin, the APC/C, the Dam1/DASH complex, the Ras pathway and the microtubule-associated proteins Alp14 and Dis1 (Garcia et al., 2002a; Li and Chang, 2003; Sanchez-Perez et al., 2005; Tange et al., 2004; West et al., 2002; West et al., 2001).

1.5.1.1 Synthetic interactions

Synthetic interactions occur when a double mutant has a phenotype different from either single mutant parent (Forsburg, 2001). The term 'synthetic lethal' was introduced by Dobzhansky in 1946 in a study about genetic variation in a natural population of *Drosophila pseudoobscura* (Dobzhansky, 1946). It was not until the 1980s that synthetic lethality/sickness was more commonly used as a genetic tool in budding yeast (Guarente, 1993). There are several reasons for synthetic lethality between two genes (reviewed in Boone et al., 2007; Guarente, 1993; Tucker and Fields, 2003). First of all, the proteins encoded by the genes could be functionally redundant and act in parallel pathways that can compensate for the loss of the respective other (between-pathway interaction) (Figure 1.6A). Between-pathway interactions can also be observed when one of the proteins is part of a pathway that prevents a potentially harmful event that is corrected by another pathway (Boone et al., 2007; Pan et al., 2006). Similarly, abolishing a cell cycle checkpoint could become lethal for the cell when the genes involved in the process that is under surveillance by this checkpoint are mutated concurrently (Hartwell and Weinert, 1989). Synthetic lethality can also occur between genes that act in the same pathway (within-pathway interaction) (Figure 1.6B). Within-pathway synthetic interactions are expected to occur between partial loss-of-function mutants in an essential pathway, where either mutation alone impairs the function of the pathway and the combination abolishes it completely. The proteins could even act in the same step, as a heterodimer (Guarente, 1993). If two mutations are synthetically lethal and the complete deletion of either of these genes alone is lethal, they are likely to act in the same essential pathway, rather than parallel pathways. If the null mutants are viable, they are expected to lie in parallel pathways. However, occasionally two non-essential genes show synthetic lethality even though they interact physically (Kelley and Ideker, 2005; Li and Chang, 2003). Synthetic lethality can also be the consequence of indirect effects that are caused by the cell's response to the absence of a particular gene, rather than resulting from loss of specific functions as described before for between-pathway and within-pathway interactions (Kelley and Ideker, 2005; Tucker and Fields, 2003).

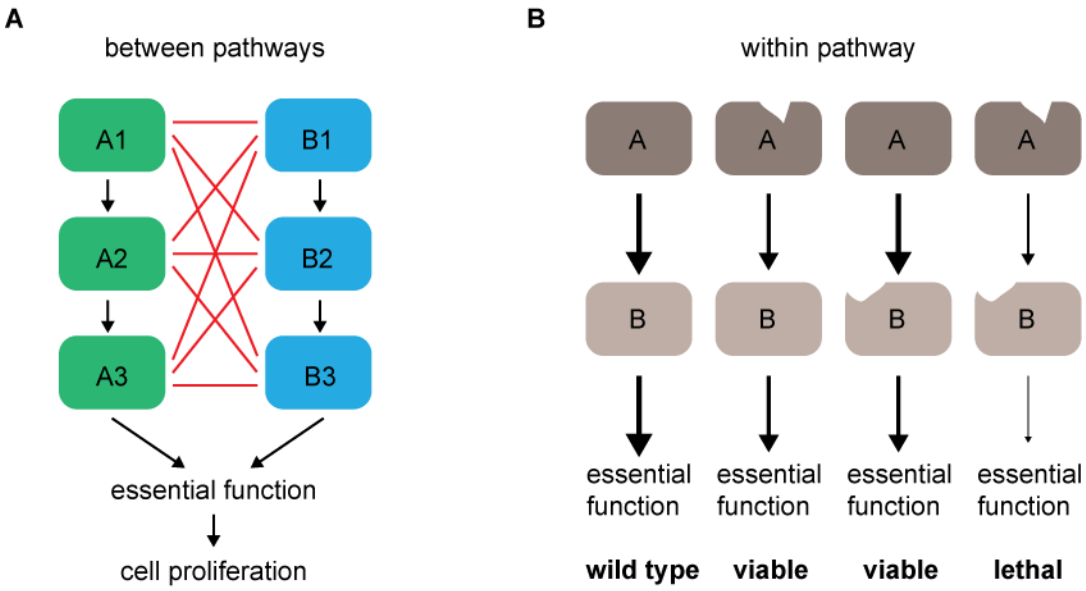

Figure 1.6 Synthetic interactions

(**A**) Synthetic lethal interactions can occur between different components of parallel pathways that share an essential function. Disturbing one of the pathways does not result in loss of viability, but loss of both pathways is lethal. Synthetic lethality between deletion mutants is indicated by red lines. (**B**) Synthetic lethality can also occur within a pathway. This is usually the case when the pathway has an essential function and the deletion mutants of its components results in loss of viability. Partial loss-of-function mutants of this pathway can be viable by themselves, but when combined result in complete loss of viability. This figure was adapted from Boone et al. (2007).

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

The APC/C is a multi-subunit ubiquitin ligase that is responsible for targeting cyclin B and securin to the 26S proteasome for degradation at the onset of anaphase (reviewed in Peters, 2006). Apart from these crucial targets, the APC/C has other substrates, both in mitosis as well as in other phases of the cell cycle. For substrate recognition, the APC/C requires co-activators. These are mainly Cdc20 (Slp1 in fission yeast, Fizzy in *Drosophila*) and Cdh1 (Ste9 in fission yeast, Fizzy-related in *Drosophila*). The temperature-sensitive APC/C mutants *nuc2-663*, *cut4-533* and *cut9-665* are all synthetically lethal with *klpΔ* (West et al., 2002). At restrictive temperature, these mutants delay progression from metaphase to anaphase (Hirano et al., 1988; Samejima and Yanagida, 1994; Yamashita et al., 1996). Nevertheless, these cells undergo septation, even though they do not separate sister chromatids and do not divide their nucleus, a typical phenotype of so-called cut (*c*ell *u*ntimely *t*orn) mutants (Hirano et al., 1986; Yanagida, 1998). The *nuc2-663* mutant is sterile, even at permissive temperature, presumably because it fails to arrest in G_1 upon nitrogen starvation, which is a prerequisite for conjugation (Kumada et al., 1995). Why kinesin-8 and APC/C mutants show a negative genetic interaction has not been investigated and remains an open question.

1.5.1.3 The Dam1/DASH complex

The Dam1/DASH complex is required for correct kinetochore microtubule attachments. It is essential in budding yeast, where only one microtubule attaches to each kinetochore, but not in fission yeast (Cheeseman et al., 2001; Hofmann et al., 1998; Liu et al., 2005; Sanchez-Perez et al., 2005). No homologs have been described in mammals, but it is assumed that the human Ska complex performs similar functions (Hanisch et al., 2006). The Dam1/DASH complex interacts directly with microtubules and can connect them to kinetochores, where it interacts with the Ndc80 complex (Janke et al., 2002; Lampert et al., 2010; Shang et al., 2003; Tien et al., 2010). In fission yeast, the Dam1/DASH complex localizes to microtubule-associated kinetochores and to the plus tips of astral microtubules (Sanchez-Perez et al., 2005). The budding yeast complex has been shown to form rings around microtubules *in vitro* and to move processively with microtubule plus ends (Lampert et al., 2010; Miranda et al., 2005; Westermann et al., 2005; Westermann et al., 2006). In fission yeast mitosis, the Dam1/DASH complex is required for retrieval of chromosomes to the spindle pole body (SPB) if they have become unclustered from the spindle pole bodies, for example due to treatment with microtubule-depolymerizing drugs (Franco et al., 2007; Gachet et al., 2008). Deletion mutants of *klp5* or *klp6* have been shown to be synthetically sick with several deletion mutants of the Dam1/DASH complex (*dam1Δ*, *duo1Δ*, *spc34Δ*, *ask1Δ*, *dad1Δ*) (Sanchez-Perez et al., 2005).

1.5.1.4 Dis1 and Alp14

Dis1 and Alp14 (also called Mtc1) are members of the Dis1/TOG family of microtubule-associated proteins, which are known to play a role in spindle formation and to promote microtubule polymerization at microtubule plus ends (reviewed in Ohkura et al., 2001). The two fission yeast proteins are not essential, but the double mutant *alp14Δ dis1Δ* is synthetically sick, and ectopic expression of *dis1* can suppress instability of cytoplasmic microtubules in *alp14Δ* mutants, indicating that they are functionally related (Nakaseko et al., 2001). Dis1 and Alp14 localize along cytoplasmic microtubules in interphase; in mitosis, the proteins relocate to mitotic spindles and get enriched at kinetochores in a microtubule-dependent manner (Garcia et al., 2001; Nakaseko et al., 2001). Both *alp14Δ* and *dis1Δ* show synthetic lethality with *klp5Δ* and *klp6Δ* (Garcia et al., 2002a), which is unexpected because Dis1/TOG proteins promote microtubule polymerization whereas kinesin-8 proteins promote microtubule depolymerization.

1.5.1.5 The Ras pathway in fission yeast

The G protein Ras lies upstream in a conserved signaling pathway that is involved in regulating the cytoskeleton, cell growth, differentiation and other fundamental cellular processes (Boguski and McCormick, 1993; Kolch, 2005). In fission yeast, the Ras pathway bifurcates. One downstream effector of the single fission yeast Ras homolog, Ras1, is the kinase Byr2, which controls pheromone signaling and is essential for mating and sporulation. The other branch involves Scd1, a direct interactor of Ras1 and a guanine-nucleotide-exchange factor of the Rho-GTPase Cdc42 (Chang et al., 1994). Cdc42 in turn activates the PAK kinase Pak1/Shk1/Orb2 (Marcus et al., 1995;

Verde et al., 1998). The scaffold protein Scd2 is required for efficient interaction between the proteins of this branch (Chang et al., 1994). The Ras1-Scd1 pathway is required for cytoskeletal organization, spindle formation, cytokinesis, morphogenesis and mating (Li and Chang, 2003; Li et al., 2000; Segal and Clarke, 2001). Only mutants of this Ras pathway branch show synthetic lethality with *klp5* and *klp6* mutants, whereas deletion of *byr2* has no synthetic effect (Li and Chang, 2003).

1.5.1.6 α-tubulin

Microtubules are composed of heterodimers of α- and β-tubulin subunits. Fission yeast has two α-tubulin genes, *nda2* and *atb2*, and one β-tubulin gene, *nda3*. Whereas *nda3* and *nda2* are essential, *atb2* is dispensable for viability (Adachi et al., 1986; Hiraoka et al., 1984; Schatz et al., 1986a; Schatz et al., 1986b). Cells lacking *klp5* or *klp6* are synthetically lethal with the cold- and TBZ-sensitive *nda2-KM52* mutant, even at permissive temperature (Toda et al., 1983; Umesono et al., 1983; West et al., 2001). In contrast, no synthetic growth defect was observed with the also cold-sensitive but TBZ-resistant β-tubulin mutant *nda3-KM311* (Hiraoka et al., 1984; Umesono et al., 1983; West et al., 2001).

1.5.1.7 γ-tubulin

The third type of tubulin is γ-tubulin. It is an integral part of a multisubunit complex, the γ-tubulin complex, which is required for microtubule nucleation and is part of microtubule-organizing centers. In fission yeast, γ-tubulin is essential for spindle formation (Horio et al., 1991; Stearns et al., 1991). A cold-sensitive γ-tubulin mutant, *gtb1-93*, was identified in a screen for synthetic lethal interactors of a deletion mutant of *pkl1*, the gene encoding for a fission yeast kinesin-14 protein (Paluh et al., 2000). Tange et al. (2004) report that it also shows synthetic lethality with *klp5Δ* and *klp6Δ*, even at permissive temperature. The mutant shares some phenotypes with kinesin-8 mutants, further indicating that they share a function. The *gtb1-93* mutant has elongated interphase microtubule bundles (Paluh et al., 2000; Tange et al., 2004), longer mitotic spindles, abnormal distribution of chromosomes along the spindle (Tange and Niwa, 2007), and it shares a synthetic sick phenotype with *dis1Δ* mutants (Tange et al., 2004). However, it is elusive how γ-tubulin and kinesin-8 proteins could have related functions, as γ-tubulin localizes to spindle pole bodies and other microtubule-organizing centers, whereas Klp5 and Klp6 localize to microtubule plus ends.

Overall, the genetic interactions between kinesin-8 mutants and various genes indicate that Klp5 and Klp6 have multiple roles during the cell cycle. However, the detailed mechanisms involved remain elusive.

1.6 Aim of this study

The kinase Bub1 is a spindle assembly checkpoint component, but also has checkpoint-independent functions in mitosis. It acts upstream of shugoshin proteins, which are required for protection of sister chromatid cohesion and bi-orientation of chromosomes. The observation that deletion of the kinesin-8 genes results in synthetic lethality with *bub1Δ*, but not with *sgo2Δ* and *mad2Δ*, pointed in the direction that *bub1* has additional, as yet undescribed functions in fission yeast. The aim of this study was to examine these uncharacterized functions of Bub1 and to investigate their potential role in chromosome segregation. Another aim was to investigate the role of the Bub1 binding partner Bub3 in mitosis. Surprisingly, I found Bub3, in contrast to what had been reported before, not to be essential for spindle assembly checkpoint activity in fission yeast. However, it shares with *bub1* the synthetic interaction with *klp5Δ*, and interaction of the two proteins is required for survival of *klp5Δ* cells, indicating that Bub1 and Bub3 share a function that is essential in the absence of *klp5*. I could show that Bub1 and Bub3 contribute to chromosome bi-orientation. The mechanistic basis for this function remains to be investigated.

2 Results

2.1 Contributions

Ryan Fallt assisted me technically with the synthetic lethal screen. Maria Langegger and Silke Hauf conducted and analyzed the live cell imaging experiments depicted in Figures 2.10, 2.15, 2.16 and 2.21. I performed all the other experiments shown.

2.2 Bub1 shares a function with the kinesin-8 family proteins Klp5 and Klp6

2.2.1 A so far uncharacterized function of Bub1 is required for growth of kinesin-8-deleted cells

In fission yeast, deletion mutants of the genes coding for the kinesin-8 proteins Klp5 and Klp6 have been reported to require the spindle assembly checkpoint gene *bub1* for survival, especially at higher temperatures (West et al., 2002), and we confirmed this result (Figure 2.1). The single deletion mutants of kinesin-8 grew healthily at all three temperatures tested, *bub1*-deleted cells grew normally at 30 °C and 34 °C, but had a slight growth defect at 25 °C (Figure 2.1A). Combining *bub1*-deletion with deletion of either *klp5* or *klp6* led to a synthetic growth defect, which was most prominent at 34 °C. This was slightly stronger in *klp5Δ bub1Δ* compared to *klp6Δ bub1Δ* (Figure 2.1B).

Given that kinesin-8 mutants have defects in chromosome congression (Garcia et al., 2002b; West et al., 2002), the most likely explanation at first sight is that a functional spindle assembly checkpoint is required to prevent erroneous chromosome segregation and that Bub1 is crucial for survival of cells lacking *klp5* or *klp6* for this reason. Surprisingly, deleting *mad2*, which is essential for spindle assembly checkpoint activity, does not impair growth of *klp5*-deleted cells (West et al., 2002) (Figure 2.1A). This indicates that the defective spindle assembly checkpoint in *bub1*-deleted cells is not the cause of the synthetic sickness with kinesin-8. Similar to *mad2*, also the SAC gene *mph1* (*MPS1* in *S. cerevisiae*) has been reported to be dispensable for healthy growth of *klp5* and *klp6* deletion mutants (West et al., 2002) (see also Figure 2.13).

The other described, conserved function of Bub1 is to localize shugoshin proteins to the pericentromeric region in mitosis (Kawashima et al., 2009; Kitajima et al., 2005; Kitajima et al., 2004; Tang et al., 2004b). Fission yeast has two shugoshin proteins, Sgo1 and Sgo2 (Kitajima et al., 2004). The *sgo1* gene is expressed solely in meiosis, whereas *sgo2* is expressed in meiosis as well as throughout the vegetative cell cycle (Kitajima et al., 2004; Mata et al., 2002). Sgo2 is required for correct chromosome segregation and SAC activity in the absence of tension (Kawashima et al., 2007; Kitajima et al., 2004). I therefore tested whether *klp5*-deleted cells require Sgo2 for survival. Surprisingly, this was not the case, and also the triple mutant *klp5Δ sgo2Δ mad2Δ* did not show a growth defect (Figure 2.1A). The latter result excludes the possibility that the

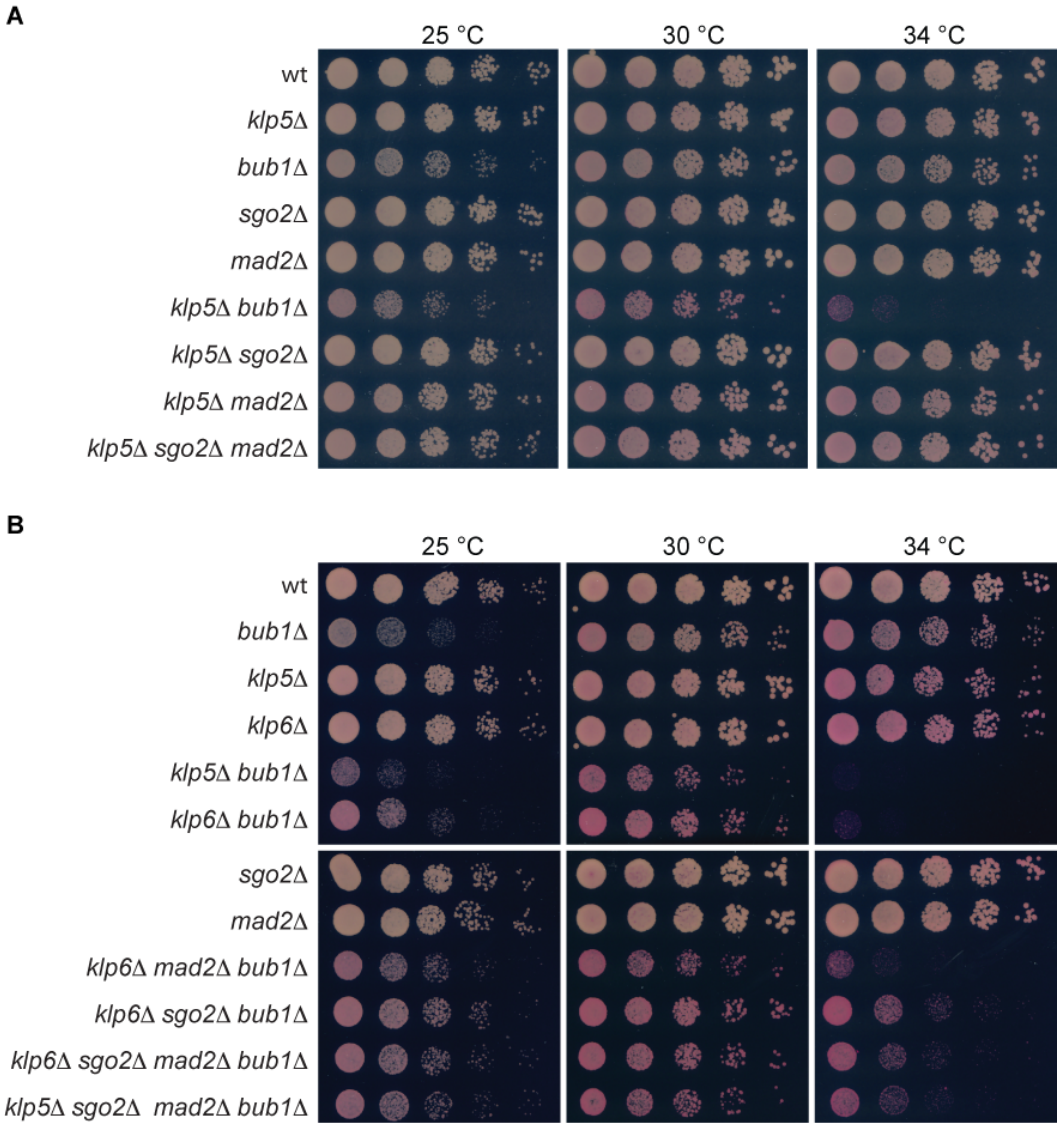

Figure 2.1 Sgo2- and SAC-independent function(s) of Bub1 are required for growth of kinesin-8 mutants

Growth assay with the indicated strains. 5-fold serial dilutions were spotted on full medium containing Phloxin-B, a stain that specifically accumulates in dead cells. Plates were incubated at the indicated temperature for 3 d. (**A**) Cells lacking Klp5 and Bub1 showed a growth defect, whereas *klp5Δ* cells lacking Sgo2 and Mad2 grew healthily. (**B**) The double mutant *klp6Δ bub1Δ* had a similar growth defect as *klp5Δ bub1Δ* cells. Impaired growth of kinesin-8 *bub1* double deletion mutants was partially suppressed by additional deletion of *sgo2*.

synthetic sickness of *klp5Δ bub1Δ* is due to a synergistic growth defect resulting from simultaneous loss of both of the two known Bub1 functions, SAC activity and Sgo2 localization. This result indicated that neither the checkpoint defect nor the loss of Sgo2 localization caused the growth defect in *klp5Δ bub1Δ* cells. It was still possible that the concomitant loss of these Bub1 functions together with one or more other Bub1 functions caused the growth defect in kinesin-8-deficient cells. I therefore tested growth of the quadruple *klp5Δ sgo2Δ mad2Δ bub1Δ* and *klp6Δ sgo2Δ mad2Δ bub1Δ* mutants. The additional deletion of *sgo2* and *mad2* in the *klp5Δ bub1Δ* and *klp6Δ bub1Δ* background did not impair viability any further; on the contrary, it rescued growth perceptibly

(Figure 2.1A,B). This suppression was mainly, but not exclusively, due to deletion of *sgo2*, as *klp6Δ sgo2Δ bub1Δ* cells grew much better than *klp6Δ mad2Δ bub1Δ* and *klp6Δ bub1Δ* cells (Figure 2.1B). We concluded that Bub1 must have additional functions, apart from ensuring spindle assembly checkpoint activity and Sgo2 localization, which are crucial for cell survival when kinesin-8 function is abolished, and it is specifically loss of these rather than concomitant loss of all Bub1 functions that causes the impaired growth in kinesin-8-deficient cells.

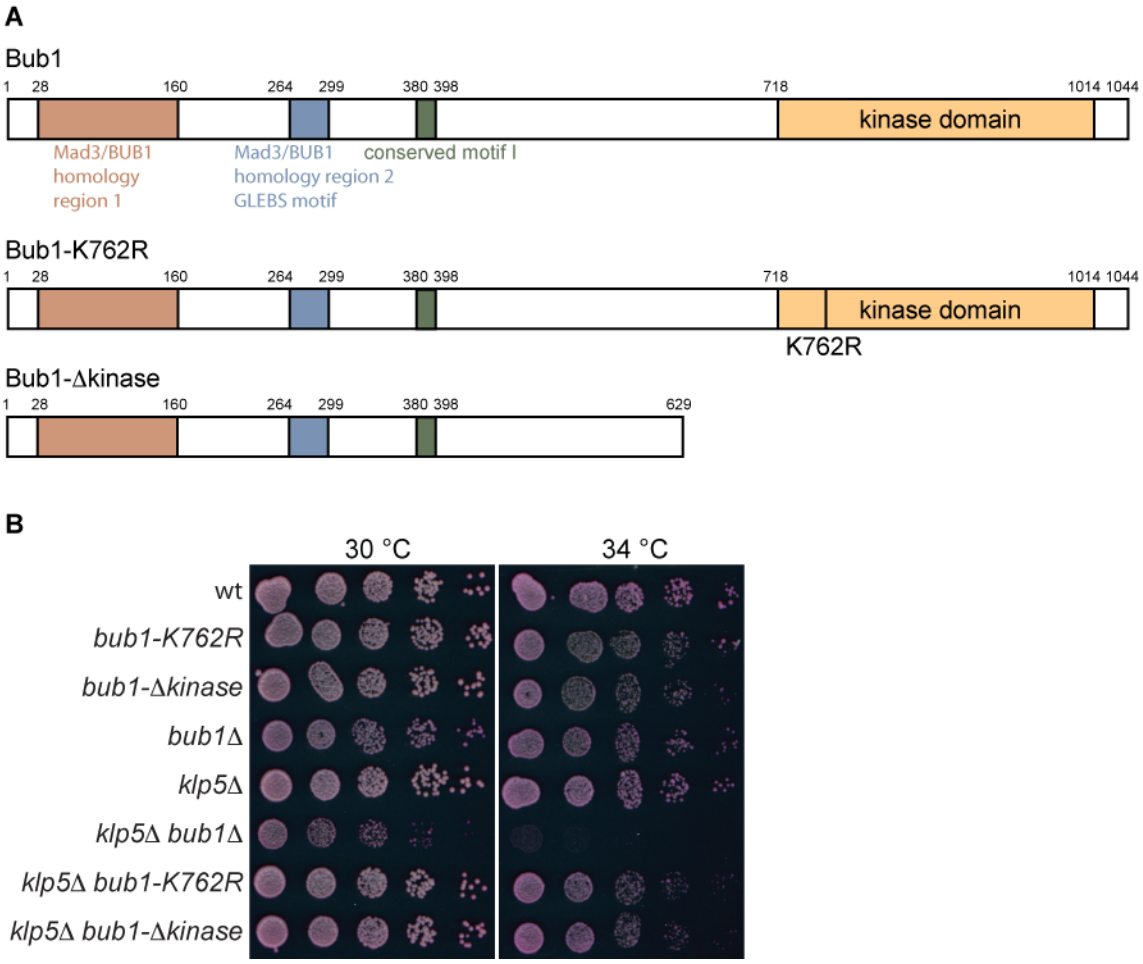

Figure 2.2 The kinase function of Bub1 is dispensable for growth of kinesin-8 mutants
(**A**) Schematics representing Bub1 and the Bub1 kinase mutants Bub1-K762R and Bub1-Δkinase. (**B**) Growth assay with Bub1-kinase mutants. 5-fold serial dilutions were spotted on full medium containing Phloxin-B and incubated at the indicated temperature for 3 d. The combination of *klp5Δ* with the indicated Bub1-kinase mutants *bub1-K762R* and *bub1-Δkinase* did not result in a synthetic growth defect.

We wanted to know whether the kinase activity of Bub1 is required to fulfill the undescribed function that is required for growth of *klp5Δ* cells. Bub1 kinase activity has a minor role in spindle assembly checkpoint activity (Fernius and Hardwick, 2007; Klebig et al., 2009; McGuinness et al., 2009; Perera and Taylor, 2010; Sharp-Baker and Chen, 2001; Vanoosthuyse et al., 2004; Warren et al., 2002; Yamaguchi et al., 2003), but it is essential for targeting Sgo2 to centromeres (Boyarchuk et al., 2007; Fernius and Hardwick, 2007; Kawashima et al., 2009; Kitajima et al., 2004;

Klebig et al., 2009). I assayed growth of *klp5*-deleted cells that additionally carried Bub1-kinase mutations (Figure 2.2). Neither a kinase-dead mutant (*bub1-K762R*) nor a mutant lacking the entire kinase domain (*bub1-Δkinase*) showed impaired growth in conjunction with *klp5*-deletion. Hence, a kinase-independent function of Bub1 is required for viability of *klp5Δ* cells.

2.2.2 Phenotype of *klp5Δ bub1Δ* mutants

In order to get a hint on the nature of the additional Bub1 function(s) required in kinesin-8-deficient cells, I tried to assess the cause of the lethality in *klp5Δ bub1Δ* cells. In spite of severe abnormal chromosome movements in mitosis (West et al., 2002), *klp5Δ* cells segregate their chromosomes remarkably well, with only a slight increase in chromosome missegregation (Garcia et al., 2002b). Although abolishing the spindle assembly checkpoint by deleting *mad2* increases the chromosome missegregation rate, this does not result in an obvious growth defect (Garcia et al., 2002a)(see chapter 2.2.1, Figure 2.1). Deletion of *bub1* in fission yeast also causes an increased rate of chromosome loss (Bernard et al., 1998; Kitajima et al., 2004; Vanoosthuyse et al., 2004), which is much higher than the chromosome loss rate in mutants of *mad2* or other spindle assembly checkpoint genes (Tange and Niwa, 2008). Combining *klp5Δ* with *bub1Δ* might therefore lead to a very high missegregation rate, which might be the cause of reduced viability in the double mutant. To determine the missegregation rate in *klp5Δ bub1Δ* cells, I used a lacO/lacI-GFP system marking a region close to the centromere of chromosome II (*cen2*-GFP; Yamamoto and Hiraoka, 2003). I assayed segregation of chromosome II in fixed cells that had just undergone mitosis, judged by septation (Figure 2.3). Compared to *bub1Δ* alone, the double deletion of *klp5Δ* and *bub1Δ* did not cause a severe increase in missegregation (Figure 2.3A) and there was no synergistic effect in the *klp5Δ bub1Δ* mutant compared to both single mutants alone (Figure 2.3C). We conclude that chromosome missegregation is unlikely to be the major reason for the synthetic growth defect of *klp5Δ bub1Δ* cells.

In addition to missegregation, *klp5Δ bub1Δ* cells often showed abnormal shapes and septation, such as two septa, asymmetric septation and failure in cell separation, indicating that these cells also have a septation defect and possibly a cytokinesis defect (Figure 2.3). The *bub1Δ* mutant alone also often showed two septa in one cell. Thus, Bub1 might be directly involved in the septation process. Some cells had one septum very close to one pole of the cell. These cells could have derived from a mother cell with two septa, at one of which cytokinesis took place (Figure 2.3B, see *bub1Δ*, upper panel, lower cell).

The combination of missegregation and septation/cytokinesis defects might be sufficient to explain the loss of viability in *klp5Δ bub1Δ* cells. A function of Bub1 in cytokinesis has not been described so far, and further studies are required to draw a conclusion. It would have to be investigated, whether Bub1 is directly involved in septation/cytokinesis, or whether the septation defects are increased in the absence of *bub1* due to secondary effects, such as the presence of DNA material at the site of septation (lagging chromosomes), which is a characteristic phenotype of *bub1Δ* mutants (Vanoosthuyse et al., 2004).

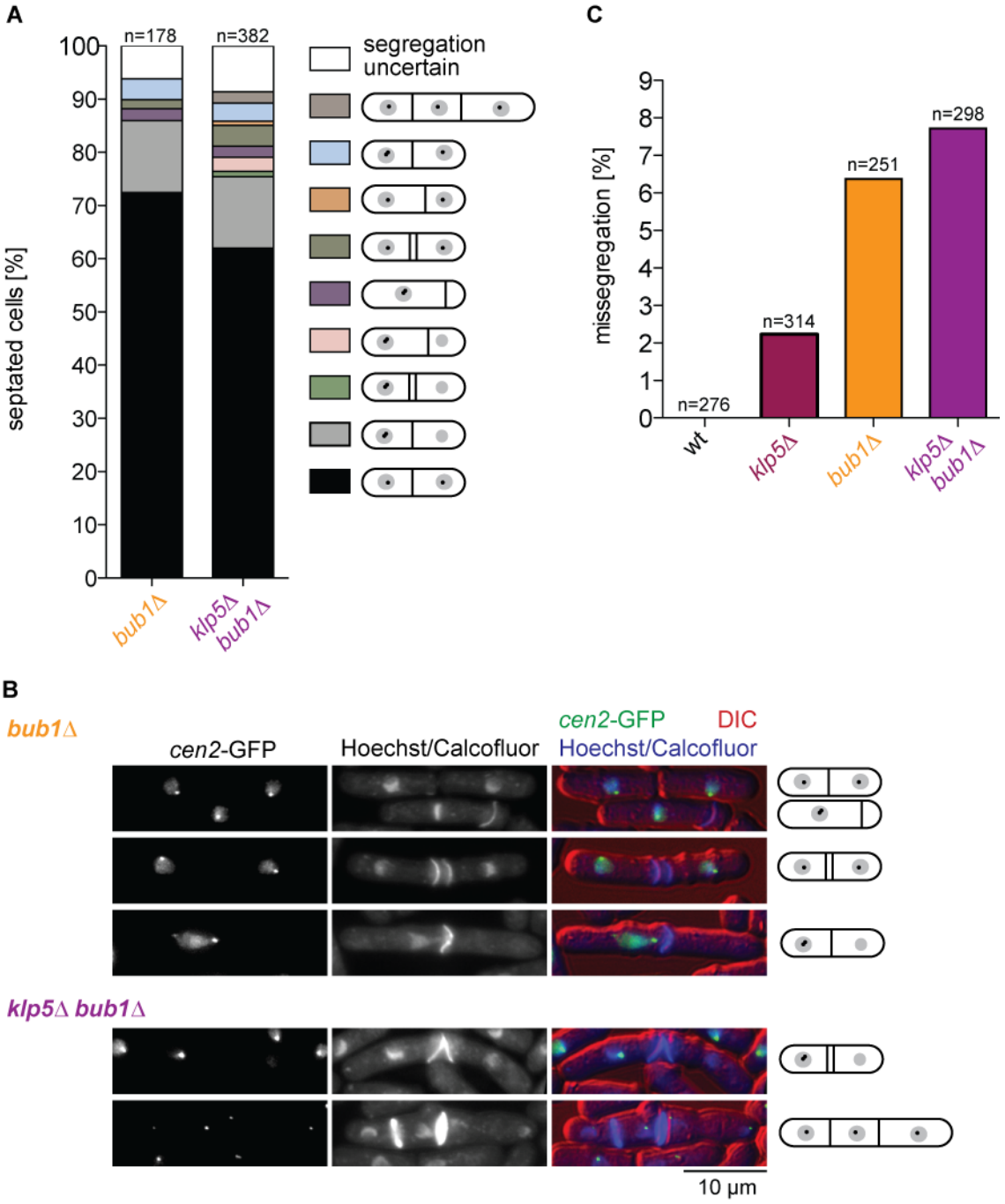

Figure 2.3 *klp5Δ bub1Δ* cells show chromosome missegregation and septation defects

(**A, B**) Cells from indicated strains carrying *cen2-GFP* were synchronized with hydroxyurea (HU) at 30 °C. After release from HU arrest, cells were incubated for 90 min at 34 °C. Fixed cells were stained with Hoechst and Calcofluor to visualize DNA and septa, respectively. (**A**) Percentage of septated cells in *bub1Δ* and *klp5Δ bub1Δ* mutants showing the indicated chromosome segregation and septation defects. (**B**) Example pictures of cells analyzed in (A). (**C**) Cells from indicated strains carrying *cen2-GFP* were synchronized with HU at 30 °C. After release from HU arrest, cells were incubated for 60 min at 34 °C and subsequently fixed. Segregation of chromosome II was analyzed. The missegregation rate in *bub1Δ* cells was much higher than in *klp5Δ* cells, and additional deletion of *klp5* in *bub1Δ* cells did not increase missegregation much further.

2.2.3 Proteins that could act together with Bub1 to exert the unknown function

2.2.3.1 *klp5* synthetic lethal screen

To identify genes that could act together with Bub1 to fulfill the function that is required for growth in the absence of kinesin-8 proteins, we screened for mutants that are synthetically sick or lethal with *klp5Δ*. To this end, I constructed a strain that carried the *klp5+* wild type gene on a plasmid (*pREP4-klp5+*; Maundrell, 1993) and had the endogenous *klp5* gene deleted (Figure 2.4A). The presence of the plasmid in the *klp5Δ* strain abolished its TBZ resistance, showing that this plasmid can rescue the endogenous *klp5* deletion (Figure 2.4B). The plasmid carried the *ura4+* gene as a selectable marker. This marker can also be used for counterselection of the plasmid on plates containing the drug 5-FOA if the endogenous *ura4+* gene is deleted (Boeke et al., 1987; Grimm et al., 1988). Plasmids can be lost stochastically during mitosis, and in the absence of selective pressure, cells that have lost the plasmid continue to grow. In contrast, cells without endogenous *ura4+* that have lost the *pREP4-klp5+(ura4+)* plasmid are unable to grow on medium lacking uracil, but they are able to grow on 5-FOA medium (supplemented with uracil), whereas a cell that still harbours the plasmid would not survive on this medium (Figure 2.4A). To verify that the plasmid can indeed be lost, I streaked *ura4Δ* cells that carried the plasmid *pREP4-klp5+* on 5-FOA medium. They were able to grow, indicating that the plasmid carrying the *ura4+* marker could be lost. Furthermore, *klp5Δ* cells carrying the *pREP4-klp5+* plasmid were resistant to high amounts of TBZ when grown on full medium, which released the selective pressure for the plasmid and allowed for plasmid loss, resulting in the typical TBZ-resistant *klp5Δ* phenotype (Figure 2.4C).

After mutagenizing the described strain with EMS, the cells were screened for mutants that do not grow on 5-FOA (Figure 2.4A). If the introduced mutation(s) do not cause synthetic lethality with *klp5Δ*, cells that have lost the plasmid are viable and able to grow on 5-FOA. In contrast, cells with synthetic lethal mutations will not grow on 5-FOA, because they cannot survive in the absence of the plasmid. We screened about 10^5 mutagenized cells and obtained 43 synthetically lethal or sick candidates. Figure 2.4C shows a streaking assay of two candidates that were obtained in the screen. Note that candidate a7 clearly shows a synthetic growth defect with *klp5Δ* and in addition confers benomyl sensitivity. In contrast, the streaking assay revealed that the a3 candidate has only a slight or no synthetic growth defect with *klp5Δ*. It is possible that this candidate first showed synthetic sickness with *klp5Δ*, but then acquired a suppressor mutation during the screening procedure.

Some of the candidates showed a different morphology than the wild type in that they were much rounder (Figure 2.4D). This is a known phenotype of mutants of the Ras pathway (Fukui et al., 1986; Fukui and Yamamoto, 1988; Nadin-Davis et al., 1986), and it has been noticed that mutants of the Ras pathway are synthetically lethal with *klp5Δ* (Li and Chang, 2003) (see below). Thus, we probably obtained mutants of these genes in the screen, indicating that the screen efficiently isolated *klp5Δ* synthetic lethal/sick interactors. The next step would be to identify the genes that are mutated by complementation using a genomic library. Having other valuable data in hand (see chapter 2.3), I did not pursue this further.

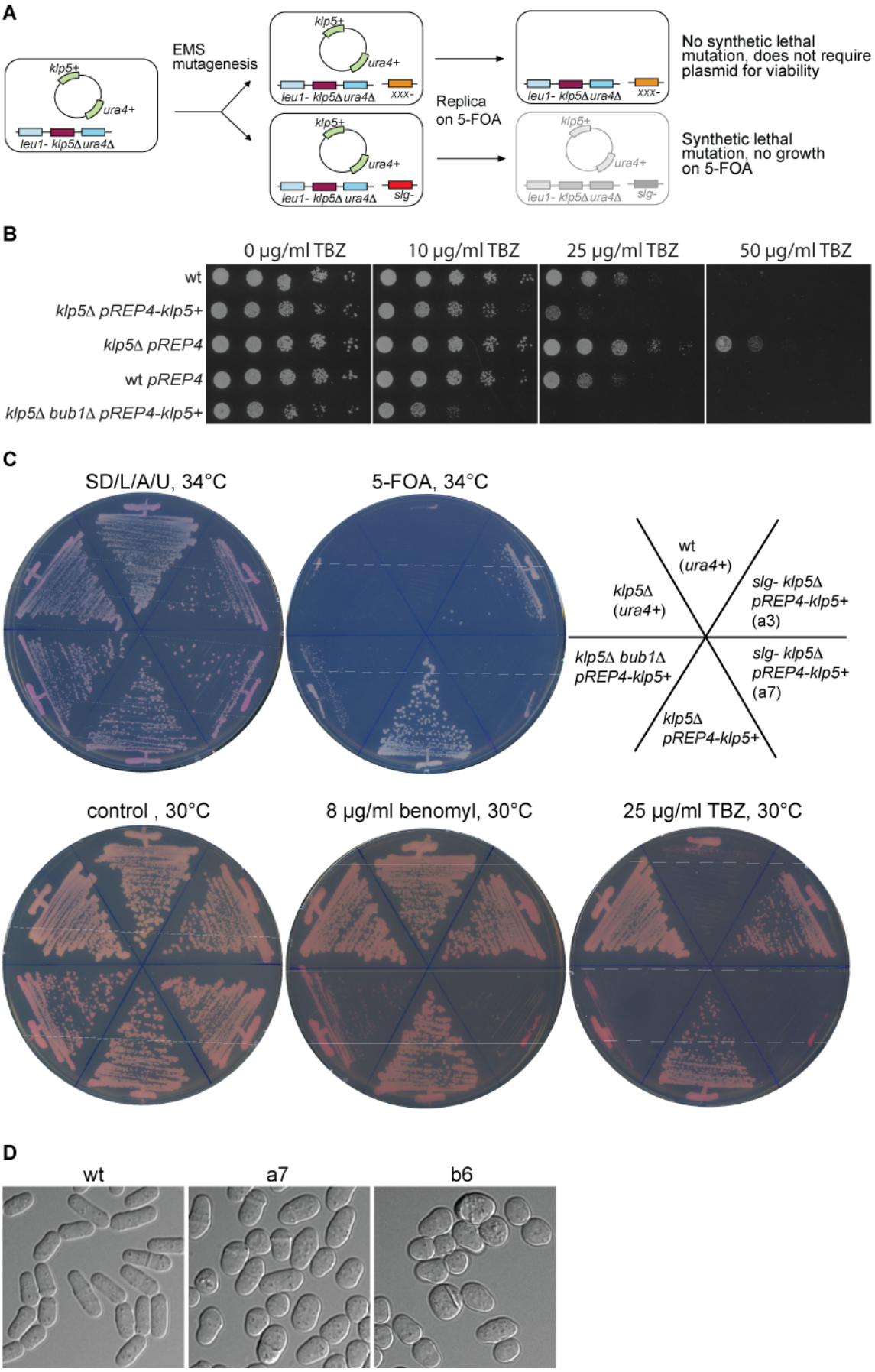

Figure 2.4 *klp5* synthetic lethal screen

2.2.3.2 Known synthetic lethal interactors of *klp5Δ*

It has been described for several genes that they have a synthetic growth defect in conjunction with *klp5Δ*. Apart from *bub1Δ*, this has been reported for mutants of the anaphase-promoting complex/cyclosome (APC/C), *nuc2-663*, *cut4-533*, *cut9-665* (West et al., 2002), the α-tubulin mutant *nda2-KM52* (West et al., 2001), the γ-tubulin mutant *gtb1-93* (Tange et al., 2004), mutants of genes encoding for the microtubule-associated proteins Dis1 and Alp14 (Mtc1), *dis1-1*, *dis1Δ* and *alp14Δ* (Garcia et al., 2002a; West et al., 2002), mutants of Dam1/DASH complex, *dam1Δ*, *duo1Δ*, *spc34Δ*, *ask1Δ* and *dad1Δ* (Sanchez-Perez et al., 2005), as well as mutants of the Ras-pathway, *ras1Δ*, overexpression of the dominant negative *cdc42-T17N*, *scd2Δ* and *orb2-34* (Li and Chang, 2003).

It is possible that the proteins encoded by these genes act together with Bub1 to fulfill a function that is required for survival of *klp5Δ*. In the lab, a specific *klp5* mutant (*klp5-T224D*) has been characterized that similar to *klp5Δ* shows resistance toward microtubule-depolymerizing drugs, delays in mitosis and has longer spindles (Julia Kamenz and Silke Hauf, unpublished data). It also has longer astral microtubules than the wild type during early mitosis, but my colleague Yu-Hua Huang and I found that the mutant protein still possesses a functional plus-end directed motor activity (data not shown). This indicates that the mutation mostly affects the depolymerase activity of Klp5. Remarkably, it does not show a strong synthetic sick interaction with *bub1Δ* (Julia Kamenz, unpublished data), which implies that the depolymerizing function of Klp5 plays a minor role for survival of the *bub1Δ* mutant. This mutant provided a convenient tool to shed light on which of the known synthetic lethal interactors of *klp5Δ* could be involved in the same process as Bub1. If *klp5-T224D* combined with the mutants mentioned above had no or very little growth defect, it was possible that they act in the same pathway as *bub1* (Figures 2.5).

Figure 2.4 *klp5* synthetic lethal screen

(**A**) Schematic outline of the screening procedure. The strain used for the *klp5* synthetic lethal screen had the endogenous *klp5* and *ura4* genes deleted and carried a wild type *klp5+* copy on a plasmid with *ura4+* as a marker (*pREP4-klp5+*). Cells were mutagenized with EMS, grown on non-selective plates and replica plated to 5-FOA plates. The plasmid gets lots stochastically during cell divisions. On 5-FOA plates, only those cells that have lost the plasmid can grow, however, if they carry a mutation that confers synthetic lethality with *klp5*, they cannot grow on 5-FOA medium at all, as they also die when they lose the plasmid carrying the *klp5+* gene. *slg*: synthetic lethal gene. (**B**) Growth assay with the indicated strains on plates lacking uracil. The *pREP4-klp5+* plasmid can complement the endogenous *klp5* deletion as judged by lack of resistance toward the microtubule-destabilizing drug TBZ in the *klp5Δ pREP4-klp5+* strain, as compared to the *klp5Δ pREP4* (empty vector) strain. (**C**) Streaking assay of two candidate synthetic lethal mutation (*slg-)* containing strains as well as control strains on full medium (lower plates), containing the indicated drugs, and on minimal medium (upper plates), without 5-FOA (SD/L/A/U) or with 5-FOA (5-FOA). The *klp5Δ pREP4-klp5+* strain was able to grow on 5-FOA plates and showed resistance toward 25 µg/ml TBZ, hence the plasmid *pREP4-klp5+* can be lost when there is no selective pressure for the *ura4+* gene present on the plasmid. Two exemplary candidates, a3 and a7, that were obtained in the screen are shown. Whereas a3 turned out to be not synthetically lethal with *klp5Δ*, the mutation(s) present in a7 resulted a clear synthetic lethal interaction with *klp5Δ*, and, in addition, turned out to cause sensitivity toward microtubule-destabilizing drugs. (**D**) DIC images of living cells. The synthetic lethal candidates a7 and b6 showed a round morphology as compared to wild type cells.

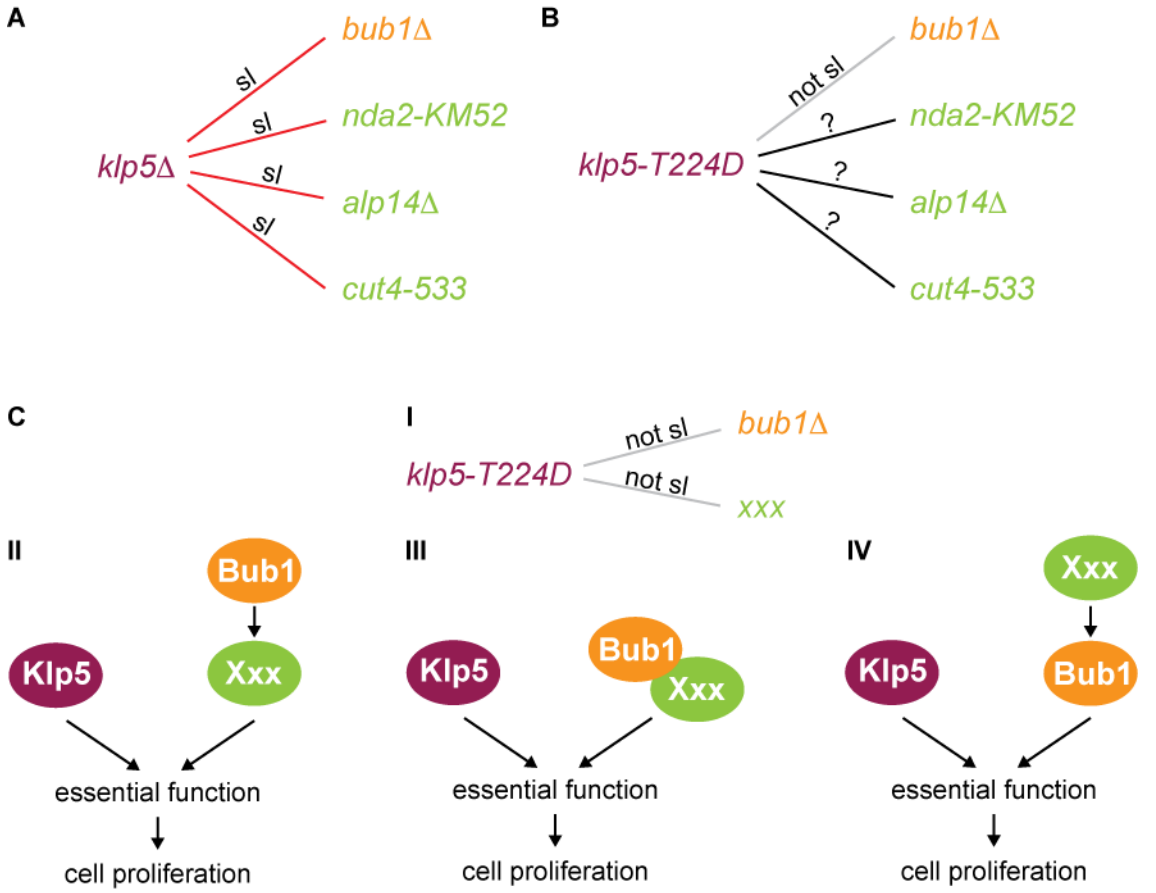

Figure 2.5 Bub1 possibly shares a function with a known synthetic lethal interactor of *klp5Δ*
(**A**) Some of the known synthetically lethal or sick (sl) interactions with *klp5Δ*. (**B**) The *klp5-T224D* mutant does not show synthetic sickness with *bub1Δ* and it was not known whether this *klp5* mutant has a synthetic sickness with other known synthetic interactors of the *klp5* deletion mutant. (**C**) If a known synthetic interactor of *klp5Δ* (*xxx*) is not synthetically sick or lethal with *klp5-T224D* (**I**), it is possible that it functions together with Bub1 in the same pathway, either downstream of Bub1 (**II**), together with Bub1 (**III**) or upstream of Bub1 (**IV**).

I analyzed synthetic interactions between *klp5-T224D* and the APC/C mutant *cut4-533* (Yamashita et al., 1996), the α-tubulin mutant *nda2-KM52* (Toda et al., 1983) as well as *alp14Δ* (Figure 2.6). No *klp5-T224D cut4-533* double mutants were obtained (16 tetrads), indicating that they are synthetically lethal (Figure 2.6A). Similarly, the combination of *klp5-T224D* with *alp14Δ* showed synthetic lethality (15 tetrads) (Figure 2.6B). Thus, the *klp5-T224D* mutation abolishes the functions of Klp5 that are required for survival of the *cut4-533* and the *alp14* mutant, whereas it does not abolish the function of Klp5 that is required for efficient growth of *bub1Δ* cells. Furthermore, in contrast to *bub1Δ*, the *dam1Δ* mutant is synthetically lethal with *klp5-T224D* (Julia Kamenz, unpublished data). Together with the fact that Dam1 and Bub1 do not show localization dependencies (see below, paragraph 2.2.3.2.1, Figure 2.7 and Sanchez-Perez et al., 2005), this points in the direction that Dam1 and Bub1 perform different functions that are – independently from each other - important in the *klp5* deletion mutant.

In contrast to *cut4-533*, *alp14Δ* and *dam1Δ*, spore colonies carrying the cold-sensitive *nda2-KM52* allele in addition to the *klp5-T224D* mutation were able to grow at 30 °C (Figure 2.6C), even though the *klp5Δ nda2-KM52* double mutant does not form colonies at the permissive temperature for *nda2-KM52* (32 °C) (West et al., 2001). However, one has to note that the *klp5-T225D nda2-KM52* mutants formed somewhat smaller colonies than either single mutant alone, indicating that there is a synthetic growth defect.

The published data taken together with these results suggest that the depolymerase function of Klp5 is not essential for survival of *bub1Δ*, and given that the *klp5-T225D nda2-KM52* mutant has a much weaker synthetic growth defect than *klp5Δ nda2-KM52*, the Bub1 function required for efficient growth of *klp5Δ* could be tightly connected to microtubule function.

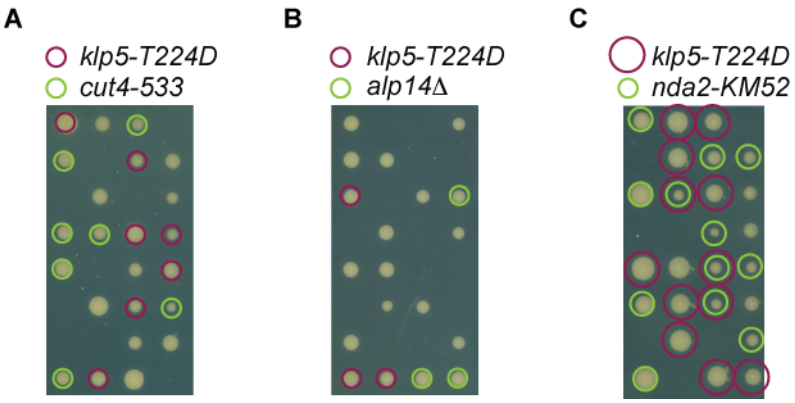

Figure 2.6 Genetic interactions between *klp5-T224D* and known synthetic lethal interactors of *klp5Δ*

Tetrad analysis of a *klp5-T224D* strain crossed with *cut4-533* (**A**), *alp14Δ* (**B**) and *nda2-KM52* mutants (**C**). Tetrads were incubated at 30 °C. The *klp5-T224D cut4-533* and *klp5-T224D alp14Δ* double mutants were synthetically lethal, whereas the *klp5-T224D nda2-KM52* mutant showed merely a synthetic sick phenotype.

2.2.3.2.1 Dam1 localization to kinetochores is not perturbed in the absence of Bub1

It has been reported that double deletion mutants of *klp5* or *klp6* and genes encoding components of the Dam1/DASH complex are synthetically lethal (Sanchez-Perez et al., 2005) (see above). However, the reason for this synthetic lethality is not known. The Dam1/DASH complex localizes to kinetochores and to plus ends of spindle microtubules in mitosis and is required for correct kinetochore microtubule attachment (Sanchez-Perez et al., 2005). We considered the possibility that Bub1 is involved in recruiting the Dam1/DASH complex to kinetochores. If this was the case, the synthetic growth defect in *klp5Δ bub1Δ* cells could be due to loss of Dam1/DASH at the kinetochore. The *bub1Δ dam1Δ* double deletion mutant is viable (Sanchez-Perez et al., 2005), which was concurrent with the notion that Bub1 and Dam1 act in the same pathway. Moreover, localization of Bub1 to kinetochores had been shown to be independent of Dam1 (Sanchez-Perez et al., 2005), indicating that Bub1 does not act downstream of the Dam1/DASH complex at the

kinetochore. I checked mitotic localization of Dam1-GFP to kinetochores in *bub1Δ* cells and could not see a reduction compared to the wild type (Figure 2.7). This excludes that mislocalization of the Dam1/DASH complex in the absence of Bub1 is the cause for the synthetic sickness of *klp5Δ bub1Δ* cells.

A

	cells	metaphases with Dam1-GFP on kinetochores (% of metaphases)	anaphases with Dam1-GFP on kinetochores (% of anaphases)
wt	111	17 (90%)	88 (96%)
bub1Δ	65	20 (95%)	41 (93%)

B

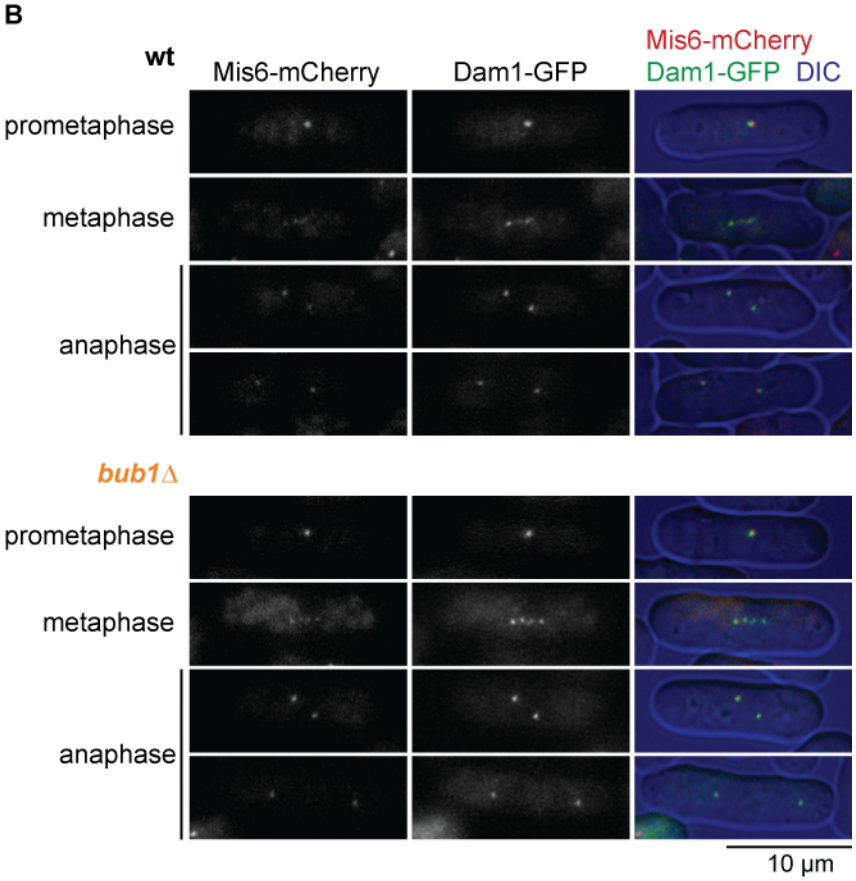

Figure 2.7 Kinetochore localization of Dam1 is Bub1-independent
Living cells from strains expressing *mis6-mCherry* as a marker for kinetochores and *dam1-GFP* were imaged by fluorescence microscopy. (**A**) Percentage of cells in wild type and *bub1Δ* cells showing Dam1-GFP signals at kinetochores. Dam1 localization was not perturbed in the absence of Bub1. (**B**) Example pictures of cells analyzed in (A).

2.2.4 Bub1 separation-of-function mutants

2.2.4.1 Genetic screen for *bub1* separation-of-function mutants

In order to characterize the role of Bub1 further, we wanted to screen for mutants that are specifically defective in the function(s) required in *klp5Δ* cells, i.e. that have normal Sgo2 localization and a functional checkpoint, but show synthetic lethal interaction with *klp5Δ*. The phenotypic analysis of the complete deletion of *bub1* is difficult because of its pleiotropic effects, hence specific alleles would facilitate analysis of the function required for survival of *klp5Δ*. To obtain specific *bub1* alleles, I mutagenized the *bub1* gene by random PCR mutagenesis, replaced the endogenous wild type copy and screened for synthetic sickness with *klp5Δ* using the 5-FOA counterselection method described above (see chapter 2.2.3.1). I obtained nine alleles that were synthetically lethal with *klp5Δ* (Figure 2.8A), four of which showed normal Sgo2 localization (Figure 2.8B) (*bub1-m3*, *bub1-m5*, *bub1-m8* and *bub1-m9*), indicating that these *bub1* alleles are expressed, and that the mutant proteins are at least partially functional. The five other mutants (*bub1-m2*, *bub1-m4*, *bub1-m6*, *bub1-m7* and *bub1-m10*) had premature stop codons, all of which terminated the open reading frame before the region coding for the kinase domain, which is essential for Sgo2 localization (Kitajima et al., 2004) (Figure 2.9). As expected for the loss of the kinase domain, none of these truncation mutants showed normal Sgo2 localization (Figure 2.8B and data not shown). The four alleles that preserved Sgo2 localization (*bub1-m3*, *bub1-m5*, *bub1-m8* and *bub1-m9*) had up to three amino acid changes, none of which were situated in the C-terminal kinase domain (Figure 2.9).

2.2.4.2 Spindle assembly checkpoint activity of *bub1* mutants

We tested whether the checkpoint is functional in cells carrying the *bub1-m3*, *bub1-m5*, *bub1-m8* and *bub1-m9* alleles (which showed normal Sgo2 localization) in the *nda3-KM311* mutant background. The *nda3-KM311* allele is a cold-sensitive β-tubulin allele that at restrictive temperature leads to a spindle assembly checkpoint-mediated arrest in mitosis, due to microtubule instability and the resulting presence of unattached kinetochores (Hiraoka et al., 1984; Umesono et al., 1983). To judge whether cells have a functional spindle assembly checkpoint and delay anaphase onset in the presence of chromosome attachment errors, we used Plo1-GFP as a mitotic marker. It localizes to spindle pole bodies when the cell enters mitosis and disappears from there in early anaphase B (Mulvihill et al., 1999). Cells carrying the *bub1-m5* and *bub1-m8* alleles showed a strong delay in mitosis at 17 °C, indicating that under these conditions the checkpoint is still functional (Figure 2.10A). In contrast, *bub1-m3* and *bub1-m9* failed to arrest, thus at least some of the mutations present in these strains disrupt checkpoint activity (see discussion, chapter 3.2).

The *bub1-m8* allele, which seems to have a functional spindle assembly checkpoint, has a tryptophan (W) 53 to arginine (R) mutation in the Mad3/Bub1 homology region I. The tryptophan is conserved among vertebrates, whereas other yeasts either have a phenylalanine or a tyrosine at this position, with fission yeast being an exception (Figure 2.11). The other two mutations affect residues that are not well conserved (Figure 2.11).

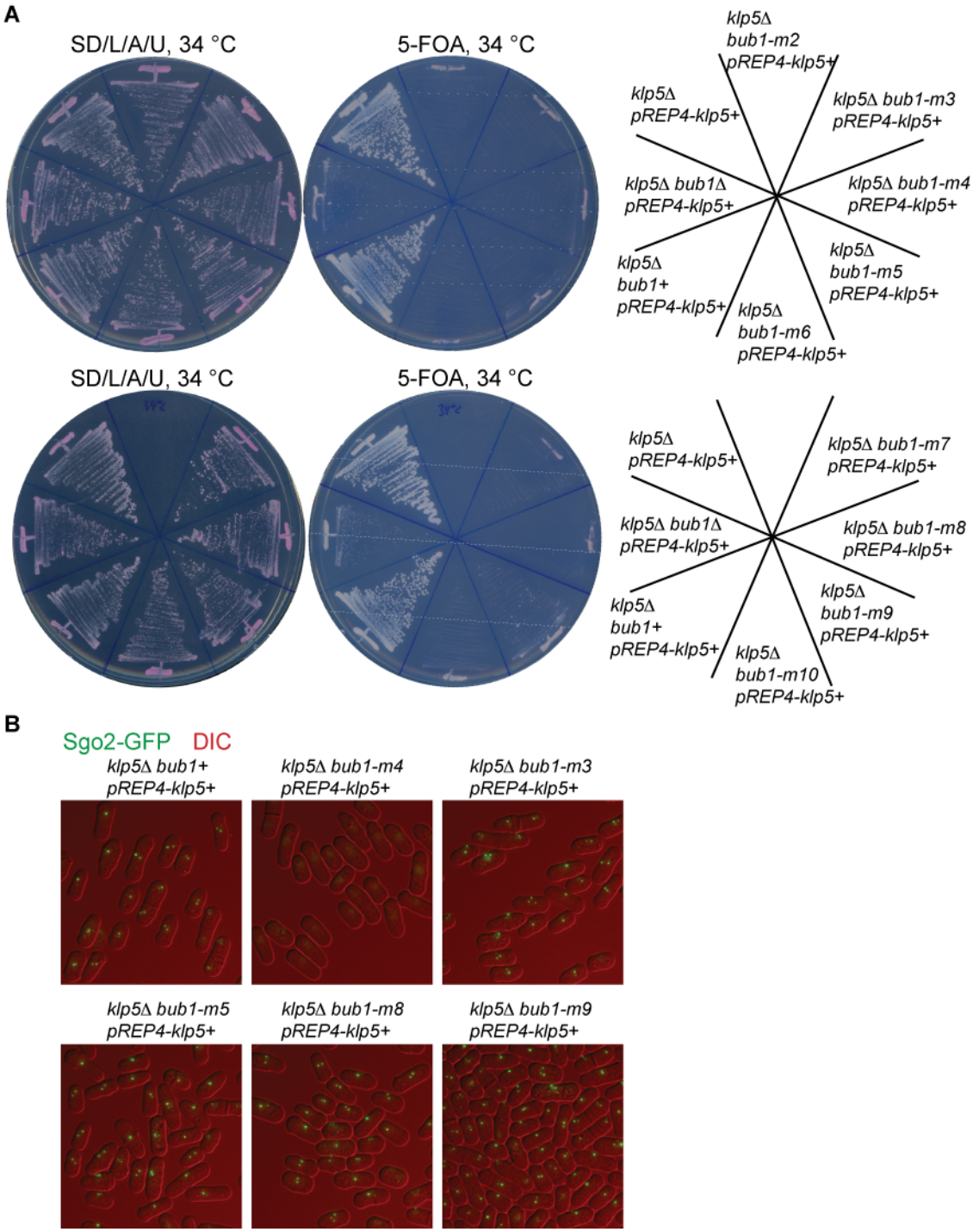

Figure 2.8 Screen for *bub1*-separation-of-function mutants

(**A**) Streaking assay of *bub1* mutants that were identified in a screen for synthetic sickness with *klp5Δ* by counterselection on 5-FOA plates. The screen revealed nine mutants (*bub1-m1* to *bub1-m9*) that were not able to grow in the absence of *klp5+*. (**B**) Living cells from the indicated strains expressing *sgo2-GFP* were imaged by fluorescence microscopy. Sgo2-GFP localization in the *bub1-m3*, *bub1-m5*, *bub1-m8* and *bub1*-m9 mutants was comparable to wild type (*klp5Δ bub1+ pREP4-klp5+*), whereas the *bub1-m4*, *bub1-m6*, *bub1-m7* and *bub1-m10* mutations abolished Sgo2 localization (data shown for *bub1-m4* only). All *klpΔ bub1-m pREP4-klp5+* strains and the control strain *klp5Δ bub1+ pREP4-klp5* in (A) and (B) carried *sgo2+* tagged with GFP at the endogenous locus.

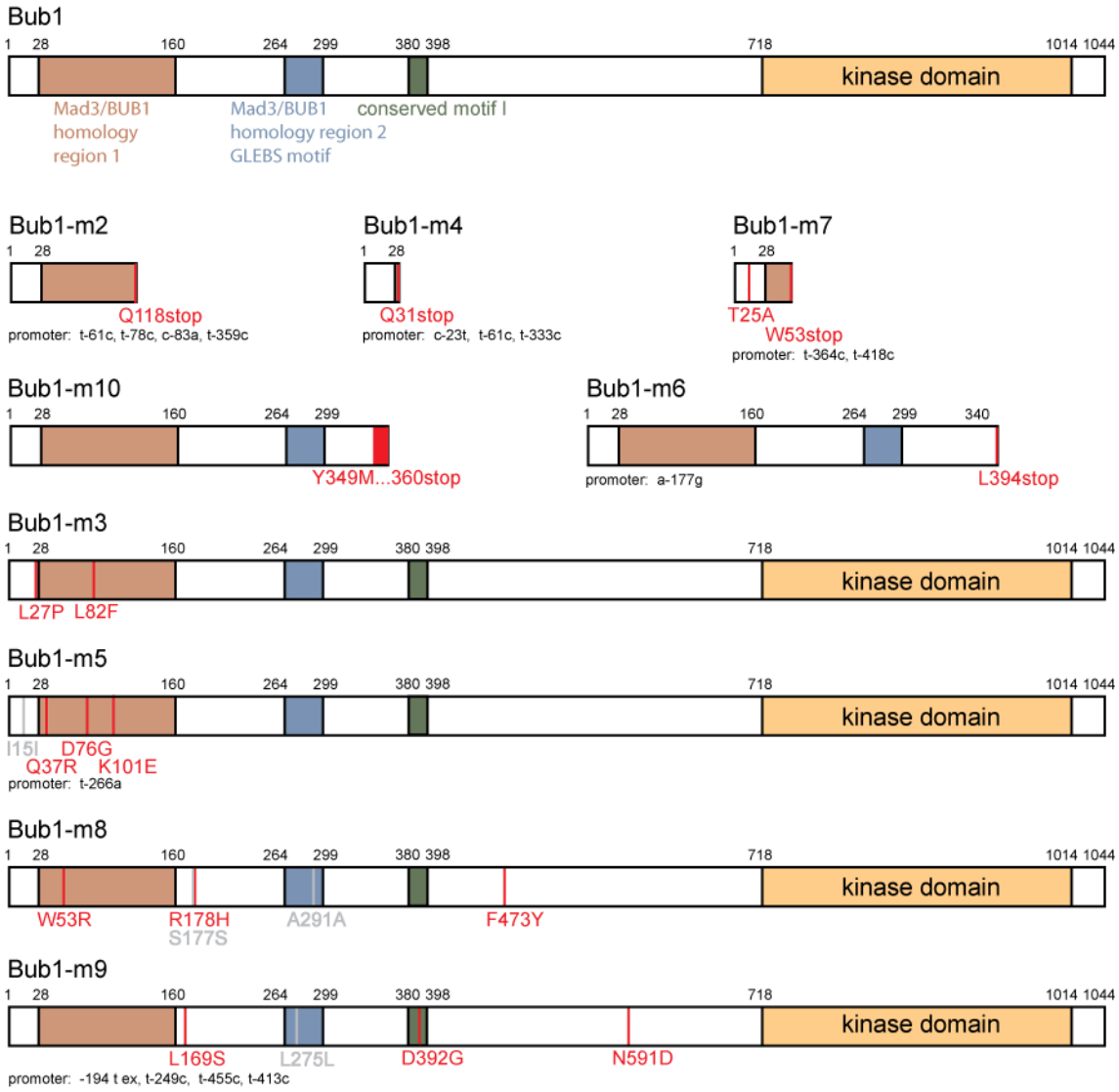

Figure 2.9 Bub1 mutants that show synthetic sickness with *klp5Δ*

Schematics of the Bub1 mutants that were identified in the screen for synthetic sickness in conjunction with *klp5Δ*. Amino acid changes are shown in red, silent mutations are indicated in grey, and mutations in the putative promoter region are indicated below the respective schematic.

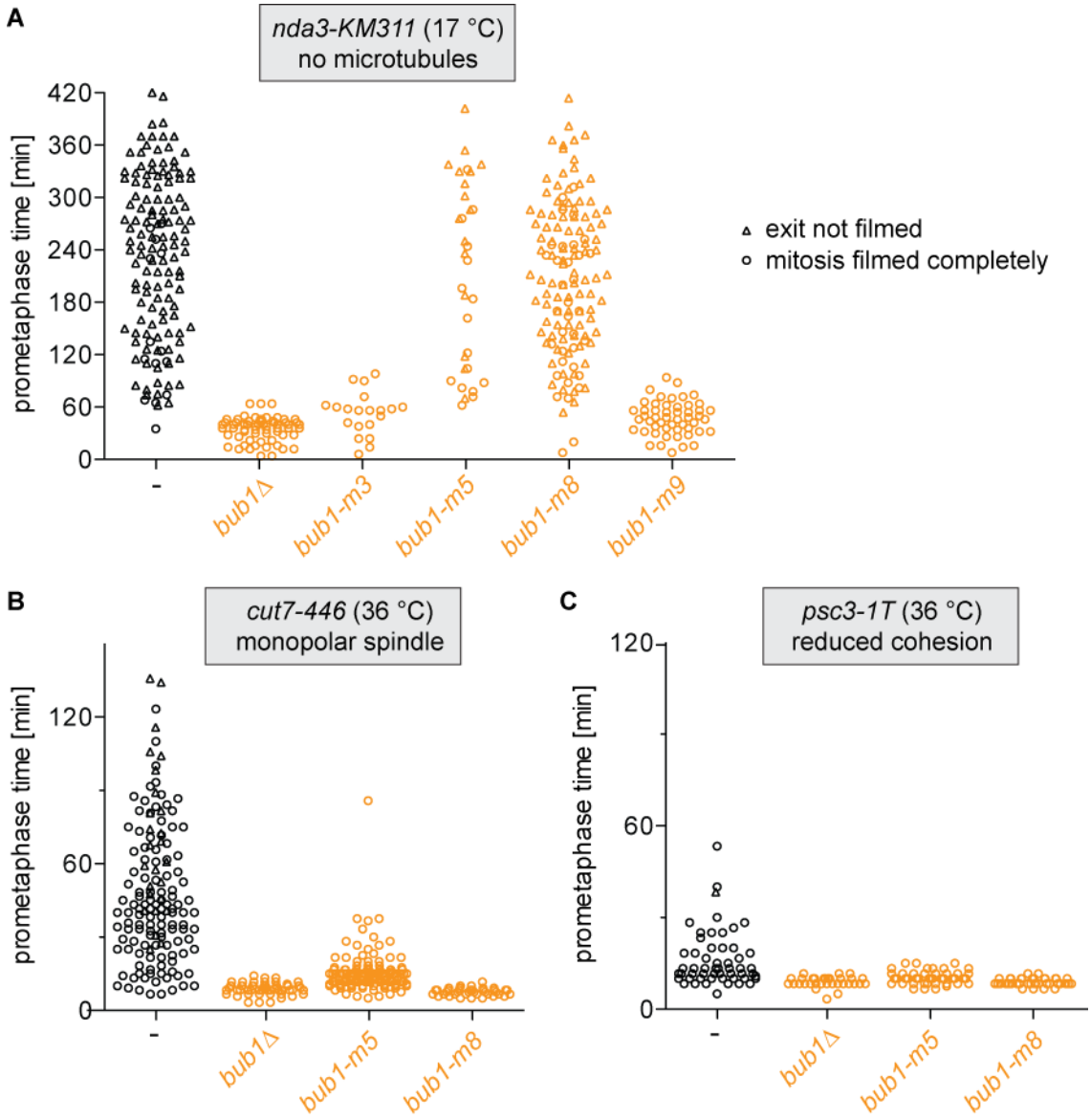

Figure 2.10 SAC activity of *bub1* separation-of-function mutants

Cells expressing *plo1-GFP* and the β-tubulin *nda3-KM311* allele (**A**), the *cut7-446* allele (**B**) or the *psc3-1T* allele (**C**) were followed by live cell microscopy at the restrictive temperatures (indicated above the diagrams). The duration of prometaphase was determined by the presence of Plo1-GFP on the SPBs. The *bub1-m3* and *bub1-m9* mutants were not able to delay in mitosis. In contrast, the *bub1-m5* and the *bub1-m8* mutants were able to delay in the *nda3-KM311* background (A). However, they failed to prolong mitosis efficiently in the *cut7* and *psc3* temperature-sensitive mutants, see (B) and (C).

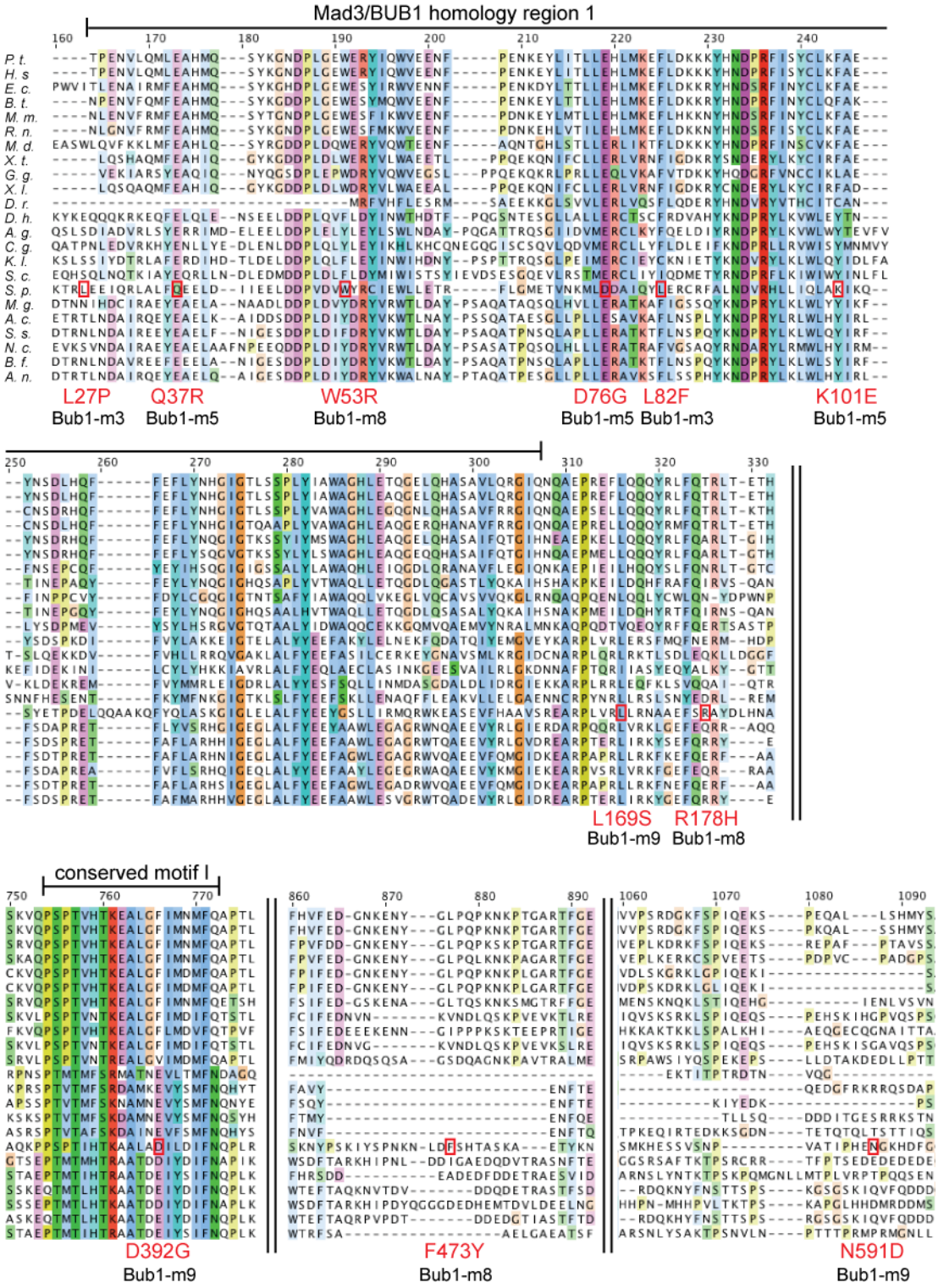

Figure 2.11 Alignment of Bub1 from different species with indicated mutations of the *bub1* separation-of-function alleles

Fragments of an alignment of Bub1 protein sequences from the indicated species (see below for complete names of species). The alignment was prepared with Jalview 2.5 using Muscle Protein Sequence Alignment (Waterhouse et al., 2009). Red boxes in the *S. pombe* sequence indicate the mutations present in the *bub1* separation-of-function mutants that showed synthetic sickness with *klp5Δ* but normal Sgo2 localization. Conserved regions are indicated above the alignment. Species, in order of the sequences from top to bottom: *Pan troglodytes*, *Homo sapiens*, *Equus caballus*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Monodelphis domestica* (gray short-tailed

Since *bub1-m5* and *bub1-m8* mutants show synthetic lethality with *klp5Δ* but are able to localize Sgo2 to kinetochores and to activate the spindle assembly checkpoint in the presence of attachment errors, they were of interest for further studies. To ensure that the observed phenotypes are caused by the mutations in *bub1* and are not due to additional, second site mutations that the strains had acquired spontaneously, I decided to reconstruct the mutants. As mentioned above, the seemingly most interesting mutation in *bub1-m8* is W53R, which resides in the Mad3/BUB1 homology region 1. All of the mutations in *bub1-m5* are found in this region. Thus, the Mad3/BUB1 homology region 1 seems to be of particular importance for the function of Bub1 that is required for healthy growth of kinesin-8-deleted cells. I reconstructed the mutations present in *bub1-m5*, in different combinations of the three single amino acid changes, including the triple mutant, and checked for synthetic lethality with *klp5Δ*. The triple mutant showed synthetic lethal interaction, as expected, ruling out that the observed phenotype was due to other mutations the strain could have acquired (Figure 2.12). Surprisingly, the mutation of the conserved aspartate (D) 76 to glycine (G) as a single mutant did not have a strong effect on survival of *klp5Δ* cells, but mutants containing the lysine (K) 101 to glutamate (E) mutation were very sick in combination with *klp5Δ* (Figure 2.12). However, the D76G mutation certainly also contributes to the synthetic growth defect. The glutamine (Q) 37 to arginine (R) mutation is peculiar in that all other organisms that are shown in the alignments in Figure 2.11 have a glutamate at this position. However, the Q37R mutation does not seem to have a strong effect on synthetic sickness with *klp5Δ*. Together these data suggest that the N-terminal Mad3/BUB1 homology region 1 is not only crucial for checkpoint activity, but also for the function that is required for survival of *klp5Δ* cells.

We also assayed checkpoint activity of the four *bub1* mutants using temperature-sensitive alleles of a cohesin subunit (*psc3-1T*, *scc3* homolog) and the kinesin-5 (*cut7-446*), which do not disturb kinetochore microtubule attachment *per se*, but cause reduced tension at kinetochores, due to lack of resisting cohesion-mediated forces of the two sister chromatids or due to formation of monopolar spindles, respectively (Hagan and Yanagida, 1990; Nonaka et al., 2002). Under these conditions, none of the mutants showed a robust mitotic delay (Figure 2.10B,C). There are two possible explanations for this. The first one is that *bub1-m5* and *bub1-m8* are temperature-sensitive mutants of *bub1*. This is plausible, since synthetic sickness with *klp5Δ* was assayed at 34 °C, whereas functionality of the 'no-attachment' checkpoint was assayed at 17 °C. Alternatively, these mutants could be checkpoint-proficient only in the presence of severe checkpoint defects, whereas under milder 'no-tension' conditions they might not be able to create or maintain a robust signal.

Figure 2.11 continued

opossum), *Xenopus tropicalis*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, and various fungi: *Debaryomyces hansenii*, *Ashbya gossypii*, *Candida glabrata*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Schizzosaccharomyces pombe*, *Magnaporthe grisea*, *Aspergillus clavatus*, *Sclerotinia sclerotiorum*, *Neurospora crassa*, *Botryotinia fuckeliana*, *Aspergillus nidulans*.

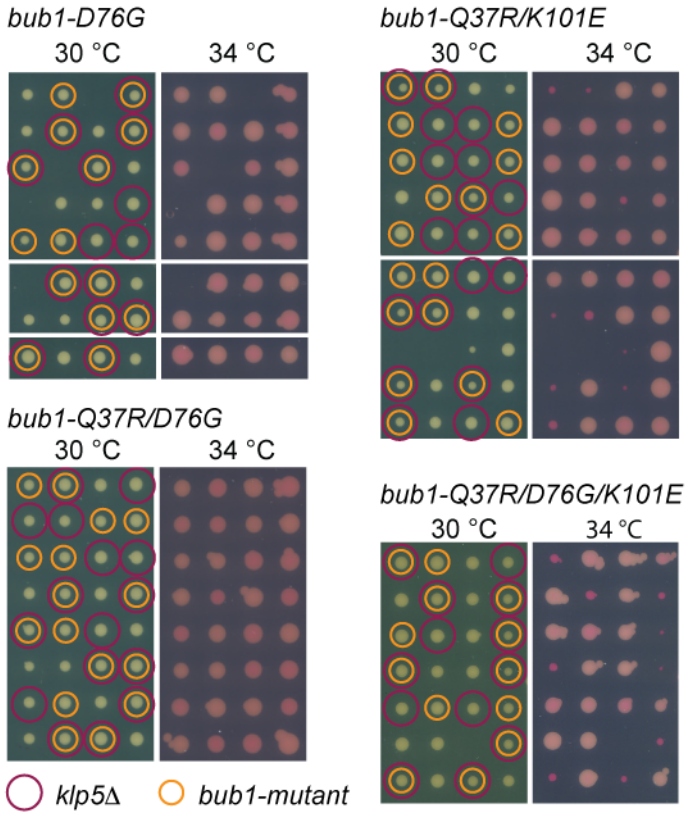

Figure 2.12 Synthetic interactions of reconstructed *bub1-m5* mutants with *klp5*Δ
Tetrad analysis of strains carrying reconstructed *bub1-m5* mutations crossed with a *klp5Δ* strains. Tetrads were incubated at 30 °C and 34 °C. The reconstructed *bub1-m5* mutant showed synthetic sick interaction with *klp5Δ*. Of the three amino acid changes present in Bub1-m5, the K101E mutation seemed to have the strongest negative influence on growth in the *klp5Δ* background.

2.3 Bub1 and Bub3 share function(s) outside the spindle assembly checkpoint

2.3.1 Deletion of *bub3* causes synthetic lethality with *klp5Δ*

I wanted to examine more carefully how the other checkpoint genes, apart from *bub1*, interact genetically with *klp5*. Interestingly, the *klp5Δ bub3Δ* double mutant had a growth defect that was even more severe than that of *klp5Δ bub1Δ* cells, both at 34 °C as well as at 30 °C (Figure 2.13). In contrast, *klp5Δ mad3Δ* cells did not have a visible growth defect, similar to the *klp5Δ mad2Δ* double deletion mutant, whereas combining *mad1Δ* with *klp5Δ* resulted in a slight synthetic growth defect, which was, however, not much increased at 34 °C. The *klp5Δ mph1Δ* double mutant had no or only a very slight growth defect. These results indicate that not only Bub1 but also Bub3 is required for survival of *klp5*-deleted cells. Since mammalian and budding yeast Bub1 and Bub3 are direct interaction partners (Larsen et al., 2007; Seeley et al., 1999; Wang et al., 2001), it was likely that they have to act together to fulfill the unknown function. Thus, if this interaction was abolished, the viability of *klp5Δ* should be greatly impaired. To this end, I constructed a *bub1* mutant lacking the

GLEBS motif (Mad3/BUB1 homology region 2), which is known to be required and sufficient for interaction with Bub3 (Larsen et al., 2007; Taylor et al., 1998; Vanoosthuyse et al., 2009; Wang et al., 2001) (Figure 2.14A). Indeed, the *klp5Δ bub1-ΔGLEBS* double mutant showed a synthetic growth defect that was similar to the *klp5Δ bub3Δ* double mutant (Figure 2.14B).

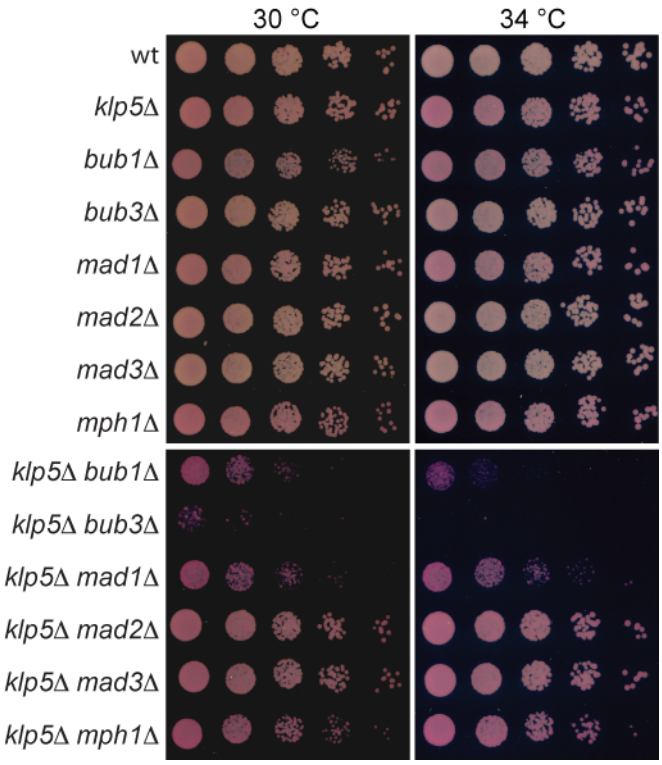

Figure 2.13 Bub3 is required for viability of *klp5*-deleted cells
Growth assay with the indicated strains. 5-fold serial dilutions were spotted on full medium containing Phloxin-B, a stain that specifically accumulates in dead cells. Plates were incubated at the indicated temperature for 2 d. Additional deletion of *klp5* in *bub3Δ* cells resulted in a growth defect that was even more prominent than that in *bub1Δ* cells, whereas deletion of other SAC components had no or much milder effects.

Furthermore, I asked whether the other Mad3/BUB1 homology region (1) was required for survival of *klp5Δ*. A fission yeast *bub1* mutant lacking this region (*bub1-Δ28-160*) has been described to be defective in spindle assembly checkpoint activity and localization of Bub1 to kinetochores, whereas interaction with Bub3 is not impaired (Vanoosthuyse et al., 2007; Vanoosthuyse et al., 2004). The *klp5Δ bub1-Δ28-160* double mutant had a slight but much weaker growth defect than the *klp5Δ bub1Δ* mutant (Figure 2.14B). Checkpoint activity is not crucial for survival of *klp5Δ* cells (Figure 2.1 and 2.13), but it is possible that kinetochore localization plays a role (see chapter 2.2.4.2).

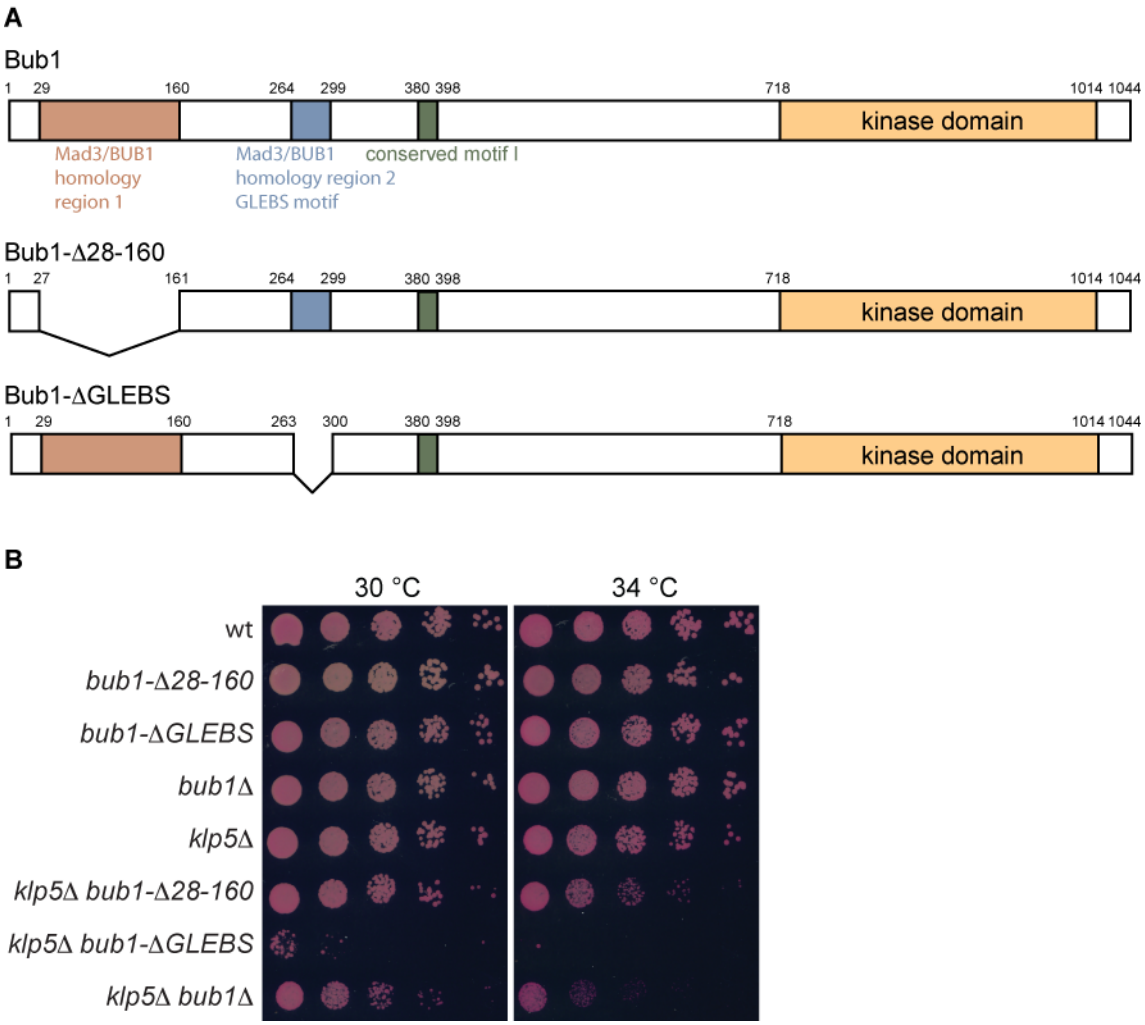

Figure 2.14 The Bub1 GLEBS motif is required for viability of *klp5*-deleted cells
(**A**) Schematics representing Bub1 and mutants lacking the Mad3/BUB1 homology regions, Bub1-Δ28-160 and Bub1-ΔGLEBS. (**B**) Growth assay with the indicated *bub1* mutants. 5-fold serial dilutions were spotted on full medium containing Phloxin-B and incubated at the indicated temperature for 3 d. *klp5Δ bub1-ΔGLEBS* cells showed a synthetic growth defect that was even stronger than that in *klp5Δ bub1Δ* cells, whereas the *klp5Δ bub1-Δ28-160* mutants showed only a slight synthetic growth defect.

2.3.2 Bub3 is not essential for the mitotic delay in the presence of chromosome attachment errors in fission yeast

For fission yeast, progression through mitosis in SAC deletion mutants had not been analyzed in much detail. The most extensive study of deletion mutants assayed septation of cells that came out of an HU arrest in the presence of a microtubule-destabilizing drug (Millband and Hardwick, 2002). This is not very precise given that, whereas cells complete S-phase synchronously, by the time they enter mitosis they have lost synchrony, which makes time-course experiments after HU release difficult. Moreover, there might be differences between the mutants in how efficiently they come out of the HU arrest and how fast they enter mitosis. Mutants lacking Bub1, for example,

have a growth defect on HU containing plates, whereas *mad2Δ* cells grow normally in the presence of HU (data not shown). In addition, *bub1Δ* cells are sensitive toward CPT (camptothecin), a topoisomerase I inhibitor (Kawashima et al., 2009), indicating that Bub1 has a function outside mitosis and that it might react differently to release from an HU arrest than other spindle assembly checkpoint mutants. Furthermore, fission yeast, in contrast to budding yeast, does not respond to microtubule drugs with a tight arrest. Even wild type cells undergo mitosis in the presence of microtubule-destabilizing drugs (data not shown); and thus, only slight differences in progression through mitosis between the wild type and the checkpoint mutants are expected in this type of experiment. Septation can be used to judge cell cycle progression, however, it is not ideal for assaying progression through mitosis. Not much is known about how the SAC and the SIN (*s*eptation *i*ntitiation *n*etwork) are coordinated (Krapp and Simanis, 2008). In *nda3-KM311* cells at restrictive temperature, which gives a tight metaphase arrest compared to drug treatment, septation can occur even though the cells are still in mitosis, as judged by the presence of Plo1-GFP on the SPBs (data not shown). In addition, *bub1* mutants might have cytokinesis defects (see chapter 2.2.2), which could further aggravate analysis of checkpoint activity using septation as a reference for progression through mitosis. Moreover, it is known that in fission yeast 'cut' (*c*ell *u*ntimely *t*orn) mutants, septation can occur even though the nucleus has not been divided, and some of these mutants septate although anaphase has not even been initiated (Hirano et al., 1986; Samejima et al., 1993; Yanagida, 1998). The cut phenotype can be caused by mutations in genes encoding for, among others, topoisomerases, condensin, APC/C components, a kinesin required for spindle formation (*cut7*), separase and securin (Yanagida, 1998).

In order to clearly judge spindle assembly checkpoint activity, we therefore employed an assay where individual cells are followed by live cell microscopy, eliminating the need for synchronization. As described before (see chapter 2.2.4.2), we used Plo1-GFP as a marker for mitosis and the cold-sensitive β-tubulin mutant *nda3-KM311* to disturb kinetochore microtubule attachment. We followed progression through mitosis in spindle assembly checkpoint deletion mutants (Figure 2.15, see Figure 2.30 for *mph1Δ*). Interestingly, in contrast to what had been reported previously (Millband and Hardwick, 2002; Vanoosthuyse et al., 2004), we found that *bub3*-deleted cells can delay mitosis in the presence of unattached kinetochores (Figure 2.15). To exclude that this was due to a defect in mitotic exit, we deleted *mad2* in addition to *bub3*. This resulted in a complete abrogation of the delay and a similar short mitosis as in the *mad2* deletion alone, indicating that Bub3 is not essential for progression into anaphase (Figure 2.15A).

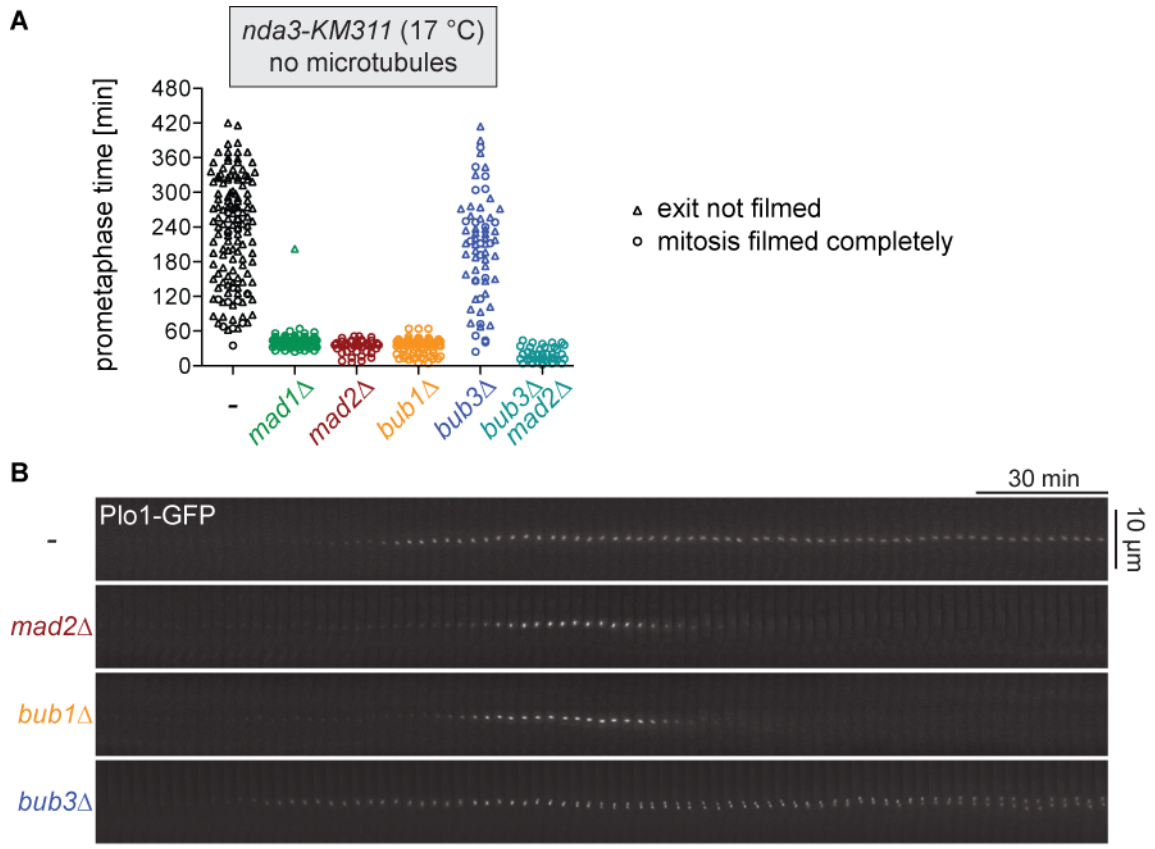

Figure 2.15 Bub3 is not essential for the SAC in *nda3-KM311* mutants (Windecker et al., 2009)

Cells expressing *plo1-GFP* and the β-tubulin *nda3-KM311* allele were followed by live cell microscopy at 17 °C. (**A**) The duration of prometaphase was determined by the presence of Plo1-GFP on the SPBs. Cells lacking Bub3 were able to delay in mitosis. Circles indicate cells where the entire prometaphase took place within the recording time. Triangles indicate cells that had not exited prometaphase when recording stopped, thus the actual time of prometaphase must be longer than this value. (**B**) Kymographs of exemplary cells that were analyzed in (A).

To rule out that this *bub3Δ* phenotype was specific to the *nda3-KM311* mutation, we assayed spindle assembly checkpoint functionality under two other conditions, using mutants that reduce establishment of tension at the kinetochores. To this end, we used a temperature-sensitive allele of the fission yeast kinesin-5, *cut7-446*, and the temperature-sensitive mutant *psc3-1T*, a component of the cohesin complex (*scc3* homolog). Both conditions gave similar results as the *nda3-KM311* experiment (Figure 2.16), showing that Bub3 is indeed not essential for generating a spindle assembly checkpoint-dependent delay in mitosis. These results are in accordance with studies from Vanoosthuyse et al. (2009) as well as Tange and Niwa (2008).

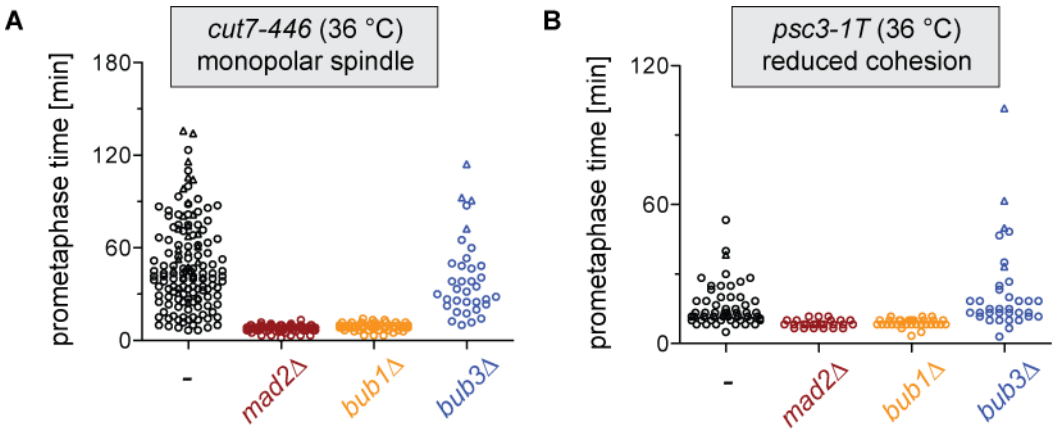

Figure 2.16 Bub3 is not required for the SAC in *cut7-446* and *psc3-1T* mutant cells (Windecker et al., 2009)

Cells expressing *plo1-GFP* and carrying the *cut7-446* (**A**) or *psc3-1T* (**B**) allele were followed by live cell microscopy at restrictive temperature (36 °C). The time in prometaphase was determined by the presence of Plo1-GFP on the SPBs. Cells lacking Bub3 were able to delay in mitosis. Circles indicate cells where the entire prometaphase took place within our recording time. Triangles indicate cells that had not exited prometaphase by the end of recording. Cells deleted for *bub3* are able to delay anaphase onset in the presence of monopolar spindles (*cut7-446*) or cohesion defects (*psc3-1T*).

2.3.3 Sgo2 localization in *bub1-ΔGLEBS* and *bub3Δ* cells

Sgo2 is the sole fission yeast shugoshin protein that is expressed in the vegetative cell cycle (Kitajima et al., 2004; Mata et al., 2002). ChIP data revealed that Sgo2 localizes predominantly to euchromatic subtelomeric regions in interphase, whereas in mitosis it is mainly found at centromeres (pericentromeric heterochromatin) (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004). Sgo2 localization is abolished in *bub1Δ* cells (Kawashima et al., 2009; Kitajima et al., 2005; Kitajima et al., 2004; Tang et al., 2004b), which has been attributed to loss of Bub1 kinase-dependent histone H2A phosphorylation (Kawashima et al., 2009).

It had never been investigated, whether Bub3 shares the Sgo2 localization function of Bub1. To address this question, we measured Sgo2-GFP intensities in the nuclei of wild type, *bub1Δ*, *bub1-ΔGLEBS* and *bub3Δ* cells expressing *mCherry-tubulin* (*atb2+*) as a marker for cell cycle stages (Figure 2.17). As expected, in the absence of Bub1, Sgo2 localization was abolished - no or only very faint dot-like Sgo2-GFP signals were observed. Interphasic Sgo2-GFP intensities in *bub1-ΔGLEBS* and *bub3Δ* on the other hand were not significantly different from those of the wild type, but in metaphase Sgo2-GFP maximum intensities were reduced in these mutants, albeit not as strongly as in *bub1Δ* cells (Figure 2.17). However, in many *bub1-ΔGLEBS* and *bub3Δ* cells, the number of dot-like Sgo2-GFP signals was increased in metaphase (Figure 2.17A,C). This effect was slightly stronger in *bub3Δ* compared to *bub1-ΔGLEBS* cells.

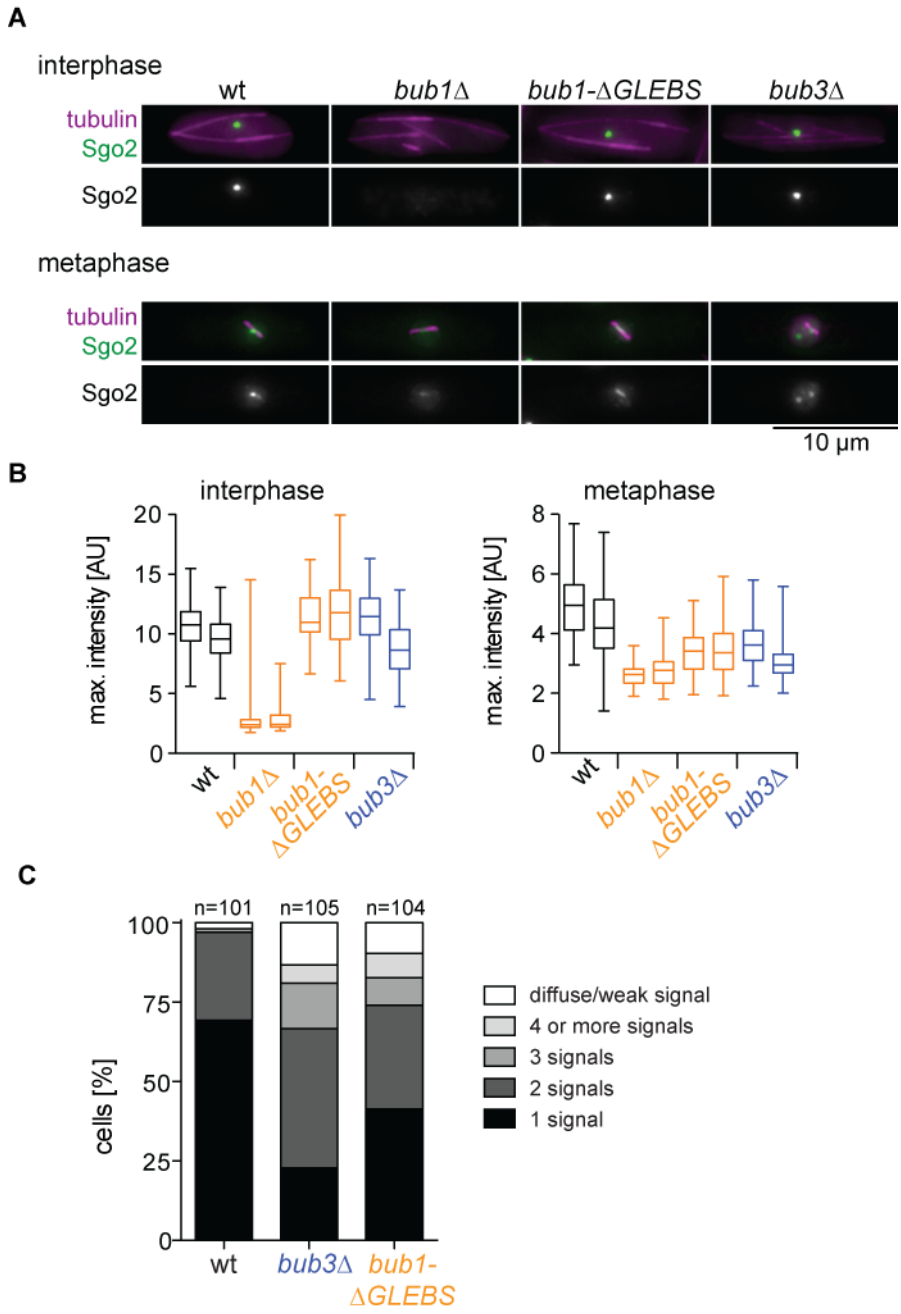

Figure 2.17 Localization of Sgo2 in *bub3Δ* and *bub1-ΔGLEBS* cells (Windecker et al., 2009)

Living cells from strains expressing *mCherry-atb2(tubulin)* and *sgo2-GFP* were imaged by fluorescence microscopy. (**A**) Example pictures of Sgo2-GFP localization in the indicated strains. In interphase, Sgo2-GFP localization was comparable to wild type in *bub1-ΔGLEBS* and *bub3Δ* cells, whereas signal intensities were reduced in mitotic cells of these mutants. The quantification of maximum signal intensity in the nucleus is shown in (**B**). Interphase or metaphase cells were identified by the characteristic microtubule signal. For each genotype, two independent strains were used. (**C**) Graph showing the number of Sgo2-GFP signals in the mitotic wild type, *bub3Δ* and *bub1-ΔGLEBS* cells used for analysis in (B). Whereas most of the wild type cells showed one Sgo2-GFP signal in the mitotic nucleus, *bub3Δ* and *bub1-ΔGLEBS* cells often had more than one signal.

Bub3 and its interaction with Bub1 are required for localization of Bub1 to kinetochores in mitosis (Klebig et al., 2009; Vanoosthuyse et al., 2004; Windecker et al., 2009). Therefore, Bub3 might not be involved in Sgo2 localization directly, since in *bub3Δ*, in contrast to *bub1Δ*, Sgo2 clearly is able to localize, albeit at reduced levels in metaphase. Instead, Bub3 might be required for restricting Sgo2 localization to centromeres in mitosis via targeting Bub1 to kinetochores. Further investigations using the appropriate markers or ChIP experiments are required to determine whether Sgo2 fails to relocalize efficiently from telomeres to centromeres in mitosis in *bub1-ΔGLEBS* and *bub3Δ*. It remains an open question whether residual localization of Sgo2 in *bub3Δ* and *bub1-ΔGLEBS* is sufficient to perform its function (further results are presented in chapter 2.4).

2.3.4 Chromosome segregation and spindle defects in the *klp5Δ bub3Δ* mutant

The observation that double deletion of *klp5* and *bub3* also causes a severe growth defect, especially at higher temperatures, and that in fission yeast, the spindle assembly checkpoint is functional in the absence of Bub3, further confirmed that loss of spindle assembly checkpoint function is not the major reason for loss of viability of *klp5Δ* cells. However, the striking result that Bub3 is dispensable for checkpoint activity opened the question which role Bub3 plays in fission yeast. There must be at least one function that is crucial for survival of *klp5Δ* cells. Bub3 co-immunoprecipitates with Mad3 in fission yeast (Millband and Hardwick, 2002) and can interact directly with Mad3 in budding yeast (Larsen et al., 2007), but since the *klp5Δ mad3Δ* double mutant is healthy, a possible joint function of Bub3 with Mad3 that is required for survival of *klp5Δ* can be excluded. In contrast, the interaction of Bub1 with Bub3 seems to be crucial, given that the *ΔGLEBS* mutant of *bub1*, which has a functional spindle assembly checkpoint but fails to interact with Bub3 (Vanoosthuyse et al., 2009), has a growth defect in conjunction with *klp5Δ* similar to the *klp5Δ bub3Δ* double deletion mutant.

The intact checkpoint of *bub3*-deleted cells gave us the opportunity to analyze mitosis in the *klp5Δ bub3Δ* mutants more carefully, without having the pleiotropic effects of a *bub1* deletion. To this end, I used *klp5Δ bub3Δ* strains that carried the wild type *klp5*+ gene on a plasmid. As a selectable marker, this plasmid carried the budding yeast *LEU2* gene, which is able to complement fission yeast *leu1* mutations (Kikuchi et al., 1988; Maundrell, 1993). When fission yeast cells are released from nitrogen starvation (G_1 arrest), plasmids are lost with a higher frequency (Ohkura and Yanagida, 1991). I used this method to observe cells that enter mitosis in the absence of Klp5 or with low levels of Klp5, due to loss of the plasmid carrying the *klp5*+ gene.

I observed many chromosome segregation defects and spindle abnormalities in the double deletion mutant (Figures 2.18A and 2.19E-F). The spindles often shrank after having been established (Figures 2.18A and 2.19G), and sometimes they collapsed and broke in the middle (Figure 2.19E). This could either be due to defects in spindle formation and stabilization, or it could be the result of unstable kinetochore microtubule attachments (see chapter 2.4.3). Shrinkage of the spindle occasionally also occurred in the wild type and was observed slightly more frequently in the single mutants (Figures 2.18A and 2.19C,D). Collapse and bending of late anaphase spindles occurred in

11% of the double mutant cells, but was never observed in the wild type or in the single mutants (data not shown).

A

	cells	spindle shrinkage	unattached chromosomes	chromosome switches SPBs
wt	29	1 (4%)	0 (0%)	0 (0%)
bub3Δ	33	3 (14%)	3 (9%)	0 (0%)
klp5Δ (pREP1-klp5+)	33	3 (9%)	0 (0%)	1 (3%)
klp5Δ bub3Δ (pREP1-klp5+)	114	54 (50%)	11 (10%)	16 (14%)

B

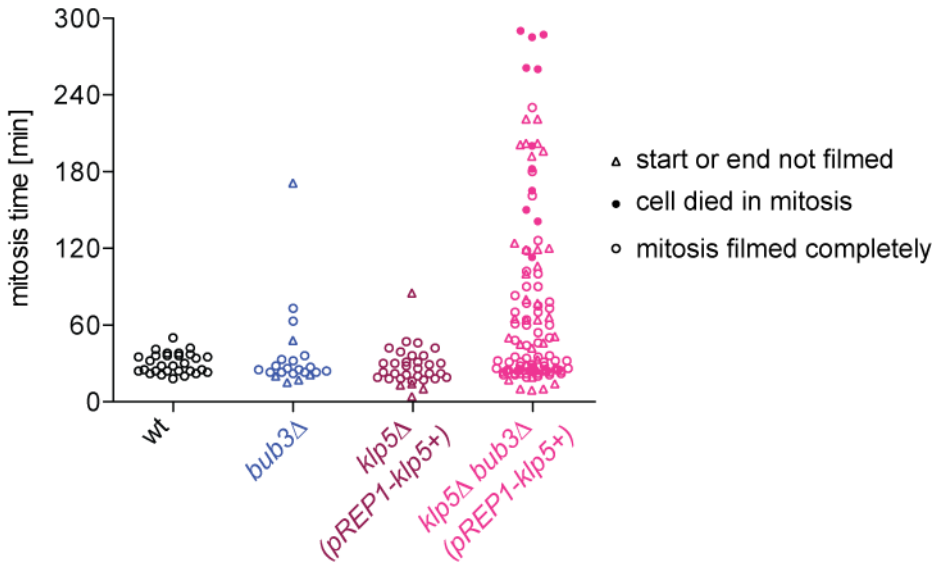

Figure 2.18 The *klp5Δ bub3Δ* double mutant shows spindle and chromosome bi-orientation defects

Cells carrying *cen2-GFP* and expressing *mCherry-atb2(tubulin)* as markers were arrested in G_1-phase by nitrogen starvation. The *klp5Δ bub3Δ* double mutant and the *klp5Δ* control strain additionally carried the *pREP1-klp5+* plasmid, which enabled normal growth of the *klp5Δ bub3Δ* double mutant. 5 h after release from G_1 arrest, which results in frequent plasmid loss (Ohkura and Yanagida, 1991), mitosis was followed by live cell microscopy at 30 °C. The wild type and *bub3Δ* control strains did not carry a plasmid, but were treated identically to the plasmid-carrying strains. (**A**) Quantification of spindle defects and chromosome attachment errors after plasmid loss. The *klp5Δ bub3Δ* double mutant had a high incidence of spindle shrinkage events and a higher number of unattached chromosomes, as well as abnormal chromosome movements from pole to pole. (**B**) The mitotic errors in the *klp5Δ bub3Δ* double mutant resulted in a prominent mitotic delay. The time in mitosis was measured from spindle formation to appearance of the post anaphase array.

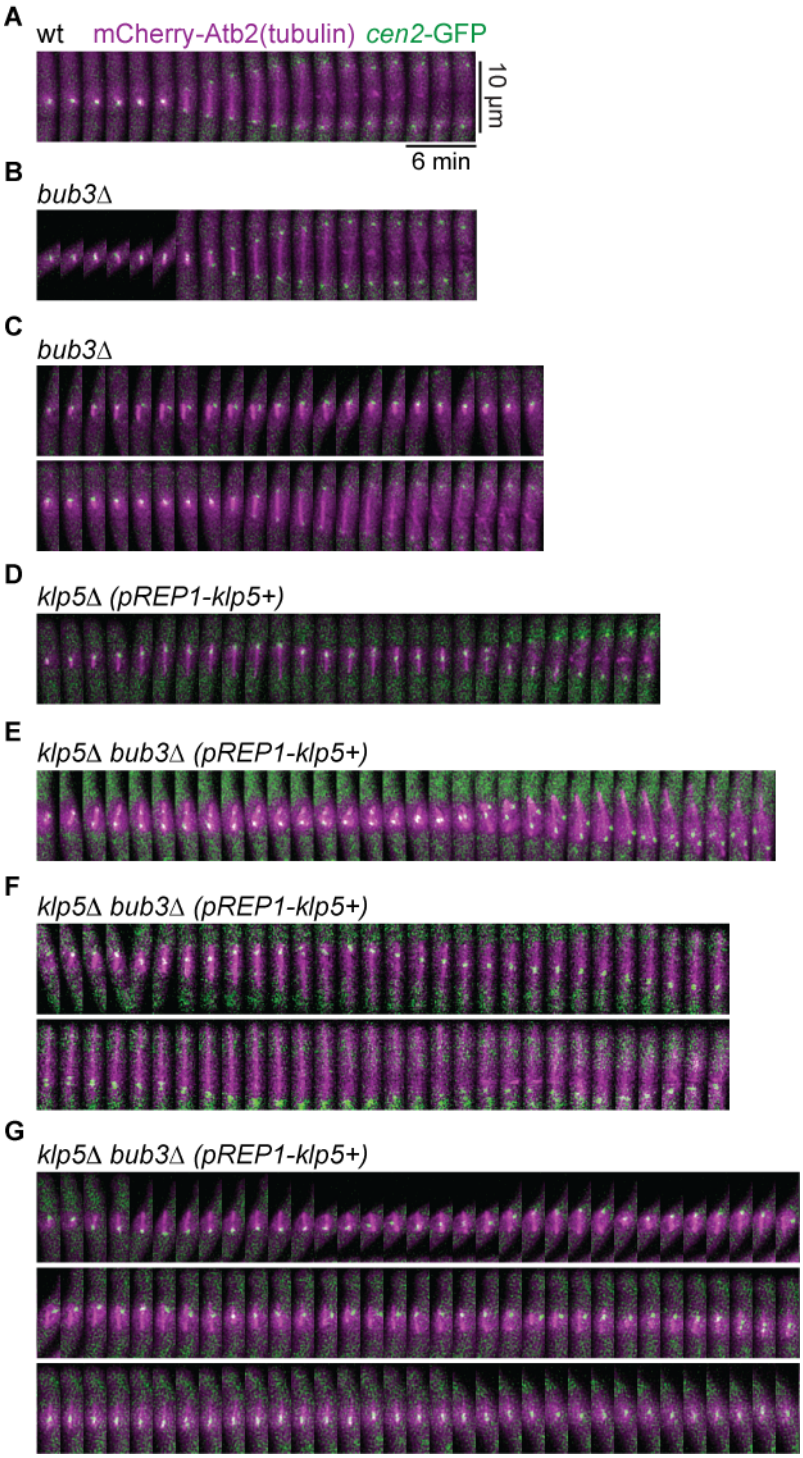

Figure 2.19 Kymographs of cells analysed in Figure 2.18

Cells carrying *cen2-GFP* and *mCherry-atb2(tubulin)* as well as the indicated gene deletions were followed by live cell microscopy at 30 °C, 5 h after release from G_1 arrest, as described in Figure 2.18. (**A**) and (**B**) Normal mitosis in a wild type and a *bub3Δ* cell, respectively. (**C**) Chromosome II of this *bub3Δ* cell was unattached for a prolonged period, which resulted in a delay of anaphase onset, confirming the SAC proficiency of *bub3Δ* cells. (**D**) Abnormal chromosome movement of chromosome II on the spindle in a *klp5Δ* cell that presumably had lost the *pREP1-klp5+* plasmid. (**E**)-(**G**) Mitotic errors in *klp5Δ bub3Δ* double mutant cells that presumably had lost the *pREP1-klp5+* plasmid. Spindle shrinkage (E), missegregation (F) and chromosome attachment defects (G) were among the most prominent phenotypic abnormalities.

Mitosis in *bub3Δ* mutants mostly resembled wild type mitosis (compare Figure 2.19A with Figure 2.19B), but sometimes *bub3Δ* cells showed chromosome attachment defects (Figures 2.18A and 2.19C); however, these were again much more prominent in the double mutant (Figures 2.18A and 2.19G). Abnormal chromosome movements, a known phenotype of kinesin-8 mutants in fission yeast (West et al., 2002), were also observed. Chromosomes often 'switched' SPBs, they first stayed close to one SPB while the spindle elongated, then they moved along the spindle to the opposite spindle pole (Figure 2.19F). Whereas only 3% of *klp5Δ* cells showed this phenotype, it was observed in 14% of *klp5Δ bub3Δ* cells (Figure 2.18A).

The *klp5Δ bub3Δ* cells after plasmid loss often had a prolonged mitosis, probably due to chromosome bi-orientation and spindle defects (Figure 2.18B), which is yet another indication that the spindle assembly checkpoint can be active in the absence of Bub3. In the plasmid loss experiment, no missegregation events of chromosome II were recorded in the single mutants, whereas the *klp5Δ bub3Δ* double mutant showed a chromosome missegregation rate of 8% (data not shown).

Overall, the *klp5Δ bub3Δ* double mutant showed severe chromosome segregation and spindle defects, which could be the cause for the synthetic lethality. It is possible that the observed spindle defects are a secondary consequence of chromosome alignment and kinetochore attachment problems or *vice versa* (see chapter 2.4.3).

2.3.5 Ase1 localization is not perturbed in *klp5Δ* and *bub3Δ* cells

Ase1 is a microtubule-associated protein that is required for microtubule bundling (Yamashita et al., 2005). It localizes to regions of overlapping antiparallel microtubules and in the vicinity of microtubule-organizing centers throughout the cell cycle (Loïodice et al., 2005; Yamashita et al., 2005). Accordingly, in mitosis, Ase1 localizes to microtubules close to the SPBs and to the spindle midzone, where microtubules emanating from the opposite spindle poles interdigitate (Loïodice et al., 2005; Yamashita et al., 2005). Ase1-deleted cells show an increased rate of chromosome loss, abnormal septation and often spindle collapse in anaphase B (Loïodice et al., 2005; Yamashita et al., 2005), all traits reminiscent of the *klp5Δ bub1Δ* and *klp5Δ bub3Δ* phenotypes. We therefore considered the possibility that Ase1 localization could be influenced or controlled by Klp5 or Bub1/Bub3, or both. I analyzed localization of Ase1-GFP by live cell microscopy, but could not see a significant localization defect in *klp5Δ* or *bub3Δ* cells (Figure 2.20). Similar results were observed for *bub1Δ* (data not shown). In *klp5Δ* cells, the region on the spindle to which Ase1 localized was slightly elongated compared to wild type. However, this is probably a consequence of the general increase in microtubule and spindle length in kinesin-8 mutants (Garcia et al., 2002b; West et al., 2002), rather than a localization defect.

The absence of either Klp5 or Bub3 (or Bub1) does not influence localization of Ase1 significantly, indicating that mislocalization of Ase1 is not the reason why the *klp5Δ bub1Δ* and *klp5Δ bub3Δ* (and *klp5Δ bub1Δ*) show a similar phenotype as the *ase1Δ* mutant does. However, the result does not rule out the possibility that the double mutants have an Ase1 localization defect.

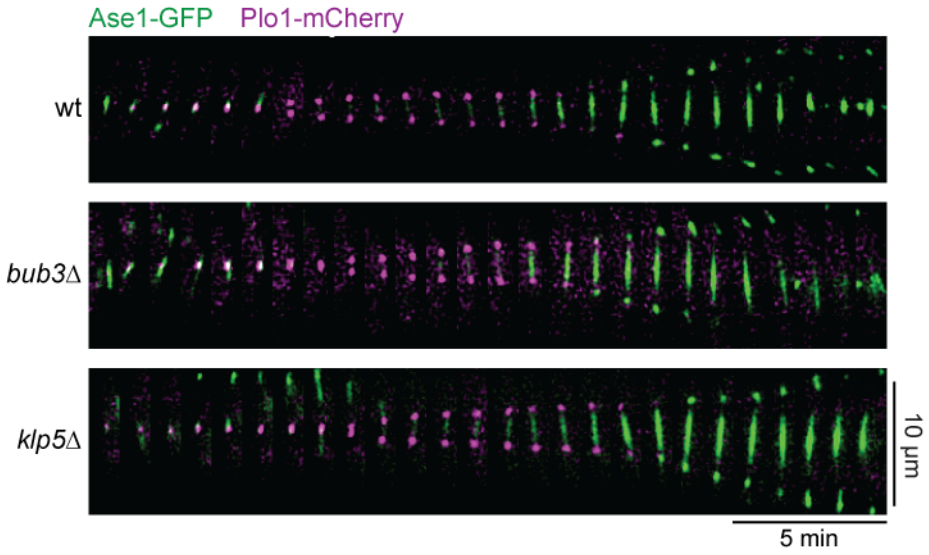

Figure 2.20 Ase1-GFP localization in *bub3Δ* and *klp5Δ* cells
Kymographs of exemplary *bub3Δ*, *klp5Δ* and wild type cells expressing *plo1-mCherry* as a marker for mitotic SPBs as well as *ase1-GFP*. Mitosis was followed by live cell microscopy at 30 °C. There is no obvious Ase1-GFP localization defect in *bub3Δ* and *klp5Δ* cells.

2.3.6 Bub1 and Bub3 have a spindle-destabilizing activity

When we assayed spindle assembly checkpoint functionality using the *nda3-KM311* strains (see chapter 2.3.2), we observed that slightly increasing the temperature to 19 °C had a prominent effect on *bub1Δ* and *bub3Δ* cells compared to the *nda3-KM311 mad2Δ* and the *nda3-KM311* mutant alone. In the absence of either of the two proteins, the spindle pole bodies, which were marked by Plo1-GFP, moved apart as if a spindle was formed, despite the presence of the microtubule-disrupting β-tubulin Nda3-KM311 (Figure 2.21A and B). Presumably, Bub1 and Bub3 contribute to microtubule destabilization in the *nda3-KM311* mutant, so that when these proteins are absent, the spindles can be formed and spindle pole bodies separate. This apparently can even sometimes lead to a reduction of spindle assembly checkpoint activity, since *nda3-KM311 bub3Δ* cells show a much shorter mitotic delay under these conditions than the *nda3-KM311* mutant alone (Figure 2.21C). This opens the question whether Bub1 and Bub3 have generally an influence on microtubule dynamics. If they indeed have a microtubule-destabilizing function, this could be the function they share with the kinesin-8 proteins and therefore the reason why they are synthetically sick.

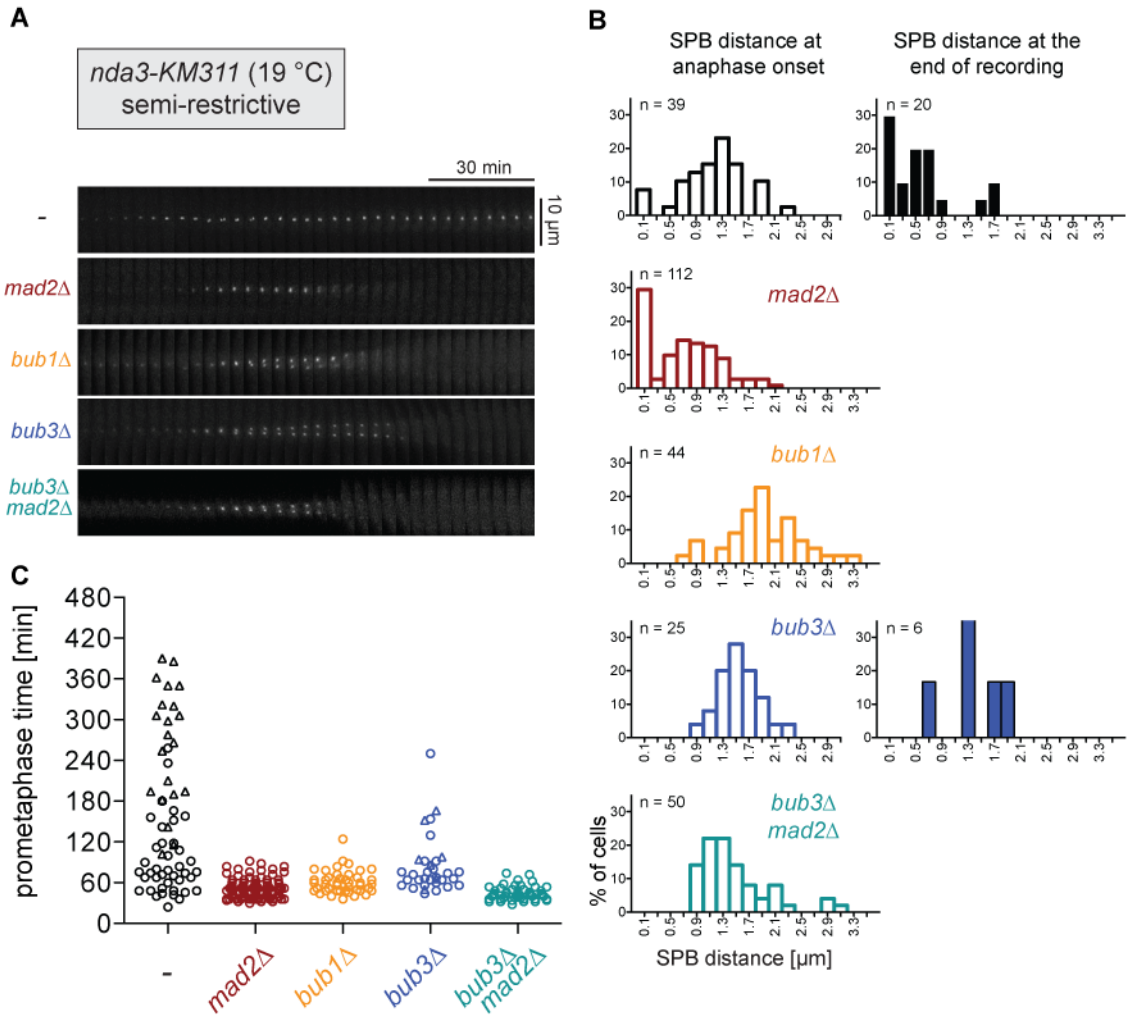

Figure 2.21 Deletion of *bub1* or *bub3* counteracts the *nda3-KM311* mutation at semi-restrictive temperature (Windecker et al., 2009)

Cells expressing *plo1-GFP* and the β-tubulin *nda3-KM311* allele were followed by live cell microscopy at the semi-restrictive temperature of 19 °C. (**A**) Kymographs of exemplary *nda3-KM311* cells also carrying the indicated SAC gene deletions. In contrast to the same experiment at 17 °C (Figure 2.15B), pronounced separation of SPBs is observed predominantly in *bub1Δ* and *bub3Δ* cells. (**B**) Quantification of SPB distance from the experiment in (A). The SPB distance was either determined at anaphase onset (before disappearance of the Plo1-GFP signal from SPBs; left side) or, in the case where anaphase was not captured in our recording, was determined in the last picture from the recording (right side). (**C**) The time in prometaphase was determined by the presence of Plo1-GFP on the SPBs. Prometaphase was shortened in the *nda3-KM311* mutant and considerably shortened in the *nda3-KM311 bub3Δ* cells compared to 17 °C (compare to Figure 2.15A).

It was formally possible that the spindle pole body separation we observed was not due to spindle formation but to other mechanisms that could result in a drift of the two spindle pole bodies. To investigate this, I performed anti-tubulin immunofluorescence with *nda3-KM311 plo1-GFP* cells that had been incubated at 18 °C for four hours. Whereas the *mad2Δ* cells hardly ever had spindle-microtubule staining, this was very often the case for *bub1Δ* and *bub3Δ* cells (Figure 2.22). For the wild type, more cells showed spindle staining than in the *mad2Δ* mutant (Figure 2.22B), however, it is unlikely that the deletion of *mad2* has a spindle-destabilizing effect, since the double deletion mutant of *bub3Δ mad2Δ* in the *nda3-KM311* background did not abolish spindle pole body separation (Figure 2.21).

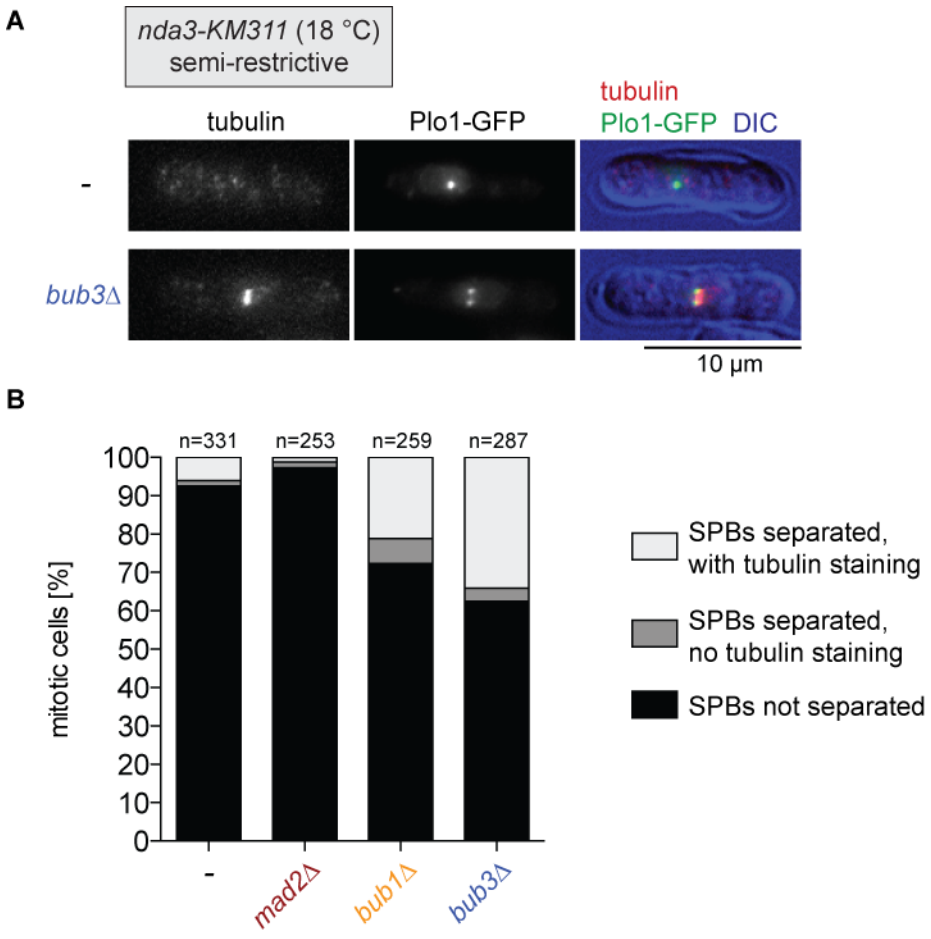

Figure 2.22 *bub1Δ* and *bub3Δ* cells show spindle formation and SPB separation in the *nda3-KM311* mutant at semi-restrictive temperature

Cells from indicated strains carrying the β-tubulin *nda3-KM311* allele and expressing *plo1-GFP* as a marker for mitosis were cultured for 4 h at 18 °C, fixed, stained with anti-tubulin antibody and imaged by fluorescence microscopy. (**A**) Example pictures of mitotic *nda3-KM311* and *nda3-KM311 bub3Δ* cells. In the *bub3Δ* cell, SPBs separated and microtubules were visible between the SPBs. (**B**) Quantification of *nda3-KM311* cells showing SPB separation and tubulin staining between the SPBs in mitosis. Cells lacking Bub1 or Bub3 often formed a small spindle and separated their SPBs.

2.3.7 Cells lacking Bub3 show normal spindle elongation but delay in mitosis

Given their influence on microtubules in the *nda3-KM311* mutant, we wanted to know whether Bub1 and Bub3 have a general effect on spindle formation. Therefore, I checked whether spindle elongation in early mitosis was disturbed in the absence of Bub3, as it is the case for kinesin-8 mutants (Garcia et al., 2002b). I measured the distance between the spindle pole bodies over time, using live cell microscopy and Plo1-GFP as a mitotic SPB marker (Figure 2.23A). Phase 1 spindle elongation was indistinguishable from wild type, indicating that spindle formation *per se* is not substantially affected by deletion of *bub3* in unperturbed mitosis. However, about seven minutes after the spindle pole bodies had separated, the spindle seemed to be shorter in *bub3Δ* cells compared to the wild type, indicating that phase two of spindle elongation is prolonged (Figure 2.23A). This is consistent with a study from Tange and Niwa (2008), who also observed a delay in mitosis, judged by spindle elongation. Whereas they report on a delay in phases 1 and 2 taken together, our data indicates that phase 1 elongation is not altered in the absence of Bub3. In addition, I measured the time of Plo1-GFP localization to SPBs. Cells lacking Bub3 delayed in mitosis even when no spindle damage was induced (Figure 2.23B). This delay is checkpoint-dependent, since it was abolished by additional deletion of *mad2* (Figure 2.23B) (Tange and Niwa, 2008).

Tange and Niwa (2008) observed an increase in intercentromere distance in *bub3*-deleted cells. They hypothesized that Bub3 could be required for efficient cohesion establishment at the centromeres. Cohesion defects result in a reduction of tension at the kinetochores, which in turn leads to a spindle assembly checkpoint-mediated delay in mitosis (Kawashima et al., 2007). Sgo2 has been shown to be required for spindle assembly checkpoint activity in the absence of tension (see discussion, chapter 3.5), whereas it is dispensable for the spindle assembly checkpoint in no-attachment situations (Kawashima et al., 2007; Vanoosthuyse et al., 2007). If the delay observed in *bub3Δ* cells in otherwise unperturbed mitosis was due to reduced tension at the kinetochore, deletion of *sgo2* should abolish this delay. This was not the case, the additional deletion of *sgo2Δ* in *bub3Δ* mutants did not rescue mitosis time substantially, indicating that the delay in *bub3Δ* mutants is not merely a result of lack of tension at kinetochores (Figure 2.23C).

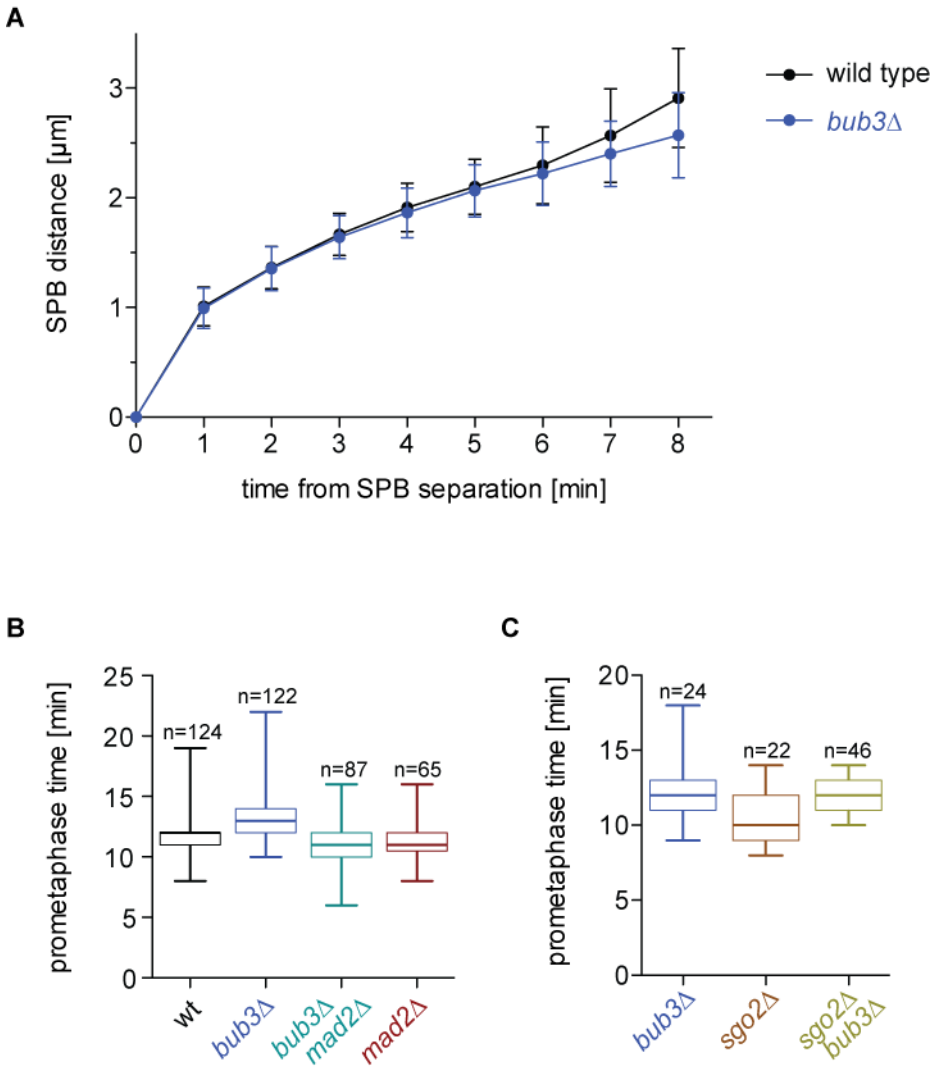

Figure 2.23 Phase 1 spindle elongation in *bub3Δ* cells is comparable to wild type, but time in prometaphase is prolonged

Localization of the mitotic marker Plo1-GFP to SPBs was followed by live cell microscopy in unperturbed mitosis at 30 °C. (**A**) The distance between spindle pole bodies in *bub3Δ* and wild type cells was measured as cells passed through mitosis. Spindle elongation of *bub3*-deleted cells was indistinguishable from wild type in early mitosis, but anaphase B spindle elongation started a bit later, reflecting the anaphase delay shown in (B) (Windecker et al., 2009). (**B**) The duration of prometaphase in *bub3Δ*, *bub3Δ mad2Δ*, *mad2Δ* and wild type cells was determined by measuring the time of Plo1-GFP localization to SPBs. Deletion of *bub3* resulted in a mitotic delay that was abolished by additionally deleting the spindle assembly checkpoint gene *mad2* (Windecker et al., 2009). (**C**) The duration of prometaphase was determined as in (B). The delay in *bub3Δ* cells was not abolished by additional deletion of *sgo2*. In all box-whisker graphs, the lines from top to bottom are maximum value, 75th percentile, median, 25th percentile and minimum value.

2.4 Bub1 and Bub3 promote bi-orientation of chromosomes

2.4.1 Deletion of *bub3* results in increased missegregation after release from MBC treatment

It seems perspicuous that defects in the spindle assembly checkpoint can lead to aneuploid daughter cells, as microtubule attachment and chromosome alignment is not monitored. This supposition has been confirmed in a number of organisms (Basu et al., 1999; Bernard et al., 1998; Dobles et al., 2000; Kalitsis et al., 2000; Tange and Niwa, 2008; Warren et al., 2002). However, even though Bub3 is not required for the spindle assembly checkpoint, *bub3*-deleted fission yeast cells have an increased chromosome missegregation rate (Tange and Niwa, 2008; Vanoosthuyse et al., 2004) (Figure 2.24), indicating that Bub3 has a function in chromosome segregation.

In fission yeast, chromosomes are closely associated with spindle pole bodies when the cell enters mitosis (Funabiki et al., 1993), which presumably facilitates capture by spindle microtubules emanating from the two spindle poles. Treatment with the microtubule-destabilizing drug MBC (*m*ethyl-2-*b*enzimidazole *c*arbamate) causes the chromosomes to uncluster from the spindle pole bodies, and hence, when the drug is washed out, the spindle has to be reformed and the chromosomes have to be re-attached to microtubules. Once chromosomes get captured by astral microtubules, they are pulled toward the SPB, from where they get aligned onto the spindle by an unknown mechanism (Franco et al., 2007; Gachet et al., 2008; Grishchuk and McIntosh, 2006)(Figure 2.24A).

When I released *bub3Δ* cells from MBC treatment (they had been synchronized with HU before release into MBC), the chromosome missegregation rate increased dramatically. This is reminiscent of *sgo2Δ* cells, which also show a strong increase in missegregation compared to the wild type when chromosomes have to be recaptured by spindle microtubules (Kawashima et al., 2007; Vanoosthuyse et al., 2007) (Figure 2.24). As it has been mentioned earlier (2.3.3), Bub3 could act upstream of Sgo2 during mitosis, so that an increase in missegregation in *bub3Δ* cells could be due to impaired Sgo2 function.

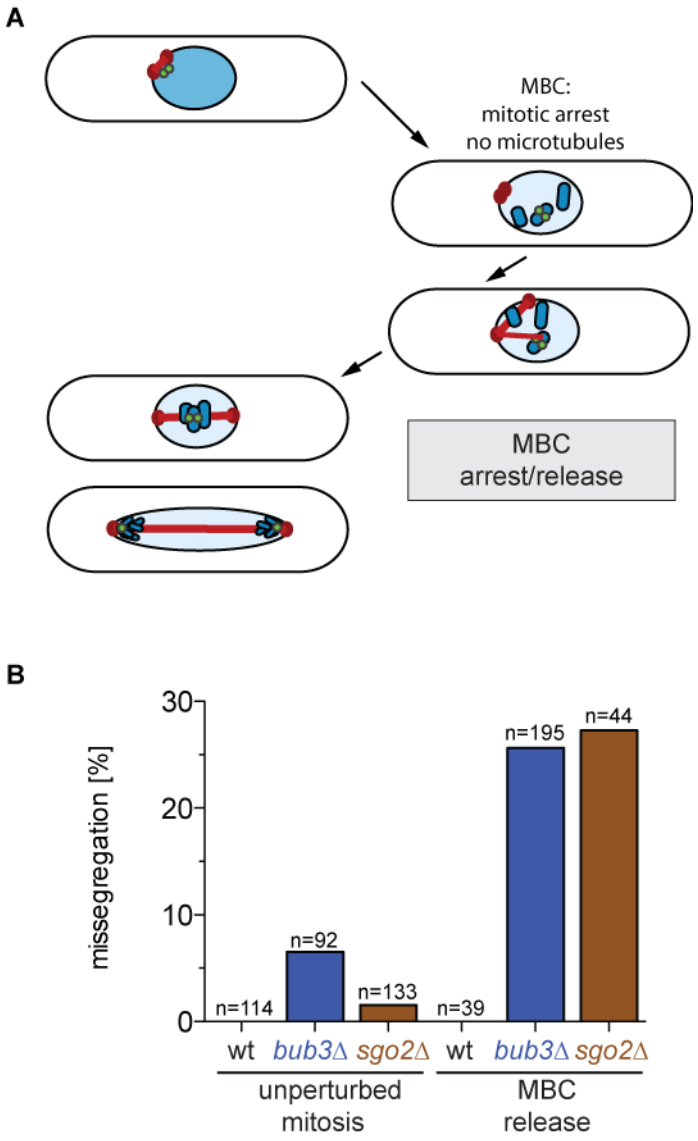

Figure 2.24 Bub3 is required for proper chromosome segregation (Windecker et al., 2009)
(**A**) Schematic showing chromosome behaviour in the MBC arrest/release assay. Centromeres are usually clustered close to the SPBs when fission yeast cells enter mitosis. In the presence of MBC, microtubules are disrupted, chromosomes uncluster and as a consequence the cells delay in mitosis. After MBC is washed out, spindles form and chromosomes are first retrieved toward the SPBs and then bi-orient on the spindle. When the SAC is satisfied, cells progress into anaphase. (**B**) Cells carrying *cen2-GFP* and *mCherry-atb2(tubulin)* were synchronized with hydroxyurea (HU), released from HU arrest and treated with the microtubule-destabilizing drug MBC for 3.5 hours. After washout of MBC, segregation of chromosome II (*cen2*-GFP) was followed by live cell microscopy at 20 °C. Only those cells that were already in mitosis when recording started were considered. Missegregation in unperturbed mitosis was determined by live cell microscopy at 20 °C after HU-release (Windecker et al., 2009). Similar to deletion of *sgo2* (Kawashima et al., 2007; Vanoosthuyse et al., 2007), deletion of *bub3* resulted in an elevated chromosome missegregation after recovery from microtubule depolymerization.

2.4.2 Bub3 and Bub1 promote chromosome bi-orientation independently of Sgo2

Sgo2 localization in metaphase is affected by deletion of *bub3* and the GLEBS motif of *bub1* (Figure 2.17) (Vanoosthuyse et al., 2009; Windecker et al., 2009). Furthermore, *sgo2Δ* and *bub3Δ* cells show a similar increase in chromosome missegregation when chromosomes have to be recaptured after they have unclustered from the SPBs (Figure 2.24) (Kawashima et al., 2007; Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2007; Windecker et al., 2009). Together, these results could indicate that Sgo2 function is lost in *bub3Δ* and *bub1-ΔGLEBS* mutant cells. Bub1 and Bub3 could act upstream of Sgo2, and their absence or mutation would abolish Sgo2 function, possibly through impaired localization. To investigate further whether loss of Bub1, Bub3, and Sgo2 leads to a similar or to distinct phenotypes, I followed all three chromosomes after release from treatment with MBC by live cell microscopy (Figure 2.25). Strikingly, *bub3Δ* and *bub1-ΔGLEBS* mutants had problems aligning the chromosomes on the spindle (Figure 2.25B-D). Chromosome capture by microtubules and subsequent retrieval to the SPBs seemed unperturbed in *bub3Δ* cells, as it has been reported recently by Vanoosthuyse et al. (2009). However, once the chromosomes had reached the SPB, they stayed there for a prolonged period. In the wild type, only 7% of all chromosomes showed mono-orientation for 10 min or longer, whereas 20% and 30% of chromosomes stayed mono-oriented for 10 min or more in *bub3Δ* and *bub1-ΔGLEBS* cells, respectively (Figure 2.25).

We wanted to know whether *sgo2Δ* cells showed a similar defect in chromosome bi-orientation after release from MBC arrest. Only 8% of chromosomes showed prolonged mono-orientation, whereas in the *sgo2Δ bub3Δ* double deletion mutant this was increased to 27%, similar to *bub3Δ* and *bub1-ΔGLEBS* mutants alone, suggesting that Sgo2 does not act downstream of Bub1 and Bub3 in promoting bi-orientation after release from MBC (Figure 2.25B-D). When it was not possible to judge attachment states of chromosomes, cells were not included in the analysis (Table 2.1).

The exact mechanism by which Bub1 and Bub3 promote bi-orientation of chromosomes that had become unclustered from SPBs when the cell entered mitosis is unclear. We proposed that under normal growth conditions, chromosomes, which are already clustered at the SPB when the cell enters mitosis, get efficiently bi-oriented by direct capture of microtubules emanating from the opposite spindle pole (Windecker et al., 2009). If, on the other hand, chromosomes have become unclustered from the spindle pole bodies, an indirect mechanism of bi-orientation is employed, and this mechanism is dependent on Bub1 and Bub3 (Figure 2.27A). Hence, the chromosome missegregation rate of *bub3Δ* mutants is much increased after release from MBC arrest (Figure 2.24). In this situation the spindle has already elongated by the time an unattached chromosome gets re-captured by microtubules and reaches one of the SPBs. In an elongated spindle, microtubules spanning from pole to pole are rare (Ding et al., 1993), and consequently, kinetochores are less likely to get captured directly by microtubules emanating from the opposite pole. Wild type cells could still bi-orient chromosomes under these conditions, whereas *bub3Δ* and *bub1-ΔGLEBS* cells had mono-oriented chromosomes (Figure 2.25 and 2.26), which is, according to our model, a result of a defect in the indirect bi-orientation mechanism (Figure 2.27A).

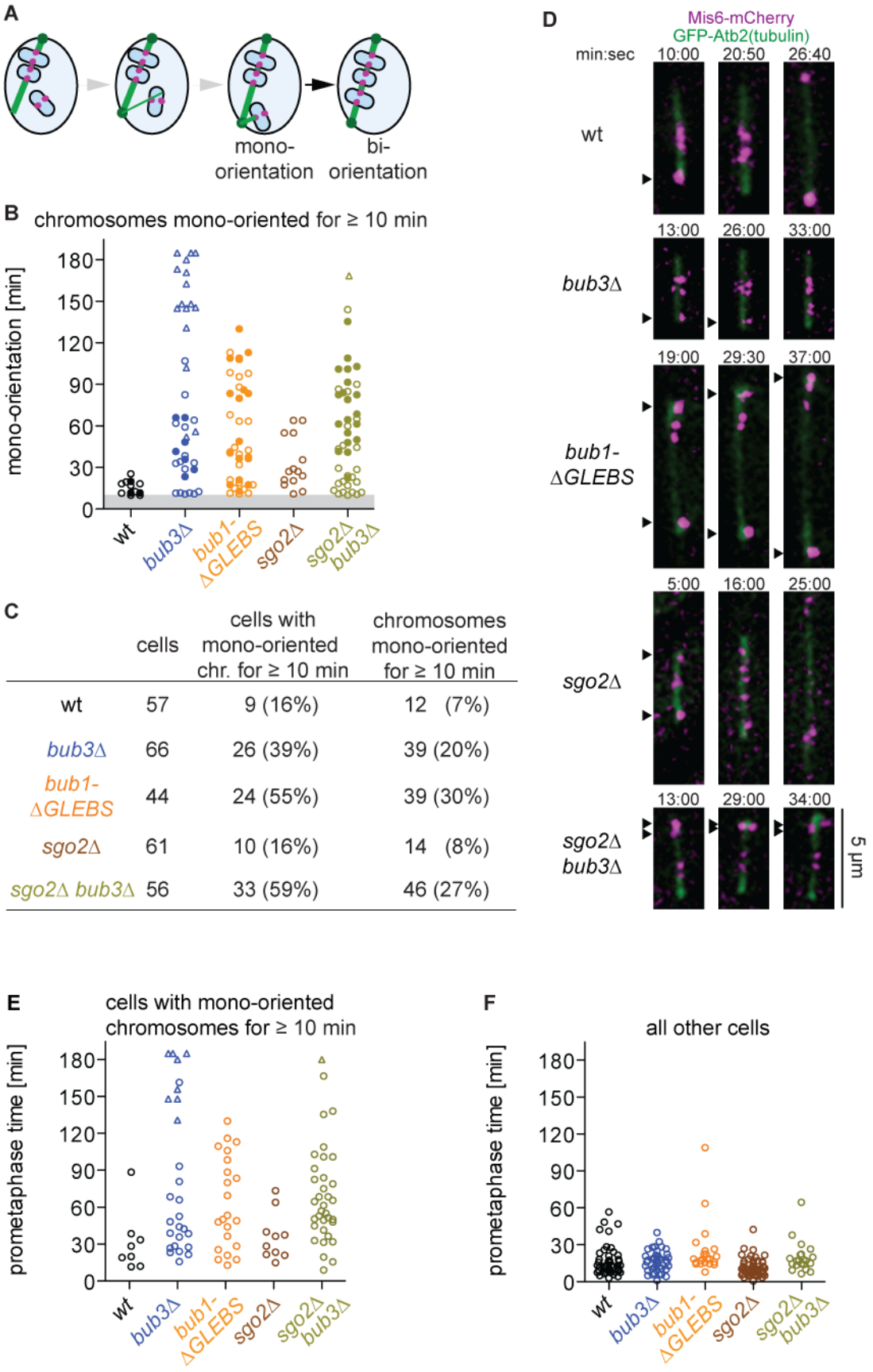

Figure 2.25 Bub3 and its interaction with Bub1 are required for chromosome bi-orientation in a Sgo2-independent manner (Windecker et al., 2009)

It was possible that Bub1 and Bub3 facilitate bi-orientation of a mono-oriented chromosome by influencing microtubule dynamics (see chapter 2.3.6), either by having a general effect on microtubule stability or by specifically influencing microtubule dynamics at the plus tips of the microtubules that are connected to the kinetochore(s) of the unaligned/mono-oriented chromosomes. If the latter was the case, Bub1 and Bub3 should localize to the kinetochores of these chromosomes. Indeed, Bub1 and Bub3 were enriched on kinetochores of unaligned chromosomes in the MBC washout experiment, and they only disappeared from there after the chromosomes had achieved bi-orientation on the spindle (Figure 2.27B), consistent with kinetochores being the location where Bub1 and Bub3 exert their function in bi-orienting chromosomes. As an alternative to influencing microtubule dynamics at the kinetochore(s) of the mono-oriented chromosome, Bub1 and Bub3 could have a role in forming a connection between the mono-oriented chromosome and the spindle, or they could even be involved in moving the mono-oriented chromosome along pre-existing spindle microtubules closer toward the center of the spindle, where capture by microtubules from the opposite pole is more likely, a mechanism that has been described in mammalian cells (Kapoor et al., 2006).

Figure 2.25 Bub3 and its interaction with Bub1 are required for chromosome bi-orientation in a Sgo2-independent manner (Windecker et al., 2009)

Cells expressing *GFP-atb2(tubulin)* and the kinetochore marker *mis6-mCherry* were presynchronized and treated with MBC as described in Figure 2.24. (**A**) After washout of MBC, bi-orientation and segregation of chromosomes was monitored by live cell microscopy at 20 °C. (**B**) Chromosomes that persisted close to an SPB for at least 10 min were followed and the time until they became bi-oriented was determined (open circles). Filled circles indicate chromosomes that apparently never achieved bi-orientation. Triangles indicate chromosomes that had failed to achieve bi-orientation by the end of recording. The time of chromosome mono-orientation is prolonged in *bub3Δ* and *bub1-ΔGLEBS* cells compared to wild type and *sgo2Δ* cells. The total number of cells observed, the number of cells displaying mono-oriented chromosomes for at least 10 min and the number of mono-oriented chromosomes are given in (**C**). (**D**) Example cells from this experiment. Arrowheads indicate mono-oriented chromosomes. For the *bub3Δ* cell, correction of mono-orientation to bi-orientation could be seen at the 33 min timepoint. In the *bub1-ΔGLEBS* cell, mono-orientation was never corrected and the cell entered anaphase after 37 min. In the *sgo2Δ* cell, bi-orientation was achieved after about 15 min. The *sgo2Δ bub3Δ* cell failed to bi-orient two chromosomes and delayed entry into anaphase for more than 1 h (Figure 2.26G). (**E**) and (**F**) Prometaphase time of the cells analyzed in (B)-(D). After washout of MBC, progression through mitosis was followed. Only cells that had entered mitosis when recording started were analyzed. The time in prometaphase was measured from this point to the onset of anaphase, judged by separation of sister chromatids (Mis6-mCherry). (**E**) Time of prometaphase in cells which showed mono-orientation of chromosomes for at least 10 min was prolonged compared to cells without mono-oriented chromosomes or with mono-oriented chromosomes for less than 10 min, which is shown in (**F**). Triangles represent cells that were still in mitosis when recording was stopped.

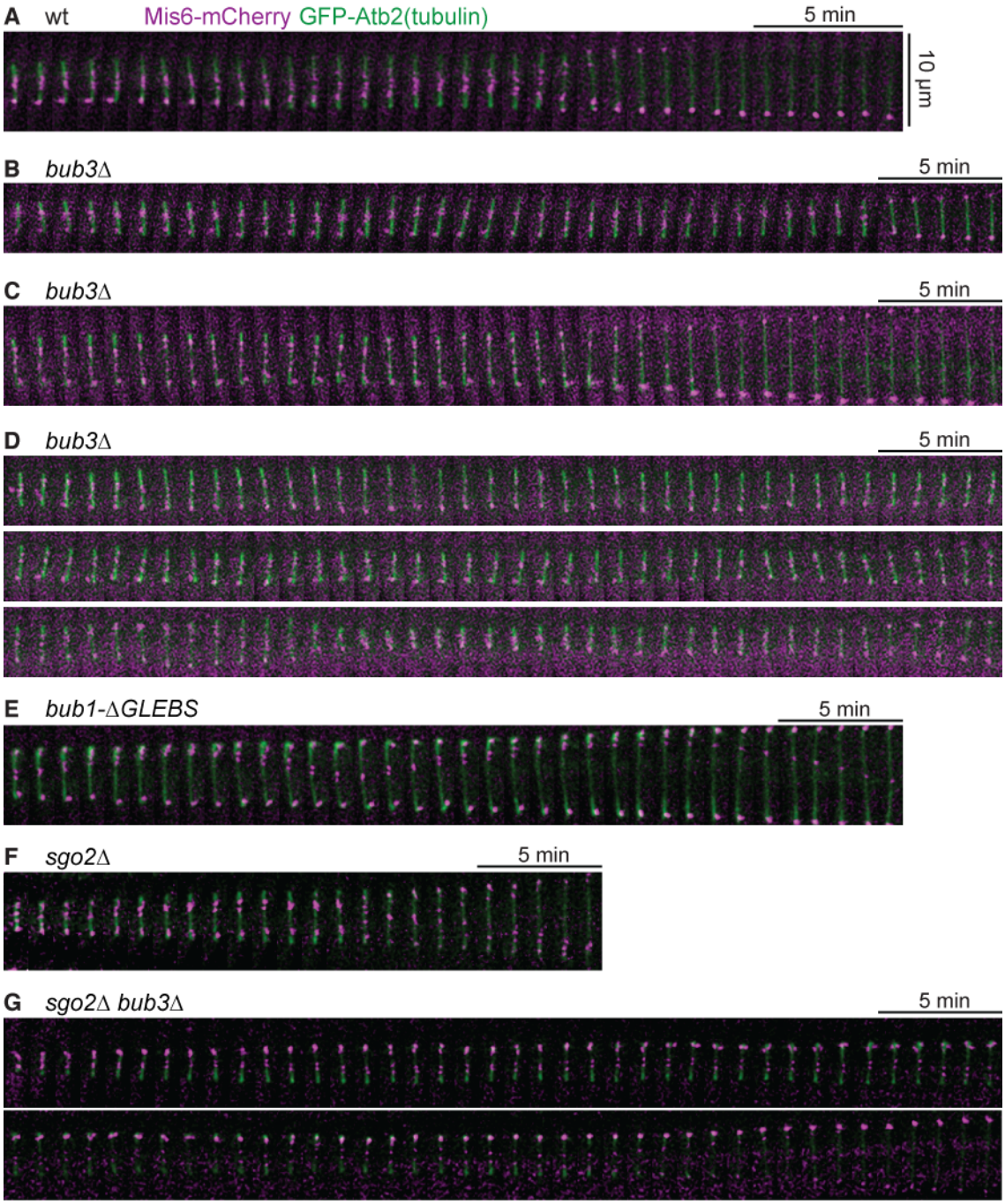

Figure 2.26 Exemplary kymographs for the experiment shown in Figure 2.25 (Windecker et al., 2009)

(**A**) Wild type cell with one chromosome initially mono-oriented. (**B-D**) Cells lacking Bub3. The cell in (B) showed one chromosome that became bi-oriented before anaphase onset. In the cell in (**C**), the mono-oriented chromosome did not achieve bi-orientation before anaphase onset. The cell in (**D**) showed a mono-oriented chromosome for more than 90 min. (**E**) Cell expressing *bub1-ΔGLEBS* in which two chromosomes were still mono-oriented when the cell entered anaphase. (**F**) Cell lacking Sgo2 with two initially mono-oriented chromosomes that became bi-oriented before anaphase onset. (**G**) Cell lacking Sgo2 and Bub3 with two mono-oriented chromosomes that had not been corrected when anaphase started (see also Figure 2.25D).

Table 2.1 Overview on all cells analyzed in Figure 2.25B-F (Windecker et al., 2009)

	total number of cells*	number of cells included	% of cells excluded from the analysis
wild type	59	57	3.4
*bub3*Δ	75	66	12
*bub1-*Δ*GLEBS*	50	44	12
*sgo2*Δ	79	61	22.8
*sgo2*Δ *bub3*Δ	65	56	13.9

*Cells that were already in mitosis when imaging started

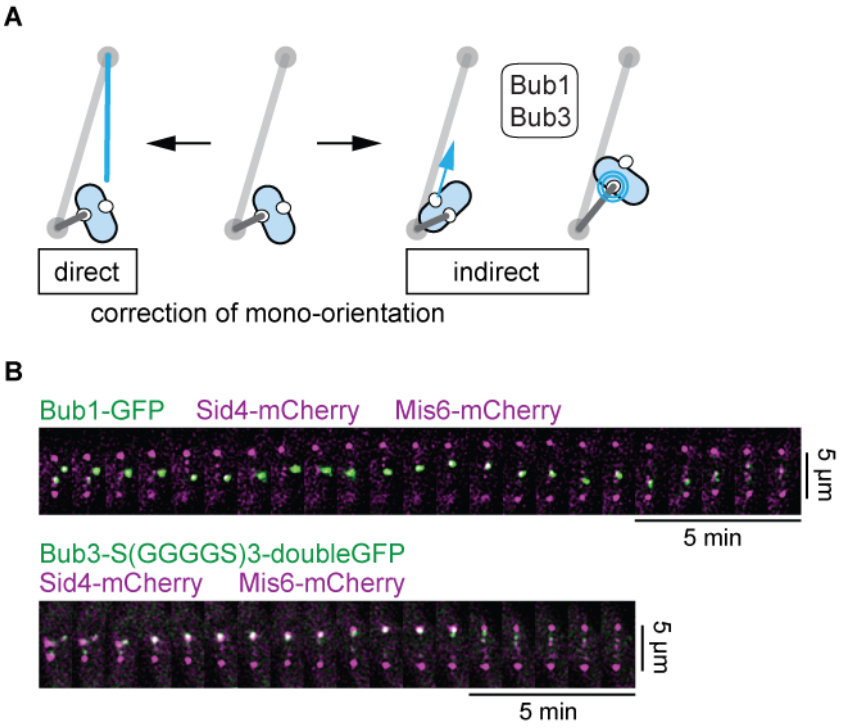

Figure 2.27 Possible pathways for correction of mono-orientation (Windecker et al., 2009)
(**A**) When chromosomes are clustered close to the SPBs at the beginning of mitosis or when spindles are short, direct capture by a microtubule from the opposite SPB may be the predominant way of correcting mono-orientation (left side). When spindles have become elongated, additional mechanisms may be needed (right side). We propose that Bub1 and Bub3 are involved in moving mono-oriented chromosomes closer to the centre of the spindle either by promoting movement along pre-existing kinetochore microtubules or by modulating kinetochore-microtubule attachment of the mono-oriented chromosome to the proximal pole. (**B**) Kymographs of cells expressing *bub1-GFP* or *bub3-GFP* and carrying *sid4-mCherry* as a marker for SPBs and *mis6-mCherry* as a marker for kinetochores. Cells were treated with MBC after release from HU-arrest as described in Figure 2.24. After MBC washout, cells were followed by live cell microscopy. Initially unattached chromosomes were captured by microtubules, pulled toward a SPB and subsequently bi-oriented. Bub1-GFP and Bub3-GFP localized to one or both kinetochore(s) of these chromosomes until the chromosomes had achieved bi-orientation.

2.4.3 Spindle shrinkage can rescue mono-orientation

In the MBC washout experiments, we frequently observed that when the spindle had elongated in the presence of unaligned chromosomes, it shrank again at some point (Figure 2.28). Upon spindle shrinkage, previously mono-oriented chromosomes often achieved bi-orientation (Figure 2.28B). This result further supported our model that direct capture of kinetochores by microtubules is more efficient when spindles are short (Figure 2.28A). We considered the possibility that spindle shrinkage events specifically occur in *bub3Δ* and in *bub1-ΔGLEBS* cells. However, shrinkage of spindles was also observed, albeit less frequently, in wild type cells, when chromosomes were unaligned for a prolonged period (Figure 2.29). We hypothesize that when not all three chromosomes are aligned, but the spindle elongates nonetheless, it becomes unstable and as a consequence sometimes shortens again.

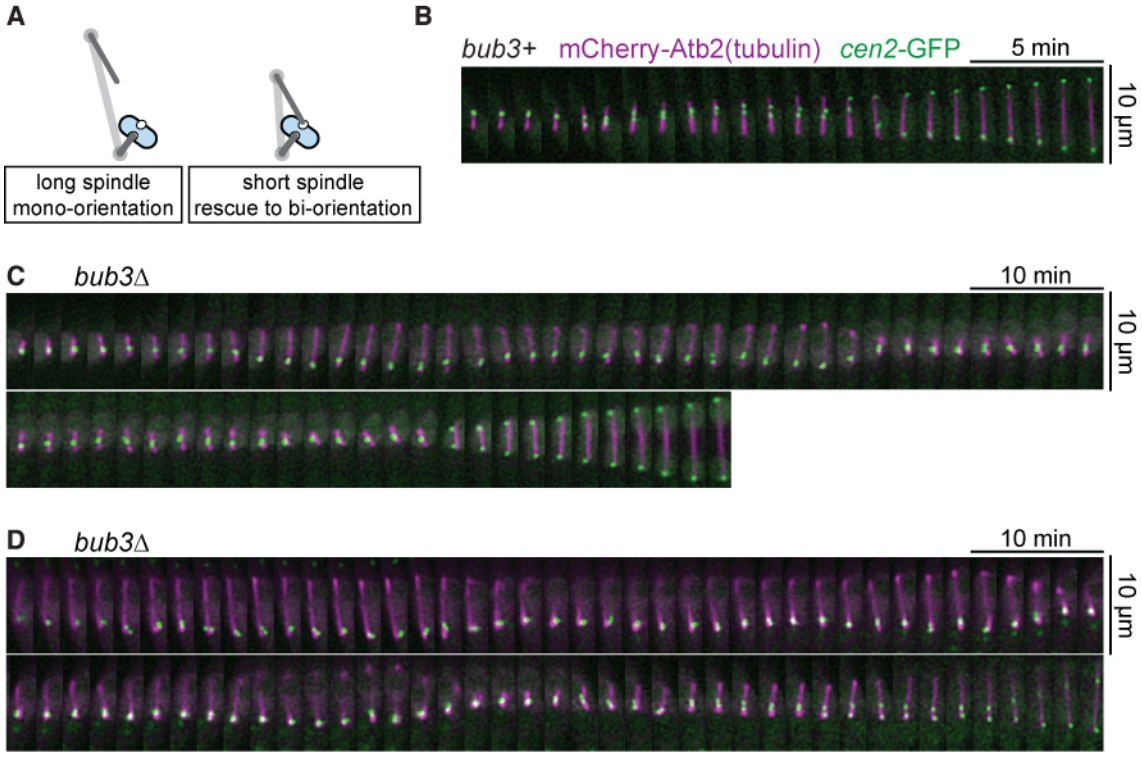

Figure 2.28 Mono-orientation of chromosomes in *bub3Δ* cells can be rescued when mitotic spindles are short (Windecker et al., 2009)

(**A**) Model for the possible mechanism of rescue: when the mitotic spindle is long, microtubules from the opposite spindle pole cannot reach the mono-oriented chromosome, whereas they can if the spindle is short. A second, indirect mechanism of rescue (Figure 2.27A) is presumably not functional in *bub3Δ* cells. (**B**-**D**) Exemplary kymographs of wild type (B) or *bub3Δ* cells (C,D) carrying *cen2-GFP* and expressing *mCherry-atb2(tubulin)* from the experiment described in Figure 2.24.

2.5 SAC activity without enrichment of SAC proteins at the kinetochore

2.5.1 Bub3 is required for localization of SAC proteins to unattached chromosomes

It had been reported previously that fission yeast Bub3 is required for enriching SAC proteins at the kinetochore (Millband and Hardwick, 2002; Vanoosthuyse et al., 2004). This was surprising in the light of our and others' (Tange and Niwa, 2008; Vanoosthuyse et al., 2009) result that Bub3 is not essential for SAC activity in fission yeast. Because cells released from MBC treatment in the absence of Bub3 obviously delayed exit from mitosis in the presence of chromosome attachment defects (Figure 2.25E), I wanted to examine the localization of the SAC protein Mad2-GFP in this situation. In wild type cells, Mad2-GFP was highly enriched on kinetochores of unaligned chromosomes that were moving freely with respect to the SPBs, and thus were probably unattached (Figure 2.29A). In contrast, no Mad2-GFP signal was detectable on kinetochores of such chromosomes in *bub3Δ* and *bub1-ΔGLEBS* cells (Figure 2.29B), indicating that Bub1 and Bub3, as well as their interaction with each other, are required for efficient recruitment of Mad2-GFP to kinetochores. This results is consistent with data published recently by Vanoosthuyse et al., who saw that localization of Mad1-GFP, Mad2-GFP and Mad3-GFP was perturbed in *bub3Δ* cells in the presence of chromosome attachment errors (Vanoosthuyse et al., 2009). Similarly, localization of Bub1 to kinetochores depends on Bub3 (Vanoosthuyse et al., 2004; Windecker et al., 2009). Overall, these results were striking, considering that Bub3 was not essential for the mitotic delay in the presence of chromosome attachment defects (Figures 2.15 and 2.16, as well as Figures 2.18B and 2.25E) (Tange and Niwa, 2008; Vanoosthuyse et al., 2009; Windecker et al., 2009). This suggests that kinetochore enrichment of the spindle assembly checkpoint proteins Mad1, Mad2, Mad3 and Bub1 is dispensable for SAC activity, which contradicts the standard model for SAC activation (Musacchio and Salmon, 2007). It is, however, possible that undetectable levels of these SAC proteins remain at the kinetochore in *bub3*-deleted cells and are sufficient for creating a robust SAC signal.

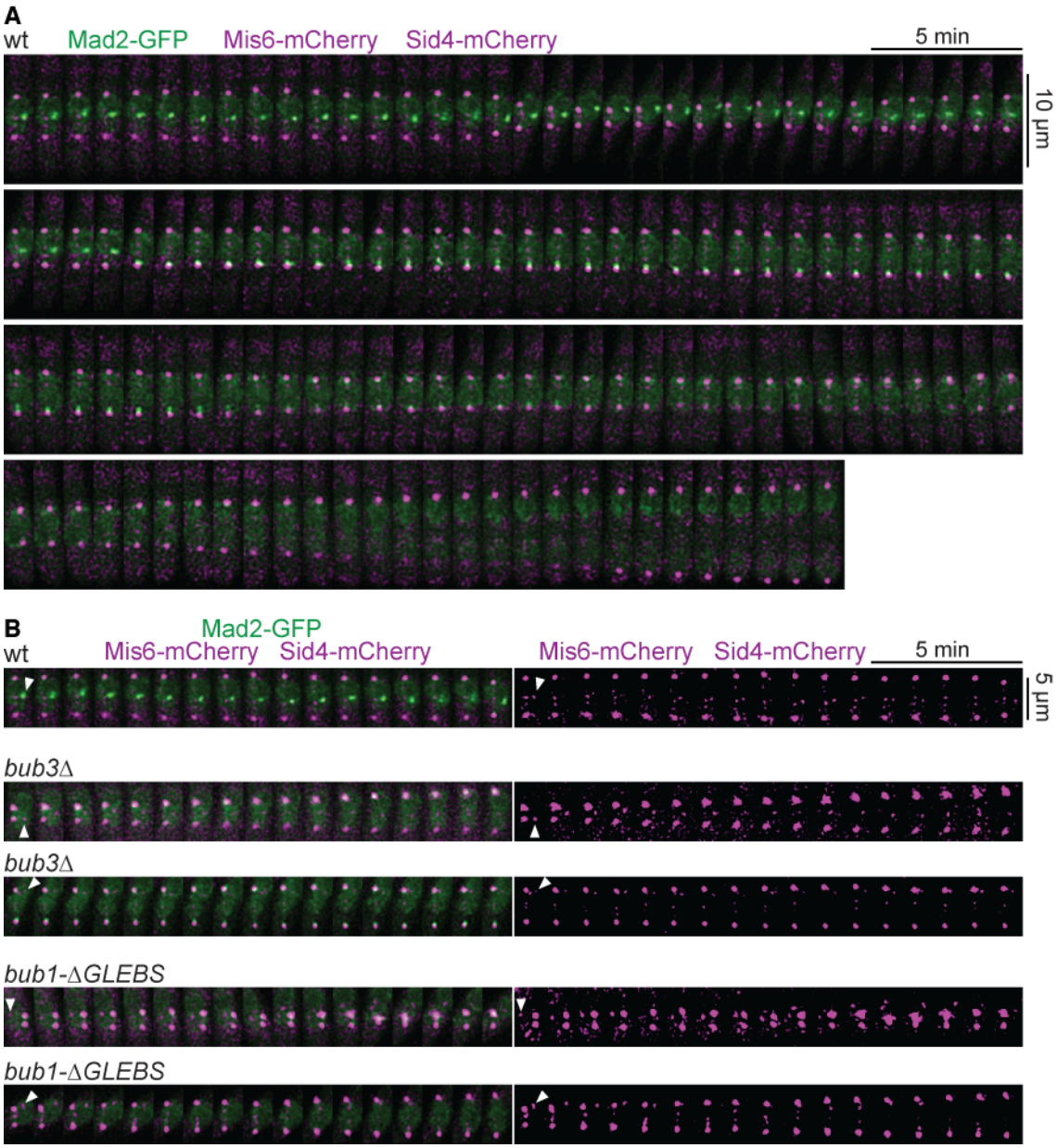

Figure 2.29 Mad2-GFP localization on kinetochores of unaligned chromosomes is perturbed in *bub3Δ* and *bub1-ΔGLEBS* cells after MBC release (Windecker et al., 2009)

Kymographs of cells expressing *mad2-GFP* as well as *mis6-mCherry* and *sid4-mCherry* as marker for kinetochores and SPBs, respectively. Synchronized cells were arrested with MBC at 20 °C and after 3.5 h released from MBC arrest and imaged by live cell microscopy at this temperature. (**A**) Kymograph of a wild type cell with one unaligned chromosome at the beginning of recording. Strong Mad2-GFP localization to one or both kinetochore(s) of this chromosome was visible. The first part of this kymograph is also shown in the first panel of (B). (**B**) Kymographs of wild type, *bub3Δ* and *bub1-ΔGLEBS* cells with unaligned chromosomes (arrowheads). In the wild type, Mad2-GFP clearly localizes to kinetochores of unaligned chromosomes, whereas in *bub3Δ* and *bub1-ΔGLEBS* cells I could not observe Mad2-GFP localization to kinetochores at any stage.

2.5.2 The N-terminal region of Mph1 is dispensable for checkpoint functionality

If kinetochore localization of Mad1, Mad2, Mad3 and Bub1 is indeed dispensable for SAC activity, the question arises of how the SAC signal is created at the kinetochore and which SAC component – if not Mad1, Mad2, Mad3 or Bub1 – is the key player at the kinetochore. A good candidate is Mph1 (Mps1 in other organisms). Genetic data indicates that Mps1/Mph1 acts upstream in the spindle assembly checkpoint (Abrieu et al., 2001; Hardwick et al., 1996; He et al., 1998), and it is known to be required for kinetochore localization of various SAC proteins in different organisms (Abrieu et al., 2001; Martin-Lluesma et al., 2002; Millband and Hardwick, 2002; Vanoosthuyse et al., 2004; Vigneron et al., 2004), again indicating that it acts upstream in the signaling pathway. Consistent with our hypothesis, Mph1 localization does not seem to be impaired in the absence of Bub3 (Stephanie Heinrich, preliminary unpublished data). We reasoned that if SAC activity depends on kinetochore localization of Mph1, disturbing its localization by disrupting the regions of the protein required for association with kinetochores would abolish SAC activity. In contrast, when specifically abolishing kinetochore localization of Mad1 and Mad2 by disrupting the region of Mad1 required for association with kinetochores, the SAC might still be active, as it is the case in the absence of Bub3. Therefore, I constructed mutants of Mad1 and Mph1 that lacked the N-terminal parts of the proteins, which are known to be required for kinetochore localization in *Xenopus* (Chung and Chen, 2002) and human cells (Liu et al., 2003; Maciejowski et al., 2010; Martin-Lluesma et al., 2002; Stucke et al., 2004) (Figure 2.30A). Spindle checkpoint activity was assayed in strains carrying the *nda3-KM311* allele, as described in 2.3.2. Whereas the Mph1 mutant lacking amino acids 1-150 was able to arrest cells in mitosis, the Mph1 mutant lacking amino acids 1-302 and the Mad1 mutant lacking amino acids 1-468 were not able to delay in mitosis. This indicates that the very N-terminal part of Mph1 is dispensable for SAC activity. However, expression levels of the mutants have not been determined yet, hence it is possible that the abrogation of the SAC in *mph1-Δ1-302* and *mad1-Δ1-468* is due to low levels of the proteins. This and whether the mutant proteins localize to kinetochores is currently under investigation.

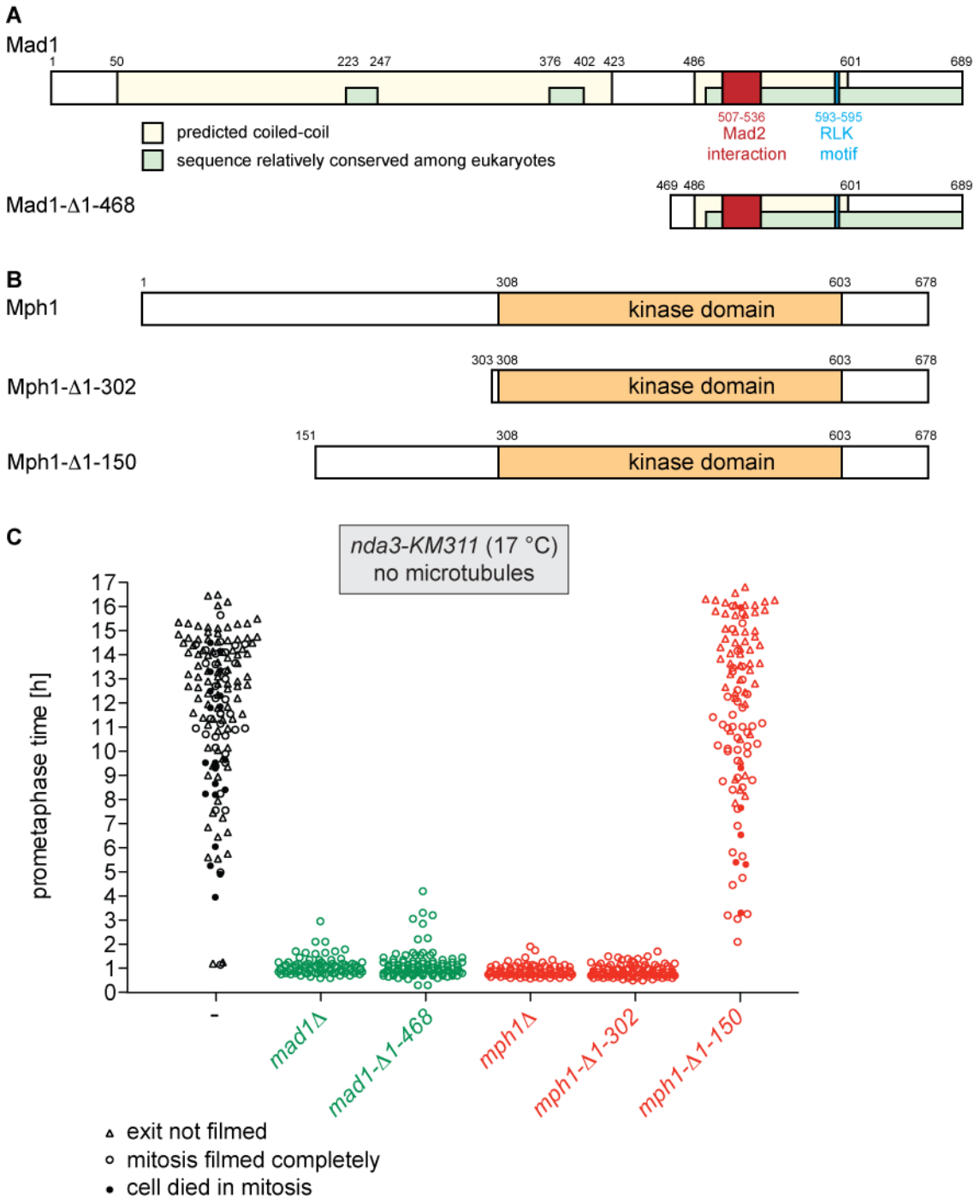

Figure 2.30 SAC functionality in Mad1 and Mph1 N-terminal truncation mutants

(**A)** Schematics representing fission yeast Mad1 and Mad1-Δ1-468. (**B**) Schematics representing fission yeast Mph1 and the N-terminal truncation mutants Mph1-Δ1-302 and Mph1-Δ1-150 (**C**) Cells of indicated strains expressing *plo1-GFP* and the β-tubulin *nda3-KM311* allele were followed by live cell microscopy at 17 °C. The duration of prometaphase was determined by the presence of Plo1-GFP on the SPBs. The *mph1-Δ1-150* mutant was able to delay in mitosis, whereas the *mph1-Δ1-302* and the *mad1-Δ1-468* mutant failed to arrest in mitosis.

3 Discussion

The aim of this study was to characterize functions that Bub1 and Bub3 have outside the spindle assembly checkpoint (SAC). I could show that the viability of fission yeast kinesin-8 (Klp5 and Klp6) mutants is much reduced in the absence of Bub1 or Bub3, or when Bub1 lacks the region required for interaction with Bub3, the GLEBS motif. This growth defect was not caused by loss of spindle assembly checkpoint activity, as we and others found Bub3 to be not essential for the SAC and deleting *mad2*, which completely abolishes SAC functionality, did not impair growth of kinesin-8 mutants perceptibly. Furthermore, viability of kinesin-8 mutants was not dependent on the kinase activity of Bub1, as both a kinase-dead mutant as well as deletion of the entire kinase domain of Bub1 did not perturb growth of a *klp5Δ* mutant. Consistently, the absence of Sgo2, whose localization depends on Bub1 kinase activity, did not impair growth of *klp5Δ* or *klp6Δ* cells. What is the molecular activity of Bub1 and Bub3 that makes them indispensable in the absence of kinesin-8? Spindle assembly checkpoint activity was the only reported function of fission yeast Bub3. However, contradictory to what had been reported before, Bub3 turned out to be not essential for a spindle assembly checkpoint-mediated delay in the presence of chromosome attachment errors. It was well established and I could confirm that Bub1 is required for spindle assembly checkpoint activity and shugoshin function. Since both of these functions are dispensable for healthy growth of kinesin-8 mutants, Bub1 and Bub3 must have at least one additional function, which they most likely share. This function is required for efficient growth of kinesin-8 mutants. I could show that Bub1 and Bub3, and presumably also their interaction, are involved in bi-orientation of chromosomes. Furthermore, I found that this function in bi-orientation is independent of Sgo2. Thus, I could identify an as yet undescribed function of Bub1 and Bub3 in mitosis. In addition, my data indicates that Bub1 and Bub3 can influence microtubule dynamics.

3.1 Do Bub1/Bub3 share a microtubule-depolymerizing function with Klp5/Klp6?

3.1.1 Genetic studies reveal an as yet uncharacterized function of Bub1 and Bub3

When West et al. (2002) observed that *klp5Δ* cells show abnormal chromosome movements in mitosis, abnormal spindle elongation and delay in mitosis, they expected that abolishing the spindle assembly checkpoint would be detrimental and result in loss of viability. They indeed observed that double mutants of *klpΔ* (*klp5Δ, klp6Δ* or *klp5Δ klp6Δ*) together with *bub1Δ* produced small colonies which failed to grow at 36 °C but, to their surprise, no synthetic sickness was observed with the SAC mutants *mad2Δ* and *mph1Δ* (West et al., 2002)(Figures 2.1 and 2.13). Thus, the synthetic sickness observed in *klp5Δ bub1Δ*, *klp5Δ bub1-ΔGLEBS* and *klp5Δ bub3Δ* cells is unlikely to be caused by loss of checkpoint activity (Figures 2.13 and 2.14). Yet, it has been proposed that there are two branches of the SAC, one being responsible for detecting the absence of kinetochore microtubule attachment and the other one for detecting the absence of tension (see chapter 1.4.3).

Bub1 has been proposed to be required for sensing the absence of tension, whereas Mad2 seemed to be mainly involved in detecting the absence of attachment (Skoufias et al., 2001). Mutation of the microtubule plus-end-tracking protein Mal3 (EB1 homolog) results in a mitotic delay that is shortened by deletion of *bub1* but not by deletion of *mad2*, from which the authors conclude that a *bub1*-dependent 'no-tension branch' of the SAC is activated in *mal3* mutants (Asakawa et al., 2005). Kinesin-8-deficient cells also show a delay in mitosis. However, this delay is abolished by deletion of *mad2* (Garcia et al., 2002a). Therefore, it is highly unlikely that a specific *bub1*-dependent SAC pathway is required for survival of kinesin-8 mutants. There is additional evidence against the requirement of a no-tension-sensing mechanism for survival of kinesin-8-deficient cells. Sgo2, whose function depends on Bub1 (see chapter 1.4.4.4), is so far the only fission yeast protein reported to be required for delaying anaphase onset exclusively in the absence of tension, but not in the absence of attachment (Kawashima et al., 2007)(see chapter 3.5 for discussion about Sgo2-dependent SAC activity). In this regard, since *klp5Δ sgo2Δ* cells grow healthily, it cannot be loss of the 'no-tension checkpoint' that causes the synthetic sickness of *klp5Δ bub1Δ* cells, nor can loss of any other Sgo2 function. In addition, the spindle assembly checkpoint is still active in *bub3Δ* and *bub1-ΔGLEBS* cells (this study; Tange and Niwa, 2008; Vanoosthuyse et al., 2009). Taken together, loss of SAC activity is highly unlikely to be the reason for the observed synthetic interaction.

My results indicate that Bub1 and Bub3 have an additional function that is independent of the SAC and does not involve Sgo2, but is required for efficient growth of kinesin-8-deficient cells. Furthermore, this function does not depend on the kinase activity of Bub1, but it depends on the presence of the Bub1 GLEBS motif (Figures 2.2 and 2.14). The Bub1 mutant lacking the GLEBS motif is expressed to similar levels as wild type Bub1 and was found to have a functional spindle assembly checkpoint, but, in contrast to the wild type protein, does not co-immunoprecipitate with Bub3 (Vanoosthuyse et al., 2009). Therefore, it is likely that the additional function of Bub1 and Bub3 is shared by the two proteins and requires their interaction.

The synthetic sickness of *klp6Δ* with *bub1Δ* can be suppressed partially by deletion of *sgo2* and slightly less by deletion of *mad2* (Figure 2.1). It is not clear why this is the case as both shugoshin function and SAC activity are expected to be abolished already by deletion of *bub1* alone. However, the presence of Sgo2 apparently has a detrimental effect on *klp5Δ bub1Δ* cells. It is possible that there is some residual localization of Sgo2 to heterochromatin in the absence of Bub1. If this is the case, it might explain why *klp5Δ bub1-ΔGLEBS* and *klp5Δ bub3Δ* have a stronger growth defect than *klp5Δ bub1Δ*, because in the *bub1-ΔGLEBS* and *bub3Δ* mutants Sgo2 localization is not completely abolished (Figure 2.17). One can only speculate why the presence of Sgo2 is a disadvantage in this situation. Sgo2 is required for efficient localization of Ark1, the fission yeast Aurora B kinase (Kawashima et al., 2007; Vanoosthuyse et al., 2007), which has been shown to destabilize erroneous kinetochore microtubule attachments (Liu and Lampson, 2009). My data indicates that kinetochore microtubule attachments in *klp5Δ bub1Δ* cells are not normal. Thus, it might be possible that when residual amounts of active Ark1 remain at the kinetochores, attachments are destabilized and as formation of correct kinetochore attachments is hindered due

to the absence of Klp5 and Bub1, chromosomes become unattached, as I observed in *klp5Δ bub3Δ pREP1-klp5+* cells after plasmid loss (Figure 2.18A). This could result in increased missegregation of chromosomes.

The growth tests showed that *klp5Δ bub1Δ* cells are slightly sicker than *klp6Δ bub1Δ* cells (Figure 2.1B). Even though most available data indicates that kinesin-8 proteins function as a heterodimer (Grissom et al., 2009), it is possible that they also have separate functions. This hypothesis is supported by the observation that *klp6Δ* cells show defects in zygote formation, whereas *klp5Δ* cells do not (West et al., 2001). Furthermore, Klp5 interacted with itself in a yeast two-hybrid assay, indicating that it might also function as a homodimer (Li and Chang, 2003). An alternative explanation for the slight difference in synthetic sickness is that the kinesins take over distinct tasks within the heterodimer, and the phenotypes of the two deletion mutants are only distinguishable when challenged, as in the *bub1Δ* cells. This hypothesis is corroborated by data from West and McIntosh (2008) as well as Unsworth et al. (2008), who analyzed the phenotypes and localization of kinesin-8 chimeras and truncation mutants. *In vivo* data suggested that Klp5 binds microtubules with higher affinity than Klp6, and, in contrast to Klp6, it can be detected on astral microtubules in mitosis when the other kinesin-8 protein is missing (Unsworth et al., 2008). If this disparity is the reason for the observed difference in synthetic sickness, residual Klp5 localization to microtubules in *klp6Δ bub1Δ* cells is beneficial for cell viability.

3.1.2 Does the *klp5Δ bub1Δ* and *klp5Δ bub3Δ* double mutant phenotype reveal the underlying cause of the synthetic growth defect?

What is the function of Bub1 and Bub3 that is required for survival of *klpΔ*? To find an answer to this question I wanted to analyze why the double mutants die. I first looked at mitotic chromosome segregation in *klp5Δ bub1Δ* cells. Chromosome missegregation was increased in the double mutant compared to the single mutants (Figure 2.3), but not to a substantially higher level, suggesting that preventing missegregation is not the sole function that they share. Live cell imaging of *klp5Δ bub3Δ* cells clearly showed that chromosome attachments are defective, much more than in the single mutants alone (Figures 2.18 and 2.19). Furthermore, spindle morphology was very often abnormal. As explained in 2.4 this could be caused by defects in chromosome bi-orientation and alignment. In addition to missegregation, I observed defects in septation and cell separation in the *klp5Δ bub1Δ* double mutant (Figure 2.3). Klp5 and Klp6 have been implicated in septation and cytokinesis, because deletion mutants of *klp5* and *klp6* are synthetically sick with mutants of the Ras1-Scd1 pathway, and cytokinesis and septation defects can be observed in *klpΔ scd1Δ* double mutants (Li and Chang, 2003). Furthermore, like components of the Ras pathway and other proteins involved in septation/cytokinesis, Klp5 and Klp6 localize to the spindle midzone in anaphase (Bähler et al., 1998a; Garcia et al., 2002b; Li et al., 2000; West et al., 2002). However, for Bub1 and Bub3, no such function has been reported and no localization to microtubules or even to the midspindle can been seen. In addition, in contrast to the observation I made in *klp5Δ bub1Δ*, I could not observe a defect in septation or cell separation in the live cell imaging experiment with *klp5Δ bub3Δ* cells, except for an occasional mispositioned spindle with respect to the plane of

septation. This could indicate that Bub1 has a function in septation/cytokinesis that is not shared by Bub3, but then lack of this function is unlikely to be the cause for synthetic sickness with *klpΔ*. As mentioned in 2.2.2, the septation defects in *bub1Δ* cells could be due to secondary effects, such as lagging chromosomes, which are presumably the consequence of lack of shugoshin function (Kawashima et al., 2009), and are probably even more frequent due to the also abolished SAC activity. Such a secondary effect would also explain why septation/cytokinesis defects were not frequently observed in the *klp5Δ bub3Δ* double mutant, as *bub3Δ* mutants have a functional SAC and are less impaired in shugoshin localization than *bub1Δ* mutants (Figure 2.17). To my knowledge, whether the absence of Bub3 causes lagging chromosomes has never been examined.

3.1.3 Can genetic interaction analysis reveal the cause of the synthetic sick interaction between kinesin-8 mutants and *bub1Δ*/*bub3Δ*?

Except for *bub1* and *bub3*, there are several other mutant genes known to show synthetic sickness with kinesin-8 mutants. These genes are potentially involved in the same 'pathway' as *bub1* and *bub3*. The encoded proteins could act together with Bub1 and Bub3 to fulfill the function required for efficient growth of kinesin-8 mutants (Figure 2.5).

Klp5 and Klp6 are known to show synthetic lethal interactions with components of the Scd1-branch of the Ras1 pathway (Li and Chang, 2003). Mutants of this branch have a round cell shape, are sterile and show sensitivity toward microtubule-destabilizing drugs, all consequences of defects in microtubule organization (Chang et al., 1994; Fukui and Yamamoto, 1988; Li et al., 2000; Qyang et al., 2002). It has been shown that Scd1 interacts with mitotic spindles (Li et al., 2000), and double mutants of the α-tubulin gene *nda2* and *scd1* (*scd1Δ nda2-KM52*) block spindle formation at 23 °C, a semi-restrictive temperature for the *nda2-KM52* mutant (Li et al., 2000). Furthermore, *scd1Δ* cells have a higher rate of chromosome loss (Li et al., 2000). All this indicates that the Ras-pathway in fission yeast not only controls the cytoskeleton in interphase, but also has mitotic functions. The synthetic interaction between Klp5/6 and components of the Ras pathway was discovered in a study that screened for mutants that suppress TBZ sensitivity of *scd1Δ* mutants, which revealed kinesin-8 mutants (Li and Chang, 2003). Kinesin-8 proteins are known to be involved in microtubule depolymerization (Gupta et al., 2006; Mayr et al., 2007; Varga et al., 2006), and, consistently, mutants of *klp5* and *klp6* show resistance toward microtubule-destabilizing drugs such as TBZ (Garcia et al., 2002a; West et al., 2001). It is therefore not surprising that they were identified in a screen for suppression of TBZ sensitivity, and this could be completely independent of the TBZ-sensitivity-conferring nature of the initial mutation. However, in a second step, Li and Chang (2003) screened for those candidates that showed a synthetic growth defect with the *scd1Δ* mutant and again obtained kinesin-8 mutants. Apparently, components of the Ras pathway and kinesin-8 share an essential function. In spite of this negative genetic interaction, the double mutants have a growth advantage over the single *scd1Δ* mutant in the presence of microtubule-destabilizing drugs. Similarly, *klp5* or *klp6* deletion partially rescues TBZ sensitivity of *bub1Δ* cells at 30 °C (Figure S1). If Klp5-dependent microtubule depolymerization was the important function

for viability of *bub1Δ* and *scd1Δ* cells, it was possible that microtubule-destabilizing drugs rescued the synthetic sickness. TBZ did not rescue the growth defect of *klp5Δ scd1Δ* at higher temperatures, which could indicate that hyperstable microtubules in the absence of *klp5* are not the major reason for the synthetic sickness (Li and Chang, 2003)(see also below). Similarly, I could not observe rescue of the synthetic sickness of *klp5Δ bub1Δ* and *klp5Δ bub3Δ* by benomyl (Figure S2). However, note that for benomyl, in contrast to TBZ, there was no suppression of drug sensitivity by deletion of *klp5* in *bub1Δ* cells. It is known that cells respond differently to TBZ than to MBC (*m*ethyl-2-*b*enzimidazole *c*arbamate), which is similar to benomyl, as TBZ not only destabilizes microtubules but also disrupts the actin cytoskeleton (Sawin, 2004; Sawin and Nurse, 1998). However, it is unclear whether its effect on actin plays a role in this case.

Similarly to *klp5Δ bub1Δ*, also *klp5Δ scd1Δ* show a temperature-dependent synthetic sick phenotype. In general, microtubule stability is reduced concomitantly with decreasing temperature. If the synthetic sick interaction is partially caused by loss of microtubule destabilization due to kinesin-8 mutation, then an increase in temperature might even further stabilize microtubules and thus enhance the detrimental effect. It has been reported that changes in morphology caused by absence of kinesin-8 proteins are enhanced at higher temperature, consistent with the hypothesis that the phenotype results from hyperstable microtubules (West et al., 2001). However, as described before, it is not very certain, neither for synthetic interaction with *bub1*/3 nor with components of the Ras pathway (see above) that loss of the depolymerizing function of *klp5* contributes substantially to the synthetic sick interaction. The synthetic interaction of kinesin-8 mutants with *bub1* and *bub3* resembles the synthetic interaction with *scd1*. However, there is no additional data indicating that Bub1 and Bub3 share a function with Scd1 or are even involved in the Ras pathway.

Neither *bub1* or *bub3* deletion nor kinesin-8 deletion alone results in a strong growth defect, indicating that the genes are not involved in an essential pathway, hence the synthetic sickness of the null mutants presumably does not represent a within-pathway interaction, but rather a between-pathway interaction (see chapter 1.5.1.1). However, Scd1 also physically interacts with Klp5 and Klp6. This is somewhat unexpected as the synthetic sickness was observed between deletion mutants that on their own are viable, as it is the case for the genetic interaction of *klp5Δ* with *bub1Δ* or *bub3Δ*. The genetic data indicates that the kinesin-8 proteins act in a separate pathway that is partially redundant to the Ras pathway, whereas the physical interaction indicates that they act in the same pathway, in one complex. There is the theoretical option that both is true, if several functions are involved, or, if they act in a bigger complex which is still stable when one component is missing, but not when two components are missing. In the latter case, the functionality deteriorates, resulting in synthetic sickness. The fact that the genetic interaction is sickness not lethality and that it is dependent on certain growth conditions (temperature) additionally complicates interpretation. For Bub1 and Bub3 it has not been investigated whether they physically interact with Klp5 or Klp6. They both localize to kinetochores in early mitosis, but in ChIP analysis Bub1 was mapped to the inner centromere (Vanoosthuyse et al., 2004), whereas Klp5 was

mapped to the outer repeats of the centromere (Garcia et al., 2002b), indicating that they are not closely associated with each other.

Deletion mutants of *klp5* or *klp6* are synthetically lethal with mutants of the Dam1/DASH complex (Sanchez-Perez et al., 2005). The Dam1/DASH complex is required for correct kinetochore microtubule attachments, and deletion mutants show lagging chromosomes and have an increased rate of chromosome loss (Sanchez-Perez et al., 2005). Similar to the kinesin-8 proteins, the Dam1/DASH complex can bind to microtubule plus ends and to microtubule-associated kinetochores, where it presumably shares a function with Klp5/Klp6 in regulating kinetochore microtubule attachment (Sanchez-Perez et al., 2005). The function of the Dam1/DASH complex seems to be independent of Bub1 and Bub3, as Dam1 and Bub1 do not show localization dependencies (Figure 2.7 and Sanchez-Perez et al., 2005). Furthermore, in contrast to *bub1Δ*, the *dam1Δ* mutant is synthetically lethal with *klp5-T224D* (Julia Kamenz, unpublished data), indicating that *bub1Δ* and *dam1Δ* have different requirements in respect to kinesin-8 function. The *Klp5-T224D* mutant protein presumably has lost its depolymerization activity, but Yu-Hua Huang and I found that it still possesses a functional plus end-directed motor activity (data not shown). Hence, the depolymerizing function of Klp5 is crucial for survival of *dam1Δ*, but is less important in *bub1* deletion mutants. I also checked genetic interactions between *klp5-T224D* and other mutants that are known to be synthetically lethal with *klpΔ*. I did not obtain viable colonies of the double mutant *klp5-T224D alp14Δ* (Figure 2.6), indicating that, like Dam1, Alp14 does not act within the same pathway as Bub1 and Bub3. Alp14 and the related protein Dis1 are members of the Dis1/TOG family of microtubule-associated proteins, which, like the kinesin-8 proteins and the Dam1/DASH complex, get enriched at kinetochores in a microtubule-dependent manner in mitosis (Garcia et al., 2001). Deletion mutants of both *alp14* and *dis1* are synthetically lethal with *klpΔ*, which is somewhat surprising given that Dis1 and Alp14 are known to promote microtubule polymerization at microtubule plus ends, and therefore have opposing activity to the kinesin-8 proteins (Garcia et al., 2002a; Ohkura et al., 2001).

Tetrad analysis revealed that *klp5-T224D*, like the kinesin-8 deletion mutants (West et al., 2002), is synthetically lethal with the APC/C mutant *cut4-533* (Figure 2.6), indicating that the function of Bub1 and Bub3 which is required for viability of kinesin-8-deficient cells is independent of APC/C activity. It is not known why kinesin-8 mutants show a synthetic lethal interaction with mutants of the APC/C. It is possible that degradation of a certain APC/C substrate is essential for survival of kinesin-8-deficient cells. The APC/C mutants could be defective in ubiquitinating a certain substrate, which then escapes degradation by the 26S proteasome. A second possibility is that Klp5 and Klp6 are somehow involved in regulation of APC/C activity.

I analyzed synthetic interactions between *klp5-T224D* and the α-tubulin mutant *nda2-KM52*. In contrast to the *klp5Δ nda2-KM52* double mutant (West et al., 2001), the *klp5-T224D nda2*-KM52 double mutant was viable and only had a slight growth defect (Figure 2.6). It remains to be determined whether the *nda2-KM52* mutant is indeed defective in the same function as *bub1Δ*, *bub3Δ* and *bub1-ΔGLEBS* cells. But this result supports the notion that there is a connection to microtubule function.

3.1.4 Bub1 and Bub3 are involved in regulating microtubule dynamics

The genetic interaction of *bub1* and *bub3* with the kinesin-8 genes is not the only observation that connects them to microtubule function. Our results indicate that Bub1 and Bub3 are involved in regulating microtubule dynamics. In the absence of Bub1 or Bub3, we observed separation of SPBs in the *nda3-KM311* β-tubulin mutant background at semi-restrictive temperature, which occurred seldom in the *nda3-KM311* single mutant. Tubulin immunostaining revealed that spindles are formed (Figure 2.22). Spindle formation was independent of checkpoint activity, as in *bub3Δ* cells the checkpoint is active, whereas in *bub1Δ* cells it is not (Figure 2.15) (Bernard et al., 1998; Tange and Niwa, 2008; Vanoosthuyse et al., 2009). Furthermore, SPB separation hardly ever occurred in the absence of Mad2. The results could indicate that Bub1 and Bub3 have a microtubule-destabilizing activity, which would be a plausible explanation for the synthetic sickness with the kinesin-8 genes. In favor of this hypothesis is the observation that *klp5Δ nda3-KM311* cells also showed SPB separation at semi-restrictive temperature (data not shown). However, cells lacking Bub1 or Bub3 are, in contrast to kinesin-8-deficient cells, not resistant toward microtubule-destabilizing drugs, they even show a strong sensitivity (Bernard et al., 1998; Vanoosthuyse et al., 2004) which speaks against a function of Bub1 and Bub3 in microtubule destabilization. Furthermore, an obvious increase in spindle length, as it is the case for *klp5Δ* cells, has not been observed in *bub3Δ* cells, and localization of Bub1 and Bub3 to microtubules has not been reported. Thus a general microtubule destabilizing activity of Bub1 and Bub3 seems unlikely, but it is possible that they have a specific regulatory role in microtubule dynamics at the kinetochore. However, it is still unclear whether this would explain the SPB separation in the *nda3-KM311* mutant, since these very short spindles often do not include kinetochore microtubules, as chromosomes frequently uncluster from the SPBs under these conditions (Grishchuk and McIntosh, 2006). It remains to be determined whether the microtubule stabilization in the absence of Bub1 or Bub3 is specific to the *nda3-KM311* mutant or whether it occurs also in other situations in which spindle formation is perturbed, e.g. by drug-treatment or in other tubulin mutants.

3.1.4.1 Genetic data indicating that Bub1 and Bub3 influence microtubule dynamics

Apart from the synthetic sickness with kinesin-8 mutants, there are other genetic interactions pointing in the direction that Bub1 and Bub3 are connected to microtubule function. The temperature-sensitive α-tubulin mutant *atb2-V260I* in *S. pombe* shows synthetic lethality with *bub1Δ* and *bub3Δ*, but not with *mad2Δ* or with *bub1* kinase mutants (Asakawa et al., 2006). This mutant has slower spindle elongation velocity in anaphase B and reduced chromosome oscillations in prometaphase compared to wild type, indicating that microtubule dynamics are altered (Asakawa et al., 2006). Furthermore, the genetic interaction between kinesin-8 genes and *bub1/bub3* seems to be conserved. High-throughput genetic screens in *S. cerevisiae* identified a genetic sick interaction between *bub3* and *kip3*, the kinesin-8 protein in budding yeast (Collins et al., 2007; Costanzo et al., 2010; Daniel et al., 2006). There are also other genetic interactions reported for *bub1* and *bub3* which indicate that these genes are involved in microtubule function. In *S. cerevisiae, bub1* and *bub3* were identified in a *ber1* synthetic lethal screen (Fiechter et al., 2008).

The deletion of *BER1* results in resistance toward microtubule destabilizing drugs, indicating that the encoded protein is involved in regulation of microtubule dynamics (Fiechter et al., 2008). Furthermore, overexpression of either *BUB1* or *BUB3* can rescue growth of a budding yeast cold-sensitive α-tubulin mutant, whereas overexpression of *MAD2* does not have an effect (Guénette et al., 1995). Rescue by overexpression of *BUB1* or *BUB3* is independent of *MAD2* and hence independent of SAC activity (Abruzzi et al., 2002). At restrictive temperature, microtubule formation is disrupted in the mutant, and Abruzzi et al. (2002) propose that interaction with the kinetochore is weakened. Spindle formation can be partially rescued by overexpression of *BUB3* (Guénette et al., 1995). Thus, Abruzzi et al. (2002) propose that Bub1 and Bub3 contribute to stabilizing microtubule kinetochore attachment and suppress the tubulin mutant by stabilizing the attachments when overexpressed. However, overexpression of *BUB3* did not have a detectable influence on microtubule dynamics in wild type cells (Guénette et al., 1995).

In *A. nidulans*, *bub1* and *bub3* were found in a screen for synthetic lethal interactors of a dynein mutant, but the cause of synthetic lethality has not been investigated (Efimov and Morris, 1998). A direct interaction between Bub3 and a dynein light chain has been reported for mammalian cells (Lo et al., 2007). In metazoans, dynein promotes silencing of the SAC by transporting SAC components from the kinetochore toward the spindle poles (Musacchio and Salmon, 2007). In fission yeast, dynein is apparently not involved in this SAC silencing mechanism but is required for efficient bi-orientation of chromosomes (Courtheoux et al., 2007; Gachet et al., 2008; Grishchuk et al., 2007). Dynein might also have additional functions in early mitosis of mammalian cells, as RNAi of the dynein light chain causes defects in chromosome congression (Lo et al., 2007).

All these genetic interactions indicate that Bub1 and its binding partner Bub3 have an influence on microtubule dynamics, but they probably do not have a general microtubule-depolymerizing function as the kinesins Klp5 and Klp6 do. I propose that Bub1 and Bub3 are involved in regulating kinetochore microtubule attachments. They could directly interact with microtubules, as they localize to the outer kinetochore, but no localization to microtubules and no direct interaction with tubulin has been reported so far. Even when budding yeast *BUB1* and *BUB3* were overexpressed, no tubulin localization of Bub1 was detected (Roberts et al., 1994). Thus, they probably do not influence microtubules directly but via downstream factors, which remain to be discovered.

3.2 Functional requirements for different regions of Bub1

Bub1 has several domains and regions, and performs various tasks. The available data indicated that specific regions are more important for specific functions of Bub1 than others, which was confirmed by my results. In my screen for specific Bub1 mutants, I first selected for synthetic lethality with *klp5Δ* at 34 °C, then assayed Sgo2 localization and the mutants that revealed normal Sgo2 localization were also tested for SAC functionality (see chapter 2.2.4). Two candidates showing normal Sgo2 localization, *bub1-m3* and *bub1-m9*, were not able to delay anaphase onset in the presence of chromosome attachment errors (Figure 2.10A). Interestingly, in the *bub1-m3* allele, leucine (L) 82 is converted to a phenylalanine (F), which is the amino acid that is present in

most organisms at this position (Figures 2.8 and 2.11). The only other mutation of *bub1-m3* is L27P, which does not affect a conserved residue. Nonetheless, this mutant has a checkpoint defect, indicating that either both of these residues or one of them is important for Bub1 function in fission yeast. The mutations lie within or close to the N-terminal Mad3/Bub1 homology region I of Bub1. The region is known to be required for checkpoint activity and localization of Bub1 to kinetochores (Vanoosthuyse et al., 2004). It could be that the function of this domain is disrupted by the mutations. The *bub1-m9* allele has three mutations, two of which affect conserved residues. Aspartate (D) 392 lies within the conserved motif I, which has been shown to be essential for Mad1 localization to kinetochores and spindle assembly checkpoint activity in human cells (Klebig et al., 2009). Similar results were obtained for budding yeast, where a region encompassing conserved motif I is required for spindle assembly checkpoint activity and co-immunoprecipitation of Mad1 with Bub1 (Warren et al., 2002) (Figure 2.9). It is possible that this region is also required for spindle assembly checkpoint activity in fission yeast. In fungi, there is generally an acidic residue at this position (392), either a glutamate (E) or an aspartate. In contrast, in vertebrates, there can be a phenylalanine (human, mouse, *Xenopus* and chicken) or a valine (zebrafish) (Klebig et al., 2009). L169, the other conserved amino acid mutated in *bub1-m9*, is situated close to the Mad3/Bub1 homology region 1 (Figure 2.9). The third mutation, asparagine (N) 591 to aspartate (D), lies within a less conserved region. The *bub1-m9* mutant has additional mutations in the putative promoter region, so that the expression level could be influenced. However, Sgo2-GFP still localizes to kinetochores in *bub1-m9* (Figure 2.8B), indicating that *bub1-m9* is expressed. Furthermore, given that Bub1 levels can be reduced dramatically in mammalian cells without SAC perturbation (Johnson et al., 2004; Meraldi and Sorger, 2005; Tang et al., 2004b) it is rather unlikely that reduced levels of Bub1-m9 are the sole cause for checkpoint deficiency (and synthetic sickness with *klp5Δ*) of this mutant.

The mutants *bub1-m5* and *bub1-m8* delayed anaphase onset in the *nda3-KM311* mutant background at restrictive temperature. In contrast, no or only a slight delay was observed in the *psc3-1T* and *cut7-446* mutant background at the restrictive temperature of 36 °C (Figure 2.10B,C). It is possible that a specific function of Bub1 in the 'no-tension' checkpoint is abolished in these mutants - if such a checkpoint pathway exists (see also chapter 1.4.3). However, the only Bub1 mutant for which a similar phenotype has been reported is a Bub1 kinase mutant; it fails to delay anaphase onset in the *psc3-1T* mutant at restrictive temperature but has a functional checkpoint when chromosomes are unattached (Kawashima et al., 2009) (see chapter 1.4.4.2). However, as the kinase function of Bub1 is required for shugoshin localization (see chapter 1.4.4.4) this phenotype is most likely due to loss of Sgo2 from centromeres. In the *bub1-m5* and *bub1-m8* mutants, Sgo2 localization was not perturbed, therefore loss of Sgo2 from centromeres was not the reason for lack of checkpoint activity in the *psc3-1T* and *cut7-446* mutant. As the first screening step was performed at 34 °C, it is possible that I obtained temperature-sensitive *bub1* alleles. To resolve this, expression levels should be determined also at higher temperatures and checkpoint functionality should be analyzed under conditions that disrupt kinetochore microtubule attachment at higher temperatures.

The Bub1 GLEBS motif, and thus presumably also interaction between Bub1 and Bub3, is required for viability of *klp5Δ* cells (Figure 2.14). However, the screen did not reveal any mutants with amino acid changes in this region. In contrast, the N-terminal Mad3/BUB1 homology region 1 was mutated frequently. A *bub1* mutant lacking this region, *bub1-Δ28-160*, showed a slight growth defect in combination with *klp5Δ*, indicating that kinetochore localization of Bub1 plays a role for survival of *klp5Δ* cells (Figure 2.14). Some residual levels of Bub1-Δ28-160 remain at the kinetochore (Vanoosthuyse et al., 2004), which might explain the intermediate phenotype of the *klp5Δ bub1-Δ28-160* double mutant. The *bub1-m5* mutant has three amino acid changes in this region, which do not abolish checkpoint activity but seem to abolish the function of Bub1 that is required for survival of *klp5Δ* cells (assuming that the allele is not merely temperature-sensitive). It would be of interest to determine whether this mutant is able to localize to kinetochores. If kinetochore localization of Bub1 is indeed crucial for survival of *klp5Δ* cells, reduced localization of Bub1 and Bub3 might be the reason why the *klp5Δ mph1Δ* double mutant also shows a slight growth defect (Figure 2.13), as Mph1 is required for the efficient localization of these two proteins (Millband and Hardwick, 2002; Stephanie Heinrich, unpublished data).

3.3 Bub3 is not essential for SAC signaling

Fission yeast Bub3 was long regarded as a SAC protein because its deletion mutants behaved similarly to other SAC mutants. Cells lacking Bub3 were benomyl sensitive, the septation index over time of cells that had been presynchronized with HU and then released into MBC-containing medium resembled more that of *mad2Δ* than of wild type cells and viability was lost similarly to *bub1Δ* cells in the *nda3-KM311* background when incubated at 18 °C (Millband and Hardwick, 2002; Vanoosthuyse et al., 2004). What had not been fully appreciated was that neither drug sensitivity nor loss of viability in the *nda3-KM311* mutant is a reliable sign for loss of checkpoint activity, but could be the result of other defects, for example in kinetochore microtubule attachment. Septation indices of presynchronized cultures are also not an ideal tool for determining SAC activity (see chapter 2.3.2). More careful analysis by others (Tange and Niwa, 2008; Vanoosthuyse et al., 2009) and us (Windecker et al., 2009) revealed that *bub3*-deleted cells arrest in mitosis in the presence of chromosome attachment errors (Figures 2.15, 2.16, 2.18 an 2.25) and even delay in unperturbed mitosis (Figure 2.23). Consistent with these results, Bub3 is not part of the mitotic checkpoint complex (MCC) in fission yeast and not required for interaction of the MCC with the APC/C (Sczaniecka et al., 2008; Vanoosthuyse et al., 2009). Nonetheless, Bub3 remains tightly linked to SAC proteins, as its interaction with Bub1 seems to be conserved in fission yeast (Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2004) and it is required for enrichment of Mad1, Mad2, Mad3 and Bub1 at the kinetochores in mitosis (Millband and Hardwick, 2002; Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2004; Windecker et al., 2009) (Figure 3.1). Another indication that Bub3 is still connected to the SAC in fission yeast is that the protein seems to become essential for SAC activity when Mad2 levels are slightly reduced (Stephanie Heinrich, preliminary unpublished data). Thus, even though Bub3 is not absolutely required for SAC activity, it possibly still has a role

in amplifying the signal. Furthermore, even though the delay in mitosis in the presence of unattached chromosomes is quite prominent in *bub3Δ* cells, it seems to be slightly weaker than in the wild type (Figures 2.15 and 2.21, see also Vanoosthuyse et al., 2009). This could be due to a weakened spindle assembly checkpoint. On the other hand, it is possible that the shortened delay in the absence of Bub3 is the result of spindle formation, which was observed in the *nda3-KM311* background (see chapter 3.1.4), and that occasionally chromosomes get correctly attached to microtubules and thus the spindle assembly checkpoint is silenced. However, the delay was also slightly weaker in the *cut7-446* background, indicating that SAC activity is slightly reduced in the absence of Bub3, whereas in the *psc3-1T* background the delay was even stronger (Vanoosthuyse et al., 2009; Windecker et al., 2009) (Figure 2.16). The cohesin mutant *psc3-1T* alone does not lead to a strong delay in mitosis compared to *psc3*+ cells, which has been proposed to result from reduced Ark1 localization to centromeres (Kawashima et al., 2007). If reduced Ark1 localization was the reason for a short delay in *psc3-1T* cells, an even shorter delay would be expected in *bub3Δ psc3-1T* cells, as Ark1 localization is also slightly perturbed in the absence of Bub3 (Stephanie Heinrich, unpublished data), which is probably the result of loss of kinetochore localization of Bub1 and consequently impaired Sgo2 localization (Figures 1.5 and 3.1) (Windecker et al., 2009). I propose that the absence of Bub3 in the *psc3-1T* background results in more severe chromosome attachment defects than is caused by the cohesin mutant alone. As a consequence, the activity of the SAC is high and causes a prolonged delay. This is consistent with the observations that unperturbed mitosis is delayed when Bub3 is absent (Figure 2.23) (Tange and Niwa, 2008) and that the mitotic delay caused by other weak attachment defects, in a kinetochore mutant and in cells lacking Dam1, was similarly exacerbated by deletion of *bub3* (Vanoosthuyse et al., 2009). Vanoosthuyse et al. (2009) report that *bub3Δ* cells have difficulties to exit mitosis efficiently when the checkpoint has been activated. For their experiment, they used an ATP analog-sensitive Ark1-kinase, which can be inactivated specifically by adding an inhibitor to the medium (Hauf et al., 2007). Cells carrying the *nda3-KM311* mutation were arrested in mitosis, and upon Ark1 inhibition, cells exited mitosis quickly, whereas when Ark1 was inhibited in *bub3*-deleted cells, exit from mitosis was delayed. The same was true for the *bub1-ΔGLEBS* mutant, indicating that Bub1 and probably also its interaction with Bub3 is required for checkpoint silencing (Vanoosthuyse et al., 2009). Vanoosthuyse and Hardwick (2009) observed a similar phenotype when the protein phosphatase 1 Dis2 was mutated and proposed that Bub3 promotes checkpoint silencing by recruiting SAC components for dephosphorylation by Dis2 to the kinetochore (Vanoosthuyse and Hardwick, 2009; Vanoosthuyse et al., 2009).

We showed that Bub3 is not required for exit from mitosis, as *bub3Δ mad2Δ* exited mitosis as efficiently and precociously as *mad2Δ* cells. However, in contrast to the Ark1 inhibition experiment, initial SAC activation was prevented due to the deletion of *mad2* and thus no conclusion can be drawn from this experiment as to whether Bub3 is required for silencing of the checkpoint once the checkpoint has been activated. To confirm that Bub3 is required for silencing of the SAC using a different experimental setup, one could employ an ATP analog-sensitive *mph1* allele instead of the analog-sensitive *ark1* allele, to exclude that the observed effect is Ark1-specific.

If Bub3 indeed is required for switching off the checkpoint, the duration of metaphase, i.e. the time from when chromosomes have achieved bi-orientation to separation of sister chromatids, should be prolonged. In the MBC release experiment (Figure 2.25), I determined the time it took until cells entered anaphase after the last chromosome had been bi-oriented. I did not observe an obvious delay in *bub3Δ*, *bub1Δ-GLEBS* and *bub3Δ sgo2Δ* cells compared to wild type and *sgo2Δ* cells (data not shown). However, the number of cells looked at was too small and the intervals between the single pictures in the time-lapse were too long to unequivocally determine whether *bub3Δ* and *bub1-ΔGLEBS* cells have defects in checkpoint silencing under these conditions.

It remains to be investigated whether Bub3 plays a role in SAC silencing in other organisms. It is possible that it is required for both efficient activation and silencing of the SAC. However, fission yeast Bub3 seems to have largely lost its function in SAC signaling, which evokes the question whether Bub3 is also not essential for the SAC in other organisms. To my knowledge, the only data pointing in that direction comes from *C. elegans*, where Bub3 was found to be dispensable for SAC activity when Mad2 was slightly overexpressed (Essex et al., 2009). However, also Mad3 was dispensable under these conditions. As fission yeast appears to be an exception in comparison with other organisms with respect to Bub3, a difference in the sequence or the structure of the protein seems likely. However, protein sequence comparisons did not reveal an obvious difference in fission yeast Bub3 (Reddy et al., 2008). In contrast, budding yeast Bub3, which is essential for the SAC, differed from Bub3 of other eukaryotic species and was grouped together with prokaryotic proteins (Reddy et al., 2008). If the difference in the SAC signaling pathway cannot be found in Bub3, Bub3-interacting proteins are likely to show differences. Indeed, fission yeast Mad3 seems to be different from Mad3 and BubR1 proteins in other organisms in that it lacks a canonical GLEBS motif (Millband and Hardwick, 2002; Musacchio and Salmon, 2007). Yet, Bub3 was found to co-immunoprecipitate with Mad3 (Millband and Hardwick, 2002), which, however, does not necessarily mean that the interaction is direct (as it is in other organisms). If interaction of Bub3 with Mad3 is not conserved in fission yeast, this might be the explanation why fission yeast Bub3 is not part of the MCC and not essential for SAC activity.

Data from mouse experiments revealed that Rae1, which is structurally similar to Bub3, performs spindle checkpoint functions, suppresses Bub3 haplo-insufficiency and interacts with the Bub1 GLEBS motif (Babu, 2003; Jeganathan et al., 2005; Wang et al., 2001) (see chapter 1.4.4.5). Thus, it was possible that in *bub3Δ* fission yeast cells, Rae1 performs spindle checkpoint functions that are normally carried out by Bub3, but such redundancy could not be confirmed. The fission yeast temperature-sensitive mutant *rae1-167* and the double mutant *rae1-167 bub3Δ* were able to delay exit from mitosis in the presence of MBC (Tange and Niwa, 2008). However, this experiment was performed at (semi-)permissive temperature for the *rae1-167* mutant. Therefore, it is possible that Rae1 was functional under these conditions. In a different study, overexpression of *rae1* did not efficiently suppress TBZ sensitivity of *bub3Δ* cells (Vanoosthuyse et al., 2004). However, as TBZ sensitivity in *bub3Δ* cells cannot be the result of loss of SAC function, no strong conclusion can be drawn from this experiment. Thus, careful additional studies are required to resolve whether fission yeast Rae1 is involved in SAC signaling.

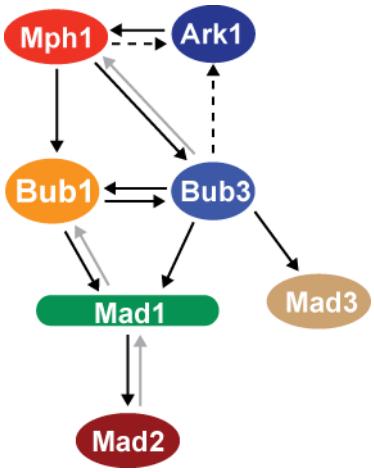

Figure 3.1 Kinetochore localization dependencies
Localization dependencies are represented by black arrows. When no localization dependency could be detected, the arrow is grey, e.g. localization of Mph1 does not depend on Bub3. In contrast, localization of Mad1, Mad2, Mad3 and Bub1 depends on Bub3. Localization of Ark1 is impaired but nor completely abolished in the absence of Bub3 or Mph1 (dashed arrows). Millband and Hardwick, 2002; Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2004; Petersen and Hagan, 2003; Windecker et al., 2009; Stephanie Heinrich and Nicole Hustedt, unpublished data.

3.4 What could be the molecular mechanism underlying the role of Bub1 and Bub3 in chromosome bi-orientation?

In fission yeast, chromosomes are usually clustered close to the SPBs when the cell enters mitosis (Funabiki et al., 1993). When this clustering has been disturbed and the chromosomes have dispersed in the nucleus, they have to be retrieved to one of the spindle poles before they can get bi-oriented on the spindle (Gachet et al., 2008; Grishchuk and McIntosh, 2006). They are captured by astral microtubules and retrieved via end-on pulling, driven by microtubule depolymerization, or via sliding on the lateral surface of microtubules (Gachet et al., 2008). The Dam1/DASH complex is essential for both retrieval mechanisms (Franco et al., 2007; Gachet et al., 2008). Retrieval can occur in the absence of all three minus end-directed motors present in fission yeast, dynein and the kinesin-14 proteins Pkl1 (Klp1) and Klp2 (Grishchuk and McIntosh, 2006). However, Klp2 contributes to the retrieval process (Gachet et al., 2008; Grishchuk and McIntosh, 2006). After reaching the spindle pole, the chromosomes get bi-oriented and move toward the center of the spindle (Gachet et al., 2008). I could show that Bub1 and Bub3 are required in this subsequent step of chromosome bi-orientation, but not for the initial retrieval of chromosomes to the spindle pole body (chapter 2.4; see also Vanoosthuyse et al., 2009). In *bub3Δ* and *bub1-ΔGLEBS* cells, the retrieved chromosomes remained mono-oriented at the spindle pole for a prolonged period and sometimes completely failed to bi-orient, whereas in the wild type chromosomes usually achieved bi-orientation fairly quickly (Figures 2.25 and 2.26). How exactly the chromosomes achieve bi-orientation after they have reached the SPB is unclear.

I observed that when a spindle had elongated while one or more chromosomes remained mono-oriented, the spindle sometimes shrank again, and subsequently chromosomes achieved bi-

orientation (Figure 2.28). As explained in 2.4 it is possible that the main mechanism of bi-orientation in normal unperturbed mitosis in fission yeast is direct capture by microtubules emanating from the opposite spindle pole. When the spindles have become already elongated before all chromosomes have aligned on the spindle, as it is the case after release from MBC treatment in my experiments, direct capture probably becomes less likely and other mechanisms, which involve Bub1 and Bub3, are needed for efficient bi-orientation. Another explanation might be that microtubules that reach over from one SPB to the other spindle half are shorter in *bub3Δ* and *bub1-ΔGLEBS* cells. However, this seems unlikely given that Bub1 and Bub3 localize to the kinetochores of mono-oriented chromosomes and cannot be detected on microtubules. Furthermore, as discussed before (chapter 3.1.4), our results suggest that Bub1 and Bub3 influence microtubule dynamics locally at the kinetochore, rather than everywhere in the spindle.
A similar bi-orientation defect as in *bub3Δ* and *bub1-ΔGLEBS* cells after release from microtubule-destabilizing conditions was observed in a dynein mutant (Gachet et al., 2008; Grishchuk et al., 2007). The chromosomes sometimes also stayed mono-oriented close to one spindle pole for a prolonged period. It is possible that Bub1 and Bub3 act together with dynein in bi-orientation of chromosomes, even though the synthetic interaction of a dynein mutant with *bub1* and *bub3* in *A. nidulans* rather indicates that they act in parallel pathways. However, it is not clear how dynein, a minus end-directed motor, can promote bi-orientation of chromosomes that have reached one spindle pole, as they should be rather transported toward the plus ends of microtubules, toward the middle of the spindle. One might rather expect involvement of a plus end-directed motor, as it has been shown for the mammalian kinesin-7 CENP-E (Kapoor et al., 2006). In mammalian cells, in contrast to budding yeast and fission yeast, centromeres are not associated with the spindle organizing centers in interphase. Therefore, also in normal unperturbed mitosis, chromosomes have to be captured and moved toward one of the spindle poles before they can achieve bi-orientation on the metaphase plate or congress via alternative mechanisms (Walczak et al., 2010). It has been shown that mono-oriented chromosomes can be moved toward the spindle center in mammalian cells. This congression mechanism requires CENP-E, which binds to kinetochores and moves along existing kinetochore microtubules of already aligned chromosomes (Kapoor et al., 2006). There are no CENP-E homologs in fission yeast, and it is unclear whether a similar mechanism exists. A good candidate for such a plus end-directed motor seemed to be Klp5/Klp6. However, I did not observe an obvious bi-orientation defect in *klp5Δ* cells in the MBC release experiment (data not shown). It is possible that a different plus end-directed kinesin performs this function. Apart from *klp5* and *klp6*, seven kinesin genes have been found in fission yeast (Fu et al., 2009). Two of these encode for the minus end-directed kinesins Pkl1 and Klp2, the others encode for putative plus end-directed kinesins, Klp3, Tea2 (Klp4), Cut7, Klp8 and Klp9. As Klp3 is a cytoplasmic protein and only interphase functions have been reported for Klp3 (Brazer et al., 2000; Rhee et al., 2005), it seems unlikely that this kinesis has a role in chromosome bi-orientation. Tea2 is required for maintaining cell polarity (Busch et al., 2004; Browning et al., 2000). Like CENP-E, Tea2 belongs to the kinesin-7 family. However, no mitotic functions for this kinesin have been reported so far. Both Klp9 and Cut7 localize to the spindle midzone. Cut7 is required for formation

of a bipolar spindle (Hagan and Yanagida, 1990), and Klp9 interacts with Ase1 at the midspindle and is required for microtubule bundling (Fu et al., 2009). The remaining kinesin, Klp8, has not been characterized, but it is known that *klp8* expression peaks in G_1 phase (Peng et al., 2005), thus it is likely to have mainly interphasic functions. Whether any of these kinesins is involved in bi-orientation remains to be determined.

Data from human cells show that Bub1, Bub3 and BubR1 are involved in chromosome congression (Johnson et al., 2004; Klebig et al., 2009; Logarinho and Bousbaa, 2008; Logarinho et al., 2008; Meraldi and Sorger, 2005). It is possible that in mammalian cells, they are involved in a similar bi-orientation mechanism as in fission yeast. The function of human Bub1 and Bub3 in chromosome congression appears to be different from the one of BubR1. Cells depleted for Bub1 and Bub3 seem to be defective in formation of stable end-on attachment, as many kinetochores are laterally attached, whereas in BubR1-depleted cells, kinetochores are frequently unattached (Logarinho and Bousbaa, 2008; Logarinho et al., 2008). However, it is impossible to differentiate between lateral and end-on attachment in fission yeast, thus it cannot be judged whether the lack of Bub1 and Bub3 causes similar chromosome attachment defects as in human cells.

As mentioned above, it is possible that motors act downstream of Bub1 and Bub3 in promoting chromosome bi-orientation. Therefore, the phenotype of cells lacking Bub3 or the GLEBS motif of Bub1 after release from microtubule-destabilizing conditions should be compared to mutants of kinesins and dynein. If there is a similar phenotype, as it has been already reported for dynein mutants, it should be tested whether there is a direct interaction and whether the kinesin/dynein is recruited to the kinetochore in a Bub1/Bub3-dependent manner. Furthermore, it remains to be determined whether the kinase activity for Bub1 is required for the bi-orientation mechanism. I analyzed the bi-orientation after release from MBC treatment. Alternative conditions might give more insight into how Bub1 and Bub3 influence bi-orientation. For example, chromosome behaviour could be followed in a *cut7-446* arrest and after release from the arrest. If kinetochore microtubule attachments are less stable in *bub3Δ* and *bub1-ΔGLEBS* cells, chromosomes might detach from the monopolar spindle microtubules more frequently and show abnormal oscillations.

3.4.1 Do Bub1 and Bub3 act independently of Sgo2 in promoting bi-orientation?

Under normal growth conditions, neither *sgo2Δ* nor *bub3Δ* cells show a high rate of chromosome missegregation, whereas missegregation is much increased after release from spindle-destabilizing conditions (Figure 2.24) (Kawashima et al., 2007; Vanoosthuyse et al., 2009; Vanoosthuyse et al., 2007), which could indicate that Sgo2 function is lost in these mutants and therefore results in a similar phenotype. In support of this notion, Vanoosthuyse et al. (2009) did not observe an increase in missegregation after release from spindle-destabilizing conditions when both genes were deleted compared to the single mutants. They conclude that Bub3 and Sgo2 act in the same pathway in bi-orienting chromosomes after release from spindle-destabilizing conditions. However, *sgo2Δ* cells did not have the same bi-orientation defect as *bub3Δ* and *bub1-ΔGLEBS* cells after release from MBC treatment (Figures 2.25 and 2.26). In addition, when *bub3* was deleted in addition to *sgo2*, prolonged mono-orientation was observed as in the *bub3Δ* mutant

alone, further supporting the notion that the defect in bi-orientation in *bub3Δ* and *bub1-ΔGLEBS* cells was not due to loss of Sgo2 function. Thus, in contrast to what has been suggested by Vanoosthuyse et al. (2009), we propose that Bub1 and Bub3 have a role in chromosome bi-orientation after release from spindle-destabilizing conditions that is independent of Sgo2.

Even though loss of shugoshin function is not the cause of the bi-orientation defect observed in *bub3Δ* and *bub1-ΔGLEBS* cells, *sgo2Δ* cells clearly also have a defect in chromosome segregation after release from MBC. Sgo2 has been reported to be involved in spindle assembly checkpoint activity in the absence of tension (Kawashima et al., 2007)(see below for discussion about Sgo2-dependent SAC activity). It is possible that some attachment defects, including the mono-orientation observed in *bub3Δ* and *bub1-ΔGLEBS* mutants under these conditions, fail to cause a mitotic arrest if *sgo2* is deleted. Chromosomes often appeared completely unattached in *sgo2Δ* cells. I excluded these cells from the analysis, as it was not possible to judge the attachment state of the chromosomes (Table 2.1). It was unclear whether these cells were still in prometaphase or had already passed through mitosis. Thus, it is possible that I missed the time period showing the bi-orientation defect. In the *bub3Δ sgo2Δ* double mutant, on the other hand, the mono-orientation could become detectable again because of a delay in exiting mitosis caused by the absence of Bub3 when the checkpoint had been previously activated (Vanoosthuyse et al., 2009)(see chapter 3.3). The checkpoint could have been activated due to the presence of unattached kinetochores, for which Sgo2 is not required (Kawashima et al., 2007; Vanoosthuyse et al., 2007). However, in the rare case that *sgo2Δ* single mutants had mono-oriented chromosomes, they stayed in mitosis for a prolonged period and did delay anaphase onset efficiently (Figure 2.24E), hence loss of SAC response to lack of tension at kinetochores is presumably not the reason why prolonged mono-orientation could not be observed frequently in *sgo2Δ* cells.

3.5 Is Bub3 required for shugoshin function?

Bub1 is crucial for shugoshin function as it is required for localization of shugoshin proteins to centromeres (see chapter 1.4.4.4). Since Bub1 and Bub3 interact and Bub3 is required for localization of Bub1 to kinetochores, it was possible that Bub3 regulates shugoshin function together with Bub1. We therefore analyzed localization of Sgo2 in *bub3Δ* and *bub1-ΔGLEBS* cells. In interphase, Sgo2 associates with outer subtelomeric regions (Kawashima et al., 2009), and this association was only marginally – if at all - reduced in *bub3Δ* and *bub1-ΔGLEBS* cells. In metaphase, Sgo2 localizes to pericentromeric heterochromatin (Kawashima et al., 2009; Kawashima et al., 2007; Kitajima et al., 2004). The mitotic Sgo2 localization was more prominently affected by deletion of Bub3 than was localization in interphase (Figure 2.17). The maximum signal intensity was reduced. In accordance, Vanoosthuyse et al. (2009) observed reduced localization of Sgo2 in the absence of Bub3 in mitotic *nda3-KM311* cells at restrictive temperature, even though Sgo2 protein levels were unaffected by *bub3* deletion. In normal mitosis, the Sgo2 signal is usually restricted to the middle of the spindle, corresponding to centromere localization, but in *bub3Δ* and also in *bub1-ΔGLEBS* cells, additional dot-like signals were visible. These were often dispersed in

the nucleus and not associated with the spindle. Therefore, overall Sgo2 levels are probably not diminished in *bub3Δ* and *bub1-ΔGLEBS* cells in unperturbed mitosis, but as Sgo2 localization is more dispersed, the maximum signal intensity is reduced.

Sgo2 is recruited to pericentromeric heterochromatin by Bub1-dependent histone H2A phosphorylation (Kawashima et al., 2009). In *bub3Δ* and *bub1-ΔGLEBS* cells, kinetochore localization of Bub1 is lost (Vanoosthuyse et al., 2004; Windecker et al., 2009). Kawashima et al. (2009) report that in a Bub1 N-terminal truncation mutant (Bub1-ΔN), which itself also does not localize to kinetochores, H2A phosphorylation is not anymore restricted to the pericentromeric heterochromatin, but occurs all along the chromosomes. As a consequence, Sgo1 localizes to not only to the pericentromeric heterochromatin, but also to other heterochromatic regions in meiosis in *bub1-ΔN* cells. Similarly, in human cells in which Bub1-ΔN was fused to H2B and ectopically localized to chromosome arms, H2A was phosphorylated on the whole chromosome, indicating that localization of Bub1 usually defines centromere-specific phosphorylation of H2A (Kawashima et al., 2009). Thus, it is likely that Sgo2 fails to translocate efficiently from the subtelomeric regions to the pericentromeric heterochromatin in mitosis when localization of Bub1 to kinetochores is abolished, as it is the case in *bub3Δ* and *bub1-ΔGLEBS* cells. Consistently, a human Bub1 mutant lacking the N-terminal Mad3/BUB1 homology region 1, which also does not localize to kinetochores, had impaired Sgo1 localization to centromeres in mitosis, indicating that localization of Bub1 itself to kinetochores is required for efficient shugoshin recruitment (Klebig et al., 2009). However, whether shugoshin function is impaired under these conditions has not been addressed.

I propose that residual shugoshin levels at centromeres in *bub1-ΔGLEBS* and *bub3Δ* fission yeast cells are sufficient to perform shugoshin function. The following observations support this hypothesis. First, *bub3Δ* cells do not have severe chromosome segregation defects in meiosis. Vaur et al. (2005) report that, in contrast to *bub1* deletion, *bub3* deletion neither resulted in an increased rate of equational segregation of sister chromatids in meiosis I, which could be attributed to loss of Sgo2 function, nor in random segregation of sister chromatids in meiosis II, which could be attributed to loss of Sgo1 function, indicating that shugoshin proteins are still functional in meiosis in the absence of Bub3. Second, Sgo2 has been reported to be required for delaying anaphase onset in *psc3-1T* cells at restrictive temperature, a situation where tension is reduced at kinetochores (Kawashima et al., 2007). In contrast, *bub3Δ* cells are able to delay anaphase onset when kinetochores are not under tension, which was examined in *cut7-446* and *psc3-1T* cells (Figure 2.16), indicating that not only the role of Sgo2 in chromosome bi-orientation, but also its role in the 'no-tension checkpoint' is unperturbed by deletion of *bub3*. However, this argument has a weak point. As mentioned in 3.4, it is possible that the lack of Bub3 in these situations causes appearance of unattached chromosomes, which could lead to a SAC-mediated delay independently of loss of tension at the kinetochores, masking potential loss of Sgo2 function. In addition, it is not absolutely clear whether Sgo2 is really essential for the 'no-tension checkpoint'. The fission yeast Aurora B kinase (Ark1), which is part of the chromosomal passenger complex (CPC), is required for activating the SAC both in response to lack of attachment and of tension (Kawashima et al., 2007; Petersen and Hagan, 2003; Vanoosthuyse and Hardwick, 2009; Nicole

Hustedt, unpublished data). Localization of the CPC to centromeres partially depends on Sgo2, yet, even though Ark1 is partially lost from centromeres in the absence of Sgo2, the checkpoint can be active in the absence of attachment in *sgo2Δ* cells (Kawashima et al., 2007; Vanoosthuyse et al., 2007). This indicates that either centromere association of Ark1 is dispensable for checkpoint activity or the residual amounts of Ark1 associated with centromeres in *sgo2Δ* cells are sufficient for checkpoint signaling. CPC localization to centromeres also depends on cohesin (Morishita et al., 2001). Consistently, Kawashima et al. (2007) found that in the cohesin mutant *psc3-1T*, localization of the CPC component Bir1 to centromeres is perturbed and is even more reduced in the *psc3-1T sgo2Δ* double mutant. Localization of Ark1 in turn depends on its binding partner Bir1 (Kawashima et al., 2007; Morishita et al., 2001), therefore also Ark1 levels at centromeres are expected to be very low in the *psc3-1T sgo2Δ* mutant. If localization of Ark1 to centromeres is required for SAC signaling, this could be the reason why *sgo2Δ* cells do not show a delay of anaphase onset in the *psc3-1T* background. Concomitant loss of two Ark1-localizing mechanisms, Sgo2-dependent localization and cohesin-dependent localization, rather than deletion of *sgo2* alone might cause the defect in SAC activity. However, a checkpoint-dependent delay in meiosis I, caused by deletion of *rec12*, which is required for recombination, was abolished by deletion of *sgo2*, indicating that it is required for checkpoint activity in the absence of tension (Kawashima et al., 2007). Yet, it is also possible that the requirements for Sgo2 in checkpoint signaling differ in mitosis and meiosis. In the *cut7-446* background, *sgo2*-deleted cells show a reduction in the mitotic delay, but not as much as *mad2*-deleted cells (Silke Hauf, unpublished data). Thus, it remains an open question whether Sgo2 is essential for activating the SAC in the absence of tension. It is possible that under these conditions, tension-less attachments are still destabilized, albeit infrequently. The resulting unattached kinetochores could lead to Sgo2-independent SAC activation.

To determine whether SAC activity in *sgo2Δ* cells is simply dependent on the level of Ark1 recruitment to kinetochores, *swi6Δ sgo2Δ* cells should be analyzed, as cohesin-mediated Ark1 localization depends on the heterochromatin protein Swi6 (Bernard et al., 2001a; Kawashima et al., 2007; Nonaka et al., 2002). The *swi6Δ sgo2Δ* double mutant is not expected to be able to delay anaphase onset, no matter whether kinetochores are unattached (e.g. in the *nda3-KM311* background) or lack tension (e.g. in the *cut7-446* background).

Additionally, it would be informative to assay checkpoint activity in *bub3Δ sgo2Δ psc3-1T* triple mutants. If deletion of *bub3* results in loss of kinetochore microtubule attachment in the *psc3-1T* background, additional deletion of *bub3* in *sgo2Δ psc3-1T* cells should rescue precocious anaphase onset and show the same delay as *bub3Δ psc3-1T* cells. If, however, *bub3Δ sgo2Δ psc3-1T* cells would, similar to *sgo2Δ psc3-1T* cells, not delay anaphase onset, this could indicate that Sgo2 indeed has a specific role in the ‘no-tension checkpoint’ and deletion of *bub3* does not result in loss of kinetochore microtubule attachment in the *psc3-1T* background.

3.6 SAC signaling at the kinetochore

The kinetochore is the multiprotein complex that assembles on centromeres and is required for connecting the chromosomes to the microtubules of the mitotic spindle apparatus. Errors in kinetochore microtubule attachment are sensed by the spindle assembly checkpoint and ultimately lead to the inhibition of the APC/C. That kinetochores are the source of the SAC signal has been revealed by laser ablation experiments (Rieder et al., 1995). Since SAC proteins get enriched specifically on kinetochores that are not correctly attached to spindle microtubules and disappear when chromosomes achieve bi-orientation, it was assumed that their kinetochore localization is essential for SAC signaling (reviewed in Musacchio and Salmon, 2007). Especially kinetochore localization of Mad1 and Mad2 has been reported to be crucial for SAC signaling, as described in the 'Mad2-template model' (see chapter 1.4.1) (reviewed in Musacchio and Salmon, 2007; Nasmyth, 2005). The importance of kinetochore localization of the SAC proteins was supported by the observation that abolishing their kinetochore localization by mutating or depleting kinetochore components also abrogated SAC activity (Essex et al., 2009; Gardner et al., 2001; Gillett et al., 2004; Kops et al., 2005; Meraldi, 2004). Having this in mind, the observation that fission yeast Bub3 is dispensable for SAC activity is astounding, as Bub3 is required for enrichment of Mad1, Mad2, Mad3 and Bub1 at kinetochores (Figure 3.1)(Millband and Hardwick, 2002; Tange and Niwa, 2008; Vanoosthuyse et al., 2009; Windecker et al., 2009). We cannot exclude that undetectable amounts of SAC components are still present at the kinetochore and are sufficient for SAC activity. However, other studies from recent years also indicate that the role of kinetochore localization in SAC signaling of at least some SAC proteins has been overestimated in the past. For example, a human Bub1 mutant lacking Mad3/BUB1 homology region 1 does not localize to kinetochores, yet the spindle assembly checkpoint is partially active, indicating that Bub1 localization to kinetochores is not an essential prerequisite for SAC activity (Klebig et al., 2009). However, as Mad1, Mad2 and BubR1 still localize to kinetochores in this mutant, the authors conclude that their kinetochore localization is crucial for SAC signaling and the reason why this Bub1 mutant still has partial checkpoint activity. Another indication that SAC signaling is not entirely kinetochore-dependent comes from the observation made in budding yeast, fission yeast and human cells that the mitotic checkpoint complex (MCC) can form in the absence of functional kinetochores or when localization of SAC proteins is abolished (Fraschini et al., 2001; Poddar et al., 2005; Sczaniecka et al., 2008; Sudakin, 2001). Meraldi (2004) reports that depletion of Mad1, which leads to loss of Mad2 from kinetochores, has less severe mitotic defects than depletion of Mad2, indicating that Mad2 functions when it is not localized to kinetochores. In addition, it has been reported that depletion of Ndc80 complex components leads to loss of SAC proteins from the kinetochore but does not disrupt the spindle assembly checkpoint, indicating that the SAC can be functional when SAC proteins are not enriched at the kinetochore (Deluca et al., 2002; Martin-Lluesma et al., 2002). However, careful analysis suggested that in these experiments the checkpoint was functional because the Ndc80 complex proteins were only partially depleted and that localization of SAC proteins to kinetochores was not completely abolished under these conditions (Meraldi, 2004). In contrast, depleting human Hec1 (Ndc80 in yeasts) with higher

efficiency led to abrogation of the SAC, which has been attributed to the loss of Mad1 and Mad2 from kinetochores (Meraldi, 2004). Since depletion of Hec1 does not impair localization of Bub1, Bub3 and BubR1, localization of these SAC proteins is not sufficient for SAC signaling (Liu et al., 2003; Meraldi, 2004; Tighe et al., 2008). However, similar to Mad1 and Mad2, Mps1 is lost from kinetochores when Hec1 is depleted (Martin-Lluesma et al., 2002; Meraldi, 2004; Stucke et al., 2004), thus it is possible that loss of checkpoint functionality is caused by lack of Mps1 at kinetochores, rather than the consequence of loss of Mad1 and Mad2 from kinetochores. Mad1 and Mad2 not only require Hec1 for kinetochore localization, but also Mps1, indicating that Mps1 acts upstream of Mad1 and Mad2 (Jelluma et al., 2008; Liu et al., 2003; Martin-Lluesma et al., 2002; and references in Lan and Cleveland, 2010). This localization dependency has been confirmed in *Xenopus* (Abrieu et al., 2001). In fission yeast, localization of Mad1, Mad3, Bub1 and Bub3 to kinetochores also depends on Mph1 (Millband and Hardwick, 2002; Stephanie Heinrich, unpublished data)(Figure 3.1), however, localization of Bub1 and Bub3 in *mph1Δ* cells seems unperturbed when Mad2 is overexpressed (Vanoosthuyse et al., 2004). Overexpression of *MPS1* in budding yeast and *mph1* in fission yeast leads to a mitotic arrest that depends on Mad2, which is another indication that Mps1/Mph1 acts upstream in the SAC (Hardwick et al., 1996; He et al., 1998).

We proposed that Mph1 is a key player at the kinetochore, so that as long as Mph1 is localized at the kinetochore, a SAC signal can be created even when the other SAC proteins do not localize to kinetchores. This hypothesis is supported by preliminary data indicating that Mph1 still localizes to kinetochores when *bub3* is deleted (Stephanie Heinrich). Thus, according to our model, in spite of loss of Mad1, Mad2, Mad3 and Bub3 from kinetochores in *bub3Δ* cells, the checkpoint is active because localization of Mph1 is unperturbed. Consistently, localization of human Mps1 was also reported to be independent of Mad1, Mad2, BubR1, Bub1 and Bub3 (Meraldi, 2004; Stucke et al., 2004). The N-terminal region of human Mps1 was shown to be crucial for its association with kinetochores (Liu et al., 2003; Maciejowski et al., 2010; Stucke et al., 2004). I constructed N-terminal truncation mutants of fission yeast Mph1 to analyze whether their localization to kinetochores correlates with checkpoint activity. I found that the N-terminal 150 amino acids are dispensable for SAC signaling, whereas amino acids 151 to 302 seem to be essential for protein function (Figure 2.30). It remains to be determined whether the truncated proteins are able to associate with the kinetochore. Further experiments are needed to draw any conclusion about which SAC proteins are required to localize to the kinetochores to ensure a robust checkpoint. It is possible that Ark1 (Aurora B) also plays a crucial role for the initial formation of a SAC signal. Fission yeast Ark1 was shown to be essential for checkpoint activity in the presence of chromosome attachment errors (Petersen and Hagan, 2003; Vanoosthuyse and Hardwick, 2009; Nicole Hustedt, unpublished data). In contrast to Mph1, however, Aurora B localizes to the inner centromere (Ruchaud et al., 2007). It has been proposed that the kinase comes in close proximity to the outer kinetochore when the kinetochores are not under tension, as it is the case in most attachment errors (Andrews et al., 2004; Liu et al., 2009; Tanaka et al., 2002). In the absence of Bub3, Ark1 localization to centromeres is perturbed, but not abolished (Vanoosthuyse et al., 2009;

Stephanie Heinrich, unpublished data)(Figure 3.1). Ark1-GFP signals were more dispersed in the nucleus with additional signals that presumably correspond to telomeres (Vanoosthuyse et al., 2009; Stephanie Heinrich, unpublished data), a localization pattern reminiscent of that of Sgo2 in *bub3Δ* cells (Figure 2.17) and probably also dependent on Sgo2. In accordance with Mph1 acting upstream of Bub3, Ark1 localization in *mph1Δ* cells resembles Ark1 localization in *bub3Δ* cells (Stephanie Heinrich, unpublished data)(Figure 3.1). However, there is data indicating that Mph1 localization depends on Ark1 (Petersen and Hagan, 2003; Nicole Hustedt and Stephanie Heinrich, unpublished data), indicating that there is not a linear localization-dependency between the two kinases, and thus it remains to be resolved whether either of them or they both together are the key players at the kinetochore/centromere in SAC signaling.

3.7 Conclusion

The synthetic lethality with kinesin-8 proteins revealed that Bub1 and Bub3 share a function which has not been described so far. It has been reported for mammalian cells that Bub1 and Bub3 are required for chromosome congression (Johnson et al., 2004; Klebig et al., 2009; Logarinho and Bousbaa, 2008; Logarinho et al., 2008; Meraldi and Sorger, 2005). I could show that fission yeast Bub1 and Bub3 are required for chromosome bi-orientation after release from microtubule-destabilizing conditions. Whether the same mechanisms are involved in mammals and in fission yeast and Bub1 and Bub3 have a conserved function in congression, remains an open question. In fission yeast unperturbed mitosis, there is no obvious chromosome alignment defect in *bub1* and *bub3* mutants, and we propose that chromosomes normally bi-orient efficiently by direct capture of kinetochores by microtubules that emanate from opposite poles. Therefore, Bub1 and Bub3 become essential for efficient bi-orientation only if the spindle has been destabilized and chromosomes have unclustered. It was not possible to unequivocally resolve whether the observed role of Bub1 and Bub3 in bi-orientation is the function that is required for viability of kinesin-8 mutants at higher temperature. However, I hypothesize that kinetochore microtubule attachments are impaired in *bub1* and *bub3* mutants and that this leads to both a bi-orientation defect after release from spindle-destabilizing conditions and to synthetic sickness with kinesin-8 mutants.

Surprisingly, fission yeast Bub3, which is a conserved SAC protein in all other organisms that have been examined, was found to be not essential for delaying anaphase onset in the presence of chromosome attachment errors (Tange and Niwa, 2008; Vanoosthuyse et al., 2009; Windecker et al., 2009; this thesis). In this respect it was even more astonishing that the SAC proteins Mad1, Mad2, Mad3 and Bub1 fail to enrich at kinetochores in the absence of Bub3, indicating that in contrast to what was believed before, enrichment of Mad1 and Mad2 at the kinetochore is not a prerequisite for SAC signaling. We propose that Mph1 localization is required for SAC signaling. This hypothesis is supported by preliminary results indicating that Mph1 remains localized to kinetochores in *bub3Δ* cells (Stephanie Heinrich, unpublished data). The fission yeast Aurora B kinase, Ark1, might also play an important role at the centromere/kinetochore in SAC signaling. Its localization is impaired, but not abolished in *bub3*-deleted cells. Further investigations are needed

to resolve these questions. I propose that kinetochore localization of Bub1 and Bub3 is important for establishing correct and stable kinetochore microtubule attachments, rather than for checkpoint activity.

It has been suggested that Bub3-dependent localization of checkpoint proteins is required for switching off the checkpoint (Vanoosthuyse et al., 2009). However, it is possible that both activation and inactivation of the checkpoint become more efficient if all SAC proteins are enriched at the kinetochore.

4 Materials and Methods

4.1 Molecular Biology techniques

4.1.1 Preparation of genomic DNA of *S. pombe*

A toothpick full of cells was suspended in 300 µL NTES (0.5 M NaCl, 0.2 M Tris HCl pH 7.5, 0.01 M EDTA, 1% SDS). About 500 µL of acid-washed glass beads (Sigma, G-8772, 500 µm) and 300 µL phenol/chloroform (1:1) were added. The mixture was vortexed vigorously for 7 min at 4 °C (Multi-Pulse Vortexer, Schütt). After subsequent centrifugation at 14,000 rpm for 10 min, the supernatant was transferred into a fresh tube, 800 µL cold ethanol was added and the sample was incubated at -20 °C for at least 30 min. The DNA was pelleted by centrifugation for 10 min at 4 °C and 14,000 rpm. The pellet was washed with 70% ethanol and after drying was resolved in 30 µL H_2O. The DNA solution was stored at 4 °C. About 1 µL of genomic DNA was used in 50 µl PCR reactions.

4.1.2 PCRs

PCRs were performed according to the manufacturers' protocols using the polymerases Herculase II (Strategene), AccuPrime Taq HiFi (Invitrogen), Taq (Eppendorf), Taq (Fisher), AccuPrime Pfx (Invitrogen) and Vent (NEB).

4.1.3 Ethanol precipitation

To a PCR reaction, 1/10 volume of 3 M Na-acetate (adjusted to pH 5.2 with glacial acetic acid) and 2 – 3 volumes of cold ethanol were added. After incubation at -20 °C for at least 30 min, the sample was centrifuged at 14,000 rpm for 10 min at 4 °C. The pellet was washed with cold 70% ethanol. The pellet was dried and resuspended in TE (10 mM Tris HCl pH 8.0 and 1 mM EDTA, adjusted to pH 8.0 with NaOH). The DNA solution was used to transform fission yeast for genomic integration.

4.1.4 Colony PCR of *S. pombe*

A small amount of yeast cells was suspended in 50 µL of a solution containing 200 µg/ml Zymolyase T100 (Medac) and 50 mM DTT. The mixture was incubated for 10 min at room temperature. After centrifugation for 3 min at 3,000 rpm, the supernatant was removed and the pellets were incubated at 95 °C for 5 min. After the pellets had cooled down on ice, a PCR reaction mix containing a Taq polymerase (Eppendorf or Fisher) was added. The PCR tubes were flicked and centrifuged briefly before the PCR reaction was started.

4.1.5 DNA oligonucleotides

The oligonucleotides used for plasmid and strain construction in this thesis are listed in Table 4.1.

Table 4.1 DNA oligonucleotides

Number	Name	Sequence	Purpose
XS056	*bub1 mut D76G and XbaI site, fw*	ggtgatgctattcagtatctagaaagatgtcgcttcg	reconstruction of *bub1-m5*
XS057	*bub1 mut D76G and XbaI site, rev*	ctagatactgaatagcatcaccaagcattttgttgacc g	reconstruction of *bub1-m5*
XS058	*bub1 mut K101E and PvuII site, fw*	cttctcatacagctggcggaaattaaacaatcatacg	reconstruction of *bub1-m5*
XS059	*bub1 mut K101E and PvuII site, rev*	cgtatgattgtttaatttccgccagctgtatgagaag	reconstruction of *bub1-m5*
XS060	*bub1 mut Q37R and NruI site, fw*	gattagcattgtttcgcgaggaactcgacattatcg	reconstruction of *bub1-m5*
XS061	*bub1 mut Q37R and NruI site, rev*	cgataatgtcgagttcctcgcgaaacaatgctaatc	reconstruction of *bub1-m5*
XS394	*mad1 fw ~500bp upstream of ATG*	tcctaaaagaaactgaaacc	construction of *mad1-Δ1-468*
XS674	*7 bp 5' of mad1 start + aa469..., fw*	tctcactatgggttacgtaacg	construction of *mad1-Δ1-468*
XS675	*mad1 aa469.. + 19 bp 5'of start, rev*	ttacgtaacccatagtgagaaa	construction of *mad1-Δ1-468*
XS678	*8 bp 5' of mph1 start + aa 303..., fw*	ctgtgtttatgaagcgtcagcaggacgttg	construction of *mph1-Δ1-302*
XS679	*mph1 aa303... + 20 bp 5' of start, rev*	ctgacgcttcataaacacagttactaaaaaac	construction of *mph1-Δ1-302*
XS680	*8 bp 5' of mph1 start + aa 151..., fw*	ctgtgtttatgggttctttatcaatttcaag	construction of *mph1-Δ1-150*
XS681	*mph1 aa151... + 20 bp 5' of start, rev*	taaagaacccataaacacagttactaaaaaac	construction of *mph1-Δ1-150*
YS034	*PstI site, klp5 5'UTR fw*	gcgctgcagttgcatattcga	cloning *klp5+* with endogenous promoter into *pREP4*
YS035	*SacI site, klp5 3'UTR rev*	gcgagctcttatgtacattgga	cloning *klp5+* with endogenous promoter into *pREP4*
YS301	*650 bp 5' of start mph1+, fw*	gtcgttattgcatagatttaag	construction of *mph1-Δ1-150* and *mph1-Δ1-302*
YS378	*pFA6a hom. + 20 bp 5' of bub1start, rev*	ttaattaacccggggatccgagtgatacgcacattga aac	*bub1* deletion
YS380	*529 bp 5' of bub1 start, fw*	tgtttggcaggagaatttgc	*bub1* deletion, construction of *bub1-ΔGLEBS*
YS381	*550 bp 3' of bub1 stop, rev*	gaagggtctgctatcactg	*bub1* deletion, *bub1* mutagenesis

YS385	*pFA6a hom. + 504 bp 5′ of bub1 start, rev*	ttaattaacccggggatccgatgcagcaaattctcctg cc	inserting *hygroR* 504 bp upstream (in *ade6*) of *bub1+* (for random mutagenesis PCR and re-insertion)
YS386	*pFA6a hom. + 504 bp 5′ of bub1 start, fw*	gtgtcgaaaacgagctcgaattcccaagatgatgcat ttgatg	inserting *hygroR* 504 bp upstream (in *ade6*) of *bub1+* (for random mutagenesis PCR and re-insertion)
YS387	*1028 bp 5′ of bub1 start, fw*	ggtctcagttgtaggataag	inserting *hygroR* 504 bp upstream (in *ade6*) of *bub1+* (for random mutagenesis PCR and re-insertion) *bub1* mutagenesis
YS388	*18 bp 3′ of start bub1, rev*	aagccgccaatcggacatag	inserting *hygroR* 504 bp upstream (in *ade6*) of *bub1+* (for random mutagenesis PCR and re-insertion)
YS391	*717 bp 5’ of bub1 start, fw*	atttgtcttgaatgcatcgc	reconstruction of *bub1-m5*
YS393	*26 bp 3′ of stop bub1, rev*	atggaaacttccctctaggc	construction of *bub1-ΔGLEBS*
YS421	*pFA6a hom. + 723 bp 5′ of bub1 stop, fw*	gtttaaacgagctcgaattccatttattgatggactatcg	*bub1* deletion
YS426	*BamHI, 517 bp 5’ of sgo2, fw*	tcgggatccaacagaggaacagatagacg	cloning *sgo2+* with endogenous promoter into *pFA6a-kanMX6-GFP,* C-terminal tagging of *sgo2+* with GFP
YS427	*SmaI, 3′ end of sgo2 without stop, rev*	gtatcgcccgggcaaattaagggtttcggag	cloning *sgo2+* with endogenous promoter into *pFA6a-kanMX6-GFP*
YS428	*523 bp 3′ of sgo2, rev*	cacgagacatattatcgccg	C-terminal tagging of *sgo2+* with GFP (amplification of 3' UTR of *sgo2*)
YS531	*GFP hom. + 20 bp 3′ of sgo2, fw*	gcatggatgaactatacaaatagtattatgtaattaaat acctttcgac	C-terminal tagging of *sgo2+* with GFP
YS659	*YS659 bub1 GLEBS del, rev*	cagtgtttggagagctcttatcatagagcagg	construction of *bub1-ΔGLEBS*
YS660	*YS660 bub1 GLEBS del, fw*	taagagctctccaaacactgctgcttctttcc	construction of *bub1-ΔGLEBS*
YS696	*1076 bp 3′ of bub1 start, rev*	tcaggagatgtagactttgc	reconstruction of *bub1-m5*
YS779	*mad1 rev downsttream region for C-terminal tagging*	cgccattcgtgatttggc	construction of *mad1-Δ1-468*
YS818	*mph1 rev downstream region for C-terminal tagging*	cgatattccattgaacctg	construction of *mph1-Δ1-150* and *mph1-Δ1-302*

4.2 Plasmids

Plasmids used in the thesis are listed in Table 4.2. I constructed plasmids v106, v107 and v108 as described in the last column of the table.

Table 4.2 Plasmids

Number	Name	Insert	Selection in *E.coli*	Selection in *S. pombe*	Construction/origin/comments
v012	*pC225*	*hygR*	*amp*		Bähler et al., 1998b
v101	*pREP81-mCherry-atb2+(LEU2)*	*mCherry-atb2+*	*amp*	*LEU2*	Hauf et al., 2007 *nmt81* promoter
v106	*pREP4-klp5+*	*5'UTR-klp5+-3'UTR*	*amp*	*ura4+*	insert: PCR YS034/YS035 from JY333, cut *PstI/SacI*; vector: vc111 cut *PstI/SacI*
v107	*pREP1-klp5+*	*5'UTR-klp5+-3'UTR*	*amp*	*LEU2*	insert: cut *PstI/SacI* from v106; vector: vc075 cut *PstI/SacI*
v108	*pFA6a-kanMX-5'UTR-sgo2+-GFP*	*5'UTR-sgo2+*	*amp*		insert: PCR YS426/YS427 from JY333, cut *BamHI/SmaI*; vector: vc185 cut *BamHI/SmaI*
vc075	*pREP1*		*amp*	*LEU2*	Maundrell, 1993 *nmt1* promoter
vc111	*pREP4*		*amp*	*ura4+*	Maundrell, 1993 *nmt1* promoter
vc185	*pFA6a-kanMX6-GFP*	*pFA6a*	*amp*		Bähler et al., 1998b
vc345	*pFA6a-NatI*	*natR*	*amp*		Bähler et al., 1998

4.3 Fission yeast culture conditions

Yeast cells were cultured on YEA, SD and 5-FOA plates or in YEA, EMM or MM liquid medium at 30 °C, if not stated otherwise. Plates were incubated in temperature-controlled incubators. Liquid cultures were incubated in shakers. If plates were used to assay growth of different strains, Phloxin-B was added to the medium (2 µg/ml). Strains were streaked on YE plates to determine which *ade6* allele they carried, which can be judged by accumulation of a red pigment in the cells. For live cell imaging, cells were grown in minimal medium, EMM (Moreno et al., 1991) or MM+N, containing the necessary supplements. The density of cultures was determined by counting cells under the microscope using a Thoma counting chamber.

4.4 Fission yeast media

All media and solutions were prepared using distilled H_2O, unless stated otherwise.

4.4.1 Liquid media

YEA liquid: 0.5% w/v yeast extract, 2% w/v glucose, 370 µM adenine

MM-N: 14.7 mM potassium hydrogen phthalate, 15.5 mM Na_2HPO_4, 1% w/v glucose, 1x salts from stock, 1x vitamins from stock, 1x minerals from stock (including citric acid)

MM+N+L+A: MM-N supplemented with 0.5% w/v NH_4Cl (N), 370 µM adenine (A), 1.52 mM leucine (L)

MM-N+L+A: MM-N supplemented with 370 µM adenine (A) and 1.52 mM leucine (L)

EMM (Edinburgh minimal medium): 0.3% w/v potassium hydrogen phthalate, 0.22% w/v NaH_2PO_4, 3% w/v glucose, 0.5% w/v NH_4Cl, 1x salts from stock, 1x vitamins from stock, 1x minerals from stock (including citric acid)

EMM + supplements: EMM supplemented with 370 µM adenine (A) and/or 1.52 mM leucine (L) and/or 446 µM uracil (U)

YEL: 0.5% w/v yeast extract, 3% w/v glucose, 380 µM leucine, 122 µM adenine, 446 µM uracil

4.4.2 Solid media

YE: 0.5% w/v yeast extract, 3% w/v glucose, 2% w/v agar granulate

YEA: 0.5% w/v yeast extract, 3% w/v glucose, 370 µM adenine, 2% w/v agar granulate

YEA/hygro, YEA/kan, YEA/nat: YEA plates containing 300 µg/ml Hygromycin B, 100 µg/ml Geneticin/G418 (kan) or 100 µg/ml ClonNAT (nourseothricin)

SD: 1% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids, 2% w/v agar granulate

SD/A, SD/L, SD/A/U, SD/L/A, SD/L/A/U: SD plates supplemented with: 370 µM adenine (A), 1.52 mM leucine (L), 446 µM uracil (U), respectively

SSA/L/A/U: 1% w/v glucose, 370 µM adenine, 446 µM uracil, 1.52 mM leucine, 1x SSA from stock, 4.2 mM NaOH, 2% w/v agar granulate

5-FOA: 2% w/v glucose, 2% w/v agar granulate, 0.7% yeast nitrogen base without amino acids, 446 µM uracil, 555 µM adenine, 1.7 mM leucine, 0.1% w/v 5-FOA (Fermentas)

4.4.3 Stocks and supplements

20x SSA stock: 300 mM $(NH_4)_2SO_4$, 300 mM KH_2PO_4, 28 mM Na_2HPO_4, 40 mM $MgSO_4$ x $7H_2O$, 75 mM aspartic acid, 13.6 mM $CaCl_2$, 20x vitamins from stock, 20x minerals from stock (including citric acid)

10,000x mineral stock (including citric acid): 8.1 µM H_3BO_3, 2.37 µM $MnSO_4$, 1.39 µM $ZnSO_4$ x $7H_2O$, 0.74 µM $FeCl_3$ x $6H_2O$, 0.25 µM MoO_4 x $2H_2O$, 0.6 µM KI, 0.16 µM $CuSO_4$ x $5H_2O$, 4.76 µM citric acid

1,000x vitamin stock: 81.2 µM nicotinic acid, 55.5 µM (meso-)inositol, 40.8 µM D-biotin, 4.2 µM pantothenic acid

200x histidine: 5 mg/mL L-histidine

50x salt stock: 5.2 mM $MgCl_2$ x $6H_2O$, 0.1 mM $CaCl_2$ x $2H_2O$, 13.4 mM KCl, 0.28 mM Na_2SO_4,

Phloxin-B: 10 mg/ml Magdala red in ethanol

Benomyl: 6.4 mg/mL stock solution in DMSO

TBZ (thiabendazole): 50 mg/ml stock solution in DMSO

MBC (carbendazim): 1 mg/ml stock solution in DMSO

4.5 Fission yeast strains

S. pombe strains with the following mutations or modifications have been described previously: *mtc1Δ::ura4+* (Nakaseko et al., 2001), *cut4-533* (Hirano et al., 1986), *nda2-KM52* (Toda et al., 1983), *bub1-Δkinase* (Yamaguchi et al., 2003), *bub1-K762R* (Yamaguchi et al., 2003), *bub1-Δ28-160* (Vanoosthuyse et al., 2004), *sgo2Δ::kanR* (Kitajima et al., 2004), *sgo2Δ::ura4+* (Kitajima et al., 2004), *mph1Δ::ura4+* (He et al., 1998), *mad1Δ::ura4+* (Vanoosthuyse et al., 2004), *mad2Δ::ura4* (He et al., 1997), *mad3Δ::ura4+* (Vanoosthuyse et al., 2004), *bub1Δ::ura4+* (Bernard et al., 1998), *bub3Δ::ura4* (Millband and Hardwick, 2002), *nda3-KM311* (Hiraoka et al., 1984; Umesono et al., 1983), *psc3-1T<<kanR* (Nonaka et al., 2002), *cut7-446* (Hagan and Yanagida, 1990), *cen2<<lacO<<ura4+<<kanR his7+<<GFP-LacI-NLS* (Yamamoto and Hiraoka, 2003), *kanR<<Pnmt41-mCherry-atb2+* (Hauf et al., 2007), *pREP81-mCherry-atb2+(LEU2)* (Hauf et al., 2007), *leu1-32::SV40-GFP-atb2[LEU1]* (Bratman and Chang, 2007), *bub1+-GFP<<kanR* (Yamaguchi et al., 2003), *plo1+-GFP<<kanR* (Kawashima et al., 2007), *mis6+-mCherry<<kanR* (Windecker et al., 2009), *sid4+-mCherry<<natR* (Windecker et al., 2009) and *ase1+-GFP<<kanR* (Loïodice et al., 2005). The strains used for each Figure in this thesis are shown in Table 4.3.

Table 4.3 Strain list

Figure 2.1A		
JY333	*h-*	*ade6-M216 leu1*
PX854	*h-*	*leu1 klp5::kanR*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
SH735	*h-*	*leu1 ura4-D18 ade6? sgo2::ura4+*
PZ285	*h-*	*mad2::ura4 leu1 ade6*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
PX924	*h+*	*leu1 ade6(-?) ura4-D18 klp5::kanR sgo2::ura4+*
SH740	*h+*	*leu1 ura4-D18 ade6? klp5::kanR mad2::ura4+*
PW343	*h+*	*leu1 ade6 ura4-D18 klp5::kanR sgo2::ura4+ mad2::ura4+*
Figure 2.1B		
JY333	*h-*	*ade6-M216 leu1*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
PX854	*h-*	*leu1 klp5::kanR*
PX840	*h+*	*leu1 ade6-M216 klp6::kanR*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
SH746	*h-*	*leu1 ura4? ade6-M216 bub1::ura4+ klp6::kanR*
SH735	*h-*	*leu1 ura4-D18 ade6? sgo2::ura4+*

PZ285	*h-*	*mad2::ura4 leu1 ade6*
SH744	*h-*	*leu1 ura4-D18 ade6-M216? mad2::ura4+ bub1::natR klp6::kanR*
SH743	*h-*	*leu1 ura4-D18 ade6-M216? sgo2::ura4+ bub1::natR klp6::kanR*
SH741	*h-*	*leu1 ura4-D18 ade6-M216? sgo2::ura4+ mad2::ura4+ bub1::natR klp6::kanR*
SH734	*h-*	*leu1 ura4-D18 ade6-M216? klp5::kanR sgo2::ura4+ mad2::ura4+ bub1::natR*

Figure 2.2

JY333	*h-*	*ade6-M216 leu1*
PN4353	*h-*	*leu1 bub1-K762R*
PN4298	*h-*	*bub1::bub1Dkinase[kanR] leu1-32*
PX854	*h-*	*leu1 klp5::kanR*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
PX942	*h+*	*leu1 klp5::kanR bub1-K762R*
PX941	*h-*	*leu1 klp5::kanR bub1::bub1Dkinase-kanR*

Figure 2.3

PX989	*h+*	*leu1 klp5::kanR cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS*
PZ809	*h-*	*leu1 ade6 cen2+<<lacO<<kanr<<ura4+ his7+<<GFP-lacI-NLS*
SH765	*h-*	*leu1 ura4? ade6-M216? cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS bub1::natR*
SH764	*h+*	*leu1 ura4? ade6-M216? klp5::kanR cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS bub1::natR*

Figure 2.4B

JY333	*h-*	*ade6-M216 leu1*
SH720	*h+*	*leu1 ura4-D18 ade6-M216 klp5::kanR pREP4-klp5*
SH724	*h-*	*leu1 ura4-D18 ade6-M210 klp5::kanR pREP4*
SH726	*h-*	*leu1 ura4-D18 ade6-M210 pREP4*
SH732	*h-*	*leu1 ura4-D18 ade6-M210/M216 bub1::natR klp5::kanR pREP4-klp5*

Figure 2.4C

JY333	*h-*	*ade6-M216 leu1*
SH732	*h-*	*leu1 ura4-D18 ade6-M210/M216 bub1::natR klp5::kanR pREP4-klp5*
SH721	*h-*	*leu1 ura4-D18 ade6-M210 klp5::kanR pREP4-klp5*
PX854	*h-*	*leu1 klp5::kanR*
a3	mutagenized SH721	
a7	mutagenized SH721	

Figure 2.4D

JY333	*h-*	*ade6-M216 leu1*
a7	mutagenized SH721	
b6	mutagenized SH721	

Figure 2.6A

PN1521	*h-*	*cut4-533 leu1-32*
SH833	*h+*	*leu1 ura4-D18 ade6-M210 hygR<<klp5-T224D*

Figure 2.6B

PV277	*h+*	*leu1 ura4-D18 mtc1::ura4+*
SI506	*h-*	*leu1 ade6-M216 ura4-D18 hygR<<klp5-T224D*

Figure 2.6C

FY9750	*h-*	*leu1 nda2-KM52*
SI152	*h+*	*leu1 hygR<<klp5-T224D*

Figure 2.7

SH821	*h+*	*leu1 ade6-M216 dam1-GFP<<natR mis6-mcherry<<kanR*
SH790	*h+*	*leu1 ade6-M216 ura4DS/E? bub1::ura4+ dam1-GFP<<natR mis6-mcherry<<kanR*

Figure 2.8

SH732	*h-*	*leu1 ura4-D18 ade6-M210/M216 bub1::natR klp5::kanR pREP4-klp5*
SH721	*h-*	*leu1 ura4-D18 ade6-M210 klp5::kanR pREP4-klp5*
SH759	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1+ sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*2.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m2 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*3.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m3 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*4.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m4 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*5.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m5 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*6.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m6 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*7.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m7 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*8.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m8 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*9.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m9 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*
bub1*10.1	*h+*	*leu1 ura4-D18 ade6?<<hph<<bub1-m10 sgo2::sgo2-GFP klp5::kanR pREP4-klp5*

Figure 2.10A

SH211	*h+*	*leu1 ade6-M216 nda3-KM311 plo1+-GFP<<kanR*
SH511	*h-*	*leu1 ade6-M216 nda3-KM311 plo1+-GFP<<kanR*
SI437	*h+*	*leu1 ade6-M216 ura4DS/E? bub1::ura4+ nda3-KM311 plo1-GFP<<kanR*
SI437'	*h+*	*leu1 ade6-M216 ura4DS/E? bub1::ura4+ nda3-KM311 plo1-GFP<<kanR*
SI431	*h+*	*leu1 ade6?<<hph<<bub1-m9 nda3-KM311 plo1-GFP<<kanR*
SI430	*h-*	*leu1 ade6?<<hph<<bub1-m9 nda3-KM311 plo1-GFP<<kanR*
SI441	*h-*	*leu1 ade6?<<hph<<bub1-m3 nda3-KM311 plo1-GFP<<kanR*
SI440	*h+*	*leu1 ade6?<<hph<<bub1-m3 nda3-KM311 plo1-GFP<<kanR*
SI448	*h+*	*leu1 ade6?<<hph<<bub1-m8 nda3-KM311 plo1-GFP<<kanR*
SI448'	*h+*	*leu1 ade6?<<hph<<bub1-m8 nda3-KM311 plo1-GFP<<kanR*
SI442	*h+*	*leu1 ade6?<<hph<<bub1-m5 nda3-KM311 plo1-GFP<<kanR*
SI443	*h-*	*leu1 ade6?<<hph<<bub1-m5nda3-KM311 plo1-GFP<<kanR*

Figure 2.10B

SI178	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI179	*h-*	*leu1 ade6-M216 cut7-446 plo1+-GFP<<kanR*
SI192	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI194	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI197	*h+*	*leu1 ade6-M210/M216 cut7-446 bub1Δ::natR plo1+-GFP<<kanR*
SI198	*h-*	*leu1 ade6-M210/M216 cut7-446 bub1Δ::natR plo1+-GFP<<kanR*
SH235	*h+*	*leu1 ade?<<hph<<bub1-m8 cut7-446 plo1-GFP<<kanR*
SH235'	*h-*	*leu1 ade?<<hph<<bub1-m8 cut7-446 plo1-GFP<<kanR*
SI199	*h+*	*leu1 cut7-446 ade6?<<hph<<bub1-m5 plo1-GFP<<kanR*
SI200	*h-*	*leu1 cut7-446 ade6?<<hph<<bub1-m5 plo1-GFP<<kanR*

Figure 2.10C

SH604	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI187	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI188	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI182	*h+*	*leu1 ade6-M216(?) psc3-1T<<kanR plo1+-GFP<<kanR bub1Δ::natR*

SI183	*h-*	*leu1 ade6-M216(?) psc3-1T<<kanR plo1+-GFP<<kanR bub1Δ::natR*
SH231	*h+*	*leu1 ade?<<hph<<bub1-m5 psc3-1T<<kanR plo1-GFP<<kanR*
SH231'	*h+*	*leu1 ade?<<hph<<bub1-m5 psc3-1T<<kanR plo1-GFP<<kanR*
SH233	*h-*	*leu1 ade?<<hph<<bub1-m8 psc3-1T<<kanR plo1-GFP<<kanR*
SH233'	*h+*	*leu1 ade?<<hph<<bub1-m8 psc3-1T<<kanR plo1-GFP<<kanR*

Figure 2.12

SI475	*h+*	*leu1 ade6-M210/216<<hph<<bub1-Q37R/D76G*
SI480	*h-*	*leu1 ade6-M216<<hph<<bub1-D76G*
SI484	*h+*	*leu1 ade6-M210/216<<hph<<bub1-Q37R/K101E*
SI500	*h+*	*leu1 ade6-M210/216<<hph<<bub1-Q37R/D76G/K101E*
PX854	*h-*	*leu1 klp5::kanR*
PX859	*h+*	*leu1 ade6-M216 klp5::kanR*

Figure 2.13

JY333	*h-*	*ade6-M216 leu1*
PX854	*h-*	*leu1 klp5::kanR*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
KP106	*h-*	*bub3::ura4+ leu1-32 ade6-M210 ura4-D18*
AE247	*h-*	*mad1::ura4+ leu1*
PZ285	*h-*	*mad2::ura4 leu1 ade6*
DM001	*h-*	*mad3::ura4+ ade6-M210*
SS560	*h-*	*mph1::ura4+ ade6-M216 leu1-32 ura4-D18*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
SH760	*h+*	*leu1 ade6-M216 ura4-D18 klp5::kanR bub3::ura4+*
SH768	*h+*	*leu1 mad1::ura4+ ura4-D18? klp5::kanR*
SH740	*h+*	*leu1 ura4-D18 ade6? klp5::kanR mad2::ura4+*
SH762	*h+*	*leu1 ade6-M210 ura4-D18? klp5::kanR mad3::ura4+*
SH783	*h+*	*klp5::kanR mph1::ura4+ ade6-M216 leu1 ura4-D18*

Figure 2.14

JY745	*h-*	*leu1 ura4-D18 ade6-M210*
JPJ1821	*h+*	*leu1 ara4 lys1 his7 arg3-D4? bub1D28-160 ade6-M216*
SH789	*h-*	*leu1-32 ade6-M216 ura4DS/E his1-102 bub1ΔGLEBS*
SH729	*h-*	*leu1 ura4-D18 ade6-M216? bub1::natR*
PW320	*h-*	*leu1 ura4-D18 ade6-M210 klpr::kanR*
SH752	*h-*	*leu1 ura4 ade6-M216 bub1D28-160 klp5::kanR*
SH793	*h+*	*leu1 ade6-M216 ura4D bub1ΔGLEBS klp5::kanR*
SH778	*h+*	*leu1 ura4-D18 ade6-M216? bub1::natR klp5::kanR*

Figure 2.15

SH211	*h+*	*leu1 ade6-M216 nda3-KM311 plo1+-GFP<<kanR*
SH511	*h-*	*leu1 ade6-M216 nda3-KM311 plo1+-GFP<<kanR*
SI463	*h-*	*leu1 nda3-KM311 mad1Δ::ura4+ plo1+-GFP<<kanR*
SH515	*h+*	*leu1 ade6-M216 (ura4-D18) nda3-KM311 mad2Δ::ura4+ plo1+-GFP<<kanR*
SI437	*h+*	*leu1 ade6-M216 ura4DS/E? bub1Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI438	*h-*	*leu1 ade6-M216 ura4-D18? bub3Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI439	*h+*	*leu1 ade6-M210 ura4-D18? bub3Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI456	*h+*	*leu1 ura4-D18? ade6-M216 bub3Δ::ura4+ mad2Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI457	*h+*	*leu1 ura4-D18? ade6-M210 bub3Δ::ura4+ mad2Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*

Figure 2.16A

SI178	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI179	*h-*	*leu1 ade6-M216 cut7-446 plo1+-GFP<<kanR*
SI192	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI194	*h-*	*leu1 cut7-446 plo1+-GFP<<kanR*
SI191	*h-*	*leu1 ade6-M216 cut7-446 mad2Δ::ura4+ plo1+-GFP<<kanR*
SI189	*h-*	*leu1 cut7-446 mad2Δ::ura4+ plo1+-GFP<<kanR*
SI197	*h+*	*leu1 ade6-M210/M216 cut7-446 bub1Δ::natR plo1+-GFP<<kanR*
SI198	*h-*	*leu1 ade6-M210/M216 cut7-446 bub1Δ::natR plo1+-GFP<<kanR*
SI193	*h-*	*leu1 cut7-446 bub3Δ::ura4+ plo1+-GFP<<kanR*
SI196	*h-*	*leu1 ade6-M210 cut7-446 bub3Δ::ura4+ plo1+-GFP<<kanR*

Figure 2.16B

SH604	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI187	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI188	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR*
SI184	*h+*	*leu1 ade6-M216(?) psc3-1T<<kanR plo1+-GFP<<kanR mad2Δ::ura4+*
SI185	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR mad2Δ::ura4+*
SI182	*h+*	*leu1 ade6-M216(?) psc3-1T<<kanR plo1+-GFP<<kanR bub1Δ::natR*
SI183	*h-*	*leu1 ade6-M216(?) psc3-1T<<kanR plo1+-GFP<<kanR bub1Δ::natR*
SI801	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR bub3Δ::ura4+*
SI180"	*h+*	*leu1 ade6-M210 psc3-1T<<kanR plo1+-GFP<<kanR bub3Δ::ura4+ (ura4-D18?)*

Figure 2.17

SI803	*h-*	*leu1 sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SI803'	*h-*	*leu1 sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SI805	*h-*	*leu1 ade6-M216? bub1Δ::natR sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SI806	*h-*	*leu1 ade6-M216? bub1Δ::natR sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SI807	*h-*	*leu1-32 (ura4-D18?) bub3Δ::ura4+ sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SI809	*h-*	*leu1-32 ade6-M210 (ura4-D18?) bub3Δ::ura4+ sgo2::sgo2+-GFP pREP81-mCherry-atb2+(LEU2)*
SK154	*h-*	*leu1 ade6-M216 bub1::bub1-ΔGLEBS sgo2::sgo2+-GFP pR81-m-Cherry-Tubulin*
SK155	*h-*	*leu1 ade6-M216 bub1::bub1-ΔGLEBS sgo2::sgo2+-GFP pR81-m-Cherry-Tubulin*

Figures 2.18 and 2.19

SI454	*h+*	*leu1 ade6 ura4-D18? bub3::ura4+ cen2<<lacO<<kanR<<ura4+ his7+<<GFP-lacI-NLS kanR<<Pnmt41-mCherry-atb2+*
SI446	*h+*	*leu1 ade6-M216 ura4-D18? bub3::ura4+ kanR<<Pnmt41-mCherry-atb2+ klp5::kanR cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS pREP1-klp5*
SI446'	*h+*	*leu1 ade6-M216 ura4-D18? bub3::ura4+ kanR<<Pnmt41-mCherry-atb2+ klp5::kanR cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS pREP1-klp5*
SI455	*h+*	*leu1 kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanr his7+<<GFP-LacI-NLS*
SI436	*h+*	*leu1 ade6-M216 kanR<<Pnmt41-mCherry-atb2+ klp5::kanR cen2<<lacO<<ura4+<<kanR his7+<<GFP-lacI-NLS pREP1-klp5*

Figure 2.20

SK101	*h+*	*leu1 ura4D-18 ade6-M210 bub3Δ::ura4+ plo1+-mCherry<<natR ase1-GFP:kanR*
SK102	*h-*	*leu1 ura4D-18 bub3Δ::ura4+ plo1+-mCherry<<natR ase1-GFP:kanR*
SK108	*h+*	*leu1 ura4-D18? klp5Δ::ura4+ plo1-mCherry<<natR ase1-GFP:kanR*
SK108''	*h+*	*leu1 ura4-D18? klp5Δ::ura4+ plo1-mCherry<<natR ase1-GFP:kanR*
SK106'	*h-*	*leu1 ura4-D18 plo1-mCherry<<natR ase1-GFP:kanR*
SK107	*h+*	*leu1 ura4-D18 ade6-M210 plo1-mCherry<<natR ase1-GFP:kanR*

Figure 2.21

SH511	*h-*	*leu1 ade6-M216 nda3-KM311 plo1+-GFP<<kanR*
SH515	*h+*	*leu1 ade6-M216 (ura4-D18) nda3-KM311 mad2Δ::ura4+ plo1+-GFP<<kanR*
SH515'	*h+*	*leu1 ade6-M216 (ura4-D18) nda3-KM311 mad2Δ::ura4+ plo1+-GFP<<kanR*
SI437	*h+*	*leu1 ade6-M216 ura4DS/E? bub1Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI437'	*h+*	*leu1 ade6-M216 ura4DS/E? bub1Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI438	*h-*	*leu1 ade6-M216 ura4-D18? bub3Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*
SI456	*h+*	*leu1 ura4-D18? ade6-M216 bub3Δ::ura4+ mad2Δ::ura4+ nda3-KM311 plo1+-GFP<<kanR*

Figure 2.22

SH511	*h-*	*leu1 ade6-M216 nda3-KM311 plo1-GFP<<kanR*
SH515	*h+*	*leu1 ade6-M216 (ura4-D18) nda3-KM311 mad2::ura4+ plo1-GFP<<kanR*
SI437	*h+*	*leu1 ade6-M216 ura4DS/E? bub1::ura4+ nda3-KM311 plo1-GFP<<kanR*
SI438	*h-*	*leu1 ade6-M216 ura4-D18? bub3::ura4+ nda3-KM311 plo1-GFP<<kanR*

Figure 2.23A and B

PX746	*h-*	*leu1 ade6-M216 plo1+-GFP<<kanR*
SH584	*h+*	*leu1 ade6-M210 plo1+-GFP<<kanR*
SI842	*h-*	*leu1 ade6-M210 bub3Δ::ura4+ plo1+-GFP<<kanR*
SI842'	*h-*	*leu1 ade6-M210 bub3Δ::ura4+ plo1+-GFP<<kanR*
SK123	*h+*	*ade6-M210 (ura4-D18?) bub3Δ::ura4+ mad2Δ::ura4+ plo1+-GFP<<kanR*
SK124	*h+*	*(ura4-D18?) bub3Δ::ura4+ mad2Δ::ura4+ plo1+-GFP<<kanR*
SK120	*h+*	*ade6-M210 (ura4-D18?) mad2Δ::ura4+ plo1+-GFP<<kanR*
SK121	*h-*	*ade6-M210 (ura4-D18?) mad2Δ::ura4+ plo1+-GFP<<kanR*

Figure 2.23C

SI842	*h-*	*leu1 ade6-M210 bub3::ura4+ plo1+-GFP<<kanR*
SI900	*h-*	*leu1 ade6-M210 bub3::ura4+ sgo2::kanR plo1+-GFP<<kanR*
SI900'	*h-*	*leu1 ade6-M210 bub3::ura4+ sgo2::kanR plo1+-GFP<<kanR*
AI901	*h+*	*leu1 ade6-M216 bub3::ura4+ sgo2::kanR plo1+-GFP<<kanR*
SI902	*h+*	*leu1 ade6-M216 sgo2::kanR plo1+-GFP<<kanR*

Figure 2.24

SI455	*h+*	*leu1 kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanR his7+<<GFP-LacI-NLS*
SI461	*h+*	*leu1 ade6-M216 kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanR his7+<<GFP-LacI-NLS*
SI461'	*h+*	*leu1 ade6-M216 kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanR his7+<<GFP-LacI-NLS*
SI454	*h+*	*leu1 ade6 ura4-D18? bub3Δ::ura4+ cen2<<lacO<<kanR<<ura4+ his7+<<GFP-lacI-NLS kanR<<Pnmt41-mCherry-atb2+*
SI454'	*h+*	*leu1 ade6 ura4-D18? bub3Δ::ura4+ cen2<<lacO<<kanR<<ura4+ his7+<<GFP-lacI-NLS kanR<<Pnmt41-mCherry-atb2+*
SI117	*h-*	*leu1 sgo2Δ::kanR kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanr his7+<<GFP-lacI-NLS*
SI117'	*h-*	*leu1 sgo2Δ::kanR kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanr his7+<<GFP-lacI-NLS*

Figures 2.25 and 2.26

SI893	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18?) mis6+-mCherry<<kanR*
SI903	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] mis6+-mCherry<<kanR*
SI894	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18?) bub3Δ::ura4+ mis6+-mCherry<<kanR*
SI894'	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18?) bub3Δ::ura4+ mis6+-mCherry<<kanR*
SK130	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] ade6-M216 bub1::bub1-ΔGLEBS mis6+-mCherry<<kanR*

SK130'	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] ade6-M216 bub1::bub1-ΔGLEBS mis6+-mCherry<<kanR*
SK133	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18) sgo2Δ::ura4+ mis6+-mCherry<<kanR*
SK133'	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18) sgo2Δ::ura4+ mis6+-mCherry<<kanR*
SK135	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18?) bub3Δ::ura4+ sgo2Δ::kanR mis6+-mCherry<<kanR*
SK135'	*h-*	*leu1-32::SV40-GFP-atb2[LEU1] (ura4-D18?) bub3Δ::ura4+ sgo2Δ::kanR mis6+-mCherry<<kanR*

Figure 2.27

SK146	*h+*	*ade6-M216 bub3+-S(GGGGS)3-double-myeGFP<<kanR mis6+-mCherry<<kanR sid4+-mCherry<<natR*
SK138	*h-*	*leu1 bub1-GFP<<kanR mis6-mCherry<<kanR sid4-mCherry<<natR*

Figure 2.28

SI455	*h+*	*leu1 kanR<<Pnmt41-mCherry-atb2+ cen2<<lacO<<ura4+<<kanR his7+<<GFP-LacI-NLS*
SI454	*h+*	*leu1 ade6 ura4-D18? bub3Δ::ura4+ cen2<<lacO<<kanR<<ura4+ his7+<<GFP-lacI-NLS kanR<<Pnmt41-mCherry-atb2+*

Figure 2.29

SK132	*h+*	*mad2+-GFP<<kanR mis6+-mCherry<<kanR sid4+-mCherry<<natR*
SK144'	*h+*	*leu1 ade6-M216 bub3Δ::ura4+ mad2+-GFP<<kanR mis6+-mCherry<<kanR sid4+-mCherry<<natR*
SK136	*h+*	*ade6-M216 bub1::bub1ΔGLEBS mad2+-GFP<<kanR mis6+-mCherry<<kanR sid4+-mCherry<<natR*

Figure 2.30

SH211	*h+*	*leu1 ade6-M216 nda3-KM311 plo1-GFP<<kanR*
SI463	*h-*	*leu1 ura4-D18? mad1::ura4+ nda3-KM311 plo1-GFP<<kanR*
SI463'	*h-*	*leu1 ura4-D18? mad1::ura4+ nda3-KM311 plo1-GFP<<kanR*
SK197	*h-*	*leu1 ura4-D18? ade6-M216 mph1::ura4+ nda3-KM311 Plo1-GFP-kanR*
SK197'	*h-*	*leu1 ura4-D18? ade6-M216 mph1::ura4+ nda3-KM311 Plo1-GFP-kanR*
SK191	*h+*	*leu1 ade6-M216 nda3-KM311 mph1::mph1-D1-150 plo1-GFP<<kanR*
SK193	*h-*	*leu1 ade6-M216 nda3-KM311 mph1::mph1-D1-150 plo1-GFP<<kanR*
SK198	*h+*	*leu1 ade6-M216 mph1::mph1-D1-302 nda3-KM311 plo1-GFP<<kanR*
SL305	*h-*	*leu1 ade6-M216 nda3-KM311 mph1::mph1-D1-302 plo1-GFP<<kanR*
SL307	*h-*	*leu1 ura4-D18 ade6-M216 mad1::mad1-D1-468 nda3-KM311 plo1-GFP<<kanR*
SL310	*h+*	*leu1 ura4-D18 ade6-M216 mad1::mad1-D1-468 nda3-KM311 plo1-GFP<<kanR*
SL311	*h-*	*leu1 ura4-D18 ade6-M216 mad1::mad1-D1-468 nda3-KM311 plo1-GFP<<kanR*

Figure S1

JY333	*h-*	*ade6-M216 leu1*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
PX854	*h-*	*leu1 klp5::kanR*
PX840	*h+*	*leu1 ade6-M216 klp6::kanR*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
SH746	*h-*	*leu1 ura4? ade6-M216 bub1::ura4+ klp6::kanR*

Figure S2

JY333	*h-*	*ade6-M216 leu1*
PX854	*h-*	*leu1 klp5::kanR*
PZ334	*h-*	*bub1::ura4+ ade6-M216 leu1-32 ura4DS/E his1-102*
KP106	*h-*	*bub3::ura4+ leu1-32 ade6-M210 ura4-D18*
PX960	*h+?*	*leu1 ade6-M216 (ura4DS/E) klp5::kanR bub1::ura4+*
SH760	*h+*	*leu1 ade6-M216 ura4-D18 klp5::kanR bub3::ura4+*

Strains used for strain construction and mentioned in Materials and Methods, but not used for experiments:

SH723	*h-*	*leu1 ura4-D18 ade6-M210/216<<hph<<bub1+*
JY336	*h+*	*leu1 ade6-M210*
SH748	*h+*	*leu1 ura4-D18 ade6? sgo2::sgo2+-GFP klp5::kanR pREP4-klp5*

4.6 Construction of fission yeast strains

4.6.1 Constructing strains by crossing

The two parental strains were mixed in 10 µL sterile H_2O and spotted on SSA/L/A/U or SSA/L/A/U plates supplemented with histidine and incubated for 2 d at the appropriate temperature. Subsequently, spores were analyzed by tetrad dissection or selected by random spore analysis.

4.6.1.1 Random spore analysis

Some cell material containing asci was picked with a toothpick from the SSA/L/A/U plate and suspended in 1 mL sterile H_2O. After adding 1.2 µL glusulase (NEE-154, Perkin Elmer, > 10,000 U/ml), the mix was incubated at 30 °C for 4 h or at 25 °C over night when temperature-sensitive strains were crossed. Subsequently, the cell debris and spores were pelleted by centrifugation and resuspended in 100 µL sterile H_2O. The spores were plated onto appropriate selective medium in a density that should results in growth of about 500 colonies per plate. If additional selection steps using media was possible, plates were replica plated to other selective media after incubation for 2 d. The spore colonies were further analyzed by PCR to test for the presence of certain mutations and/or examined under the microscope for the presence of the desired fluorescent markers.

4.6.1.2 Tetrad dissection

Some cell material containing asci was picked with a toothpick from the SSA/L/A/U plate and streaked in the middle of a YEA plate. Asci were picked from this region and dissected using a dissecting microscope (Singer). After incubation for 2 d, plates were replica plated to selective medium or to plates that were incubated at different temperatures to assay growth of the double mutants compared to the parental single mutants. If necessary, the spore colonies were further analyzed by PCR to test for the presence of certain mutations.

4.6.2 Transformation for genomic integration of resistance cassettes

Cells were grown to mid log phase. About 1 x 10^8 cells were spun down for 3 min at 3,000 rpm. Cells were washed once with an equal volume of H_2O and a second time with 1 mL of 0.1 M Li-acetate in TE (pH 7.5). The pellet was resuspended in 0.1 M Li-acetate in TE (pH 7.5) to a cell concentration of 2 x 10^9 cells/ml. To 50 µL of cell suspension, up to 5 µL DNA and 10 µg Herring sperm DNA (Sigma, D-7290) were added. The suspension was mixed and incubated at room temperature for 10 min. 130 µL 40% PEG 4000 in 0.1 M Li-acetate in TE (pH 7.5) were added, the suspension was mixed gently and incubated for 30 min at 30 °C. After adding 21 µL DMSO, cells were heat-shocked at 42 °C for 5 min. Cells were centrifuged, resuspended in 500 µL medium and

plated on 2 YEA plates, which were incubated at 30 °C over night and replica plated to selective plates (YEA/hygro or YEA/nat, depending on the resistance cassette) the following day. After 2 days, colonies had grown and were restreaked to obtain single colonies. Subsequently, the presence of the correct genotype was determined by PCR and, if necessary, sequencing.

4.6.3 Transformation with a plasmid

The transformation protocol was the same as for integration of a resistance cassette, as described above, but only about 50 ng were transformed. After the transformation, cells were directly plated on selective SD medium lacking leucine or uracil, as plasmids carried either *LEU2* or *ura4+* as selectable markers.

4.6.4 Transformation for *ura4+* replacement

Replacement of the *ura4+* gene was used to insert a mutant allele of a gene at the endogenous locus (Grimm et al., 1988). The transformation protocol described in 4.7.2 was followed, but instead of plating cells on YEA, they were incubated in 1 L YEL over night at 30 °C. The following morning, cells were centrifuged and resuspended in H_2O to a concentration of 2.5 x 10^8 cells/ml. Cells were plated on 5-FOA plates or on 5-FOA plates supplemented with histidine; each plate was inoculated with about 5 x 10^7 cells. After 4 days, colonies were picked and analyzed for the presence of the desired allele by PCR and sequencing.

4.6.5 Construction of *hygroR<<bub1+* and *bub1Δ::natR*

The *bub1Δ::natR* and *hygroR<<bub1+* strains were created by PCR-based gene targeting (Bähler et al., 1998b). Homology fragments were amplified from genomic DNA of the wild type strain JY333 with primers YS421 and YS381 as well as YS378 and YS380 (for *bub1Δ::natR*), and YS387 and YS385 as well as YS388 and YS386 (for *hygroR<<bub1+*). For *bub1Δ::natR*, the resistance cassette was amplified from the linearized plasmid *pFA6a-NatI* (vc345; Bähler et al., 1998b), using the corresponding homology fragments and primers YS380 and YS381. For *hygroR<<bub1+,* the resistance cassette was amplified from linearized plasmid *pC225* (v012; Bähler et al., 1998b), using the corresponding homology fragments and primers YS387 and YS388. Yeast cells were transformed with the PCR fragments as described in 4.7.2.

4.6.6 Construction of *bub1* mutant strains using random PCR mutagenesis

The PCR mutagenesis was performed with a Taq polymerase (Eppendorf) using primers YS381 and YS387. A PCR fragment amplified with the same primers from genomic DNA of strain SH723, which carried the *hygroR<<bub1+* construct, was used as a template. Strain SH748 (carrying *sgo2+-GFP klp5Δ and pREP4-klp5+*) was transformed with the resulting PCR product.

4.6.7 Reconstruction of *bub1-m5*

For reconstruction of the *bub1-m5* allele, wild type strains were transformed with *hygroR<<bub1* fragments. For the D76G mutation, DNA fragments were amplified from genomic DNA of strain SH723, which carries the *hygroR<<bub1+* construct, using primers YS696 and XS056 as well as YS391 and XS057. Strain JY333 was transformed with the overlap PCR of these fragments, amplified with primers YS696 and YS391. Genomic DNA of the resulting strain was used to construct double mutants using the primers YS696 and XS058 as well as YS391 and XS059 for the K101E mutation, and YS696 and XS060 as well as YS391 and XS061 for the Q37R mutation. The overlap PCRs were amplified with primers YS391 and YS696. Strain JY336 was transformed with the resulting PCR products. I used genomic DNA of SI475 (*bub1-Q37R/D76G*) as a template for construction of the triple mutant. For amplifying the *bub1-Q37R/D76G/K101E* fragments, I used the primers described above and transformed strain JY336 with the resulting PCR product.

4.6.8 Construction of *bub1-ΔGLEBS*

The *bub1-ΔGLEBS* mutant was constructed by deleting the bases corresponding to amino acids 264 – 299 (GKRV...SSIQ). Primers YS659 and YS380 were used to amplify the *bub1* fragment 5' of the GLEBS motif and primers YS660 and YS393 and were used to amplify the *bub1* fragment 3' of the GLEBS motif. With the overlap PCR of these fragments a strain carrying *bub1Δ::ura4+* (PZ334; Bernard et al., 1998) was transformed, and the modified *bub1* gene was integrated into the endogenous locus by replacement of the *ura4+* cassette (4.7.4).

4.6.9 Construction of *sgo2-GFP*

The *sgo2::sgo2+-GFP* strain was created by replacing the *ura4+* cassette of *sgo2Δ::ura4+* (SH735). First, the 3'UTR was amplified with primers YS428 and YS531, then this fragment and the linearized plasmid *pFA6a-kanMX-5'UTR-sgo2+-GFP* (v108) were used to amplify the fragment for transformation, using primers YS428 and YS426.

4.6.10 Construction of *mph1Δ-1-302* and of *mph1-Δ1-150*

The *mph1Δ-1-302* and the *mph1-Δ1-150* mutants were constructed by replacing the *ura4+* cassette of *mph1Δ::ura4+* (SS560; He et al., 1998). To construct *mph1Δ-1-302,* primers XS679 and YS301 were used to amplify the 5' fragment and primers XS678 and YS818 and were used to amplify the 3' fragment. To construct *mph1Δ-1-150,* primers XS681 and YS301 were used to amplify the 5' fragment and primers XS680 and YS818 were used to amplify the 3' fragment. Strain SS560 was transformed with the overlap PCR of these fragments.

4.6.11 Construction of *mad1-Δ1-468*

The *mad1-Δ1-468* mutant was constructed by replacing the *ura4+* cassette of *mad1Δ::ura4+* (AE247; Vanoosthuyse et al., 2004). Primers XS675 and XS394 were used to amplify the 5'

fragment and primers XS674 and YS779 were used to amplify the 3' fragment. Strain AE247 was transformed with the overlap PCR of these fragments.

4.7 Synthetic lethal screen

4.7.1 EMS mutagenesis

Cells of strain SH721 were grown to mid log phase in MM+N+L+A. Cells were centrifuged at 3,000 rpm for 5 min and resuspended in MM-N+L+A to a cell density of 1 x 10^8 cells/ml. EMS (ethyl methane sulfonate) was added to 2% to a 1 mL culture. The culture was incubated at 30 °C for 1.5 h. Subsequently, cells were harvested and washed 3 times with 1 mL of MM-N+L+A. The EMS-mutagenized cells showed a survival rate of 36% as determined by plating onto YEA plates.

4.7.2 Screening procedure

Mutagenized cells were plated on YEA plates and incubated at 34 °C until the following day. The YEA plates were replica plated to SD/L/A/U containing Phloxin-B and after incubation for one day these plates were replica plated to 5-FOA plates containing Phloxin-B. Subsequently, both SD/L/A/U and 5-FOA plates were incubated for 2 days at 34 °C. Colonies that appeared to grow on SD/L/A/U but not on 5-FOA were picked and streaked on YEA to obtain single colonies. After incubation for 3 days at 34 °C, single colonies were picked and a streaking assay was performed on SD/L/A/U and 5-FOA, both containing Phloxin-B. After 3 to 4 days at 34 °C, synthetic sickness could be assayed. The streaking assay was repeated to confirm the result, and synthetic sick/lethal candidates were stored at –80 °C in YEA medium containing 20% glycerol.

4.8 Growth test

Cultures (YEA) that had grown to mid log phase at 30 °C, were diluted to 2 x 10^6 cells/ml in H_2O. From this first dilution four 1:5 serial dilutions were prepared. Drops of 4 µL or 7 µL of all five dilutions per strain were spotted on plates containing 2 µg/ml Phloxin-B. Typically, some of the plates contained a microtubule destabilizing drug, benomyl or TBZ, and plates were usually incubated at several temperatures, to assay growth under different conditions.

4.9 Synchronization of cells using hydroxyurea (HU)

Cells were synchronized in early S-phase by addition of 12 mM hydroxyurea (HU; Sigma, H-8627) to the medium and incubation for 4 h at 30 °C. To release from HU, cells were washed three times with medium and cultured in fresh medium.

4.10 Fluorescence microscopy

Single images of living or fixed cells were taken with an AxioImager M1 microscope (Zeiss) using a 63x/1.4 Plan Apochromat oil objective (Zeiss) driven by a Piezo motor. Time-lapse live cell imaging was performed as described in 4.11.5.

4.10.1 Fixation of *S. pombe* cells for microscopy

4.10.1.1 Methanol fixation

Cells of about 1 mL of an exponentially growing culture were pelleted. The supernatant was removed completely and the cells were resuspended in 1 mL cold methanol (-20 °C). The cells were incubated for at least 2 h at -80 °C. Subsequently, the cells were washed once with 1 mL PEM/MetOH (1:1), then once with 1 mL PEM (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, adjusted to pH 6.8 with KOH).

4.10.1.2 PFA fixation

To 9 mL of an exponentially growing culture, PFA ('para'formaldehyde)/PEM was added to 3.7% (1 mL 37% PFA/PEM). Cells were fixed in a shaking incubator at 18 °C for 1 hour. 37% PFA/PEM was prepared the following way: 90 mL H_2O were added to 45.6 g PFA and stirred on a 60 °C heating block. 30 mL 4x PEM (400 mM PIPES, 4 mM EGTA, 4 mM $MgSO_4$, adjusted to pH 6.8 with KOH) were added after 15 min and stirred for another 15 min. The mixture was incubated in a 60 °C water bath over night. If a small amount of PFA remained undissolved the solution was cleared by centrifugation. PFA/PEM was stored in aliquots at -20 °C.

4.10.2 Anti-tubulin immunostaining

Cells were grown to exponential phase in YEA at 30 °C, diluted in YEA to a concentration of 4 x 10^6 cells/ml and incubated in a shaker for 4 h at 18 °C, the restrictive temperature for the *nda3-KM311* allele. Cells were fixed with PFA as described above and washed 3 times with 10 mL PEM + 0.1% Triton X-100 at room temperature for 20 min. Cells were resuspended in 1 mL PEMS (1.2 M Sorbitol in PEM), and 5 µL β-mercaptoethanol was added. After mixing for 30 sec, 400 µg zymolyase 100-T (Medac, 20 mg/ml stock containing 5% glucose) were added, and the mixture was incubated for 1 hr at 37 °C. Cells were washed once with 1 mL PEMS for 20 min and twice with 1 mL PEM + 0.1% Triton X-100 for 15 min, then they were resuspended in 1 mL blocking solution (PEM-NL: 5% normal goat serum and 100 mM L-lysine monohydrochloride in PEM) and incubated for 30 min at room temperature. Subsequently, they were incubated over night at 4 °C with the TAT1 anti-tubulin antibody (1:300, mouse, Biomol, UG9510-0100) in 300 µL PEM-NL. Cells were washed 3 times with PEM + 0.1% Triton X-100 for about 20 min at room temperature, resuspended in 300 µL PEM-NL containing anti-mouse Alexa 568 (1:1,000, goat, Invitrogen) and incubated on a rotating wheel at room temperature for 1.5 h protected from light. After 3 additional washing steps with PEM + 0.1% Triton X-100, cells were resuspended in 300 µL PEM + 0.1% Triton X-100 and imaged with an AxioImager M1 microscope (Zeiss).

4.10.3 Staining with Calcofluor and Hoechst

To methanol-fixed cells that had been resuspended in PEM, Hoechst 33342 and Calcofluor were added to 1 µg/ml and 1.75 µg/ml, respectively. Cells were incubated, protected from light, at room temperature for 5 min on a rotating wheel.

A 2,000x Calcofluor stock was prepared the following way. To 35 mg Calcofluor white (Sigma F-3543, Fluorescence Brightener) 7 mL H_2O and 2 - 3 drops of 10 N NaOH (to increase pH to 10 - 11) were added. Calcofluor was dissolved and volume was adjusted to 10 mL with H_2O. The solution was stored protected from light at -20 °C

4.10.4 Quantification of Sgo2-GFP

A z-stack with 14 images spaced by 0.3 µm was recorded and three pictures that were the least in focus were discarded. The resulting z-stack was projected using the 'maximum intensity' algorithm of MetaMorph software (Molecular Devices). The maximum signal intensity was determined in a circle centered on the nucleus of interphase or metaphase cells, which were identified based on the characteristic appearance of microtubules.

4.10.5 Time-lapse microscopy

Live cell imaging was performed on a DeltaVision Core system (Applied Precision) equipped with a climate chamber. Cells were mounted in # 1.5 glass-bottom culture dishes (MatTek or Ibidi) that had been coated with lectin (Sigma, L-2380). Cells were incubated on the microscope stage at the appropriate temperature for at least 30 min before imaging was started. Pictures were taken using a 60x/1.4 Plan Apo oil objective (Olympus) and the 'optical axis integration' algorithm of the SoftWorx software (Applied Precision). All images were deconvolved using SoftWorx software. GraphPad Prism software was used to visualize the results. Kymographs were assembled with the help of Adobe ImageReady and Photoshop software.

4.10.5.1 Coating of glass bottom dishes

The glass was covered with a 25 µg/ml lectin (Sigma, L-2380) solution, which was reused 3 to 4 times. After incubation at room temperature for 10 min protected from light, the solution was sucked off and the dish was dried at 30 °C for 10 min to 30 min.

4.10.5.2 Live cell imaging after plasmid loss

Cells carrying the *pREP1-klp5*+ plasmid were grown in minimal medium lacking leucine (MM+N+A), cells without plasmid were grown in MM+N+L+A. Cells of cultures that were grown to mid log phase were harvested, washed and subsequently cultured in minimal medium without additional ammonium chloride to starve the cells for nitrogen and induce an arrest in G_1 phase. The medium contained ¼ of the normal amount of leucine and ½ of the normal amount of adenine, because fission yeast can use these amino acids as a nitrogen source. The cultures were incubated at 30 °C for 19 h. Subsequently, the cells were cultured for 4 h at 30 °C in MM+N+L+A in order to obtain cells that have lost the *pREP1-klp5*+ plasmid and grown in the absence of Klp5. It

has been reported that cells released from G_1 arrest lose plasmids more frequently (Ohkura and Yanagida, 1991). Cells were mounted in a MatTek glass-bottom culture dish and after incubation for 1 h on the microscope stage at 30 °C, cells were filmed at this temperature.

4.10.5.3 Live cell imaging for visualization of Ase1-GFP

Cells were grown to mid log phase at 30 °C in EMM containing the necessary supplements. Cells were mounted, and filming was started after incubation for about 1 h on the stage at 30 °C. Ase1-GFP localization was followed in mitotic cells, which were identified by the appearance of the Plo1-mCherry signal on SPBs.

4.10.5.4 Live cell imaging for assaying SAC activity

Cells carrying *nda3-KM311* were grown to mid log phase at 30 °C in minimal medium. Cells were mounted in minimal medium and incubated at 17 °C or 19 °C for 2 h on the microscope stage before imaging started. To determine the time of appearance and disappearance of Plo1-GFP at SPBs, the background intensity in the cytoplasm was measured, multiplied with 1.2, and this value was taken as a threshold for positive Plo1-GFP signals.

4.10.5.5 Live cell imaging to determine time in prometaphase

Cells were grown and imaged at 30 °C in EMM containing the necessary supplements. To determine the time of appearance and disappearance of Plo1-GFP at SPBs, I measured the background intensity in the cytoplasm, multiplied it with 1.2, and took this value as a threshold for positive Plo1-GFP signals.

4.10.5.6 Live cell imaging of cells after MBC release

Cells were grown to mid log phase in EMM containing the necessary supplements. Cells were synchronized in early S-phase by addition of 12 mM HU to the medium. After release from HU arrest, 20 µg/mL MBC/carbendazim (Sigma, 45368) was added to the cultures. Cells were mounted and incubated on the microscope stage at 20 °C. After 3.5 h the medium was exchanged to fresh medium, not containing MBC, and imaging was started at 20 °C. To analyze missegregation (Figure 2.24), I judged the segregation of cen2-GFP signals on the spindle by the time of spindle break-down at the end of mitosis. Errors in distribution of chromatids to the daughter cells resulting from mispositioned spindles were not categorized as missegregation events. In the experiment shown in Figures 2.25 and 2.26, cells where chromosome attachment could not be judged clearly were excluded from the analysis (Table 2.1).

4.10.5.7 Live cell imaging for visualization of Bub1-GFP, Bub3-GFP and Mad2-GFP

Cells were grown, pre-synchronized with HU and treated with MBC as described above. After 3.5 h of incubation on the microscope stage at 20 °C, the medium was exchanged to fresh medium, not containing MBC, and imaging was started at 20 °C.

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6 Supplemental data

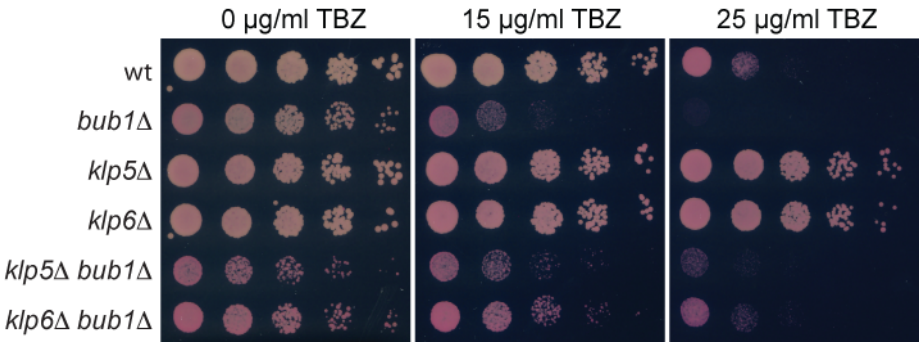

Figure S 1 Deletion of *klp5* or *klp6* partially rescues TBZ sensitivity of *bub1Δ*

Growth assay with the indicated strains. 5-fold serial dilutions were spotted on full medium containing Phloxin-B. Plates containing the indicated concentration of thiabendazole (TBZ) were incubated at 30 °C for 3 d. Deletion of kinesin-8 genes results in resistance to TBZ. Deletion of *bub1* results in TBZ sensitivity, which is partially rescued by additional deletion of a kinesin-8 gene.

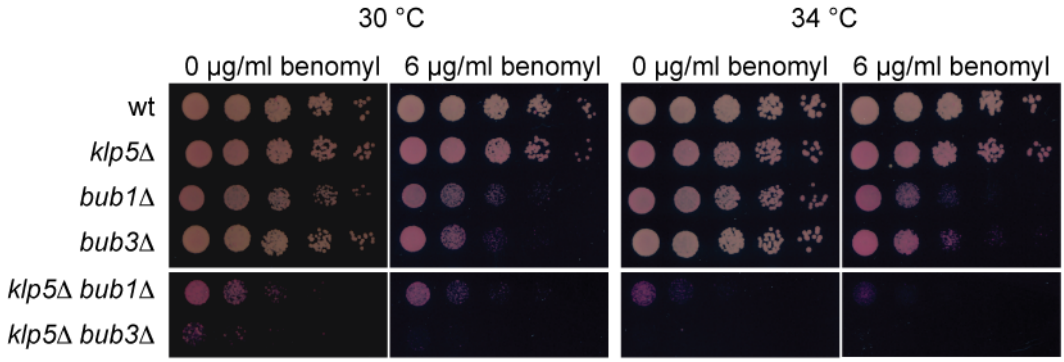

Figure S 2 Benomyl does not rescue the synthetic sickness between *klp5Δ* and *bub1Δ* or *bub3Δ*

Growth assay with the indicated strains. 5-fold serial dilutions were spotted on full medium containing Phloxin-B. Plates containing the indicated concentration of TBZ were incubated at the indicated temperature for 2 d. At 34 °C, the double mutants *klp5Δ bub1Δ* and *klp5Δ bub3Δ* show a growth defect compared to the single mutants that is not rescued by benomyl.