Molecular Mechanisms of Spindle Positioning in Budding Yeast *Saccharomyces cerevisiae*

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
for the degree of
Doctor of Natural Sciences

presented by
Justine Kusch
Dipl. Biol., University of Konstanz
born September 18, 1972
citizen of Germany

accepted on the recommendation of
Prof. Dr. Yves Barral, examiner
Prof. Dr. Monica Gotta, co-examiner
Prof. Dr. Matthias Peter, co-examiner

2004
Table of contents

Summary..................................................................................................................................................7
Zusammenfassung...................................................................................................................................8

CHAPTER I .............................................................................................................................................11

General introduction..........................................................................................................................11
How do cells coordinate chromosome segregation with cell cleavage and cell polarity?..................11
Microtubules.........................................................................................................................................11
Features...............................................................................................................................................11
Dynamic instability..............................................................................................................................12
Microtubule function............................................................................................................................13
Microtubule regulatory proteins...........................................................................................................14
Stabilizing factors: Microtubule Associated Proteins (MAPs)...............................................................14
Destabilizing factors: kinesins and Op18/Stathmin.............................................................................15
+TIPs..................................................................................................................................................15
+TIP activity........................................................................................................................................16
EB1......................................................................................................................................................16
CLIP170...............................................................................................................................................16
Plus end tracking: treadmilling...........................................................................................................16
Preferential binding to plus end lattice...............................................................................................17
Compolymerization...............................................................................................................................17
+TIP interaction..................................................................................................................................18
+TIP complexes: how to specify microtubule function.......................................................................19
+TIP complexes in leading edges of migrating cells.........................................................................19
+TIP complexes at kinetochores..........................................................................................................20
Regulation of microtubule regulatory proteins...................................................................................20
Role of microtubules in coordination of spindle function with cell cleavage and cell polarity........21
Role of microtubules in spindle orientation........................................................................................21
Role of microtubules in spatial coordination of cleavage apparatus and mitotic spindle..............22
Models for cleavage site placement in animals...................................................................................23
Astral microtubule stimulation at the equator....................................................................................23
Astral microtubule inhibition at cell poles............................................................................................23
Equatorial stimulation by differential dynamics of astral microtubules............................................24
Midzone microtubule stimulation at equator......................................................................................24
Molecular insight into cleavage furrow induction............................................................................25
# Table of contents

- Chromosomal passengers ................................................................. 25
- Small GTPase regulators ..................................................................... 26
- Interaction between microtubules and cortical actin ........................ 27
- Budding yeast as a model system to study microtubule function .......... 27
- Cell polarity in yeast ........................................................................... 28
- Cleavage site organization yeast ......................................................... 29
- Spindle positioning in yeast ................................................................. 29
- Two genetically redundant pathways ................................................. 30
  - Preanaphase attachment .................................................................. 30
  - Anaphase attachment ...................................................................... 30
- Open questions for spindle positioning in yeast ................................. 31
- Spatial coordination of the spindle and cleavage site ......................... 32
- Spindle alignment ............................................................................... 32
- Aim of the thesis ................................................................................. 33

## CHAPTER II ......................................................................................... 35

Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast ............................................ 35

### Abstract ......................................................................................... 36

### Introduction ..................................................................................... 36

### Results ............................................................................................. 38
  - The septin cytoskeleton is required for proper nuclear positioning .... 38
  - Nuclear migration defect of septin mutants is not due to actin polarity defects 40
  - Nuclear positioning is independent of the assembly of the actomyosin ring 41
  - Microtubules interact with the septin ring ............................................. 41
  - Microtubule plus-end capture at the bud neck is associated with the establishment of pulling forces on the spindle ........................................... 44
  - SDK are required for the shortening of microtubule interacting with the neck 47
  - Septins act in the Kar9 pathway ............................................................ 48
  - Kar9 links microtubules to the septin ring ............................................ 50
  - Septins are not involved in spindle alignment .................................... 52

### Discussion ....................................................................................... 52

### Methods ............................................................................................ 55
  - Quantification of nuclear migration .................................................... 55
  - Strains and growth conditions ............................................................ 55
  - Fluorescence and time-lapse Microscopy ............................................ 56

## CHAPTER III ......................................................................................... 59
Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment

Abstract

Introduction

Results

Kar9 associates with the spindle pole destined to the bud
Cdc28 activity is required for the maintenance of Kar9 asymmetry
Phosphorylation of Kar9 by Cdc28/Clb4 controls its asymmetry
Clb4 localizes to the mother-bound pole
Kar9 is loaded onto microtubules at the spindle pole
Microtubules emanating from the bud-bound pole are actively guided towards the bud
Microtubule guidance depends on Kar9, Myo2 and actin
Symmetric loading of Kar9 onto spindle poles orient both poles towards the bud
Kar9 asymmetry is specifically required for its function in spindle positioning

Discussion

Spindle asymmetry is required for proper spindle orientation
A model for Kar9 function in spindle orientation
Spindle poles as loading machines
A compass theory for spindle alignment

Experimental Procedures

Strains, Plasmids and Growth Conditions
Protein and Immunological Techniques
Fluorescence and time-lapse Microscopy
Photobleaching

CHAPTER IV

General discussion

In general
Spatial coordination of the spindle and the cleavage site relative to each other
The role of septins in spindle positioning in yeast
Septins: a cortical cue for spatial coordination of the spindle relative to the cleavage site?
Is spatial coordination of the mitotic spindle and the cleavage site conserved?
Interaction between astral microtubules and the cleavage cortex in animals
Maybe
Maybe not
Molecular nature of neck attachment complex
Conservation of interaction between cleavage site components and microtubules
The role of SDKs Hsl1 and Gin4 in microtubule attachment at the neck
Role of phosphorylation in microtubule attachment
Table of contents

Which substrates of SDK mediate microtubule capture-shrinkage?.........................89
Kar9?.....................................................................................89
A kinesin related motor?..........................................................89
The shmoo tip and the bud neck are similar attachment sites.................................90
A MAP/+TIP?..........................................................................90
Kar9: a new +TIP......................................................................92
Localization of endogenously expressed Kar9.........................................................92
Binding partners for Kar9.................................................................................93
Kar9 binding to microtubule plus ends.................................................................93
Kar9 dynamic localization...............................................................................93
Kar9 binding to SPB .................................................................................94
Parallels between Kar9 and APC........................................................................94
How is Kar9 localization regulated?.................................................................95
Kar9 localization during the cell cycle ..................................................................95
Role of phosphorylation......................................................................................95
Guidance: a new mechanism for microtubule attachment in yeast..........................96
Guidance: a way to efficiently target microtubules to specific cortical sites in yeast...96
Guidance: a way to target microtubules to different attachment sites......................96
Guidance: a way to coordinate different microtubule attachments in yeast?...........97
Different ways to polarize the microtubule cytoskeleton......................................98
In yeast.........................................................................................98
In animal cells......................................................................................99
Yeast as a model for +TIP function and regulation...............................................100
The different +TIP complexes in yeast...............................................................100
Conservation.........................................................................................100

References for Chapters I through IV ...................................................................103

CHAPTER V ..................................................................................111

Spindle asymmetry: a compass for the cell..............................................................111
Abstract..........................................................................................112
Introduction.......................................................................................112
Spindle positioning: the role of the cortex............................................................113
Translation of cortical cues into spindle orientation..............................................115
The role of cell geometry..............................................................................115
Response to cortical cues in S. cerevisiae...........................................................116
Kar9 localizes asymmetrically on spindle poles and microtubules.........................116
How is Kar9 asymmetry established?...............................................................120
Microtubule guidance: is it conserved?.............................................................120
Table of contents

...and spindle asymmetry? ................................................................. 121
Role of cell cycle regulators in asymmetric cell division ......................... 122
Concluding remarks .................................................................. 123
References .............................................................................. 124

CHAPTER VI ................................................................................. 129

Appendix .................................................................................... 129
Appendix 1: Role of MARK-related kinases in regulation of microtubule attachment ................................................. 129
Appendix 2: Kar9 is hyperphosphorylated in SDK mutants ....................... 130
Appendix 3: Localization of Kar9 .................................................... 131
Appendix 4: Microtubule guidance .................................................. 132
Table of contents
Summary

Proper positioning of the mitotic spindle relative to the site of cleavage and the polarity axis of the cell is critical for correct chromosome segregation. This process depends on astral microtubules that emanate from the spindle poles and establish contacts with specific cortical sites. A variety of proteins including microtubule Plus end Tracking Proteins (+TIPs) and other Microtubule Associated Proteins (MAPs) are involved in regulation of microtubule behaviour. However, little is known about action and regulation of these proteins during these events.

We used budding yeast as a model system to understand microtubule function during spindle positioning in *Saccharomyces cerevisiae*.

We show that the cleavage site, the mother-bud neck, represents a new microtubule capture site in budding yeast. While the septin ring links microtubules to this site, the two septin dependent kinases (SDK) Hsl1 and Gin4 induce microtubule shrinkage. Genetic analysis revealed that neck attachment is essential to pull the spindle to the bud neck prior to anaphase. We propose direct astral microtubule interaction with the cleavage site as a mechanism to restrict spindle position to this site in budding yeast.

Furthermore, we found that the protein Kar9 mediates microtubule attachment to the bud neck besides linking it to other cortical sites in the bud. Identification of Kar9p as a strictly microtubule-localized protein lead to the discovery of a new mechanism for how microtubules reach their attachment sites. Interaction of microtubule ends with cortical actin cables through a Kar9-myosin complex provides a tracking platform for directional microtubule transport towards cortical capture sites at the neck and in the bud.

We demonstrated an asymmetric recruitment of Kar9 to microtubules that emanate from the bud proximal spindle pole body. We demonstrate, that this polarization of the microtubule cytoskeleton served to align the spindle along the mother bud axis, the polarity axis of budding yeast prior to anaphase.

These results demonstrate for the first time how in budding yeast microtubule attachment is regulated on a molecular level first, to spatially coordinate the mitotic spindle with a predetermined cleavage site, and second, to align the spindle with the polarity axis of the cell. Our studies also add new parallels to the analogy between Kar9 and the tumor suppressor Adenomatous polyposis coli. This gives rise to the perspective to utilize Kar9 function and features in yeast as a model system to uncover further potential properties of the human analog.
Zusammenfassung

Während eines Zellteilungszyklus wird die in Chromosomen verpackte genetische Information verdoppelt und auf die beiden Zellnachkommen übertragen. Die genomischen Kopien werden mittels des Spindelapparates voneinander getrennt und zu den zwei Spindelpolen gezogen. Damit jede Tochterzelle genau einen Chromosomensatz erhält, muss sichergestellt werden, dass die Teilung der Zelle zwischen den voneinander getrennten Chromosomen erfolgt. Dafür muss die Position der Spindel und damit die Richtung der Chromosomentrennung, mit der Zellteilungsebene koordiniert werden und im Falle von polaren Zellen muss sich die Spindel zudem korrekt zur Symmetrieachse der Zelle orientieren.


Ziel dieser Arbeit war es, in der Bäckerhefe *Saccharomyces cerevisiae* die Funktionsweise dieser Proteine zur Positionierung der mitotischen Spindel zu verstehen. Während des Zellzyklus bildet dieser einzellige Organismus eine Knospe an einer Seite der Mutterzelle. Diese wächst zur Tochterzelle heran und trennt sich an der Schnittstelle zum Mutterzellkörper ab.


Die einseitige Lokalisierung von Kar9 auf einem der beiden Spindelpole und damit auf den Mikrotubuli, die von diesem Pol gebildet werden, hat zur Folge, dass nur dieser Pol, und damit nur eine genomische Kopie in die Tochterzelle gezogen werden kann. Dieser Mechanismus bringt gleichzeitig eine richtige Orientierung der Spindel entlang der Polaritätsachse mit sich.

Die vorliegende Arbeit ermöglicht die Sicht auf einen neuen Mechanismus, wie Mikrotubuli und deren assoziierte Proteine innerhalb einer asymmetrischen Zellteilung sicherstellen, dass die zwei genomischen Kopien korrekt auf Mutter- und Tochterzelle verteilt werden. In diesem Zusammenhang eröffnen mittels der Hefe als Modellsystem die Parallelen des Proteins Kar9 mit dem Tumorsuppressor Adenomatous Polyposis Coli eine neue Perspektive zur Untersuchung der Funktionsweise dieses wichtigen menschlichen Proteins.
Chapter 1

General introduction

How do cells coordinate chromosome segregation with cell cleavage and cell polarity?

When cells divide the genomic information of one cell has to be duplicated and distributed onto two cells. Mitosis describes separation of duplicated genomes by a bipolar spindle apparatus whereas cytokinesis is the process that physically cleaves the cell into two daughters. To ensure proper segregation of the genome onto the two daughter cells mitosis and cytokinesis have to be spatially and temporally coordinated. Chromosome segregation and its orientation according to cellular architecture rely on the function of microtubules that emanate from spindle poles and interact with various cellular sites. A variety of proteins involved in regulation of microtubule behaviour during this process.

My work investigated the molecular mechanism of microtubule function to position the mitotic spindle relative to the cleavage site and to orient it within the polarity axis of the cell in budding yeast *Saccharomyces cerevisiae*. These two topics are introduced for budding yeast in the respective publications.

This introduction gives first a general insight into regulation of microtubule behaviour by different proteins. The second part of the introduction gives an overview about the current knowledge of microtubule regulation in the two specific aspects of spindle positioning in higher eukaryotes: coordination between spindle position and cleavage site in higher eukaryotes is described in more detail, while spindle orientation within cell polarity is introduced and discussed in (Kusch et al., 2003). The last part describes the background of spindle positioning in budding yeast and formulates open questions that gave rise to this work.

Microtubules

Features

Microtubules are dynamic polymers, consisting of 13 laterally associated protofilaments that close up into a hollow tubule. Protofilaments are composed of alpha- and beta-tubulin heterodimers attached head to tail to each other. Both subunits of the heterodimer bind GTP, while only the beta subunit hydrolyses GTP. This organization confers an intrinsic polarity to...
the whole tubule which is defined by a minus end, marked by alpha subunits and a plus end, marked by beta subunits (Desai and Mitchison, 1997). In animal cells microtubule are nucleated from their minus ends from centrosomes, the major microtubule-nucleating center in these cells (Doxsey, 2001). They mostly stay anchored with the minus end at the centrosome. With their plus ends they explore the cytoplasm in astral arrays. Microtubules predominantly grow at their plus ends by addition of new heterodimers, which is accompanied by GTP hydrolysis (Desai and Mitchison, 1997).

**Dynamic instability**

Many microtubule dependent functions are based on the ability of the microtubule cytoskeleton to undergo polar and dynamic rearrangements. This property is due to an intrinsic dynamic feature of the polymer, termed dynamic instability. Dynamic instability has been observed in vivo and in vitro as stochastic interconversions between microtubule growth and shrinkage. This behaviour is based on the rate of GTP hydrolysis compared to the rate of GTP-subunit addition. Under conditions of relatively high polymerization rates - as is the case at high local GTP-subunit concentrations - microtubule ends are covered by one or several layers of GTP-bound tubulin, called the GTP cap, while the shaft of the microtubule is covered with GDP-bound tubulin. The GTP cap is thought to stabilize microtubule ends by conferring a straight conformation to protofilaments at the end. These ends are characterized by straight lattices that close upon addition of new subunits (Gigant et al., 2000; Janosi et al., 2002). As soon as the rate of hydrolysis exceeds that of GTP-subunit addition, the stabilizing GTP cap is hydrolysed before new GTP-subunits can be added. Protofilaments are released into a relaxed, bend conformation and depolymerize quickly (Arnal et al., 2000; Desai and Mitchison, 1997; Howard and Hyman, 2003; Janosi et al., 2002; Mandelkow et al., 1991). Transition from microtubule growth to shrinkage is called catastrophe whereas the reverse is called rescue (Mitchison and Kirschner, 1984). Before undergoing a transition microtubule ends can reside in a stabilized state, termed pause. Microtubule dynamics are commonly characterized by four parameters: Rates of microtubule growth and shrinkage and frequencies of catastrophe and rescue (Desai and Mitchison, 1997) (Fig 1).
General introduction

Figure 1: Dynamic instability of microtubules: The GTP-cap model. Adapted from Carvalho et al., 2003 and Desai and Mitchison, 1997.

Microtubule function

In animal cells microtubules participate in processes as diverse as intracellular transport, cell morphogenesis, chromosome segregation, cytokinesis and spindle orientation. The stochastic behaviour of microtubule dynamics is specifically modified by a variety of factors to regulate specific microtubule functions.

In dividing cells for instance, an increase in overall microtubule dynamics at the onset of mitosis is crucial for facilitated capture of microtubules to chromosomes and efficient spindle assembly (Desai and Mitchison, 1997; Hayden et al., 1990).

In other processes microtubule dynamics are used to generate force to move structures on microtubule plus ends (Howard and Hyman, 2003). This mechanism is utilized for instance for chromatid separation, where microtubule ends, attached to kinetochores, are induced to shrink while staying captured. This “capture-shrinkage” results in tearing the chromatids apart (He et al., 2001). A similar mechanism accounts for spindle positioning in yeast. In this process microtubules are captured at cortical sites and shrinkage results in movement of the spindle towards these sites (Adames and Cooper, 2000).

In migrating cells, a subfraction of microtubule ends is specifically stabilized in certain cortical regions, which leads to the generation of asymmetric microtubule arrays. Specific microtubule dynamics in adhesive regions of a migrating cell is thought to modulate actin based substrate adhesion complexes to regulate cell motility. In this process microtubules are thought to exert their effect indirectly by controlling the local balance between the two actin regulatory GTPases Rho and Rac (Rodriguez et al., 2003; Small and Kaverina, 2003).
Chapter 1

In mature neurons, microtubule dynamics are rather kept at a steady state to provide a stable platform for directed vesicle trafficking between cell body and the synapse (Desai and Mitchison, 1997).

Regulation of microtubule dynamics is also crucial to target microtubule ends to specific sites, where they either are captured or modulate the actin cytoskeleton, as demonstrated for migrating cells (Carvalho et al., 2003; Desai and Mitchison, 1997; Small and Kaverina, 2003).

Proper microtubule behaviour is essential for the cell and therefore has to be tightly controlled. In the case of chromosome segregation, cells have developed a mechanism that elicits a cell cycle arrest in response to errors in microtubule function at kinetochores. Disruption of this control mechanism leads to defects caused by microtubule dysfunction, e.g. genomic instability and aneuploidy, resulting ultimately in cell death or cancer (Biggins and Walczak, 2003)

**Microtubule regulatory proteins**

A number of microtubule binding proteins has been implicated in modulating microtubule dynamics and in mediating microtubule attachment to different targets.

**Stabilizing factors: Microtubule Associated Proteins (MAPs)**

This group of proteins comprises the neuronal MAPs, MAP1, MAP2, tau, and the ubiquitous MAP4, which is conserved from Drosophila to humans. MAPs bind to microtubule lattices and are believed to stabilize them by crosslinking several tubulin subunits (Fig. 2). In studies with purified tubulin, MAPs promote microtubule assembly and rescue, increase growth rates and suppress catastrophes to different extents (Desai and Mitchison, 1997). In dividing cells, MAP activity accounts for stabilization of the microtubule array during interphase. In young neurons, MAPs regulate microtubule polymerization during neuronal outgrowth while in mature neurons MAPs keep microtubule dynamics low to provide a stable platform for vesicle trafficking (Desai and Mitchison, 1997; Drewes et al., 1998).

XMAP215, isolated from Xenopus egg extracts, represents another group of MAPs, conserved from budding yeast to mammals (Kinoshita et al., 2002). XMAP215 specifically influences microtubule plus ends and increases the turnover of microtubules by increasing both polymerization and depolymerization rates and by reducing rescue frequencies. It is proposed to account for rapid rearrangements of microtubules in vivo (Desai and Mitchison, 1997).
General introduction

Destabilizing factors: kinesins and Op18/Stathmin

MAPs are counteracted by a group of destabilizing motor proteins represented by the family of mitotic centromere-associated kinesins, which involve the Xenopus XKCM1 and mammalian MCAK (Desai and Mitchison, 1997; Howard and Hyman, 2003). These catastrophe promoting factors preferably bind to bent protofilaments that are present in low numbers at growing plus ends. Its destabilization activity is due to induction of further protofilament bending at these ends (Moores et al., 2002; Niederstrasser et al., 2002). XKCM1/MCAK are involved in microtubule attachment at kinetochores by increasing overall microtubule dynamics at the onset of mitosis and locally around chromosomes (Walczak, 2000). Role of kinesin-related motors in modification of microtubule dynamics is conserved. In budding yeast, the kinesin related proteins Kar3 and Kip3 exhibit microtubule destabilizing activity to direct nuclear position (Miller et al., 1998; Stearns, 1997).

Op18/Stathmin is a catastrophe promoting protein first isolated in tumor cells, that has been implicated in regulating microtubule dynamics near chromosomes and in leading edges of migrating cells. Two mechanisms have been proposed for Op18/Stathmin destabilizing action: It could either act as a GAP to accelerate GTP hydrolysis or as a subunit sequestering factor (Walczak, 2000) (Fig.2).

+TIPs

A group of plus end binding proteins with microtubule stabilizing effects has recently been defined as plus end tracking proteins or +TIPs (Mimori-Kiyosue and Tsukita, 2003; Schuyler and Pellman, 2001a). Founding members of this group are the human proteins CLIP170, and EB1 (Brunner and Nurse, 2000; Perez et al., 1999; Tirnauer and Bierer, 2000). These proteins track and stabilize growing microtubule plus ends. They only fall off microtubule plus ends upon microtubule shrinkage. CLIP170 and EB1 are highly conserved and have homologues in Drosophila and Xenopus, in budding yeast, (Bik1 and Bim1), (Berlin et al., 1990; Pellman et al., 1995; Schwartz et al., 1997) and in fission yeast (Tip1 and Mal 3) (Mimori-Kiyosue and Tsukita, 2003; Schuyler and Pellman, 2001a).

Other +TIP representatives include CLASP (CLIP170 associated protein); the tumor suppressor Adenomatous Polyposis Coly (APC), an EB1 binding partner; the dynactin subunit p150Glued; Lis1, a protein involved in several dynein/dynactin dependent processes and its homolog in budding yeast Pac1; Stu2, budding yeast homologue of the XMAP215 (Howard and Hyman, 2003; Mimori-Kiyosue and Tsukita, 2003; Schuyler and Pellman, 2001a).
+TIP activity

+TIPs are implicated in a large variety of microtubule dependent processes. Besides regulating general microtubule dynamics in the cell, +TIPs have been shown to be involved in modulation of locally specific microtubule behaviour, in microtubule targeting to specific sites, and in microtubule interaction with their targets.

EB1

EB1 was shown to regulate overall microtubule dynamics during the cell cycle in animal cells. During mitosis EB1 is required to stabilize microtubules whereas in interphase cells it reduces microtubule pauses by affecting both rescue and catastrophe frequencies. (Rogers et al., 2002; Tirnauer et al., 2002b). EB1 is also implicated in microtubule stabilization at leading membrane edges of migrating cells, and in microtubule attachment to kinetochores and at cortical sites during spindle orientation (Lu et al., 2001; Palazzo et al., 2001a; Tirnauer and Bierer, 2000; Tirnauer et al., 2002a). EB1 homologue Bim1 in budding yeast is also involved in regulation of microtubule dynamics during the cell cycle. Similarly to EB1 it increases microtubule dynamics specifically during G1 by decreasing pauses indicating a conserved mode of action (Tirnauer et al., 1999). During mitosis Bim1 is involved in microtubule attachment at cortical sites to orient the mitotic spindle indicating, that +TIPs are conserved in their function (Korinek et al., 2000; Lee et al., 2000).

CLIP170

CLIP170 exhibits rescue activity on shrinking microtubule ends in the cell periphery, thereby maintaining microtubule ends in the periphery. (Komarova et al., 2002a; Komarova et al., 2002b). This is different to the anti-catastrophe function of its yeast homologs Tip1 and Bik1. (Brunner and Nurse, 2000; Lin et al., 2001). Tip1 is important to stabilize microtubules while they grow from the center towards the cell poles by preventing catastrophe when microtubule ends meet the lateral cortex. Only when microtubules reach the cell poles they undergo catastrophes (Brunner and Nurse, 2000). In mammalian cells, overall microtubule outgrowth from cell center to the periphery appears to be CLIP170 independent. (Komarova et al., 2002a).

Plus end tracking: treadmilling

Important questions to understand effects of individual +TIPs on microtubules are, how +TIPs specifically track growing ends, how they interact with microtubule targets, how they regulate microtubule dynamics and how they are regulated themselves in localization and activity.
General introduction

**Preferential binding to plus end lattice**

It has remained mainly unclear, by which mechanism +TIPs regulate microtubule dynamics. In analogy to MCAK which preferably binds to bent protofilaments, +TIPs have been proposed to bind with high affinity to straight open lattices at microtubule plus ends and to specifically stabilize these. Decrease of binding affinity upon closure of open lattices to a tubule would result in +TIP dissociation and this difference in affinity binding would account for plus end tracking appearance of GFP-tagged +TIPs (Diamantopoulos et al., 1999; Howard and Hyman, 2003). GFP-CLIP170 binding along plus end stretches that resemble open lattices could be indicative of such a preferred binding to specific plus end structures (Arnal et al., 2000; Diamantopoulos et al., 1999; Perez et al., 1999). Treadmilling e.g. association of individual molecules with plus ends and dissociation from tubule walls, was supported for CLIP170 and EB1 by Fluorescence speckle analysis (FSA) (Carvalho et al., 2003) (Fig.2).

**Compolymerization**

Specific accumulation of +TIPs on growing microtubule plus ends was also proposed to be the result of copolymerization with tubulin subunits, as several +TIPs were demonstrated to bind tubulin subunits in vitro (Carvalho et al., 2003). In combination with rapid dissociation from tubule walls this could also lead to relative accumulation at plus ends (Carvalho et al., 2003). Recent FRAP data with EB1 revealed, that this +TIP dissociates from microtubule shafts and ends with comparable kinetics. This indicates, that accumulation of GFP-EB1 fluorescence at the ends is the result of copolymerization with subunits (Tirnauer et al., 2002b) (Fig.2). Selective dissociation from tubule walls as a result of phosphorylation was demonstrated for p150Glued. (Vaughan et al., 2002). This modification could also account for fast dissociation from tubule walls for other +TIPs like CLIP 170.

Further elucidation of the mechanism of +TIP binding to growing microtubule plus ends could help to assess their mechanism of regulation of microtubule dynamics. For instance has it remained unclear, how CLIP170 could exert its rescue effect on shrinking microtubules. Other mechanism for plus end tracking include plus end-directed motor transport and “hitchhiking” along with other +TIPs (Carvalho et al., 2003).
Figure 2 Models for mechanisms of microtubule binding, stabilizing and destabilizing activities of different microtubule regulatory proteins. Adapted from Carvalho et al., 2003

**+TIP interaction**

Effects of +TIPs on microtubule dynamics vary with different cell types, cellular regions or cell cycle phases, as has been described at the examples of EB1 and CLIP170 and their homologues. This suggests that +TIPs are subjected to complex spatial and temporal regulation by various factors inside the cell. A complex regulation of +TIPs in vivo is also supported by the fact that in vitro properties of purified proteins often deviate from those in living cells (Popov and Karsenti, 2003; van Breugel et al., 2003).

Different +TIPs colocalize at microtubule plus ends suggesting that these proteins could cooperate and influence each other in their effect on microtubule dynamics and attachment. Moreover, multiple interactions among +TIPs and between +TIPs and microtubule targets have recently been revealed biochemically. This indeed suggest the existence of microtubule plus end complexes (Galjart and Perez, 2003; Gundersen, 2002; Mimori-Kiyosue and Tsukita, 2003).

Indication for mutual regulation of +TIPs in their effects on microtubule dynamics come for instance from studies with p150Glued and EB1. These +TIPs colocalize on microtubule plus ends. Microtubule binding occurs independently of each other. However EB1 and p150Glued
were shown to physically interact and thereby could influence each other on plus ends (Ligon et al., 2003; Tirnauer et al., 2002b). Indeed, disruption of p150Glued in cultured cells augmented the characteristic effect of EB1 to promote microtubule elongation. This indicated that p150Glued influences EB1 activity in vivo. (Ligon et al., 2003).

CLIP170 also physically interacts with p150Glued and has been proposed to target dynein to microtubule plus ends (Goodson et al., 2003; Komarova et al., 2002a). Although no direct interaction has been described for CLIP170 and EB1 they could influence each other indirectly via their common interaction with p150Glued (Galjart and Perez, 2003).

+TIP complexes: how to specify microtubule function

Versatile interaction properties between +TIPs could be utilized to specify and localize their regulatory effects on microtubule behaviour.

+TIP complexes in leading edges of migrating cells

While for instance overall localization of EB1 accounts for its individual effect on general dynamic changes in response to cell cycle regulators in dividing cells, its specific stabilizing effect to target microtubule ends to specific regions such as leading edges of migrating cells depends on its interaction with APC (Mimori-Kiyosue et al., 2000b; Nakamura et al., 2001). Local interaction between APC and EB1 is based on asymmetric localization of APC to microtubule ends in these regions. Likewise, specific function of CLIP170 in leading edges of migrating cells could be mediated by interaction with CLASP. Like APC, CLASP is asymmetrically recruited to microtubule ends in these regions (Gundersen, 2002; Schuyler and Pellman, 2001a).

Both +TIP complexes, APC-EB1 and CLIP170-CLASP, are involved in targeting microtubule polymerization to leading edges of migrating cells (Schuyler and Pellman, 2001a). At the same time these complexes are proposed to act synergistically to activate the small GTPase Rac in these regions (Small and Kaverina, 2003). Rac activity is responsible for actin based cell motility. Rac in turn promotes further microtubule stabilization in this cortical region by inhibition of microtubule destabilizer Stathmin/Op18 (Daub et al., 2001).

This example also illustrates the complex interplay between the actin and microtubule cytoskeleton. In addition, EB1 and CLIP170 have recently been shown to directly interact with effectors of the small GTPases Cdc42/Rac and Rho. These interactions suggest a complex network in which the small GTPase Cdc42 acts as a key regulator to coordinate formation of actin structures and microtubule dynamics in polar cortical regions. (Gundersen, 2002; Small and Kaverina, 2003).
+TIP complexes at kinetochores

Similar +TIP complexes are involved in microtubule attachment at the kinetochores in mammalian cells (Mimori-Kiyosue and Tsukita, 2003). However, during this process these complexes fulfill complementary roles. The EB1-APC complex functions to specifically attach polymerizing microtubules at kinetochores during prophase/prometaphase (Tirnauer et al., 2002a). At the onset of mitosis other +TIPs including Lis1, CLIP170 and CLASP and dynein are recruited microtubule independently to kinetochores. While CLASP is proposed to counteract MCAK to stabilize microtubule ends, CLIP170 regulates dynein/dynactin function via its interaction with Lis1 (Coquelle et al., 2002; Maiato et al., 2002; Mimori-Kiyosue and Tsukita, 2003).

The complex interaction network of +TIPs and cortical components provides a broad range of specification possibilities. To understand the physiological functions of +TIPs it will be important to further clarify how different +TIP interactions influence each other in specific cell types and functions and how activity of different complexes and interactions is locally regulated to account for specific microtubule behaviour and function.

Regulation of microtubule regulatory proteins

To allow proper dynamic rearrangements of the cytoskeleton during cell division and at specific sites, MAPs and +TIPs themselves have to be tightly regulated. Controlling microtubule association of MAP and +TIP provides one way to regulate their activity. Kinases called MARKs (Microtubule Affinity-regulating Kinases) have been identified in human neurons that decrease affinities of the stabilizing MAPs tau, MAP2 and MAP4 by phosphorylating them in their microtubule binding domains (Drewes et al., 1998; Drewes et al., 1997; Trinczek et al., 2004). MARK belong to a family of Ser/Thr kinases with four human members, MARK 1 to MARK 4, the yeast homologue Kin1 and2, C.elegans Par1 and kin1 in fission yeast, all implicated in cell polarity (Barral et al., 1999; Bohm et al., 1997; Drewes and Nurse, 2003).

Also interaction of +TIP with microtubules has been shown to be regulated by phosphorylation. CLIP170 association with microtubules is enhanced when phosphorylated by mTOR (rapamycin sensitive kinase) (Carvalho et al., 2003; Choi et al., 2002), while p150Glued is released from microtubules when phosphorylated by PKA (Carvalho et al., 2003; Vaughan et al., 2002). Also EB1 affinity for microtubule could be regulated by a balance between kinase and phosphatase activity, as dissociation from microtubules occurs with lower dynamics during interphase than during mitosis (Tirnauer et al., 2002b).

Stathmin/Op18 has also been shown to be regulated by phosphorylation. Its activity is high in interphase cells (Gavet et al., 1998), and inhibited by phosphorylation during mitosis.
General introduction

Multiple kinases have been implicated in Stathmin phosphorylation and gave rise to a model in which Stathmin rearranges the microtubule cytoskeleton in response to a variety of signal transduction pathways (Walczak, 2000).

Role of microtubules in coordination of spindle function with cell cleavage and cell polarity

To ensure proper chromosome segregation cell cleavage must only occur between sister chromatides and after they have been separated by the mitotic spindle at anaphase. In addition, chromosome separation has to be oriented properly according to cell architecture. During asymmetric cell division, spindle orientation according to the polarity axis of a cell decides if a cell divides symmetrically or asymmetrically. It thus determines cellular fates and has to be tightly regulated to ensure for instance proper tissue homeostasis or generation of cell diversity during development.

Microtubules function in both processes by establishing a communication network to transduce spatial information between spindle poles and different cortical structures.

Role of microtubules in spindle orientation

In the case of spindle orientation microtubules act to transduce cortical topology to the spindle. Asymmetrically distributed cortical components are supposed to serve as microtubule attachment sites and differentially regulate microtubule dynamics to adjust spindle orientation according to cortical polarity. According to a conventional view, microtubule capture at polar sites is mediated by a random search and capture mechanism. This model implicates, that microtubules from both spindle poles undergo frequent dynamic switches to efficiently scan the whole cortex for specific capture sites. However, this model does not explain, how such a stochastic capture mechanism prevents attachment and subsequent positioning of both spindle poles in the same part of a cell, which would result in misorientation of chromosome segregation.

How spindle orientation is thought to be coordinated with cell polarity during asymmetric cell division is discussed at the example of the model organisms C.elegans and Drosophila in (Kusch et al., 2003) page 562-563.

In general, not much is known about the molecular link between cell polarity and microtubule regulation to achieve proper spindle orientation in higher eukaryotes.

Only recently have +TIPs such as APC and EB1 been implicated in spindle orientation during Drosophila development (Lu et al., 2001; McCartney et al., 1999; McCartney et al., 2001).
However, it has remained unclear, how these proteins are involved in regulation of microtubule behaviour according to specific cortical cues.

**Role of microtubules in spatial coordination of cleavage apparatus and mitotic spindle**

In many eukaryotic cells the mitotic spindle itself determines the site of cleavage. In this process, microtubules transmit positional information from the spindle towards the cortex to direct the position of the cleavage machinery to the cortex between separated spindle poles. This is different to plant and yeast cells where the cleavage site is determined prior to mitosis and the mitotic spindle has to adjust its position accordingly (Guertin et al., 2002) (Fig. 3).

![Mechanistic comparison of cleavage site placement](image)

*Figure 3* Mechanistic comparison of cleavage site placement: In higher plants, budding and fission yeast the cleavage site is determined prior to mitosis: in plants by microtubules that first position the nucleus in the center and then condense into a structure called phragmosome which marks the future cleavage site at the cortex; similarly in fission yeast, by a protein Mid1, that diffused out of a centrally (microtubule-dependently) positioned nucleus; in budding yeast by a bud scar derived from the former division that serves as a landmark to direct the septin ring and polar cell growth. In animals cleavage site is determined by microtubules emanating from the spindle at the onset of anaphase (see below). Taken and adapted from Guertin et al., 2002.
Most somatic eukaryotic cells divide by fission at their equatorial plane giving rise to two equal daughter cells. With exception of plant cells cleavage is accomplished by constriction of a contractile ring that is composed of filamentous actin and myosin typeII units. Furrow ingression upon ring contraction is facilitated by targeted vesicle fusion. In yeast additional septum formation is essential for cytokinesis. In a final step called abscission daughter cells are physically separated (Field et al., 1999; Glotzer, 2001; Guertin et al., 2002; Scholey et al., 2003; Wang et al., 2003).

Assembly of the cleavage apparatus and the subsequent actomyosin ring contraction and cell separation are well conserved from fungi to humans. However, positioning of the cleavage apparatus with respect to the mitotic spindle is supposed to rely on different mechanisms in different organisms. However in all organism this process depends on microtubule function (Glotzer, 2001; Guertin et al., 2002; Nanninga, 2001) (Fig. 3).

**Models for cleavage site placement in animals**

Animal cells rely on a versatile microtubule cytoskeleton which signals position of the actomyosin contractile ring to the cortex. Based on results obtained in several experimental systems different microtubule subsets, e.g. astral and midzone microtubules, may be responsible for cleavage apparatus placement in different systems.

**Astral microtubule stimulation at the equator**

Indication for a stimulatory effect of astral microtubules on cortical contractility and furrow induction at the equator initially came from studies in sand dollar eggs performed by Rappaport in 1961 (Rappaport, 1961). These cells were manipulated such that they contained two mitotic spindles. Ectopic furrow formation between microtubule asters belonging to different spindles gave rise to the astral stimulation model (Oegema and Mitchison, 1997) (Fig. 4).

**Astral microtubule inhibition at cell poles**

The astral relaxation/inhibition model considers astral microtubules to transduce an inhibitory signal onto the contractile potential of the cortex (Oegema and Mitchison, 1997; White and Borisy, 1983). This model is consistent with computer simulations that demonstrated a relative high microtubule density at cell poles compared to the equator in sae urchin eggs implicating a maximal inhibition of contraction at the cell poles. (Yoshigaki, 1999). Given that the contractile potential is equally distributed over the cortex, maximal inhibition at the poles would cause relative high contractility in low microtubule density areas like the equator.
Chapter 1

(Mandato et al., 2000; Oegema and Mitchison, 1997). The idea of inhibitory signaling by astral microtubules is also supported by observations in cells that exhibit contractility all over the cortex upon disruption of microtubules (Canman et al., 2000). This is also consistent with recent indications that low microtubule density at the equator is crucial for furrow induction in *C. elegans* embryos (Dechant and Glotzer, 2003). A low microtubule density at the equator is achieved through tight bundling of midzone microtubules in conjunction with spindle elongation (Dechant and Glotzer, 2003) (Fig. 4).

**Equatorial stimulation by differential dynamics of astral microtubules**

In contrast to differential distribution of microtubule densities over the cortex, a recent study implicates a differential in microtubule dynamics to induce furrowing in cultured cells. A specific subset of astral microtubules has been observed to establish stable interactions with cortical sites at the equatorial cortex while other astral microtubules contact the cortex only transiently and stay highly dynamic (Canman et al., 2003). Alternatively these authors propose a model combining density and dynamics gradient. Accordingly, a steep gradient of stabilized microtubules contacting flanking regions of the equatorial cortex together with a low microtubule density at the equator itself would signal furrowing at the equator (Canman et al., 2003; Dechant and Glotzer, 2003). Stabilization of the specific microtubule subset is thought to be due the factors derived from chromosomes, as these microtubules are associated with separated chromosome masses at their minus ends (Canman et al., 2003). However, nature of these factors awaits elucidation (Fig. 4).

**Midzone microtubule stimulation at equator**

A large number of studies has implicated microtubules at the spindle midzone in signaling furrow induction directly to the overlying equatorial cortex. Supporting this idea, in various experimental systems furrow positions correlated with displaced spindles or midzone microtubule bundles, suggesting a tight correlation between these structures (Alsop and Zhang, 2003; Cao and Wang, 1996; Maddox and Oegema, 2003; Oegema and Mitchison, 1997; Savoian et al., 1999; Wheatley et al., 1998; Wheatley and Wang, 1996). Midzone microtubules are organized in antiparallel interdigitating bundles that become tightly packed at the onset of anaphase to form a central spindle. This process depends on plus end directed kinesin-like motor proteins and a group of proteins called chromosomal passengers (Gatti et al., 2000b; Guertin et al., 2002; Severson et al., 2000).

The central spindle has been proposed to signal furrow position to the overlying cortex and at the same time to keep nuclei apart (Wheatley et al., 1998). A similar function has been proposed for a presumed midzone microtubule equivalent in *S. pombe*, the Post Anaphase
General introduction

Array (PAA). This cortical microtubule array is required to keep the contractile ring in its position after anaphase to ensure cleavage between separated genomes (Pardo and Nurse, 2003) (Fig. 4).

Figure 4 Models for cleavage site determination in animal cells. Arrows indicate the source of signaling for cleavage furrow induction. Adapted from Kaltschmidt 2002.

Molecular insight into cleavage furrow induction

The apparently controversial results implicating different microtubule subsets in furrow induction lead to the opinion that cleavage site placement is not conserved among different cell types and systems (Wang, 2001).

One of the reasons for little elucidation in this field might also be the difficulty to temporally dissect furrow induction and furrow formation in animal cells. So far, no specific furrow displacement mutants have been identified which would help to experimentally separate the two cytokinetic steps and to isolate molecules and microtubule subtypes responsible for each step. Thus, with the nature of the molecular signal for furrow induction also the microtubule subtype, that would transport this signal have remained unclear.

So far two types of molecules have been suggested as candidates for furrow signaling, however, evidence for their roles are rather indirect.

Chromosomal passengers

Chromosomal passengers, implicated in central spindle formation, have been envisioned as molecular signals for cleavage furrowing due to their relocalization from centromeres to midzone microtubules at the onset of anaphase and later to the equatorial cortex (Adams et al., 2000; Guertin et al., 2002; Severson et al., 2000; Wang, 2001). However, studies in echinoderm eggs and insect spermatocytes demonstrated that chromosomes might not play a
Chapter I

role in furrow induction, contrasting the view of chromosomal passengers as furrow signals (Alsop and Zhang, 2003; Wang, 2001). Surprisingly, recent studies revealed presence of chromosomal passengers both on the midzone microtubules and on astral microtubules (Murata-Hori et al., 2002; Wang, 2001). This finding could reconcile the role of astral microtubules in furrow signaling in the absence of chromosomes and midzone microtubules (Alsop and Zhang, 2003; Canman et al., 2000; Wheatley et al., 1998). Cell type differences in the proportions between astral microtubules and spindle midzone microtubules as well as different in relations between cell size and spindle size could then account for the role of either microtubule subtype in cleavage site placement (Fig. 5).

Small GTPase regulators

Recruitment of actomyosin units to the equatorial cortex involves rearrangement of the whole cortical actin network in a process called cortical flow (Mandato et al., 2000). Given, that RhoA is essential for actomyosin ring formation and contraction, and is activated early in cytokinesis, also regulators of RhoA were implicated in spatiotemporal control of furrow initiation (Glotzer, 2001; Glotzer, 2003). Interestingly, RhoA GEF and Rac GAP have been colocalized at the cleavage furrows in a microtubule dependent way. Chromosomal passenger kinesin KLP3 was shown to be responsible for Rac GAP and Rho GEF localization and interaction at the equatorial cortex implicating midzone microtubules in this process (Somers and Saint, 2003). However, it will have to be clarified, if this particular chromosomal passenger also localizes to astral microtubules as has been described for AuroraB (Murata-Hori et al., 2002).

Figure 5 Different proportions of midzone and astral microtubules in different cell types could account for contribution of either microtubule subtype on cleavage furrow signaling, transmitting the same or different molecular signals. Adapted from Wang, 2001.
Interaction between microtubules and cortical actin

Astral microtubules were suggested to participate in cleavage furrow induction by controlling the distribution of actomyosin contractile potential over the cortex (Mandato et al., 2000). Suggestions for a regulatory role of astral microtubules are based on studies, in which different microtubule dynamics were shown to have different effects on the cortical actin cytoskeleton. Microtubules are proposed to modulate the actin cytoskeleton indirectly by regulating the activity of small GTPases Rho and Rac. While microtubule polymerization activates Rac GTPase, which is responsible for actin polymerization and inhibition of contractility (Waterman-Storer et al., 1999) microtubule depolymerization activates Rho GTPase, counteracting the Rac effect (Ren et al., 1999). In turn, small GTPases can regulate microtubule dynamics. Rac GTPase for instance activates PAK (p21 activated kinase) which inhibits the microtubule destabilizer Stathmin/Op18 (Daub et al., 2001; Rodriguez et al., 2003).

As suggested by Canman 2003 (see above) stabilization of microtubules at regions flanking the equatorial cortex might specifically trigger contractility at the cell equator by eliciting a small GTPase signaling cascade. These authors propose a differential in microtubule dynamics between equatorial cortex and its flanking regions to cause cortical contractility at the equator. This could occur in parallel to actin modulation during cell migration. In this process a differential in microtubule dynamics between the rear and the leading edge of a migrating cells is proposed to allow cell motility (Wittmann and Waterman-Storer, 2001). This relation between microtubule and the actin cytoskeleton has been shown to be mediated by various +TIPs such as APC, EB1, CLIP and CLASP (Small and Kaverina, 2003) (see above). In analogy, these +TIPs could as well account for a modulative effect of microtubule polymerization on contractile ring assembly.

Alternatively, direct transport of actomyosin units at the ends of polymerizing astral microtubules has been proposed to accumulate these at the equatorial cortex between cell poles (Foe et al., 2000).

Altogether, the correlation between microtubule dynamics and actomyosin contractility integrates several models in cleavage furrow induction and might reflect the in vivo situation best.

Budding yeast as a model system to study microtubule function

The microtubule cytoskeleton of budding yeast *Saccharomyces cerevisiae* comprises only few astral microtubules that emanate from the Spindle Pole Bodies (SPB), the yeast equivalents of
centrosomes. Due to closed fungal mitosis the SPBs stay embedded inside the nuclear envelope and nucleate two classes of microtubules: astral or cytoplasmic microtubules and nuclear microtubules that assemble the mitotic spindle upon SPB duplication and separation. Experiments disrupting specifically cytoplasmic microtubules using conditional-lethal mutant alleles of the beta-tubulin subunit (tub2-401) (Read et al., 1992; Sullivan and Huffaker, 1992) revealed that spindle positioning relies on the function of cytoplasmic microtubules. The low number of astral microtubules and amenability of yeast to genetics as well as the fact, that in yeast microtubule function is restricted to spindle positioning render this organism a convenient system to dissect the mechanism how microtubule attachment achieves coordination of the spindle position with cortical polarity and the prefixed cleavage site.

Budding yeast represents an extreme case of asymmetric cell division and cell polarity. It divides asymmetrically by forming a bud, the future daughter cell, at one site of the mother cell. Cleavage takes place at the junction between mother and bud, the mother bud neck. In budding yeast the cleavage plane is physically linked to the budding site, which at the same time determines the polarity axis of the cell.

**Cell polarity in yeast**

Similar to higher eukaryotes in budding yeast cell polarity and cleavage site organization are controlled by Rho like small GTPases, Cdc42 and Rho1, that regulate organization of the actin cytoskeleton through different effectors (Glotzer, 2001; Somers and Saint, 2003). During the cell cycle actin dependent polarized cell growth is first restricted to the incipient bud site and the bud cortex. During cytokinesis it becomes redistributed to the bud neck to direct formation of the actomyosin ring and synthesis of the septum (Casamayor and Snyder, 2002). While in higher eukaryotes the actin cytoskeleton can be controlled by microtubules either directly or indirectly via regulation of small GTPases in processes like furrow formation and cell migration in animal cells (see above), this is not the case in budding yeast (see also Fig 3).

Cdc42 acts through its effectors Bnr1 and Bni1, two formin family members, to polymerize actin cables. These originate at branched actin assemblies in the bud called actin patches and transverse the bud neck in a polar manner. Polarized cell growth involves type V myosin based vesicle transport along polarized actin cables towards the bud followed by vesicle fusion with the membrane (Casamayor and Snyder, 2002; Kozminski et al., 2003). The polar actin cytoskeleton in the bud also provides a platform to localize a variety of daughter specific determinants to the bud cortex, like Lte1, a component of the MEN pathway, Ash1 mRNA,
the product of which is involved in mating type switching, and proteins important for microtubule attachment (Beach et al., 2000; Jensen et al., 2002; Yin et al., 2000).

Cleavage site organization yeast

Before cytokinesis Cdc42 reorients the actin cytoskeleton to the bud neck to target vesicle delivery here and to localize factors important for actomyosin ring formation. Assembly of this ring depends on IQGAP homolog Iqgl that recruits actin filaments to the neck, and on Rho1-activated formins, which help to assemble the contractile ring (Palmieri and Haarer, 1998; Tolliday et al., 2002). Recruitment of other cleavage apparatus components depends on the septins, conserved GTP binding proteins, that are localized to the incipient bud site in a Cdc42 dependent way and here form a ring. Septins were found in a genetic screen to be essential for cytokinesis (Glotzer, 2001). The septin ring has been proposed to provide a scaffold for proteins that are required for both actomyosin ring assembly and septum formation. These proteins include type II myosin Myo1, that in turn recruits Iqgl and actin filaments to the neck, as well as factors involved in cell wall synthesis (Longtine and Bi, 2003; Longtine et al., 1996).

Spindle positioning in yeast

Yeast cells face the challenge that they have to position their spindle from a random position in the mother cell to an predetermined, eccentriclly placed cleavage site. At the same time the spindle has to align along the mother bud axis to target elongation through the narrow mother-bud neck to allow proper spindle pole segregation (see red arrows below. Adapted from Guertin et al., 2002).

Time lapse microscopy with GFP-labeled microtubules revealed transient microtubule capture to cortical sites as a mechanism to pull or push the spindle around. Microtubules were proposed to randomly attach to specific cortical sites in the bud in a so called search and capture mode ((Adames and Cooper, 2000; Yeh et al., 2000).
Chapter 1

Two pathways

Genetic studies revealed that spindle positioning depends on two redundant pathways, one acting during preanaphase to achieve spindle movement towards the future cleavage site at the neck and its alignment along the mother bud axis. The second pathway ensures maintenance of the right orientation and pulling the spindle through the bud neck, completed by spindle elongation in the direction of the mother bud axis. Disruption of components of one pathway results in nuclear migration defects, which are partially overcome by the second pathway, rendering the cells sick but viable. Deletion of both pathways results in cell death (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Miller et al., 1998) (Fig.6). These two pathways involve two major types of microtubule attachment at the bud cortex.

Preanaphase attachment

The major component of microtubule attachment responsible for preanaphase spindle movements is the protein Kar9. Kar9 had been proposed to provide a capture site at the bud cortex. Localization studies with overexpressed forms of Kar9 visualized it as cortical dots in the bud, along microtubule arrays and on the spindle pole (Beach et al., 2000; Miller et al., 1998; Miller et al., 1999). Biochemical studies confirmed its ability to interact with the cortical actin cytoskeleton via the myosin type V Myo2 (Yin et al., 2000). Myo2 dependent transport along polarized actin cables has been proposed to account for asymmetric cortical localization of Kar9. (Beach et al., 2000; Yin et al., 2000). Interaction with the +TIP Bim1, the yeast EB1 homologue, at microtubule ends mediates transient microtubule capture which induces microtubule shrinkage and pulling of the spindle near the bud neck and alignment with the mother bud axis (Adames and Cooper, 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000; Yeh et al., 2000) (Fig6).

Anaphase attachment

During anaphase, the +TIP Bik1 is proposed to interact with cortical dynein/dynactin complex, that mediates lateral microtubule gliding and final spindle pulling inside the neck (Adames and Cooper, 2000; Berlin et al., 1990; Carminati and Stearns, 1997; Heil-Chapdelaine et al., 2000a; Heil-Chapdelaine et al., 2000b; Yeh et al., 2000) (Fig6).
**General introduction**

**Kar9 pathway**

- Spindle positioning and alignment prior to anaphase

**Dhc1 pathway**

- Spindle pulling into neck during anaphase

**Figure 6** The two genetically redundant pathways for spindle positioning in budding yeast:
The preanaphase pathway is characterized by Kar9 mediated microtubule capture shrinkage (top panel left: microtubules in pink, arrow indicates the direction of spindle movement following microtubule attachment) while dynein mediates lateral microtubule gliding at the bud cortex during anaphase (top panel right). The micrographs (low panel) illustrate typical nuclear migration phenotypes of the respective mutants. Cells are arrested before mitosis with hydroxyurea. DNA is stained with DAPI to visualize nuclei.

**Open questions for spindle positioning in yeast**

While in animal cells an abundant number of dynamic microtubules could provide efficient targeting of specific capture sites budding yeast relies on a low number of astral microtubules or microtubule bundles, that transmit cortical information onto the spindle. Given that yeast microtubules are less dynamic too, the question arises, how these few microtubules efficiently reach distant attachment sites in the bud.

However, little is known about the molecular basis for regulation of microtubule attachment. Neither has it been clarified, how the process of capture shrinkage at the bud cortex is controlled. Also the molecular constitution of bud cortex attachment has remained obscure. Actin cables and their nucleators such as the formins Bni1 or Bnr1 perform a rather indirect role by providing localization platforms for capture factors such as Myo2 and Kar9. How these are regulated and which role they play in microtubule dynamic has neither been clarified.
**Spatial coordination of the spindle and cleavage site**

Another open question is, how a single type of attachment at one proposed site, namely the bud cortex, could achieve diverse aspects of spindle positioning such as spindle coordination with the bud neck and spindle alignment along the mother bud axis. It is for instance not clear, how bud attachment prevents pulling of the whole spindle into the bud. The spatial coordination between the cleavage site and the mitotic spindle in budding yeast has not been unraveled yet. While in animal cells actomyosin ring position is directed by the mitotic spindle in a microtubule dependent process, in yeast the spindle has to respond to predetermined cortical sites. Cleavage site placement and spindle position occur at different time points and rely on different mechanisms. Cleavage site placement depends on cortical components, whereas spindle positioning depends on microtubule contacts with the bud cortex. No direct interaction between these structures have been observed so far.

**Spindle alignment**

Yeast SPB duplicate once per cell cycle by a conservative mechanism resulting in a pre-existing “old” and a newly formed SPB. In this way the yeast spindle gains intrinsic polarity. Old and new SPB can be visually distinguished by labelling a SPB component with a slow folding RFP variant. Recent experiments have revealed that the different SPB are not randomly distributed between mother and daughter. The mother always inherits the newly synthesized SPB while the bud inherits the old SPB (Pereira et al., 2001). This indicates existence of a mechanism that specifies microtubule attachment for one spindle pole to achieve its destination. However, in yeast like in higher eukaryotes, it has not been elucidated yet, which mechanism underlies asymmetric spindle pole behaviour. Microtubule have long been thought to probe the cortex in a random fashion to search for attachment sites at the bud cortex where they were incidentally captured (Adames and Cooper, 2000; Desai and Mitchison, 1997; Schuyler and Pellman, 2001b; Shaw et al., 1997; Yeh et al., 2000). How would random search and capture of microtubule at the bud cortex prevent positioning of both spindle poles in the bud, and how is spindle alignment maintained?

Tolerated spindle orientation is restricted to few coordinates. Slight misorientation off the mother bud axis can result in spindle elongation inside the mother cell and cell death as a result of accumulation of two sets of DNA in the mother. Such limitations provoke a stringent and specific mechanism to achieve pronounced and at the same time precise spindle reorientation to ensure survival of descendant cells.

These questions could shed light on basic regulatory mechanisms for microtubule behaviour. Given also a high conservation of implicated +TIPs among eukaryotes, studying microtubule function in yeast could help to elucidate these principles.
**Aim of the thesis**

The aim of my thesis was to understand the molecular mechanism of microtubule function underlying spatial regulation of spindle positioning in budding yeast. The problems the mitotic spindle faces can be formulated in the following questions that were addressed in the two parts of the work:

1. how do microtubules achieve proper spindle positioning at the cleavage plane prior to anaphase, or more specifically, what kind of signaling mechanism exists between spindle and bud neck in this respect?

2. how do microtubules ensure that the spindle aligns within the mother bud axis and maintains this orientation until spindle elongation occurs?

3. what is the molecular basis for microtubule function in these processes?

At the time the thesis started, not much was known about how budding yeast specifically achieves spatial coordination between the mitotic spindle and the predefined cleavage site at the mother bud neck. Neither had spindle alignment along the polarity axis in itself been addressed. Spindle pulling from the bud cortex was the only clue how to imagine movement of the mitotic spindle towards the bud before anaphase extends it through the neck into the bud. While spindle positioning had been thought to be predominantly controlled by microtubule interaction with the bud cortex - no interaction between the spindle and the cleavage plane had been described. Since in yeast cleavage site determination occurs microtubule independently through bud site selection in G1/S these processes had been thought to occur independently of each other.

However, we imagined tight regulation of microtubule function in specific aspects of spindle positioning - coordination with cleavage site and polarity axis- to be of particular importance in asymmetrically dividing budding yeast to ensure proper chromosome segregation.

Preliminary observations by Yves Barral indicated a role of the septins at the mother bud neck in spindle positioning. These lead to the question, if septins could constitute an interaction site for microtubule as a communication platform between the mitotic spindle and cleavage site to guide their spatial coordination. Interestingly, septins localize three kinases Hsl1, Nim1 related kinases, Gin4 and Kcc4 to the bud neck, which have sequence homology to MARK like kinases. This indicates that these kinases could play additional roles in microtubule dependent processes besides their function in sensing septin structure (Barral Y et al., 1999). As microtubule function determines spindle positioning in budding yeast, kinases could play a role in this process.

I applied high speed 4D fluorescence microscopy in combination to a genetic approach to test, if and how mutants of septin and SDK influence microtubule function in preanaphase cells. Analysis of microtubule dynamics in cells with a 1µm spindle have been performed.
Aim of the thesis

using GFP-alpha-tubulin. Colabeling of the cortical microtubule capture factor Kar9 was instrumentalized to facilitate the assessment of microtubule attachment. Observations of a strikingly new localization for the presumed cortical capture factor Kar9 when expressed at endogenous levels, using a chromosomal Kar9-GFP construct, lead me to reinvestigate new parameters of Kar9 localization in the second part of my thesis. For this I applied two color 4D microscopy and Fluorescence Recovery After Photobleaching (FRAP). In the course of this study I aimed at elaborating the consequences of Kar9 localization for the mechanism of microtubule attachment to the bud to specifically ensure proper spindle alignment with the mother bud axis.
Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast

1Justine Kusch, 2Anne Meyer, 2Michael P. Snyder and 1Yves Barral

1Institute of Biochemistry, Federal Institute of Technology, Universitätsstrasse 16, CH-8092 Zürich, Switzerland.
2Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut, USA.

Correspondence should be addressed to Y.B.
E-mail: yves.barral@bc.biol.ethz.ch

The work described in this Chapter was published in:
GENES&DEVELOPMENT, Vol 16, 1627-1639, in July 2002
Abstract

Cell division is the result of two major cytoskeletal events: partition of the chromatids by the mitotic spindle and cleavage of the cell by the cytokinetic apparatus. Spatial coordination of these events ensures that each daughter cell inherits a nucleus. Here we show that capture and shrinkage of yeast astral microtubules at the bud neck is required to position the spindle relative to the cleavage apparatus. Capture required the septins and the microtubule-associated protein Kar9. Like Kar9-defective cells, cells lacking the septin ring failed to position their spindle correctly and showed an increased frequency of nuclear mis-segregation. Microtubule attachment at the bud neck was followed by shrinkage and a pulling action on the spindle. Enhancement of microtubule shrinkage at the bud neck required the Par-1-related, septin-dependent kinases (SDK) Hsll and Gin4. Neither the formin Bnr1 nor the actomyosin contractile ring was required for either microtubule capture or microtubule shrinkage. Together, our results indicate that septins and septin-dependent kinases may coordinate microtubule and actin functions in cell division.

Introduction

In animal cells, the cleavage apparatus, composed of septins and an actomyosin contractile ring, assembles at the cell cortex during anaphase (Field et al. 1999a, b; Hales et al. 1999). The plane of this ring defines the plane of cleavage. Proper positioning of the cleavage plane between the two spindle poles ensures the segregation of the replicated chromosomes between the daughter cells. Previous studies showed that spatial coordination of spindle and cleavage apparatus relative to each other depends on astral microtubules and the central spindle (see for review Strome 1993; Gatti et al. 2000).

In budding yeast, the nuclear envelope does not break down during mitosis. The microtubule organizing centers, or spindle pole bodies, are embedded in the nuclear envelope and the spindle forms inside the nucleus. Spatial coordination of the spindle and the cleavage apparatus is achieved through migration of the nucleus to the bud neck (Shaw et al. 1998; Segal et al. 2001). As in animal cells, this coordination event is mediated by astral microtubules (Sullivan et al. 1992; Segal and Bloom 2001). These microtubules, also called cytoplasmic microtubules, are nucleated on the cytoplasmic side of the spindle pole bodies and interact with the cellular cortex with their plus ends. Mutations in tubulin have been isolated that specifically disrupt cytoplasmic but not intra-nuclear microtubules. These mutations prevent proper positioning of the nucleus (Huffaker et al. 1988; Sullivan and Huffaker 1992) and lead to the formation of binucleated mother cells and anucleated daughters. A great deal of progress has been made in understanding how astral microtubules
Microtubule capture by the cleavage apparatus

interact with the cell cortex to move and orient the intra-nuclear spindle. Two independent pathways mediate these interactions. Early in the cell cycle, the protein Kar9 facilitates microtubule capture at the bud cortex (Adames et al. 2000; Yeh et al. 2000). During anaphase, dynein mediates lateral interactions between microtubules and the cellular cortex and helps to pull the daughter nucleus into the bud (Muhua et al. 1994; Cottingham et al. 1997; Adames and Cooper 2000). However, like in animal cells, little is known about how microtubules ensure that the spindle and the cleavage apparatus are properly positioned relative to each other.

The cleavage apparatus has been particularly well characterized in *Saccharomyces cerevisiae*. Its assembly starts in late G1, concomitant with bud emergence. First, the septins Cdc3, Cdc10, Cdc11 and Cdc12 form a ring at the bud neck (Longtine et al. 1996). During bud formation, this ring serves as a template for the organization of type II myosin, Myo1, into a second ring (Epp et al. 1997; Bi et al. 1998; Lippincott et al. 1998). This myosin structure is required for the recruitment of actin filaments during mitosis to form the actomyosin contractile ring (Bi et al. 1998). Contraction of this ring allows cytokinesis (Bi et al. 1998; Lippincott and Li 1998). Additional proteins associated with the septin ring include the formin Bnr1 (Kikyo et al. 1999), Bni4, a protein involved in the spatial control of chitin synthesis (DeMarini et al. 1997), and the kinases Hsll and Gin4 (Ma et al. 1996; Carroll et al. 1998; Longtine et al. 1998a; Barral et al. 1999). Formins are involved in the nucleation and polymerization of actin filaments (Evangelista et al. 2002; Sagot et al. 2002). Both kinases Hsll and Gin4 interact physically with septins, and strictly depend on septin function for localization and activity (Longtine et al., 1998a; Carroll et al. 1998; Barral et al. 1999). For these reasons, we refer to them as septin-dependent-kinases (SDK). Hsll, and possibly Gin4, are involved in the control of the G2/M transition (Ma et al. 1996; Barral et al. 1999). Remarkably, the closest homologues of SDKs comprise both Nim1 from S. pombe (Kanoh et al. 1998) and the MARK (microtubule affinity regulatory kinase) kinases from higher eukaryotes (Drewes et al. 1998; Barral et al. 1999). MARK kinases control microtubule dynamics in mammals (Drewes et al. 1997, 1998; Ebneth et al. 1999). *C. elegans* MARK kinase Par-1 (Guo et al. 1995) is involved in spindle positioning. On the basis of this homology, Hsll and Gin4 may also have some function in microtubule related processes.

In this study, we investigated how the yeast mitotic spindle and cleavage plane are positioned relative to each other. We analyzed whether cells lacking the septin ring or some of its associated components show defects in microtubule organization, nuclear position and nuclear migration. Our results indicate that the septins, but not the actomyosin ring, are required to form a microtubule capture site at the bud neck-cortex. Upon capture, septin-
dependent kinases were required to induce microtubule catastrophe and the creation of a pulling force on the spindle, which then moved towards the bud neck.

**Results**

**The septin cytoskeleton is required for proper nuclear positioning**

In *S. cerevisiae*, the septin ring is necessary for the assembly of all components of the cleavage apparatus identified thus far. Thus, to determine whether the presence of the cleavage apparatus was necessary for the positioning of the nucleus to the bud neck, we determined whether cells lacking the septin ring positioned their nucleus properly. Two alleles of the CDC12 septin gene were used to disrupt the septin ring. Cells carrying the cdc12-1 temperature sensitive mutation fail to assemble a ring at the bud neck at restrictive temperature (Barrai et al. 2000). However, cells that have already formed the ring before the shift maintain the ring, finish their cycle and undergo cytokinesis comparably to wild type (data not shown). In the cdc12-6 allele, the septin ring becomes unstable after shift to the restrictive condition, irrespective of the position of the cell in the division cycle (Barrai et al. 2000).

After shift to restrictive temperatures (33°C) a majority of septin-defective cells arrested prior to anaphase failed to localize the nucleus to the bud neck (Fig. 1A, B: 59 % +/- 9 of cdc12-1 cells mis-localized their nucleus, N> 1200, 57% +/- 3 in cdc12-6, N>900). This nuclear positioning defect was quite similar to that observed in cells lacking either Kar9 or dynein (dhc1, Figure 1B), two proteins that have been implicated in nuclear positioning. The nuclear-positioning defect observed in cells lacking a septin ring suggests that the septin ring may be required either for nuclear migration, or to anchor already migrated nuclei to the neck region. In the first case, septins may be required as a cortical cue to direct nuclear migration. Septin defects would therefore lead to either random nuclear movements or would impair migration altogether. In contrast, if the septin ring serves to anchor already migrated nuclei migration should not be affected. The positioning defects should be due to random movements after the nucleus reached its proper position. To distinguish between these two possibilities, we characterized the effect of septin disruption on early stages of nuclear migration (figure 1C). Since nuclear migration happens in parallel to bud growth, most nuclei are generally randomly positioned in small budded cells and only a fraction of them has migrated to the bud neck in medium budded cells. Compared to wild type, small- and medium-budded cells lacking the septin ring showed an even stronger mis-positioning of the nucleus (Fig. 1C). Thus, septin function was required for proper nuclear migration.
Microtubule capture by the cleavage apparatus

Figure 1. The septin cytoskeleton is involved in the control of nuclear positioning. (A) Micrographs of wild-type and mutant cells arrested at the G2/M transition with hydroxyurea for 3 hours and stained with DAPI to visualize nuclei. The relevant genotype of the strains is indicated. Scale bar represents 2mm. (B) Quantification of nuclear positioning defects in strains mutated for components of the cleavage apparatus. kar9 and dhcl mutants are added for comparison. Cells were arrested prior to anaphase and the frequency of mislocalized nuclei (more than about 1mm away from the bud neck) was determined (N=500 for each strain, in at least two independent counts of 200-300 cells each). (C) Nuclear migration in small and medium budded cells of unsynchronized wild-type and cdc12-1-mutant strains. The percentage of cells with a nucleus in the vicinity of the bud neck (within 1mm from the bud neck) is shown. The cells were shifted for 2 hours to the restrictive temperature.
Nuclear migration defects generally result in a failure of nuclear segregation during mitosis (Huffaker et al. 1988). This leads to the formation of binucleate mothers and anucleate buds. A similar effect is expected if the spindle and the cleavage plane are not correctly positioned relative to each other. Thus, we investigated whether septin mutants have nuclear segregation problems. The cdc12-1 mutant and an isogenic wild-type strain were grown at the restrictive temperature for 3 hours, fixed, stained with DAPI to visualize nuclei, and the frequency of binucleate mother cells was determined. Septin defective cells exhibited an increased frequency of binucleated mother cells (18%, +/-10, N=253 in cdc12-1at 33°C) relative to wild type (2.8%, +/-2.7, N=355). Altogether these results indicated that septin function is required for proper positioning of the spindle relative to the cleavage plane.

**Nuclear migration defect of septin mutants is not due to actin polarity defects**

We recently showed that the septin ring forms a cortical barrier that is required specifically during isotropic bud growth to maintain polarized actin distribution (Barrai et al. 2000). During apical growth, the early phase of bud growth, actin polarity does not depend on the presence of the septin barrier (Longtine et al. 1996; Barral et al. 2000). Since actin polarity is required for microtubule orientation and nuclear migration, the nuclear migration defect observed in septin-defective cells could be due to actin polarity defects. Thus, we examined whether it correlated with actin defects. As demonstrated by the formation of elongated buds (Fig. 1A), cdc12-1 cells did not lose polarity at restrictive temperature and kept growing apically. Rhodamine-phalloidin staining (Fig. 2A) was performed to visualize actin in these cells. No defect in actin polarization and organization were found. Both actin patches and cables polarized normally upon bud emergence, similar to the situation in wild type. Thus, the nuclear positioning did not correlate with an actin polarity defect.

The role of actin in nuclear positioning is mediated by Kar9. Kar9 protein interacts with both the type V myosin, Myo2, and the microtubule associated protein Bim1, and thereby mediates interaction between microtubules and cortical actin. Since the main function of actin in nuclear positioning consists in the transport of Kar9 to the bud, we investigated if this process was affected in septin defective cells. No defect in the localization of Kar9 to and in the orientation of microtubules towards the bud cortex could be observed in cells lacking the septin ring (Fig. 3B, C and Fig. 6C, D). Thus, the nuclear positioning defect observed in the septin mutant was not due to defects in actin function. It was also not due to changes in the width of the bud neck, since this parameter was not significantly affected by the cdc12-1 mutation (Fig. 2B). Therefore, nuclear mis-positioning in cells lacking the septin ring was due to defects in the assembly of the cleavage apparatus, and not to a lack of cell polarity.
Microtubule capture by the cleavage apparatus

Figure 2. Nuclear positioning defect in the absence of septin function is not due to actin or morphological defects. (A) Actin polarity in wild type and septin defective cells. Cells grown at the restrictive temperature for 2 1/2 hours were fixed with formaldehyde and stained with rhodamine-phalloidin. (B) Measurement of the bud neck width in wild-type cells and the cdc12-1 mutant grown at 33°C for 3 hours (N = 78 for wild type and 89 for cdc12-1).

Nuclear positioning is independent of the assembly of the actomyosin ring

To determine whether any neck-specific actin structures are required for nuclear migration, we disrupted individually MYO1 and BNR1. Consistent with previous findings (Epp and Chant 1997) indicating that the actomyosin ring is not required for nuclear migration, we found that nuclei localized normally in the myo1 mutant (Fig. 1B). Similarly, elimination of the Bni4 protein, which is required for chitin synthesis, did not affect nuclear positioning (data not shown). Thus, neither the actomyosin contractile ring, nor the chitin ring at the bud neck are involved in nuclear positioning.

Cells lacking the bud neck-specific formin Bnr1 exhibited a mild defect in nuclear positioning (Fig. 1B). Thus, Bnr1 and actin structures dependent on Bnr1 may be involved in the septin-dependent positioning of the nucleus. However, because the nuclear positioning defect is small in the bnr1 strain, such actin structures cannot alone account for the nuclear mispositioning observed in septin-defective cells. Altogether our results indicated that the maintenance of actin polarity, the assembly of the contractile ring and the role of septins in nuclear migration are three distinct functions of the septin ring.

Microtubules interact with the septin ring

The movements of the nucleus in the cell depend on the interaction of microtubules with the cell cortex. Therefore, we investigated whether disruption of the septin ring affects the manner with which microtubules interact with the cortex. A GFP-TUB1 reporter construct
Chapter II

(Straight et al. 1997) was used to visualize astral microtubules in vivo. In addition, a KAR9-GFP reporter construct was used to visualize contact points between microtubules and the cell cortex. Interestingly, in our experiments Kar9 assembled into dots at the plus end of microtubules (figure 3A). To avoid synthetic effects, KAR9-GFP was expressed at endogenous levels for Kar9. Nine z-section images through the cell were recorded and the resulting images were projected using a “maximum projection” algorithm. This procedure ensured that all cytoplasmic microtubules of the cell could be observed and taken into account.

In wild type cells with a short spindle, astral microtubules interacted with the mother, bud neck and bud cortex (Adames and Cooper 2000; Segal et al. 2000; Vogel et al. 2000; Yeh et al. 2000). In yeast, where only few cytoplasmic microtubules are present, the average distribution of microtubules is highly informative about the stability of the interactions that they establish with the different parts of the cell cortex (see for example Miller et al. 1998). Thus we determined the percentage of cells with microtubules contacting either the mother cell, the bud neck or the bud cortex. This distribution was quite robust, as shown by the low variation observed between independent counts. This suggests that microtubule distribution is the result of tightly regulated processes. Importantly, a substantial number of microtubules (21% +/- 1; n>500) interacted with the bud neck with their plus end (Fig. 3A, B,C) (other authors reporting microtubule interaction with the bud neck include Snyder et al. 1991; Segal et al. 2000; Vogel and Snyder 2000; Yeh et al. 2000). This was a high frequency, since the bud neck represents only a small fraction of the overall cell surface. In most cases a Kar9 dot could be unambiguously distinguished at the microtubule end, demonstrating that the microtubule indeed touched the cell cortex at the bud neck. Microtubule interaction with the neck was not due to fusing GFP to Kar9, since contact was also apparent in the TUB1-GFP KAR9 strain (Fig. 3D). Moreover, replacing Kar9 by Kar9-GFP did not lead to any detectable nuclear positioning phenotype (data not shown), indicating that Kar9-GFP was fully functional.

In cells lacking the septin ring, the only microtubules that were less frequently observed than in wild type were those contacting the bud neck. In cdc12-1, this category dropped from 20% (+/-1) at permissive temperature (data not shown) to 9% (+/- 1%, n>500) at 33°C, the restrictive temperature. This drop was not due to difficulties in imaging GFP, since we did not observe any signal loss upon shift to 33°C. Furthermore, wild-type cells did not show substantial changes in microtubule organization at this temperature (data not shown). The effects observed appeared to be highly significant in regard to the low level of variation observed between experiments. The remaining microtubule-bud neck interactions observed in cdc12-1 most likely reflected random events. This interpretation is supported by the fact
Figure 3. Astral microtubules establish septin-dependent interactions with the bud neck. (A) Kar9 dots localize at the plus end of microtubules. Cells expressing Tub1-GFP, Tub1-GFP Kar9-GFP and Tub1-CFP Kar9-GFP are shown. Both tagged proteins were expressed at endogenous level. No dot can be seen at the plus end of microtubules when Kar9 is not tagged with GFP. Double staining shows that these dots correspond to Kar9 dots. Thus, staining of Tub1 and Kar9 both with GFP permits to visualize microtubule interaction with the cortex. (B) Micrographs of wild-type and mutant cells (genotypes are indicated) expressing GFP-Tub1 and Kar9-GFP at endogenous levels. The spindle is prominent, astral microtubules are fainter and emanate from the spindle poles (see drawing in the case of wild type). A Kar9 dot (marked with a red arrow) can be seen at the tip of many astral microtubules. Scale bar represents 1μm. (C) Quantification of microtubule organization in cells with a small spindle. The cells were sorted within three categories (see drawing): 1) no astral microtubule touching either the bud neck or the bud cortex, 2) cells with astral microtubules touching the bud neck cortex, 3) cells with astral microtubules touching the bud cortex. Cells that have astral microtubules touching both the bud neck and the bud cortex were rare (less than 3%) and excluded from the graph. Results of at least 5 independent counts per strain, with at least 100 cells per count. (D) Analysis of astral microtubule organization (as in (B)) in wild-type and cdc12-1 mutant cells that express only GFP-Tub1. The cdc12-1 and wild type control cells were shifted to 33°C for three hours.
that microtubule interaction with the bud neck was reduced to the same level in the kar9 mutant (Fig. 3B). In this mutant, microtubule interaction with the cell cortex prior to anaphase is thought to be random (Miller et al. 1998b, Miller et al. 1999). Thus, in wild type, microtubules interacting with the bud neck were transiently captured and/or stabilized. This led to a clear increase in the number of microtubules interacting with the bud neck, compared to random. The capture and/or stabilization of microtubules at the bud neck required septin function.

Remarkably, septin defects had no negative impact on the interaction of microtubules with other parts of the cell cortex. Particularly, microtubule interaction with the bud cortex was not defective. These results indicate that septins affect microtubule interaction with the cell cortex solely at the bud neck.

In the bnrl strain, changes in microtubule organization were not observed at the bud neck but at the bud cortex. This suggested that the positioning defect observed in this strain was possibly not caused by defects in the interaction of microtubules with the bud neck. Taken together our results indicated that astral microtubules established a functional interaction with the bud neck. These interactions depended on septin function, but not on the assembly of actin-related structures. At this point, our data indicated that septin-dependent interaction of microtubules with the bud neck cortex was required for proper positioning of the nucleus.

Microtubule plus-end capture at the bud neck is associated with the establishment of pulling forces on the spindle

To investigate how microtubule interaction with the bud neck could be involved in nuclear positioning, we monitored spindle movement upon microtubule attachment to the cortex of wild type cells. The behavior of microtubules in vivo was recorded by 3D-time lapse microscopy (Fig. 4A, B). This analysis was carried out in cells expressing both Tub1-GFP and Kar9-GFP. Tub1-GFP allowed visualization of microtubules, while Kar9-GFP was used as a marker for microtubule/cortex interactions. The tagging of both molecules with the same chromophore allowed very rapid acquisition of image stacks through the cell, without filter changes. With this setup we could monitor microtubule behavior with a very high time resolution. As shown in figure 3A and 4A, this set up also allowed the clear distinction of Kar9 dots at the plus ends of microtubules. An example of the movies obtained is shown in Figure 4A. In this movie an astral microtubule first grew towards the neck. Upon neck contact, a clearly identifiable Kar9 dot, which may have been faintly present at the tip of the microtubule in the previous frames, formed and gained in intensity. At the same time, the microtubule stopped growing. In the following 3 frames (8-10) the microtubule shrank and
the spindle was pulled for about 0.6 μm towards the neck. At frame 11, the spindle stopped moving. Simultaneously, the Kar9 dot faded away suggesting that the microtubule had detached from the cortex. The duration of this event was 30 sec. Altogether, these movies indicated that microtubule capture and shrinkage at the neck permits displacement of the spindle towards the bud neck. Furthermore, these movies indicated that Kar9 was present at the attachment sites at the bud neck and may be functionally involved in the capture process (see below).

To confirm that our observations were not due to isolated cases, movies of ten cells and of 15 minutes each were analyzed in detail. Contacts between microtubules and the cell cortex were considered established only when a Kar9 dot appeared at the microtubule plus-end. Also, only the cases where only one microtubule could be found contacting the cell cortex at a time were considered. This ensured that the effects observed were not due to the combination of several contact events. On a total of 150 minutes of recording, 89 contacts with the bud neck were observed, which had an average duration of 29.5 seconds. For comparison, in the same cells 43 contacts to the mother cell (average duration: 44.5 sec.) and 40 contacts to the bud cortex (average duration: 40.6 sec.) were observed. For all the contacts observed, the position of the spindle at the end of the event was compared to its position prior to the contact (Fig. 4C). Contacts with the mother cell cortex led to a displacement of the spindle towards the bud neck in only 26% of the cases. In contrast, contacts with the bud and bud neck cortex resulted in a movement towards the neck in 48% and 65% of the cases respectively. In the absence of microtubule contacts with the cortex, the spindle did not move. Thus, microtubule interaction with the bud neck led in most cases to nuclear movement towards the bud neck.

To gain more insight into the mechanism of the nuclear movement during cortical contacts, we determined the effects of contacts on microtubule dynamics. The relative duration of microtubule shrinkage and growth was measured during these events (Fig. 4B). Over 26 minutes of neck contacts (a total of 43 capture events, from 6 different cells), microtubules spent 19.6% of the time shrinking and 5% of the time growing. The net result of these contacts was microtubule shrinkage. For comparison, microtubule contacting the mother cortex (30 minutes total) spent 7.6% of the time shrinking and 16.7% of the time growing (Fig. 4B), and the net result of these events was microtubule growth. Thus, microtubule contacts with the bud neck were associated with microtubule shrinkage, leading to a pulling action on the spindle. In contrast, contacts with the mother cortex were associated with microtubule growth, leading to a pushing effect on the spindle. The observation of a pulling action on the spindle upon microtubule interaction with the bud neck is a strong evidence for microtubule attachment at this site. Thus, our results uncover a new microtubule capture site,
the bud neck, and indicate that this capture site is involved in pulling the nucleus towards the neck.

**Figure 4.** Function of microtubule attachment to the bud neck in nuclear migration. (A) Microtubule capture at the bud neck is associated with shrinkage of the microtubule and pulling of the spindle towards the bud neck. A wild-type cell expressing Kar9-GFP and GFP-Tub1 at endogenous levels is shown. Each frame was generated by projecting images of six focal planes taken through the cell. Frames are taken at 6 second intervals. The line drawn through all frames marks the position of the spindle at the beginning of the contact event. An astral microtubule grows towards the bud neck (frame 1-6) and remains attached there (defined by the presence of a clear Kar9-GFP-dot at the end of the microtubule) during 30 seconds (until frame 10). At frames 10-11 it detaches and shrinks back to the spindle pole (frame 11-12). During the second half of the attachment period (frames 6-10) the microtubule shrinks and pulls the spindle towards the bud neck. Scale bar represents 1μm. (B) Effect of microtubule attachments on spindle movement, depending on the attachment site. The position of the spindle relative to the bud neck was compared at the beginning and at the end of capture events (N= 89 at bud neck, 40 at bud cortex, 43 at mother cortex). (C) Dynamics of microtubules contacting either the mother or the bud cortex. The fraction of time spent in either growth or shrinkage is shown depending on the cortical domain contacted (mother cell or bud neck).
SDK are required for the shortening of microtubule interacting with the neck

Time laps analysis indicated that microtubules behaved differently, depending whether they contacted the mother or the bud neck cortex. This observation suggested that promoters of microtubule shrinkage might specifically localize to the bud neck. In many cases, microtubule dynamics is regulated through protein phosphorylation events. Moreover, the MARK kinases, to which the septin dependent kinases Hsll and Gin4 are related (Barrai et al. 1999), have been implicated in the stimulation of microtubule shrinkage. Thus, we investigated whether Hsll or Gin4 could be implicated in triggering microtubule shrinkage at the bud neck.

First, we analyzed what was the effect of disrupting HSL1 and GIN4 on microtubule organization. Cells expressing Tubl-GFP and Kar9-GFP were analyzed as in Figure 3B. Two striking phenomena were observed in these cells. 1) The two single mutants hsl1 and gin4 showed an enhanced interaction of microtubules with the bud neck (Fig. 5A, B); 31% (+/-3, n>500) of hsl1 cells and 35% (+/- 1, n>500) of gin4 cells contained microtubules touching the neck region. These effects were additive since the double mutant had an enhanced phenotype (43%, +/- 2, n>500 of the hsl1 gin4 cells showed microtubules connected with the bud neck). 2) In all these strains, the number of microtubules penetrating into the bud decreased proportionally to the increase in microtubules interacting with the bud neck. Two different interpretations could account for these results. On one hand, it is possible that microtubules interacting with the bud cortex are destabilized in SDK mutants. However, this hypothesis fails to explain why more microtubules would specifically attach to the bud neck. On the other hand, SDK mutations may lead to an excessive stabilization of microtubules binding the bud neck. This would increase the steady state number of microtubules attached to the bud neck, at the expense of the bud cortex. This last explanation suggests that SDKs activate microtubule dynamics at bud neck. It has the advantage to be consistent with the localization of Hsll and Gin4.

To test whether increased interaction with the bud neck was accounting for the microtubule organization phenotype of the SDK mutants, we examined the effect of disrupting the septin ring in these cells. Our rational was that, if SDK mutants led to a stabilization of microtubule/neck interactions, disrupting the septin ring should abolish these interactions. On the other hand, if the SDK are necessary for microtubule capture at the bud cortex, disrupting the septin ring should have no effect on microtubule interaction with the bud cortex. Our results (Fig. 5B) show that the cdc12-1 hsl1 gin4 cells restored wild type level of microtubule interaction with the bud. Consistent with the finding that functional septins are required for microtubule interaction with the bud neck, microtubule binding to the neck was abolished. Thus, cells lacking SDKs develop stable interactions between the microtubules and the bud
neck. This stabilization prevents the interaction of microtubules with the bud cortex, indicating that the bud neck and the bud cortex compete for microtubules. Thus, our results suggest that SDKs ensure that microtubules interactions with the bud neck remain dynamic.

Stable microtubules are generally long, while dynamic microtubules are shorter. Therefore, to assess whether microtubules interacting with the neck were less dynamic in SDK mutants, we analyzed their length. As shown in Figure 5C, microtubules contacting the neck were reproducibly longer in SDK mutants than in wild type (average length in WT = 0.75 μm, +/- 0.22, in gin4 = 1.14 μm, +/- 0.35, in hs11 = 1.24 μm, +/- 0.35, in hs11 gin4 = 1.25 μm, +/- 0.48). Thus, SDKs are required for the proper regulation of microtubule dynamics at the bud neck. Taken together, our data support the idea that microtubules binding the cleavage apparatus are induced to shrink through SDK-dependent phosphorylation events.

Disruption of HSL1 and GIN4 lead to a G2 delay, which is abolished by disruption of the SWE1 gene (Barrai et al. 1999). The stabilization of microtubule/neck interactions in SDK mutants may be due to these cells spending more time in G2. To test this possibility, we analyzed microtubule organization in the hs11 gin4 swe1 triple mutant. Disruption of SWE1 suppressed the cell cycle progression defects observed in hs11, gin4 and hs11 gin4 mutants (see methods section, Barrai et al. 1999). However, it did not suppress the enhanced microtubule interaction phenotype (Fig. 5B). Thus, the role of Hs11 and Gin4 in the regulation of microtubule/neck interaction was independent of their function in cell cycle control and not mediated by Swe1. Therefore, the effect of SDKs on microtubule dynamics at the bud neck is most likely direct.

Septins act in the Kar9 pathway

Since Kar9 was found to be present at microtubule attachment sites at the bud neck, we investigated the functional relevance of this presence. In yeast, nuclear positioning is ensured by two redundant pathways (Cottingham and Hoyt 1997; DeZwaan et al. 1997; Miller et al. 1998; Miller and Rose 1998; Adames and Cooper 2000; Yeh et al. 2000). The Kar9 pathway acts early during bud formation. It positions the spindle to the bud neck, aligned along the mother-bud axis (Adames and Cooper 2000; Yeh et al. 2000). In cells lacking Kar9, spindle positioning is very inefficient (see figure 1) and depends on the function of dynein (encoded by the DHC1 gene), a microtubule-dependent motor (Muhua et al. 1994; Cottingham and Hoyt 1997; Adames and Cooper 2000). Accordingly, dynein mutants also have a nuclear positioning defect (figure 1B). While mutants affecting either the Kar9 or the dynein pathway are viable, inactivation of both pathways is lethal (Cottingham and Hoyt 1997; DeZwaan et al. 1997; Miller and Rose 1998b). Nuclei divide in the mother cell at mitosis and fail to
Figure 5. The kinases Hs1 and Gin4 are important for proper microtubule dynamics at the bud neck. (A) and (B), see Figure 3 (A) and (B). (C) Length distribution of microtubules attached at the bud neck in wild-type and in hsl1 gin4 double mutant cells. N= 56 in wild type and 64 in the mutant.
become segregated, leading to the formation of binucleate mother cells and anucleate daughters.

We determined genetically if septins acted either in the Kar9 or in the dynein pathway. At the semi-permissive temperature of 30°C the cdc12-1 strain grew slowly (Fig. 6A) and exhibited septin organization defects (data not shown). At the same temperature cdc12-1 dhcl, cdc12-1 num1 and cdc12-1 bik1 cells were unable to do more than a few divisions (Fig. 6A, Num1 and Bik1 act in the dynein pathway, Farkasovsky et al. 2001; Pellman et al. 1995). Analysis of the cdc12-1 dhcl cells showed that the mother cells rapidly became multinucleated (data not shown). Thus, these cells were strongly defective for nuclear segregation. In contrast, the cdc12-1 kar9 and cdc12-1 kip3 strains grew as well as the cdc12-1 single mutant (Fig. 6A, Kip3 acts in the Kar9 pathway (Cottingham and Hoyt 1997; DeZwaan et al. 1997). At permissive temperature, all six strains grew at rates very similar to wild type. Thus, in septin defective cells the Kar9 pathway is deficient and unable to compensate for the loss of dynein. In contrast, in cdc12-1 kar9 and cdc12-1 kip3 cells, dynein can compensate for the loss of the Kar9 pathway, indicating that the dynein pathway does not require septins for function. Thus, septins acted in the Kar9 pathway but not in the dynein pathway.

Kar9 links microtubules to the septin ring

The observation that septins act in the Kar9 pathway suggested that microtubule capture at the bud neck may depend on Kar9. Thus, we reinvestigated the localization of Kar9 using a Kar9-GFP fusion protein expressed from the endogenous KAR9 promoter (Fig. 6B). To eliminate Kar9 dots at the spindle poles from our counts, we quantified Kar9 localization in cells in which both Tub1 and Kar9 were stained. Only Kar9 dots that are at the plus end of microtubules and Kar9 dots that are not associated with microtubules (less than 0.5%) were counted. This study indicated that Kar9 localized frequently to the bud neck (similarly to Beach et al. 2000); 37% (+/-1, n=140) of Kar9 dots localized to the bud neck, 35% (+/-1, n=140) to the bud cortex (Fig. 6C). Consistent with the microtubule distribution results, Kar9 localization to the bud neck in cdc12-1 cells was reduced to 15% (+/- 0, n=268) at restrictive temperature (Fig. 6C). In this study, it was never possible to find Kar9 dots at the bud neck independently of microtubules. This result was consistent with time lapse microscopy data showing that the Kar9 dot formed upon microtubule attachment. Thus, both septins and microtubules were necessary to recruit Kar9 at the bud neck. This may be different from the situation reported for microtubule capture at the bud tip, where Kar9 dots were found independently of microtubule capture (Miller and Rose 1998; Miller et al. 1998, 1999; Beach et al. 2000).
**Figure 6.** Role of septins in Kar9 function and localization. (A) Genetic interactions between cdc12-1 and mutations in genes participating in either the Dhcl (dhcl, bik1, num1) or the Kar9 pathways (kar9, kip3) for nuclear migration. Strains of indicated genotypes were streaked out on the same plates and were incubated at either 24 or 30°C for 3 days. See the Methods section for the construction of the strains. (B) Micrographs of wild-type cells expressing Kar9-GFP under the endogenous promoter. The two upper cells show Kar9 in the mother cell and at the cortex of the bud. The two lower cells have one dot each at the bud neck. Scale bar represents 1mm. (C) Quantification of Kar9 distribution to the mother cell, bud neck and bud cortex in wild-type, cdc12-1 and gin4 hsl1 double mutants. Quantification was made in cells stained for both tubulin and Kar9. Only the Kar9 dots associated with microtubule distal tips and the cortex were counted (N= 140 for WT, 268 for cdc12-1, 110 for hsl1 gin4; average of at least two independent counts).
In any case, our results suggested that Kar9 might facilitate microtubule capture by the cleavage apparatus. To test this possibility, we analyzed microtubule organization in cells lacking Kar9. Deletion of KAR9 strongly reduced the frequency of microtubules interacting with the bud neck in both wild type and the hsl1 gin4 double mutants (Fig. 5B). In contrast, no reduction in microtubule interaction with the bud neck was observed in the dhc1 strain compared to wild type (data not shown). Thus, our genetic and microscopy data both indicated that Kar9, and not dynein, mediated the capture of astral microtubules by the cleavage apparatus.

**Septins are not involved in spindle alignment**

Prior to mitosis, the spindle does not only need to be positioned at the bud neck but also to be aligned along, i.e., to be parallel to, the mother bud axis. Kar9 has been demonstrated to act in both spindle positioning and spindle alignment. Disruption of BNI1, which is required for Kar9 localization to the bud cortex (Lee et al. 1999; Miller et al. 1999), affects spindle alignment (Fig. 7A). In contrast, we found that spindle alignment was not defective in cells lacking the septin ring (Fig. 7A). Thus, the septin ring is specifically involved in spindle positioning to the bud neck and not in its alignment along the mother/bud axis.

**Discussion**

In this study, we demonstrate the existence of a new microtubule capture site at the yeast bud neck. Microtubule shrinkage and a pulling action on the spindle towards the bud neck followed microtubule capture at this site. This capture site was no longer present in cells lacking the septin ring, indicating that septins or other downstream components of the cleavage apparatus were required for microtubule capture. These capture events were functionally important since septin mutants exhibited nuclear positioning and nuclear segregation defects at restrictive temperature. These defects did not correlate with actin defects, but only with a decrease of microtubule interaction with the bud neck. Thus, microtubule capture at the bud neck was required for proper positioning of the nucleus. We propose that microtubule capture and shrinkage at the bud neck is required to pull the nucleus towards the cleavage apparatus.

Disruption of the actomyosin ring and the formin Bnr1 did not lead to microtubule capture defects. Since these mutations are thought to disrupt bud neck-specific actin structures, our results suggest that actin may not be required for microtubule capture at the bud neck. This is different from what has been reported for microtubule capture at the bud tip. Disruption of other septin associated proteins such as Bni4, Hs11 and Gin4 did also not abolish microtubule
interaction with the septin ring. Therefore, the protein(s) mediating microtubule interaction with the cleavage apparatus still remain to be identified. One attractive possibility is that such proteins are the septins themselves. In support to this idea, *Drosophila* septins Pnut, Sep1 and Sep2 have been reported to bind onto microtubule columns (Sisson et al. 2000). Thus, it will be important to determine whether septins can interact directly with Kar9 and/or microtubules.

Altogether, our results are consistent with the model presented in Figure 7B. In yeast, spindle positioning depends on two processes: the alignment of the spindle along the mother-bud axis and the positioning of the nucleus relative to the cleavage apparatus. The Kar9 pathway is involved in both processes. We suggest that it fulfills these functions by interacting with two distinct cortical structures, respectively, the bud cortex and the cleavage apparatus at the bud neck. Our results also implicate dynein in nuclear positioning independently of its role in spindle alignment. If dynein was only acting through its role in spindle alignment it should not be co-essential with septin defects, since in these cells Kar9 is still able to orient the spindle properly. Thus, dynein may have an additional role in nuclear positioning independent of its role in spindle alignment. As presented in Figure 7C, we propose that septin plays a pivotal role in cell division: it serves as a linker to organize and position actin and microtubule related structures relative to each other.

Microtubule capture at the bud neck was associated with microtubule shrinkage. In cells lacking SDKs, microtubules remained attached to the bud neck for longer periods of time and were increased in length. From these experiments, we propose that the regulation of microtubule dynamics upon capture at the bud neck is ensured by MARK-related septin-dependent kinases.

The involvement of SDKs in nuclear positioning is a remarkable result, since it indicates that, at least in yeast, the same kinases coordinate nuclear and cortical events of mitosis at both the temporal level, through regulation of Swe1 (Barral et al. 1999), and at the spatial levels, through regulation of microtubule function (Fig. 7C). This co-regulation of entry into mitosis and spatial coordination of spindle and cleavage apparatus may be conserved at least in *S. pombe*. Indeed, in this organism, the kinase Cdr2, the homologue of *S. cerevisiae* Gin4, influences both the timing of mitosis (Breeding et al. 1998; Kanoh and Russell 1998) and the positioning of the cleavage apparatus (Breeding et al. 1998). Thus, Cdr2 may regulate a microtubule-dependent event involved in the positioning of the cleavage apparatus in fission yeast. In summary, our results provide the first evidence that Par-1-like molecules control microtubule function also in yeast. This finding will open new possibilities to determine how this class of kinases controls microtubule function.
Septins and septin-dependent kinases are highly conserved in animal cells. Therefore, it is tempting to speculate that interactions between astral microtubules and the septin cytoskeleton may be conserved and spatially coordinate the spindle and the cleavage machinery in higher eukaryotes. Astral microtubules and microtubules from the central spindle may bind to septin-dependent structures of animal cells. Such microtubules may thereby recruit septins to the equator of the cell to position the cleavage apparatus properly relative to the spindle.

Figure 7. Role of the septins in coordination of cytoplasmic and nuclear events in cell division. (A) Role of the septins in spindle alignment. Unlike kar9 and bnl1 mutants, cells lacking the septin ring are fully competent for spindle alignment. Deletion of the formin BNR1 has no effect. Results of at least two counts with a minimum of 80 cells per count. (B) and (C) Models: (B) Astral microtubule interaction with the septin-dependent cytoskeleton participates in bringing the nucleus to the bud neck. The dynamic of microtubules at the bud neck is regulated by SDK Hs11 and Gin4. Spindle alignment occurs through microtubule interaction with the bud cortex. Both processes are Kar9-dependent. (C) The septins are involved in at least three independent processes: the recruitment of Bnl1 and Myol, which are required for cytokinesis, the recruitment and activation of Hs11 and Gin4, and the Kar9-dependent capture of microtubules. In turn, Hs11 and Gin4 regulate both entry into mitosis (via repression of Swel) and microtubule dynamics at the bud neck. Microtubule capture and shrinkage are required to position the spindle relative to the cleavage apparatus. After proper anaphase (chromosome segregation), the mitotic exit network (MEN) induces cytokinesis.
Methods

Quantification of nuclear migration

Nuclear migration was assayed as described (Yeh et al. 1995). Briefly, log phase cultures of the indicated strains were diluted in fresh complete medium in the presence of 100 mM hydroxyurea. After 2 1/2 hours at room temperature (22-24°C) more than 85% of the cells were arrested with a large bud and a single nucleus. The cells were fixed with 70% ethanol for at least 1/2 hour. After washing the cells in PBS nuclei were stained with DAPI and viewed using an Olympus fluorescence microscope. Nuclei that were more than 1 mm away from the bud neck were considered as mislocalized. In the case of septin mutants, the cells were shifted to the minimal restrictive temperature (30°C for cdc12-6 and 33°C for cdc12-1) directly after being put in presence of hydroxyurea. In the ideal case the experiment should be done by shifting the cells back to the permissive conditions to determine whether nuclear positioning was restored. However, the septin defect was not reversible, preventing this approach. Shifting the non-temperature-sensitive strains (such as the wild type, and the hs1 and gin4 mutants) to these temperatures did not significantly affect nuclear localization compared to room temperature (data not shown). For nuclear migration analysis in unsynchronized populations cells were grown in YPD at room temperature until early log phase and subsequently shifted to 33°C for 3 hours before fixation. (At this time all cells had failed to assemble a septin ring, as indicated by apically growing buds and cytokinetic defects). The fraction of cells with the nucleus within 1 mm from the bud neck was counted in cells with small, medium or large buds.

To determine whether septin-defective cells showed defects in nuclear segregation, we grew cdc12-1 cells at restrictive temperatures for 3 hours, fixed the cells and stained with DAPI to visualize nuclei. During this period, the cells did not progress through more than one cycle. Therefore, a change in the frequency of binucleated mother cells following the shift to restrictive temperature reflects the frequency of nuclear mis-segregation per cell cycle.

Strains and growth conditions

All strains were grown in rich medium (YPD) at room temperature, unless indicated otherwise. Gene disruption and GFP tagging was achieved using a PCR-derived approach as described (Longtine et al. 1998b). The transformants were subsequently screened by PCR, microscopy and phenotypic analysis. Deletion of SWE1 in hs1, gin4 and hs1 gin4 suppressed the accumulation of premitotic cells as quantified by the frequency of cells with short spindles and large budded cells with a single nucleus. It also suppressed the formation
Chapter II

of elongated cells characteristic of these strains due to prolonged apical bud growth (Barral et al. 1999). Production of the multiple mutant strain was systematically achieved through crossings. In the case of the cdc12-1 kip3 strain, 25 full tetrads where obtained, producing 26 cdc12-1 KIP3 spores and 24 cdc12-1 kip3. All spores were tested for growth at 30°C, and no growth difference was observed between cdc12-1 and cdc12-1 kip3 strains. 20 cdc12-1 and 18 cdc12-1 bik1 strains were obtained out of 19 full tetrads. At 30°C, none of the cdc12-1 bik1 spores grew. 16 cdc12-1 and 12 cdc12-1 num1 spores were obtained out of 14 full tetrads, all double mutants died at 30°C. Finally, 26 cdc12-1 dhc1 and a similar number of independent cdc12-1 kar9 spores were obtained. In 100% of the cases the cdc12-1 dhc1 spores died at 30°C, while the cdc12-1 kar9 strains grew. In all these experiments, cdc12-1 spores also grew at 30°C.

Fluorescence and time-lapse Microscopy

Cells were mounted in low autofluorescence selective drop-in medium shortly before viewing. Objects were subjected to time-lapse microscopy for a maximum of 15 min.
To visualize GFP-labeled microtubules and quantify their organization in vivo full frame pictures of fields of cells were taken at 9 focal planes (0.2 μm step size) using an Olympus BX50 fluorescence microscope, a piezo motor and the TILLVision software (TILL.photonics, Martinsried, Germany). The pictures were projected on one single plane using the maximum method. When Kar9-GFP was overexpressed from the GAL promoter, Kar9 dots were more frequently observed at the bud tip, indicating that Kar9 overexpression may affect its localization. For analysis of Kar9 distribution and microtubule organization in cdc12-1 ts-mutants, the cells were shifted to restrictive temperature (33°C) for 3 hours before microscopy.
To investigate microtubule-attachments, growth/shrinkage and spindle movements Z-stacks of 6 focal planes (0.3 μm step size) were taken at intervals of 6 sec for a total time of 15 min. Z-stacks were projected by the maximum-method. Before each movie a single transmission image was taken at the middle focal plane.
For analysis of microtubule-length and dynamics, spindle pole body movement respective to the neck and timing of microtubule-attachments we used NIH Image 1.62b7 (by Wayne Rasband, NIH). Microtubules were regarded as attached, when Kar9-GFP-dots were visible at the microtubule tips. Transmission images served to confirm microtubule-attachments at bud neck, mother cortex or bud cortex. Coordinate values of the proximal spindle pole body at the beginning and at the end of the attachments were recorded to determine whether the spindle moved towards or away from the bud neck. Attachments could last from 6 seconds to 3.5 minutes seconds and could consist of different types of microtubule end behavior including slight movements like gliding or sweeping and still capture at a single site.
Acknowledgement

We thank J. Vogel and C. Mann for providing strains and constructs. We thank Markus Aebi, A. Helenius and the members of the Barral laboratory for support and discussions and M. Bornens, R. Kroschewski, D. Liakopoulos, A. Paoletti and M. Peter for their comments on the manuscript. We are indebted with R. Moser and A. Lehman for their technical support during many phases of this work. M. P. S. is supported by a grant from the NIH. J. K. and Y. B. are supported by the Swiss Federal Institute of Technology.

References

*The references of this publication are included in the general bibliography from page 103.*
Chapter III

Asymmetric loading of Kar9 onto spindle poles and microtubules ensures proper spindle alignment

Dimitris Liakopoulos¹,³, Justine Kusch¹,³, Sandrine Grava¹, Jackie Vogel² and Yves Barral¹,⁴

¹Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
²McGill University, Montreal, Canada
³These two authors contributed equally to this work and should be considered as co-first authors
⁴To whom correspondence should be addressed,

Institute of Biochemistry
ETH-Hönggerberg
CH-8093 Zürich, Switzerland

Tel: +41 (0)1 632 0678 Fax: +41 (0)1 632 1591
Email: yves.barral@bc.biol.ethz.ch

The work described in this Chapter was published in:

My contribution to this work is presented in Figures 1, 5, 6 and 7C.
Chapter III

Abstract

Spindle alignment is the process in which the two spindle poles are directed towards pre-selected and opposite cell ends. In budding yeast the APC-related molecule Kar9 is required for proper alignment of the spindle with the mother-bud axis. We find that Kar9 localizes to the prospective daughter cell spindle pole. Kar9 is transferred from the pole to cytoplasmic microtubules, which are then guided in a myosin-dependent manner to the bud. Clb4/Cdc28 kinase phosphorylates Kar9 and accumulates on the pole destined to the mother cell. Mutations that block phosphorylation at Cdc28 consensus sites result in localization of Kar9 to both poles, and target them both to the bud. Thus, Clb4/Cdc28 prevents Kar9 loading on the mother-bound pole. In turn, asymmetric distribution of Kar9 ensures that only one pole orients towards the bud. Together, our results indicate that Cdk1-dependent spindle asymmetry ensures proper alignment of the mitotic spindle with the cell division axis.

Introduction

Alignment of the mitotic spindle with a predetermined axis of division occurs in many cell types, and plays an important role in cell morphogenesis, asymmetric cell division, embryogenesis and organogenesis. Positioning of the spindle is thought to depend on the interaction of astral microtubules with cortical cues (Kaltschmidt and Brand, 2002). However, how these interactions are established and translated into correct spindle orientation is not well understood.

A number of microtubule-binding proteins involved in microtubule-cortex interactions have been identified (reviewed in Gundersen, 2002). These proteins are proposed to both regulate microtubule dynamics and provide a link to targeting cues residing at the cell cortex. Representatives of this class of molecules in higher eucaryotes are the plus-end microtubule associated proteins CLIP170 and EB1 (Perez et al., 1999; Su et al., 1995). CLIP-170 is thought to mediate microtubule interactions with the cortical dynein/dynactin complex (Dujardin and Vallee, 2002). EB1 acts in concert with the adenomatous polyposis coli (APC) tumor suppressor (reviewed in Dikovskaya et al., 2001 and Bienz, 2002), a multi-domain protein that interacts with microtubules via a polybasic domain, and through EB1 binding. APC also interacts with cortical factors, thereby linking microtubules to the cortex (Dikovskaya et al., 2001). Studies in Drosophila show that both EB1 and APC are required for proper alignment of the mitotic spindles along the epithelial cell plane (Lu et al., 2001). Thus, these studies reveal that conserved components of the molecular machinery interacting with the cell cortex localize to the plus end of microtubules.
Asymmetric loading of Kar9

Simple unicellular organisms such as *Saccharomyces cerevisiae* also divide asymmetrically, suggesting that the molecular mechanism underlying spindle positioning was established early in evolution. Prior to cell division, *S. cerevisiae* cells undergo polarized growth to form a bud. Concomitantly, the mitotic spindle organizes in the mother cell. Inheritance of a daughter nucleus by the bud is ensured through alignment of the mitotic spindle with the mother-bud axis. As a result, spindle elongation pulls one set of sister chromatids into the bud, while the complementary set is pulled back into the mother cell.

Spindle positioning is governed through cortical capture of the plus end of cytoplasmic microtubules emerging from the spindle (Beach et al., 2000; Adames and Cooper, 2000; Segal and Bloom, 2001). Two redundant pathways are involved (Miller et al., 1998). The Kar9 pathway involves the microtubule-associated EB1 and APC homologs Bim1 and Kar9, the type V myosin Myo2 and the kinesin-related protein Kip3 (Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000; Yin et al., 2000; Miller and Rose, 1998). The Kar9 pathway acts primarily prior to anaphase. Kar9 binds indirectly to microtubules via the microtubule binding protein Bim1 and Kar9 polarized distribution is dependent on its interaction with the type V myosin Myo2 (Beach et al., 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000; Yin et al., 2000). Through these interactions, Kar9 is thought to mediate the capture of cytoplasmic microtubules at the bud cortex. In addition to its localization at the cortex, Kar9 also localizes to spindle pole bodies (SPB, Miller and Rose, 1998). However, Kar9 function at SPBs is not known. The second pathway depends on the minus-end directed motor dynein, the CLIP170 homologue Bik1, the cortical protein Num1 and the dynactin complex (Adames and Cooper, 2000; Farkasovsky and Kuntzel, 2001). This pathway acts predominantly during anaphase. Mutations affecting either pathway are viable and show only minor defects in nuclear segregation. However, when both pathways are disrupted cells are strongly defective in nuclear segregation and not viable (Miller et al., 1998).

Since plus-end binding molecules such as Kar9 and dynein are frequently observed at spindle poles (Segal and Bloom, 2000), we wished to investigate whether spindle poles play any direct role in spindle positioning. We show that asymmetric localization of Kar9 to the spindle pole and microtubules proximal to the bud is crucial for proper alignment of the spindle along the mother-bud axis. Asymmetric localization of Kar9 depends on its phosphorylation by Cdc28/Clb4 and Cdc28/Clb3. These complexes were also distributed asymmetrically on spindle poles. Altogether, our results indicate that Cdc28-dependent asymmetry of the spindle plays a key role in spindle alignment with cortical polarity.
Results

Kar9 associates with the spindle pole destined to the bud

To reinvestigate the localization of Kar9 to spindle poles, we examined cells expressing Kar9-YFP, and either CFP-Tubulin (figure 1A) or the SPB marker Spc42-CFP (figure 1B). As already reported (Kusch et al., 2002), Kar9-YFP expressed at endogenous level localized along microtubules and to microtubule plus ends (supplementary movie 1), as well as to spindle poles (figure 1). Remarkably, in metaphase cells only one spindle pole and the cytoplasmic microtubules attached to it were loaded with Kar9 (figure 1A). Asymmetric localization to the spindle pole did not depend on whether the spindle was properly oriented (examples shown in figure 1A, B). Also, treatment with nocodazole did not lower Kar9 level at the pole (figure 1C), indicating that maintenance of this localization was not dependent on the presence of cytoplasmic microtubules. Thus, Kar9 localized asymmetrically to spindle poles as well as microtubules.

Analysis of microtubule dynamics indicates that the two spindle poles are functionally distinct (Vogel et al. 2001). The pole destined for the bud actively produces microtubules, while microtubules emerge from the mother-bound pole at a lower rate. The microtubules produced by the daughter-bound pole are also longer, allowing non-ambiguous identification of this pole. We analyzed Kar9 localization in movies of cells co-expressing Kar9-GFP and GFP-Tub1, or Kar9-YFP and CFP-Tub1. In these cells Kar9 predominantly associated with the daughter-bound pole and associated microtubules; 92 % (+/-7, N=138 movies) of the active poles showed Kar9 staining, while only 15% of the cells showed a signal on the inactive pole. Accordingly, in 89% (+/- 3, N=334 cells) of the cells with a short and properly oriented spindle, Kar9 associated with the spindle pole proximal to the bud. Thus, Kar9 associated specifically with the pole destined to enter the bud.

To investigate the mechanism of Kar9 asymmetry, we first tested whether its localization depended specifically on one or more of the proteins it is known to interact with, physically or genetically. Thus, we tested whether spindle pole localization was affected in myo2-16, stu2-13, dhcl1μ or bim1μ cells. The myo2-16 allele affects Myo2 interaction with Kar9 at 37°C (Yin et al., 2000). Stu2 is the yeast homologue of XMAP215 and localizes to spindle poles and microtubules (Wang and Huffaker, 1997). Kar9 localization was unaffected in myo2, stu2 and dhcl1μ mutants (figure 1G and data not shown). In contrast, Kar9 loading on both microtubules and spindle poles was lost in bim1μ cells (figure 1D). However, Bim1 is symmetrically distributed on nuclear and cytoplasmic microtubules, and spindle poles (figure 1E), and Kar9-YFP decorated only a subset of Bim1-CFP-labeled microtubules (figure 1F).
Figure 1: Kar9 associates asymmetrically with spindle poles.
A- Pictures of wild type cells expressing CFP-Tub1 and Kar9-YFP. The chromosomal copy of KAR9 is fused with the YFP-coding sequence allowing expression of Kar9-YFP at endogenous level. B- Wild type cells with spindle poles labeled with Spc42-CFP, and expressing Kar9-YFP as in (A). C- Cells as in (B), but treated with nocodazole for 30 minutes. D- Localization of Kar9 in bim1Δ cells. No Kar9-YFP is seen on either microtubules or SPB. E- Localization of Bim1. Picture of cells where endogenous Bim1 is C-terminally tagged with GFP. F- Colocalization of Bim1-CFP and Kar9-YFP. Endogenous Kar9 and Bim1 are tagged with YFP and CFP respectively. G- Quantification of Kar9 asymmetry in cells affected in the indicated manner. All pictures are projections of at least six z-sections through the cell. Scale bars are 1 μm.
Thus, while Bim1 is required for Kar9 localization on spindle poles and microtubules, it does not account for Kar9 asymmetry. Thus, Kar9 interaction with microtubules and spindle poles must be spatially regulated.

Next, we tested whether Kar9 asymmetry was dependent on the overall asymmetry of the cell. cdc42-1 cells fail to polarize their cytoskeleton and do not form a bud at the restrictive temperature. However, they replicate DNA and assemble metaphase spindles correctly (Sia et al., 1996). In these unbudded cells, Kar9 was still distributed asymmetrically on the spindle (figure 1G). Kar9 also distributed asymmetrically on spindle poles in cells treated with latrunculin A, a drug that depolymerizes actin and disrupts the initial phase of spindle positioning (Theesfeld et al., 1999). Likewise, Kar9 asymmetry was not affected in the myo2-16 cells shifted to the restrictive temperature. Thus, Kar9 asymmetry is not dependent on cortical cues or cortical asymmetry. It may rather depend on some intrinsic asymmetry of the spindle.

Cdc28 activity is required for the maintenance of Kar9 asymmetry

Recent studies have implicated Cdc2 in asymmetric cell division in Drosophila, and Cdc28/Cdk1 in spindle orientation in S. cerevisiae (Tio et al., 2001; Segal et al., 1998). How Cdk1 promotes asymmetric cell division is not known. To investigate this question, we constructed a strain in which Cdc28 was replaced by its inhibitable variant, Cdc28-1as (Bishop et al., 2000) and endogenous Kar9 was C-terminally tagged with YFP. These cells also expressed CFP-Tub1 to visualize microtubules. Treatment of this strain with the inhibitor (1NM-PP1) led to growth arrest at G2/M, as assayed by spindle and bud morphology, as previously described (Bishop et al., 2000). Control cells (CDC28 KAR9-YFP CFP-TUB1) were not affected by treatment with 1NM-PP1. In the cdc28-1as cells, Kar9 rapidly redistributed upon drug treatment but not upon treatment with DMSO (figure 2A,B). Kar9 rapidly appeared on both poles and on cytoplasmic microtubules emanating from both sides of the spindle. Such symmetric localization increased within the first 10 minutes of treatment (figure 2B) and reached its maximum within 20-40 minutes. No such symmetry was observed in control cells treated with the drug. These results indicate that Cdc28 activity is required to maintain the asymmetric distribution of Kar9.
**Figure 2:** Asymmetric loading of Kar9 onto spindle poles and microtubules depends on Cdc28 activity

A- Cells expressing the inhibitable form of Cdc28, Cdc28-las, CFP-Tub1 and Kar9-YFP, were treated with the inhibitor (1NM-PP1) or DMSO for the indicated amount of time. Scale bar is 1 µm. B- Quantification of Kar9 asymmetry in cells arrested with a short spindle (hydroxyurea block). Partial asymmetry was scored when Kar9 fluorescence intensity on the mother-bound pole and microtubules comprised between 10% and 50% of the intensity on the daughter side of the spindle. When the difference between the two sides of the spindle was less than a factor 2, the spindle was considered as symmetric. At least 200 cells were counted each time.
To test whether Cdc28 acts directly on Kar9, we investigated whether Kar9 was modified in a Cdc28-dependent manner in vivo. The chromosomal copy of Kar9 was C-terminally fused with the TAP tag (Puig et al., 2001) and its modification was investigated by SDS-PAGE and Western-blot analysis. Kar9-TAP was fully functional (see figure 7G). Kar9 migrated as several forms (figure 3A), which were resolved to a single fast migrating form upon treatment with phosphatase. In agreement with Kar9 being a phosphoprotein, two-dimensional electrophoresis (figure 3B) revealed that Kar9 exists as a major form and three increasingly acidic isoforms (arrows, figure 3B). Thus, Kar9 is a phospho-protein in vivo. Several observations indicated that Kar9 phosphorylation is dependent on Cdc28 activity. First, Kar9 phosphorylation sharply disappeared in the cdc28-las cells upon treatment with the inhibitory drug. No such effect was observed with DMSO alone (figure 3C). Second, Kar9 phosphorylation was also strongly reduced in the cdc28-4 mutant grown at the permissive temperature and totally abolished upon shift to the restrictive temperature (figure 3D). Third, the level of Kar9 modification varied during the cell cycle, being high shortly after bud emergence and decreasing at the onset of anaphase (figure 4A). Finally, both S197A and S496A mutations, which affect the two only Cdc28 consensus sites in Kar9, inhibited Kar9 phosphorylation (figure 3E). Mutation of both sites (Kar9-AA) abolished the formation of slow migrating forms (figure 3E) and the formation of acidic isoforms (figure 3F). Therefore, we conclude that Kar9 is phosphorylated by Cdc28 at positions 197 and 496 in vivo. The site at position 496 may be conserved in APC (adenomatous polyposis coli; Bienz, 2001; figure 3E).

The pattern of Kar9 phosphorylation during the cell cycle suggested that it depends on early cyclins. Consistent with this possibility, Kar9 was hypo-phosphorylated in cells deleted for the cyclin gene CLB4, and to a lesser extend, deleted for CLB3. In cells lacking both Clb3 and Clb4, Kar9 migrated like Kar9-AA (figure 3G). In contrast, Kar9 phosphorylation was unchanged in cells deleted for either of CLN1, CLN2, CLB5, CLB1 or CLB2 (figure 3G). Thus, our results are consistent with Kar9 being phosphorylated by Cdc28/Clb4 and Cdc28/Clb3 on Ser197 and Ser496 in vivo.
Asymmetric loading of Kar9

Figure 3: Kar9 is phosphorylated in a Clb4-, Clb3- and Cdc28-dependent manner

A- Kar9 is phosphorylated in vivo. The migration of a TAP-tagged version of Kar9 was analyzed by SDS-PAGE and western analysis using IgG for the detection. Immunoprecipitations from asynchronous cells or cells arrested in S phase with hydroxyurea (HU) and treated or not with CIP phosphatase are shown. B- Analysis of Kar9-TAP by NEPHGE reveals three acidic isoforms (arrows). C- Phosphorylation is abolished upon inhibition of Cdc28. The cdc28-las cells expressing Kar9-TAP at endogenous level were treated with 1NM-PP1 or DMSO for the indicated time. The cells were arrested with HU for the entire time of the experiment. In this strain and under these conditions, Kar9 is almost completely in its phosphorylated form prior to drug treatment. D- Migration of Kar9-TAP in the cdc28-4 mutant. E- Migration of Kar9 mutants where putative Cdc28 phosphorylation sites are mutated to alanine. Sequences of the putative Cdc28 phosphorylation sites are shown. The site at position 496 is conserved in the alignment to APC proposed by . F- Analysis of Kar9-AA migration as in B. G- Migration of Kar9-TAP in cyclin mutants. Phosphorylated forms are indicated with an arrow. The position of the non-phosphorylated Kar9-TAP is indicated by a line.
Phosphorylation of Kar9 by Cdc28/Clb4 controls its asymmetry

We next investigated whether Kar9 phosphorylation affected its interaction with known partners. Wild type cells expressing Kar9-TAP and HA-Bim1 were synchronized. At regular intervals samples were taken and Kar9-TAP was immunoprecipitated (IP) from cell extracts. The amount of Kar9-TAP, HA-Bim1, Stu2 and Myo2 present in the IPs was estimated by western-blott analysis. The level of Myo2 and Stu2 co-IPing with Kar9 did not vary significantly during the cell cycle (figure 4A). In contrast, the amount of co-IPed HA-Bim1 decreased dramatically when Kar9 was maximally phosphorylated (figure 4A). Thus, phosphorylation by Cdc28 may decrease the ability of Kar9 to interact with Bim1, similarly to what is the case for APC (Trzepacz et al., 1997). By modulating its affinity for Bim1, phosphorylation might spatially regulate the ability of Kar9 to interact with microtubules and spindle poles.

To investigate if Kar9 phosphorylation regulates its distribution, mutants that inhibit Kar9 phosphorylation in vivo (Kar9-S197A, Kar9-S496A and Kar9-AA) were C-terminally tagged with YFP and their intracellular distribution was analyzed (figure 4B). Kar9-YFP associated with both poles of short spindles in only 15% of the cells. In contrast, this proportion increased to 51% for Kar9-S496A-YFP, 56% for Kar9-S197A-YFP and 58% for Kar9-AA-YFP (figure 4C). Consistent with Clb4 being the predominant cyclin for Kar9 phosphorylation, disruption of CLB4 similarly affected the asymmetric distribution of Kar9 (figure 4B and C). Disruption of either CLB2 or CLB5 had no effect (figure 4B and C). We conclude that phosphorylation of Kar9 by Clb4/Cdc28 controls its distribution by preventing its loading and/or maintenance on the mother-bound pole.

Clb4 localizes to the mother-bound pole

The observation that Clb4 prevents Kar9 loading on the mother-bound pole prompted us to investigate Clb4 distribution. A CLB4-GFP fusion gene under the control of a galactose inducible promoter (pGAL1) was transformed into wild type cells expressing Spc42-CFP. A CLB5-GFP construct was used for comparison. After three hours on galactose, Clb4-GFP was observed as a diffuse staining in the nucleoplasm and as a prominent dot at the nuclear periphery (figure 4D). This dot co-localized with Spc42-GFP in 100% of the cases. In budded cells with duplicated SPBs, Clb4 localized essentially to one pole. In cells with a properly oriented spindle, the prominent Clb4-GFP dot co-localized with the Spc42-CFP labeled pole distal to the bud (figure 4D). Clb5-GFP was only seen as a diffuse nuclear staining (data not shown). Thus, Clb4 localized asymmetrically on spindle poles and preferentially to the pole without Kar9. Similar results were obtained with Clb3 (data not
Asymmetric loading of Kar9

These results are consistent with Clb4/Cdc28 phosphorylating Kar9 at and thereby inhibiting its localization to the pole destined to the mother cell.

Figure 4: Kar9 phosphorylation is required for its asymmetric loading on spindle poles

A- Coprecipitation of Bim1 with Kar9 is reduced in extracts where Kar9 is hyperphosphorylated. Coprecipitation of Stu2 and Myo2 is not affected. Wild-type cells expressing HA-Bim1 and Kar9-TAP were synchronized with pheromone. Samples were taken every 20 minutes and Kar9-TAP was immunoprecipitated with IgG beads. The amount of Kar9-TAP, HA-Bim1, Myo2 and Stu2 in the immunoprecipitates was estimated by western. A quantification of Bim1/Kar9 ratios relative to cell cycle progression is shown. The amount of Bim1 present in each extract was used to standardize Bim1 levels. B- Localization of Kar9 and Kar9-S197A,S496A (Kar9-AA) fused to YFP is shown in cells where microtubules are visualized with the CFP-Tub1 reporter. In addition, the two bottom rows show the localization of Kar9-YFP in cells where the indicated cyclin is deleted. To ensure proper visualization of Kar9- and Kar9-AA-YFP onto spindle poles, cells were treated with nocodazole such as to depolymerize cytoplasmic microtubules without affecting the spindle. C- Quantification of the asymmetric distribution of the different Kar9 mutants and of Kar9 in cells lacking the indicated cyclins. At least 200 cells were counted for each sample. D- Localization of Clb4-GFP at the spindle pole. SPC42-CP pGAL1::CLB4-GFP cells were grown in galactose for 3 hours and analyzed by fluorescence microscopy. The GFP signal was recorded with a YFP filter to avoid cross talk with the CFP signal.

69
Kar9 is loaded onto microtubules at the spindle pole

Next, we wanted to clarify the function of Kar9. Using time-lapse microscopy we frequently observed that Kar9 dots moved from the spindle pole towards the cortex along microtubules and retracted towards the spindle pole together with shrinking microtubules (Figure 5A; supplementary movie 1). Altogether, Kar9 localized primarily to microtubules emanating from the Kar9-loaded pole (examples in figure 1A, 2A and 7A). Furthermore, in cells treated with nocodazole no Kar9-YFP dots were observed at the bud cortex (figure 1C). In these cells, Kar9 was only observed on the spindle pole. Thus, our data indicate that, unlike for overexpressed Kar9 (Miller and Rose, 1998), the localization of endogenous Kar9 to the bud cortex depends on microtubules. They also suggest that these Kar9 molecules originally come from the spindle pole.

To clarify the origin of the Kar9 molecules present in cortical dots, cells expressing GFP-Tub1 and Kar9-GFP or Kar9-GFP alone were subjected to photobleaching experiments (figure 5B,C). First, the bud was irradiated to photobleach cortical Kar9-GFP. In these cells, Kar9 dots still formed and were seen to move from the presumptive spindle pole into the bud (figure 5B, 13/14 experiments). Once in the bud, they neither disappeared nor lost intensity compared to unbleached cells, suggesting that they did not exchange material with bleached molecules at the bud cortex. In experiments where GFP-Tub1 was also present, these dots localized to the plus end of growing and shrinking microtubules. Thus, little or no Kar9 found at the tip of microtubules in the bud originated from the bud cortex.

In reverse experiments, we bleached the pool of Kar9 at the pole and monitored dot formation in the bud. In 8 out of 9 such experiments, no recovery of Kar9 was observed at the pole and no Kar9 dots formed in the bud (figure 5C). Thus, the Kar9 molecules present at the tip of microtubules in the bud all originate from the spindle pole or its vicinity. A putative cortical pool of Kar9 made no or little contribution to the formation of Kar9 dots. Together, our results indicate that Kar9 was loaded onto microtubules at the spindle pole. This conclusion is consistent with the observation that Kar9 localization was restricted to the microtubules emerging from the Kar9-loaded pole.
Figure 5. Kar9 localizes exclusively to microtubules and microtubule loading occurs at the spindle pole.

A- Movie showing a Kar9 dot migrating along a microtubule. The dot moved towards the spindle pole with the retracting microtubule. The end of the microtubule is marked with a black arrow; the Kar9 dot with a red arrow. B- Kar9 molecules present on microtubule tips is neither provided by nor exchanged with cortical Kar9. The bud of a cell expressing Kar9-GFP was photobleached by laser. Kar9 dots were observed to migrate into the bud and their intensity did not diminish as they entered the bleached area. The bleached area is outlined in blue. Kar9 dots are marked with red arrows. The elapsed time after photobleaching is indicated in each frame. C- Kar9 molecules present at microtubule tips originate from the spindle pole. The Kar9 dot observed in the mother cell (spindle pole) was photobleached as in B. No recovery was observed in the mother, nor did dots form in the bud.
Microtubules emanating from the bud-bound pole are actively guided towards the bud

Together, our results indicate that Kar9 acts predominantly on microtubules and not at the cortex, as previously proposed. Thus, we re-examined the function of Kar9. To address this issue, we first analyzed the behavior of cytoplasmic microtubules in wild-type cells by 3D time-lapse microscopy. As described, microtubule behavior unambiguously identified an active (daughter-bound pole) and a relatively inactive pole (mother-bound pole) (Vogel et al., 2001). We observed a striking difference between the microtubules coming from each pole in their ability to orient in the cell. Short microtubules (< 1μm) growing from the daughter-bound pole emanated at various angles relative to the mother-bud axis (figure 6A). However, they rapidly re-oriented towards the bud as they elongated (figure 6A), indicating that they were guided by some mechanism. In contrast, microtubules coming from the mother-bound pole did not reorient (figure 6B, C).

To confirm this observation, we monitored over time the angle that microtubules (N>120) made with the mother/bud axis (polar angle) in wild type cells. When the microtubules emerged from the daughter-bound pole, this angle dropped within few frames upon microtubule emergence (examples in figure 6B). Microtubule orientation took place in the mother cell and did not depend on whether the spindle was properly oriented or not (example in figure 6A). In contrast, the polar angle of microtubules coming from the mother-bound pole varied little over time, indicating that these microtubules failed to reorient (examples in figure 6B). This difference was obvious when we plotted polar angles at t=0 (angle upon emergence from the pole) versus t=27 seconds (three frames after emergence) for a population of microtubules coming from either the daughter-bound or the mother-bound pole (figure 6C). 98% of the microtubules coming from the daughter-bound pole (N = 81) reduced their angle within this time, and 93% of them reached a small angle (> 25°) independently of their angle of emergence. In contrast, microtubules coming from the mother-bound pole (N=34) remained on the diagonal of the graph, indicating that they barely changed orientation within 27 seconds. Accordingly, only 9% of them reached low angles. Together, our results indicate that microtubules growing out of the daughter-bound pole and loaded with Kar9 do not randomly search for the bud cortex but are guided from the start towards their target.
Asymmetric loading of Kar9

Figure 6: Kar9- and Myo2-dependent orientation of d-pole but not m-pole microtubules towards the bud

A- Movie of a wild type cell with a short spindle showing a microtubule growing from one pole and orienting towards the bud. The microtubules were visualized with GFP-Tub1. This cell also expresses Kar9-GFP, causing the increased intensity at the plus end of the orienting microtubule.

B- Measurement of polar angles over time in wild type cells. Examples are shown for single individual microtubules that emanated from either the daughter- (d-) or the mother-bound (m-) pole. A drawing defines the polar angles.

C- Plot of polar angles at t = 27 sec. versus t = 0 for d-pole (red, N=81) and m-pole (blue, N=34) microtubules. Note that within this time frame most d-pole microtubules have reoriented towards the bud (polar angle 0), while the m-pole microtubules remain along the diagonal.

D- Polar angles over time for d-pole microtubules in the kar9-A (blue) and the dhcl-A (red) mutants.

E- Polar angles over time for d-pole microtubules of a myo2-16 mutant grown at the permissive temperature (red) or after a 10 minutes shift to the restrictive temperature (blue).

F- Polar angles over time for d-pole microtubules in cells treated with latrunculin A (blue), and after washing out the drug (red).

G- Statistical analysis of d-pole microtubule reorientation towards the bud in cells of the indicated phenotypes and upon growth in the indicated conditions. 50 to 200 microtubules were traced for each quantification. Microtubules were scored as orienting if 1- they were not properly oriented at emergence, 2- reorientation towards lower angles was rapid (took place within the first three frames), and 3- the microtubule plus end actually reached the bud neck.
Microtubule guidance depends on Kar9, Myo2 and actin

We next investigated the mechanism of microtubule guidance towards the bud. This process depended on Kar9, since cells lacking Kar9 completely failed to orient microtubules (figure 6D, G). It also depended on Myo2, since the myo2-16 cells showed the same defect upon shift to the restrictive temperature (figure 6E, G). The myo2-17 allele leads to a temperature sensitive growth defect, yet does not affect either motor function or the Kar9-Myo2 interaction (Yin et al., 2000). This allele did not affect microtubule orientation (figure 6G). Thus, the function of Myo2 in microtubule guidance correlates with its ability to bind Kar9. Finally, cells treated with latrunculin A also failed to reorient microtubules towards the bud (figure 6F). In contrast, reorientation of microtubules emanating from the active pole was not affected by disruption of either dynein or BIK1 genes (figure 6D and G). We conclude that microtubules emanating from the active pole were guided towards the bud in an actin-, Myo2- and Kar9-dependent manner.

Symmetric loading of Kar9 onto spindle poles orients both poles towards the bud

Taken together, our data indicate that Kar9 loading onto microtubules at one spindle pole targets these microtubules into the bud. In turn, this may orient the spindle pole towards the bud. To test whether this is the case, we next investigated the consequence of symmetric loading of Kar9 onto spindle poles using Kar9-AA-YFP, which frequently localizes at both poles (figure 4B and 7B). Cells expressing this mutant protein together with CFP-Tub1 were monitored by time-lapse microscopy. The difference between the cells expressing wild type (supplementary movie 2) and mutated Kar9 (supplementary movie 3) was striking. In cells expressing only wild-type Kar9, most spindles (80%, N=59 movies) were properly oriented, with Kar9 localizing on the pole and microtubules pointing towards the bud (figure 7A). These spindles rarely changed position or orientation. In contrast, Kar9-AA was frequently on both sides of the spindle (poles and microtubules, 56%, N=36 movies) and the vast majority of the spindles were mis-oriented (87%, figure 7B). They performed a “dance”, in which each pole repeatedly moved towards the bud and retracted as the other moved towards the bud (supplementary movie 3). In KAR9-AA cells, microtubules coming from both poles were guided towards the bud at high frequency (figure 7C, N=142 microtubules). This was in contrast to wild type cells (figure 6B, C, N= 191 microtubules), where mainly microtubules coming from the daughter-bound pole, and to kar9μ cells, where microtubules coming from neither pole were guided towards the bud (figure 7C, N=114 microtubules). Cells expressing both Kar9-AA and endogenous Kar9 also guided microtubules coming from both poles towards the bud (figure 7C, N= 254 microtubules). Thus, Kar9-AA acted in a dominant manner to guide microtubules from both poles towards the bud. Therefore, the serine to
alanine mutations affected only Kar9 distribution but not its ability to guide microtubules towards actin nucleation sites. Accordingly, Kar9-AA was also fully functional for proper microtubule orientation during mating (figure 7D). Together our data indicate that loading of Kar9 on both poles leads to the targeting of both poles towards the bud.

Similar results were obtained in the clb4μ mutant, where Kar9 was associated with both poles of the majority of the spindles (73%, 53 movies). These spindles were mis-oriented (79%, figure 7E) and showed aberrant spindle movements similar to those of the cells expressing Kar9-AA (supplementary movie 4). In contrast, cells lacking Clb5 behaved like wild type in regards to spindle orientation (figure 7F) and movement (supplementary movie 5).

Kar9 asymmetry is specifically required for its function in spindle positioning

Inactivation of Kar9 function is not lethal in wild type cells because the dynein pathway rescues spindle positioning during anaphase. However, the kar9D dhc1D double mutant is not viable (Miller et al., 1998). To test whether asymmetric loading of Kar9 onto microtubules is required for proper Kar9 function, we tested whether the dhc1D KAR9-AA strain is viable. A dhc1D kar9D strain rescued with a URA3 based KAR9-TAP plasmid was transformed with constructs encoding either Kar9, or Kar9-AA, or with an empty vector. The transformants were then re-streaked on 5-FOA to test whether they survived in the absence of the URA3 and KAR9 containing plasmid. As shown in figure 7G, the KAR9-AA plasmid was not able to support growth of the dhc1D kar9D double deleted strain. Consistently with Kar9-AA acting dominantly and not as a null, a dhc1μ KAR9 strain transformed with a KAR9-AA containing plasmid was unable to grow at 20°C (figure 7H). The same strain was not affected for growth at this temperature when transformed with an empty vector or a KAR9 containing plasmid. Altogether, our data demonstrate that asymmetric loading of Kar9 on spindle poles and microtubules is essential for Kar9 function in spindle alignment.
Figure 7: Kar9 asymmetry is required for proper spindle positioning
A- Wild type cell expressing wild type Kar9 fused to YFP, together with CFP-Tub1. B- A wild type cell expressing Kar9-AA-YFP at endogenous levels. Kar9-AA-YFP is present at both poles, microtubules from both poles point towards the bud and the spindle is mis-aligned. Since the wild type copy of Kar9 is also present, Kar9-AA acts in a dominant manner. C- Kar9-AA causes guidance of mother-bound microtubules towards the bud, in a dominant manner. Quantification of the fraction of microtubule guided towards the bud as in figure 6G. This quantification is shown for both d- and m-pole microtubules in the strains of the indicated genotypes. D- Picture of cells treated with pheromone. GFP-TUB1 cells expressing solely the indicated from of Kar9 were monitored for their ability to orient microtubules (green arrows) towards the shmoo tip (red arrows). E- A clb4Δ KAR9-YFP CFP-TUB1 cell. F- A clb5Δ KAR9-YFP CFP-TUB1 cell. G- A dhclΔ kar9D double mutant containing a pURA3-KAR9 plasmid was transformed with TRP1 based plasmids containing wild type KAR9, KAR9-AA or no insert. Absence of growth on 5-FOA plates (on which URA3 cells cannot grow) indicates that the indicated TRP1 plasmid cannot replace wild type KAR9 for spindle orientation in the absence of dynein. H-
Discussion

Spindle asymmetry is required for proper spindle orientation

In this study, we show that asymmetric localization of spindle pole components is required for alignment of the spindle with cell polarity. This conclusion is based on the following observations.

First, the microtubule-associated protein Kar9 localized asymmetrically on the spindle poles and on microtubules. At the spindle pole, Kar9 appears to localize to the SPB rather than on short cytoplasmic microtubules, since Kar9 levels at the pole are not sensitive to nocodazole. In contrast, the localization of microtubule associated protein to spindle poles, such as dynein, is impaired by microtubule depolymerizing drugs (Shaw et al., 1997). According to the idea that Kar9 localizes to the SPB independently of microtubules, we found (similarly to Lee et al. 2000) that overexpressed Kar9 localizes to spindle poles in bim1D cells (Kusch and Barral, unpublished data). In these cells, Kar9 localization to microtubules was not restored by overexpression. Thus, Kar9 localizes asymmetrically to both SPBs and microtubules.

Second, Kar9 asymmetry depended on the activity of the Cdk1 kinase, specifically Cdc28/Clb4 and perhaps Cdc28/Clb3. Cdc28/Clb4 and Cdc28/Clb3 phosphorylated Kar9 in vivo and also localized asymmetrically onto SPBs. Finally, unphosphorylatable variants of Kar9 localized symmetrically on spindle poles and microtubules. This form of Kar9 was still active for microtubule guidance, as observed during both mating and spindle orientation. However, spindles that were symmetrically loaded with Kar9 failed to achieve and maintain proper orientation.

Kar9 has been proposed to mediate microtubule interaction with the cortex (Bloom, 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000; Miller and Rose, 1998). The model currently accepted proposes that Kar9 is localized to the bud cortex by the type V myosin, Myo2. There it forms a capture site for microtubules, due to its ability to interact with the EB1 homologue Bim1. Microtubules are thought to reach this site by random search. Our data are inconsistent with this model. First, we found that endogenous Kar9 localized to and acted on microtubules, rather than at the cortex. In fact, Kar9 was only seen at the cortex independently of microtubules when over-expressed (Korinek et al., 2000; Lee et al., 2000; Miller and Rose, 1998). Second, we show that, during their growth, microtubules are guided towards the bud in a Kar9-, Myo2- and actin-dependent manner. Therefore, orientation did not depend on random search. Finally, if Kar9 acted at the cortex, microtubules coming from both spindle poles would have the same chance to reach attachment sites. However, the analysis of spindle pole inheritance (Pereira et al., 2001) and our analysis on microtubule behavior showed that this is not the case. Not only are microtubules guided towards cortical
sites, but guidance was restricted to microtubules coming from a single pole. Therefore, our results establish that Kar9 must rather be viewed as an adapter that links microtubule plus ends to the myosin V Myo2, as already proposed by Yin et al. (2000). In this manner, Kar9 guides microtubules along actin cables and towards actin nucleation sites at the bud neck (Kusch et al., 2002, Chapter II) and in the bud (Miller and Rose, 1998).

A model for Kar9 function in spindle orientation

Altogether our results support the following model (figure 8): Kar9 loading onto microtubules takes place mainly at the spindle pole. During G1 and mating, the cell contains a single SPB. This SPB harbors Kar9, which is loaded onto microtubules emanating from this pole. In cells with unduplicated SPBs, Kar9 is crucial to orient the spindle pole towards the cortex to position the nucleus adequately for mitosis or karyogamy (Miller and Rose, 1998). However, upon spindle pole duplication, alignment of the spindle along the polarity axis requires that only one pole be directed towards actin nucleation sites. The other spindle pole needs to be inactive in regard to Kar9 loading. Regulation of Kar9 loading is ensured by the cell cycle machinery, which phosphorylates Kar9 and reduces its affinity for Bim1. This inhibition prevents efficient association of Kar9 with microtubules and spindle poles. Our results indicate that this inhibition most likely takes place locally at the mother-bound pole. Indeed, Clb4 was found to localize preferentially to the mother-bound pole at which Kar9 binding is low. Thus, we propose that it is the recruitment of Clb4 to a single pole that ensures the asymmetry of Kar9 distribution.

The mechanism of Clb4 asymmetry needs now to be elucidated. Clb4 asymmetry may depend on some structural asymmetry. Our data do not support the idea that spindle asymmetry depends on cortical polarity. Rather, Clb4 localization may be coupled to spindle pole body duplication. Clb4 and Clb3 participate in this process (Haase et al., 2001; Tyers et al., 1991). Therefore, they may associate primarily with the assembling SPB. Supporting this idea, recent studies revealed that the new SPB is directed towards the mother (Pereira et al., 2001).

Spindle poles as loading machines

Another important conclusion of our study is that Kar9 is loaded onto microtubules at the spindle pole, a conclusion supported by our photobleaching data and the observation that Kar9 distribution is spindle pole-dependent. Alternatively, Kar9 may be loaded onto microtubules at other cellular locations, but its maintenance on specific microtubules must be
Asymmetric loading of Kar9

controlled at the spindle pole. Thus, we propose that spindle poles act as loading machines for microtubule associated factors. Conceptually, this finding has at least two interesting implications. First, this ensures that only microtubules that are associated with a specific pole become loaded with given molecules. This could prove important in cells such as neurons, where microtubules frequently detach from nucleation sites (Baas, 1999), or in cells where microtubules are nucleated at various sites. Second, spindle pole-dependent loading sorts microtubules into functional classes and gives them different properties dependent on the pole they emanate from. In yeast, we show that this plays a crucial role in positioning the spindle appropriately relative to the polarity axis.

How should we conceive the loading activity of spindle poles? Spindle poles could titrate cargos from the cytoplasm and ensure that they do not interact with microtubules at random sites. Our FRAP data are consistent with this possibility. In addition, spindle poles are likely to interfere with the regulation of motor molecules. The loading of molecules like Kar9 may depend on their motorized transport to microtubule plus ends, a view supported by the observation of Kar9 dots moving along microtubules. Recent studies have shown that the +tip molecule EB1 also associates with centrosomes (Rehberg and Graf, 2002) and that some minus-end molecules, such as gamma-tubulin, affect plus-end activities (Vogel et al. 2001). Thus, microtubule organizing centers may have a conserved function as loading machines.

---

Figure 8: Model
Kar9 attaches first to the daughter-bound pole. Binding is inhibited at the mother-bound pole due to high Clb4 activity at that pole. Kar9 is loaded onto microtubules at the daughter-bound pole upon nucleation or transported to the plus end via motor activity. Kar9-loaded microtubules are then guided towards the bud along actin cables, due to Kar9-dependent recruitment of Myo2. Microtubules emanating from the mother-bound pole are not loaded with Kar9 and therefore do not orient towards the bud.
Chapter III

A compass theory for spindle alignment

Spindle alignment with polarity cues is a recurrent and crucial process during asymmetric cell division. In most cases, the question of whether the two poles are identical or not is highly debated. Here, we propose that spindle alignment functions like the alignment of a compass with the north-south axis of the earth. Orientation of the compass relies on the fact that it has its own magnetic polarity. Interaction of the polarity of the compass with that of the external magnetic field is what allows the compass to “read” the orientation of that field. The situation is very similar in the case of the mitotic spindle. Both the establishment of a spindle polarity and the interaction of one of the polarized components, Kar9, with cortical polarity, via actin cables, were crucial for the proper orientation of the spindle.

This mechanism may be conserved. Our results reveal similarities between Kar9 and APC (adenomatous polyposis coli). Like APC, Kar9 interacts with EB1, localizes to microtubule ends, and is implicated in microtubule orientation and spindle positioning. In turn, APC also interacts with the actin cytoskeleton and is regulated by phosphorylation (Tio et al., 2001; Dikovskaya et al., 2001; Bienz, 2001). Remarkably, human APC has been shown to be a substrate of Cdc2, and its phosphorylation by this cyclin-dependent kinase reduces the affinity of APC for EB1 (Trzepacz et al., 1997). The phosphorylation site involved is conserved between APC and Kar9 (Bienz, 2001; figure 3E). Thus, our findings may have implications for our understanding of spindle orientation in many other cell types.

Experimental Procedures

Strains, Plasmids and Growth Conditions

Media and genetic methods are described (Guthrie and Fink, 1991). Yeast strains are listed in Supplementary Table T1. C-terminal tags and gene deletions were generated at endogenous loci as described (Knop et al., 1999; http://www.cnrs-gif.fr/cgm/epissage). GFP-TUB1 and CFP-TUB1 cassettes were integrated into the URA3 locus. Phospho-inhibiting mutations were generated by PCR, tagged C-terminally with TAP or YFP and cloned into pRS vectors (Sikorski and Hieter, 1989). Plasmids were confirmed by sequencing.

For a-factor arrest, cells grown in YPD at 23°C to OD600~0.8 were incubated with 5 μg/ml a-factor. Hydroxyurea (HU) arrest was performed by incubating exponentially growing cells in HU (100 mM) for 2h at 30°C. To inhibit Cdc28, cdc28-1as cells arrested with HU as
described, were pelleted and re-suspended in fresh medium containing 1 μM 1NM-PP1 in DMSO, or DMSO alone (1% final).

Protein and Immunological Techniques

Extracts were prepared from 15 OD600 cells washed 3 times (W-buffer: 150 mM NaCl, 1 mM EDTA, 5 mM MgSO4, 50 mM Tris-HCl pH 7.8, 20 mM b-glycerophosphate). Cell pellets were broken with glass beads for 2 x 3 min at 4°C in 100 μl of L-buffer (W-buffer with protease inhibitors, Sigma yeast protease inhibitor cocktail, and 1% NP-40). 900 μl of W-buffer was added and extracts were clarified at 13 000 g for 10 min. Immunoprecipitations were performed as described (Yin et al., 2000). Equal amounts of total protein were used each time. After immunoprecipitation (15 μl IgG-Agarose, Sigma), and extensive washes (W-buffer + 0.1% NP-40), proteins were eluted at 90°C for 7 min in 1.5X Laemmli buffer. For immunoblots Peroxidase/anti Peroxidase (PAP, Sigma), anti-Myo2 (gift from A. Bretscher), anti-HA (Santa Cruz Biotechnology), and anti-Stu2 (gift from T. Hyman) antibodies were used at 1:2000. Immunoblots were performed as described (ECL, Amersham). Phosphatase treatment was performed with 10 U of Calf Intestinal Phosphatase (New England Biolabs) in the recommended buffer at 37°C for 30 min. Kar9 isoforms were analyzed in two-dimensional gels by NEPHGE (O'Farrell, 1977). Extracts were prepared from untagged and Kar9-TAP strains as described, and analyzed by two-dimensional PAGE (Vogel et al. 2001), focusing with 8/10:3/10 ampholytes for 1500 volt hours.

Fluorescence and time-lapse Microscopy

Fluorescence microscopy was performed as described (Kusch, 2002) with an Olympus BX50 microscope equipped with a piezo motor, TILLVision software (TILLPhotonics, Martinsried, Germany) and a HQ-lamp as light source. For the localization of GFP-, YFP- and CFP-labeled proteins, Z-stacks of 6-11 layers (step size 0.3-0.4 μm) were taken with appropriate exposure times and projected into a single plane.

Time-lapse microscopy was performed using a monochromator Polychrom IV as light source and a high speed CCD camera (Imago, TillPhotonics). Z-stacks of 9 focal planes (0.3 μm apart, 1 sec exposure, binning 2x2) were taken continuously for 450 seconds. Two color movies were performed using a Chroma CFP/YFP dual band filter. In this case, Z-stacks of 5 focal planes (0.4 μm apart) with 1 second acquisition for each channel were taken at 0 or 10 seconds intervals. Projected pictures were analyzed using NIH Image 1.62b7 (by Wayne Rasband, NIH). Microtubule angles were measured from the time of emergence until they reached the neck or shrank back.
myo2 mutants were shifted to the restrictive temperature for 5 minutes on the microscope heating table prior to observation. Latrunculin A was added at 200µM final to early log cells for 15-60 min. at room temperature. Staining with rhodamin-phalloidin confirmed the effect of the drug. For recovery, 3 washes were performed with fresh medium. The cells recovered for 30 minutes before viewing.

Photobleaching

Photobleaching experiments were performed on a Zeiss LSM510 confocal microscope using an argon laser (maximum output: 25 mW). GFP was visualized at 2% transmission. Photobleaching was carried out at full intensity for 10-20 iterations. Afterwards, the signal was detected by continuous acquisition of 10 focal planes (0.4µm apart) for 5-10 minutes. Bleaching was minimal; Kar9-GFP dots were still visible in unbleached cells. Photobleaching did not affect cell viability or microtubule dynamics as tested in cells labeled with GFP-TUB1.

Acknowledgement

We thank Markus Aebi, Antony Bretscher, Damian Brunner, Gaudenz Danuser, Michael Knop, Ari Helenius, Tony Hyman, Carl Mann, and Matthias Peter for their support, sharing of strains, fruitful discussions and help with the manuscript. We are particularly endowed with Miriam Sattler for her help in two-dimensional electrophoresis experiments. This work was supported by grants from the Swiss Federal Institute of Technology to Y.B. and a EMBO fellowship to D.L. Y.B. is member of the EMBO young investigator program.

References

The references of this publication are included in the general bibliography from page 103.
Chapter IV

General discussion

In general

The work presented in this thesis dissected the molecular mechanisms that are the bases for proper microtubule regulation in spindle positioning in budding yeast and developed new concepts of microtubule function that account for two aspects of spindle positioning: spindle coordination with the neck and spindle alignment with the polarity axis.

We found that in yeast, coordination of the mitotic spindle with the cleavage site, the mother bud neck, was achieved by septin-dependent microtubule attachment. We demonstrated that phosphorylation by MARK-related SDK regulated microtubule shrinkage upon capture at the neck. We found that Kar9 to localized and acted as a +TIP that mediated microtubule attachment to various attachment sites. Additionally we uncovered an actin-dependent guidance mechanism that allowed Kar9 to efficiently recruit microtubule to cortical attachment sites. Finally we showed that asymmetric recruitment of Kar9 to one spindle pole was necessary to ensure proper spindle alignment with the mother bud axis.

These studies define Kar9 as a general adaptor for the communication of microtubule with cortical sites. Differential microtubule dynamics upon attachment to different cortical sites translate cortical topology into different spindle movements. Thereby, Kar9 acts as a sensor of the spindle that scans the cortex for directional input.

Our data allowed us to formulate explicit models for how budding yeast coordinates the position of the mitotic spindle with that of the cleavage site and how it is oriented along the polarity axis of the cell.

In the following I discuss the general validity of the concepts that apply for proper microtubule regulation in budding yeast for similar microtubule dependent processes in other organisms. The process of spindle orientation has been reviewed in (Kusch et al., 2003) which is attached at the end of this discussion.

Spatial coordination of the spindle and the cleavage site relative to each other

We first investigated the question, how budding yeast achieves spatial coordination of its mitotic spindle with respect to the predetermined cleavages site, the mother-bud neck. In yeast, the spindle has to be positioned according to a predetermined cleavage site (Glotzer,
2001; Guertin et al., 2002). So far, no relation between the cleavage machinery and the spindle had been observed in budding yeast.

In yeast, the septin ring at the bud neck serves as a platform to localize cleavage apparatus components such as the type II myosin Myo1 and Hof1 (Kinoshita, 2003; Longtine and Bi, 2003; Longtine et al., 1996).

**The role of septins in spindle positioning in yeast**

We first tested the role of septins in spindle positioning by analysing microtubule behaviour in septin mutants expressing GFP-alpha-tubulin. Compared to wild type cells, septin mutants exhibited a decrease in microtubule attachment at the bud neck (Kusch et al., 2002) Chapter II Fig. 3C). 4D fluorescence microscopy demonstrated that capture of microtubule ends at the bud neck is accompanied by microtubule shrinkage, which causes spindle pulling to the neck (Kusch et al., 2002) Chapter II Fig. 4). Microtubule attachment to the neck contributed to a major extent to spindle movement towards the neck when compared to microtubule attachment at the bud cortex (Kusch et al., 2002) Chapter II Fig 4C). Accordingly, septin mutants lacking neck attachment showed strong nuclear migration defects (Kusch et al., 2002) Chapter II Fig 1). Synthetic lethality of septin and dynein double mutants indicated that septin dependent microtubule attachment plays an essential role in spindle positioning prior to anaphase (Kusch et al., 2002) Chapter II Fig. 6A). This is consistent with our finding, that Kar9 participates in linking microtubule to the bud neck, placing septins in the early pathway of spindle positioning (Kusch et al., 2002) Chapter II Fig. 5 B, and Fig. 6B and C).

**Septins: a cortical cue for spatial coordination of the spindle relative to the cleavage site?**

These results indicated that the septins serve not only as a template for cleavage apparatus assembly but also as a new platform for microtubule end-on attachment at the bud neck. Microtubule capture-shrinkage mechanism at this site accounts to a large extent to spindle movement towards the bud neck prior to anaphase. These findings stand in contrast to the classical view, that spindle positioning is predominantly achieved by microtubule attachment to the bud cortex (Adames and Cooper, 2000; Yeh et al., 2000). However, microtubule attachment to the bud cortex only would ultimately result in movement of the whole spindle into the daughter cell, a case, rarely observed in wild type cells.

Alternatively, we propose direct attachment of astral microtubules with the future cleavage site and major contribution of this microtubule attachment in spindle pulling as a mechanism to restrict spindle position around the bud neck. These observations suggest for the first time a model for how the spindle is spatially coordinated with the predetermined cleavage site in budding yeast.
Is spatial coordination of the mitotic spindle and the cleavage site conserved?

Interaction between astral microtubules and the cleavage cortex in animals

In animal cells, the mitotic spindle determines the position of the cleavage site in a microtubule dependent process. Various models implicate either astral microtubule or midzone microtubule in signalling placement of cleavage site with respect to the mitotic spindle. Differential distribution of microtubule density over the cortex or, alternatively, different signal qualities derived from different microtubule subtypes were thought to ultimately signal arrangement of the cleavage machinery to the site between segregated spindle poles (see also Fig. 4 in Chapter I, Introduction) (Glotzer, 2001; Guertin et al., 2002; Wang, 2001).

Recent studies in cultured cells also implicate direct interaction between astral microtubule and the equatorial cortex in positioning of the cleavage machinery at this site intersecting separated chromosomes. (Canman et al., 2003). A subset of astral microtubule that emanates from the centrosome at sites adjacent to separated chromosomes was observed to perform stable interactions with the equatorial cortex. In contrast, all other astral microtubule contact the cortex only transiently and stay highly dynamic. In these cells, a differential in microtubule dynamics between different cortical sites in combination with low microtubule density at the equator stabilization of astral microtubule at cortical sites flanking the low microtubule density area at the equator itself has been proposed to signal position of the cleavage machinery according to the spindle Fig. 4 in Chapter I, Introduction) (Canman et al., 2003).

Maybe

Interaction between astral microtubules and the cleavage site in animals is reminiscent of our findings in yeast, where microtubules exhibit specific dynamics upon attachment at the cleavage site to orient the spindle relative to this site. Thus, although in yeast the cleavage site is predetermined and directs spindle position, which is opposite to the situation in animals, spatial coordination of the spindle and the cleavage site could rely on the same mechanism. Both, in fungal and in animal cells, direct astral microtubule interaction with the future cleavage site provide a communication line between the spindle and the cortex. Differential modulation of microtubule dynamics might serve in signaling spatial information in either direction.

In animal cells elucidation of the signaling mechanism for cleavage site placement has been complicated by the simultaneous occurrence of furrow induction and furrow formation (Glotzer 2001). A clear separation between cleavage site placement and spindle positioning in
Chapter IV

yeast renders this organism an excellent model system to dissect the yet obscure signalling mechanism for cleavage site determination in higher eukaryotes.

**Maybe not**

One difficulty to extrapolate from results obtained in yeast to animal cells is represented by the circumstance, that fungal cells undergo closed mitosis. In this context, spindle microtubule never come into direct contact with cytoplasmic components (Field et al., 1999; Nanninga, 2001). Thus, yeast astral microtubule may take over functions performed by midzone microtubule in animal cells. Different models have implicated midzone microtubule in signalling position of the cleavage site to the overlying cortex (Alsop and Zhang, 2003; Bonaccorsi et al., 1998; Gatti et al., 2000a; Wheatley et al., 1998; Wheatley and Wang, 1996). However, none of these models has reached the molecular level. Neither can any of these models exclude a participation of astral microtubule in determining the cleavage site. (Canman et al., 2003; Glotzer, 2003; Murata-Hori and Wang, 2002; Wang, 2001). The idea that astral microtubule interact with component of the cleavage machinery has the advantage of proposing more readily testable hypothesis at the molecular level (see below).

Using yeast as a model system for interaction between astral microtubule and the cleavage site it will be interesting to elucidate, which other components at the bud neck are important for proper microtubule neck attachment and if these are conserved (see below).

**Molecular nature of neck attachment complex**

To further dissect the molecular basis of microtubule attachment at the neck, we assessed the role of neck components in microtubule capture at the neck, such as typeII myosin Myol, a component of the actomyosin contractile ring that recruits actin to the bud neck (Bi et al., 1998; Longtine and Bi, 2003) and the formin Bnr1, implicated in actin cable nucleation (Kikyo et al., 1999). Analysis of microtubule attachment revealed that microtubule capture at the neck is mainly based on the septins and Kar9 (Kusch et al., 2002) Chapter II Fig. 3C).

Our results demonstrate, that microtubule capture at the neck is independent of the actomyosin ring or any other actin structure (Kusch et al., 2002) Chapter II Fig. 1B, 2A, and 3C). This result stands in contrast to microtubule interaction with the cortex during guidance. Guidance is based on polar actin cables which serve as tracks for Kar9-dependent microtubule transport toward the bud neck and the bud (see below and (Liakopoulos et al., 2002) Chapter III Fig. 6D and G), This result gives rise to the yet unsolved question, how Kar9 interacts with the bud neck. No interaction between Kar9 and septins has been revealed yet. It is therefore
possible, that the decreasing effect of Kar9 deletion on microtubule attachment at the neck is due to its role in microtubule recruitment to this site rather than direct binding.

*Conservation of interaction between cleavage site components and microtubules*

Direct linkage between septins and microtubule at the neck is supported by data from mammalian cells and *Drosophila* that demonstrate biochemical interaction between septins and microtubule (Sisson et al., 2000; Surka et al., 2002). Septins are conserved components of cleavage furrows in a variety of animal cells. Their role in cytokinesis varies among different cell types (Field et al., 1999; Glotzer, 2003; Kinoshita, 2003). Conserved interaction between microtubule and septins as a major component of the cleavage cortex adds to the analogies in spindle-cleavage site interactions between budding yeast and metazoans. Other conserved cleavage apparatus components are represented by the formin family and the IQGAP family. These are effectors of small Rho like GTPases that are responsible in modulating the actin cytoskeleton during processes like cell migration and cleavage apparatus assembly (Glotzer, 2003; Rodriguez et al., 2003). Recently, the formin mDia, an effector of RhoGTPase, and IQGAP1, an effector of small GTPases Rac1 and Cdc42, have been shown to interact with microtubule via the +TIPs EB1 and CLIP170 respectively in migrating fibroblasts (Fukata et al., 2002; Ishizaki et al., 2001; Palazzo et al., 2001a; Palazzo et al., 2001b). These interactions were proposed to provide a link between microtubule polymerization and modulation of the actin cytoskeleton during cell migration (Small and Kaverina, 2003; Wittmann and Waterman-Storer, 2001). As the same factors are important to assemble the actomyosin contractile ring at the cleavage site, similar +TIPs might play a role in mediating microtubule interaction with the cleavage cortex proposed by Canman 2003. This idea is supported by our finding, that EB1 homolog in yeast Bim1 plays a role in mediating astral microtubule attachment to the bud neck, as this +TIP links Kar9 to microtubule (Korinek et al., 2000; Lee et al., 2000; Liakopoulos et al., 2003 Fig ID; Miller et al., 2000; Small and Kaverina, 2003; Wittmann and Waterman-Storer, 2001).

**The role of SDKs Hsl1 and Gin4 in microtubule attachment at the neck?**

The septins also recruit the septin-dependent kinases (SDK) Hsl1, Gin4 and Kcc4 to the bud neck. Interestingly, these kinases share sequence homology with human MARKs (Microtubule Affinity Regulatory Kinases), which play a role in the regulation of microtubule dynamics in human cells (Drewes et al., 1997; Trinczek et al., 2004) (Fig). We investigated the role of SDK kinases Hsl1 and Gin4 in spindle positioning. Disruption of SDK kinases caused a strong defect in nuclear migration to the bud neck (Appendix 1A). Analysis of microtubule attachment in kinase mutants revealed an increase of the microtubule population contacting the neck (Kusch et al., 2002) Fig 5B). At the same time, neck attached microtubule
Chapter IV

appeared to be longer in the kinase mutant than neck attached microtubule in wild type cells (Kusch et al., 2002) Chapter II Fig 5C).

These results indicated that kinases do not play a role in microtubule capture at the neck itself, but in regulation of microtubule stability. Time lapse microscopy demonstrated that kinase deletion caused microtubule pause and/or microtubule growth concomitant with microtubule capture at the neck, resulting in pushing the spindle away from the neck (Appendix 1B). This would explain the strong nuclear migration defect of the kinase mutants. Increased microtubule attachment in the kinase mutant depended on the presence the septins and Kar9, indicating, that these act upstream of the kinases in regulating microtubule attachment (Kusch et al., 2002) Chapter II Fig 5B). Together, these results indicate that SDK kinases –similar to MARKs- could be responsible to induce microtubule shrinkage at the neck while septins act as a binding platform for microtubule capture to this site (Kusch et al., 2002) Chapter II Fig 5B).

Role of phosphorylation in microtubule attachment

Similar to microtubule attachment to the bud neck, also microtubule attachment to kinetochores in animal cells is characterized by microtubule capture and shrinkage to pull sister chromatides apart. The molecular basis for microtubule attachment and regulation to kinetochores has not been elucidated yet. Recent studies also implicate a kinase, the kinase Aurora B/Ipl1 in kinetochore attachment (Biggins et al., 1999; Cheeseman et al., 2002). This parallel could hint on a similar regulation of microtubule undergoing capture-shrinkage at different attachment sites. Thus, microtubule neck attachment could serve as a model system to investigate molecular mechanisms in typical capture-shrinkage events.

Interestingly, two other MARK homologues Kin1 and Kin2 exist in yeast. Supporting a role of phosphorylation in regulation of microtubule dynamics, also disruption of these kinases caused a nuclear migration defect (Appendix 1C). Recent analysis of microtubule dynamics in kin1/kin2 deletion mutants –performed by a diploma student in our lab- have indicated a role for these kinases in regulation of microtubule dynamics at the bud cortex. The kinase mutant exhibited shorter microtubule attached to the bud than wild type cells.

The fact, that only few cytoplasmic microtubule mediate spindle pulling towards the bud neck illustrates importance of proper regulation of microtubule at attachment at different sites. Our observation, that SDK mutants exhibit an increase in microtubule attachment to the neck comparable to a decrease in attachment to the bud reflects a competitive link between different attachment sites for few cytoplasmic microtubule (Kusch et al., 2002) Chapter II Fig 5B). These sites are also linked by microtubule guidance along polarized actin cables (see below). Guidance leads microtubule first to the bud neck and only upon passage of the neck bud cortex attachment is possible. This tight relation between bud neck and bud cortex also
explains the strong nuclear migration defect in SDK mutants that stabilize microtubule contacts at the neck on cost of microtubule contacts at the bud.

Which substrates of SDK mediate microtubule capture-shrinkage?

One striking feature of microtubule capture-shrinkage is that microtubule maintain attachment with their plus ends while depolymerizing at the same end to allow force generation. Such complex attachment is either mediated by few components with multiple functions that regulate both microtubule dynamics and attachment at the same time. This attachment could also involve a complex of components that regulate either microtubule dynamics or attachment. Accordingly, each SDK could phosphorylate either one specific substrate with a specific function, or a different site of a multifunctional substrate. Specific roles of respective kinases still have to be analysed by testing single mutants with respect to microtubule dynamics. However, single kinase mutants appear to largely overlap in their respective effects on the extent of microtubule attachment to the neck and are only partially additive. (Kusch et al., 2002) Chapter II Fig. 5B). This indicates that they rather act redundantly in regulating only few components with multiple functions.

Kar9?

As Kar9 mediated microtubule attachment to the neck, we first tested biochemically, if this factor could be a substrate for SDKs. Surprisingly, disruption of the kinases resulted in hyperphosphorylation of Kar9 (Appendix 2, and Dimitris Liakopoulos, unpublished results). Due to a separate role of SDK in cell cycle regulation SDK disruption also results in prolongation of the activity of Cdc28 in complex with early cyclins such as Clb3, 4, 5 and 6. Dimitris Liakopoulos in our laboratory tested if hyperphosphorylation of Kar9 in SDK mutants is an indirect result of the cell cycle regulatory function of SDK due to prolonged Cdc28/Clb3-6 activity. He indeed found, that Kar9 hyperphosphorylation in SDK mutant depended on activity of Cdc28/Clb4 (Liakopoulos et al., 2002) Chapter III Fig 2 and 3). This indicated that Kar9 is not the direct substrate of SDK at the bud neck.

A kinesin related motor?

Minus end directed motor proteins are other candidates with multiple properties and could account for SDK controlled microtubule capture-shrinkage at the neck. Motor proteins consist of different domains, a cargo binding and a motor domain, which combine binding to cargo with movement along microtubule. In the case of binding to cortical sites motor dependent movement toward microtubule minus ends would result in relative movement of the nucleus toward these cortical sites. Some motors additionally exhibit microtubule depolymerizing activity, as has been demonstrated for several motors like dynein (Fig. 1A) (DeZwaan et al.,
Chapter IV

1997), Kar3 (Maddox et al., 2003; Saunders et al., 1997a; Saunders et al., 1997b) or in animal cells for MCAK (Moores et al., 2002; Niederstrasser et al., 2002). By combining all three properties these proteins could couple microtubule attachment at cortical sites with regulation of microtubule shrinkage. SDK could play a role in coordination of different activities of the two domains. Kar3 has recently been shown to mediate microtubule capture-shrinkage at shmoo tips of mating cells (Maddox et al., 2003). Simultaneous microtubule shrinkage and attachment depend on ATPase activity of the motor domain (Maddox et al., 2003). Such motor activity could also be involved in capture-shrinkage at the neck. To test this possibility, ATP dependency of microtubule shrinkage at the neck could be investigated. Duration of attachment could be linked to processivity of the motor on microtubule.

Another minus end directed kinesin with related properties, Kip3, is involved in spindle positioning in budding yeast (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Miller et al., 1998). This motor also localizes to the plus ends of cytoplasmic microtubule (unpublished results from our laboratory) and should be tested for a role in microtubule capture-shrinkage and as a substrate candidate for SDKs.

The shmoo tip and the bud neck are similar attachment sites

The shmoo tip reflects additional features of bud neck attachment. Interestingly, the Bim1-Kar9 complex is also implicated in shmoo attachment. It acts in a mutual exclusive manner with Kar3 which is indicated by accumulation of Bim1-GFP at microtubule growing ends, while they are captured at the shmoo tip, whereas Kar3-GFP accumulates at shrinking attached ends (Maddox et al., 2003). In parallel, in our movies, fluorescence intensity of the Kar9-GFP dot appears maximal when the microtubule reaches the bud neck. Retesting the roles of Kar3 and Kip3 in microtubule neck attachment by for instance applying a similar microscopical approach in addition to analysis of microtubule attachment in deletion mutants could help to identify substrates for SDK.

The high similarity of shmoo tip and bud neck attachment events with regard to similarities in microtubule behaviour and to components involved, supports the idea that they rely on similar molecular regulation and protein protein interactions.

A MAP/?TIP?

MARKs regulate microtubule dynamics by controlling affinity of stabilizing MAPs for microtubules. Homology of SDKs to MARKs could implicate a MAP as a SDK substrate in yeast. By analogy to conventional MAPs the yeast MAP would loose its affinity for microtubules upon phosphorylation by SDK and thereby induce shrinkage. This scenario implicates two possibilities for microtubule interaction with the neck: either the MAP itself mediates microtubule interaction with the neck or a second component is specifically responsible for this (Fig.1B). The latter possibility bears complementary functions for the two
SDKs, Hs11 and Gin4, in regulating neck attachment, each controlling attachment and microtubule dynamics separately. Bim1 represents a +TIP that is required for Kar9 association with microtubule and therefore is involved neck attachment too. Bim1 has microtubule destabilizing activity during G1 by affecting microtubule pausing, while during mitosis microtubule appear to be stabilized (Tirnauer et al., 1999). Biochemical analysis didn’t detect any SDK-dependent phosphorylation of Bim1 (Liakopoulos, unpublished results). However, the bulk of Bim1 localizes to SPB and spindle microtubule where it could be involved in microtubule attachment to kinetochores. A potentially transient SDK-dependent modification would affect only a small subfraction of Bim1 molecules and could be difficult to detect. As Kar9 is the only marker that specifically colocalizes with Bim1 on cytoplasmic microtubule only (Korinek et al., 2000; Lee et al., 2000), it could help to purify specifically cytoplasmic Bim1. Large scale purification of Kar9-bound Bim1 in wild type and SDK mutants could help to isolate SDK-dependent modification of Bim1.

Figure 1 Possible substrates for SDK that could mediate capture shrinkage at the bud neck, represented by a septin ring.
A. a minus end directed motor could mediate capture shrinkage and spindle pulling by simultaneously binding to the neck, inducing microtubule shrinkage and maintaining microtubule capture by moving to the minus end and there pulling the structure at the minus end, the nucleus towards the neck. SDK phosphorylation could regulate any of these parameters, however, transient capture might also be due to pulling force, that disrupts contact.
B. a stabilizing MAP/+TIP could mediate microtubule growth towards the neck, contact could be direct between septins and microtubule. Phosphorylation induces MAP/+TIP dissociation and microtubule shrinkage. In this case it is not clear, how capture could be maintained to produce pulling force.
Kar9: a new +TIP

Previously, Kar9 had been regarded as a prelocalized microtubule target at the bud cortex to which microtubules bind upon random search and capture (Beach et al., 2000; Miller et al., 1998). Kar9 localization here had been proposed to be based on Myo2-dependent transport along polarized actin cables (Beach et al., 2000; Miller et al., 1999; Yin et al., 2000). Our observation, that Kar9 links microtubule to the bud neck in an actin independent fashion (Kusch et al., 2002) Chapter II Fig 2 and Fig 3 C) prompted us to investigate, how Kar9 is recruited to this site.

Localization of endogeneously expressed Kar9

Surprisingly, treating cells with the microtubule depolymerising agent nocodazole restricted Kar9 localization to the SPB (Liakopoulos et al., 2002) Chapter III Fig 1C). This indicated that Kar9 recruitment to both the bud neck and the bud cortex depended on microtubule. Indeed Kar9 distribution was tightly correlated with microtubule distribution (Kusch et al., 2002) Chapter II Fig 5A and B and Fig. 6C). This is different to what others and us observed in studies with overexpressed Kar9-GFP. Here, Kar9 localized along microtubules and also microtubule independently as dots to the bud cortex (Appendix 3A, (Beach et al., 2000; Miller et al., 2000; Miller et al., 1999). Time lapse microscopy revealed that Kar9 localized as a dynamic dot on both growing and -untypically- also shrinking microtubule plus ends, or as a dot moving along microtubule shafts towards their plus ends. (Liakopoulos et al., 2002) Chapter III Fig. 5A and 6A; (Kusch et al., 2002) Chapter II Fig. 4A (Appendix 3B). Furthermore, FRAP (Fluorescence Recovery After Photobleaching) data indicated that the major Kar9 pool visible on microtubule is derived from SPB rather than from cytoplasmic sites (Liakopoulos et al., 2002) Chapter III Fig. 5B). This is consistent with SPB localization observed in two color microscopy (Liakopoulos et al., 2002) Chapter III Fig1A.B and C). FRAP analysis also indicated, that Kar9 binding is highly processive, e.g. it exchanges little or not with cytoplasmic sites (Liakopoulos et al., 2002) Chapter III Fig. 5B and C). Two-colour fluorescence microscopy also revealed asymmetric localization of Kar9 to SPB and microtubules proximal to the bud (Liakopoulos et al., 2002) Chapter III Fig1A,B, C and G) Together these results indicated that Kar9 when expressed at endogenous levels predominantly associates with microtubules and/or spindle pole bodies (SPB). This prompted us to regard Kar9 as a new +TIP.
General discussion

Binding partners for Kar9

Localization of a +TIP to microtubule plus ends is often linked to its activity to influence microtubule dynamics and/or microtubule attachment to cellular structures (Carvalho et al., 2003; Galjart and Perez, 2003). One major question is, how a +TIP is recruited to the microtubule plus end and how it maintains localization at polymerizing plus ends. Different recruitment strategies have been described for different proteins. These range from copolymerization with tubulin subunits, treadmilling, hitchhiking along with other +TIPs and motor dependent transport (Carvalho et al., 2003; Schuyler and Pellman, 2001a). Thus exploring the interaction between +TIPs and microtubule, SPB or cortical sites will be important to elucidate regulation of +TIP and ultimately microtubule behaviour.

Kar9 binding to microtubule plus ends

The complex localization pattern of Kar9 on growing and shrinking microtubule ends and along microtubule shafts suggests that this +TIP uses several mechanisms to associate with microtubule and microtubule ends. As already indicated by biochemical and genetic studies (Beach et al., 2000; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000), we confirmed Bim1 dependent Kar9 interaction with microtubule (Liakopoulos et al., 2002) Chapter III Fig 1D). This suggests that microtubule-end binding of Kar9 occurs mainly via hitchhiking with the +TIP Bim1. Another yeast +TIP, called Stu2, biochemically interacts with Kar9 and Bim1 (Miller et al., 2000) and could be another microtubule plus end tracking partner for Kar9. Occasionally, we observed Kar9-GFP as a dynamic dot moving along microtubule shafts from the spindle pole towards plus ends (Liakopoulos et al., 2002) Chapter III Fig. 5A). Recent studies by Maekawa and Schiebel 2003 suggest that Kar9 is transported by the kinesin motor Kip2 towards microtubule ends (Maekawa et al., 2003). Interestingly, Kar9 also localizes as a dot along shrinking microtubule ends. This behaviour is untypical of localization of other +TIP which are reported to specifically track growing microtubule ends. Shrinking end localization has been described already described for dynein (Carvalho et al., 2003). As dynein is not implicated in Kar9 dependent pathway of spindle positioning different minus end directed motors such as Kip3 or Kar3 could be involved in backwards transport of Kar9.

Kar9 dynamic localization

Kar9 localization on shrinking ends could provide a way to recruit Kar9 quickly back to SPB. This could underly the importance of Kar9 as a major attachment factor that needs to be quickly available for newly nucleated microtubule. Reloading form microtubule onto SPB might provide a more efficient mechanism for Kar9 recruitment than loading from cytoplasmic sites. Such a reloading mechanism is supported by FRAP data that indicate a
high processivity of Kar9 on microtubule and little exchange with cytoplasmic sites. FRAP analysis also indicates that Kar9 is recruited from the SPB onto microtubule and not from cortical or cytoplasmic sites (Liakopoulos et al., 2002) Chapter III Fig.5 B and C).

**Kar9 binding to SPB**

Overexpression of Kar9 in mutants deleted for Biml exhibit residual Kar9 localization at the SPB. (Appendix 3A). This indicates the presence of a Biml independent binding site on SPB, which is not detectable at endogenous Kar9-GFP levels (also proposed by (Miller et al., 2000). Likewise, it can’t be excluded, that Kar9 localizes on microtubule in a Biml independent way when expressed at endogenous levels, but in an undetectable fashion.

Overexpression of Kar9 could provide a way to identify other low affinity binding sites for Kar9, which are not detectable when Kar9 is expressed at endogenous levels. Thus overexpression could proof as a tool for the identification of other interaction partners and mechanisms, that link Kar9 to different sites. However, results obtained from such overexpression studies have to be interpreted with caution as they could be misleading for functional meaning of these interactions.

**Parallels between Kar9 and APC**

Interestingly, the Kar9 localization pattern resembles that of the tumor suppressor Adenomatous polyposis coli (APC) in animal cells. However, these proteins share only little sequence homology. Both proteins contain an EB1/Biml interacting motif in their C-termini. Another similarity is the presence of a coiled coil domain in the N-terminus of both proteins (Bienz, 2002; Dikovskaya et al., 2001; Miller et al., 2000). Similarly to Kar9, APC interacts with EB1 only on a subfraction of microtubule ends (Liakopoulos et al., 2002) Chapter III Fig. 1F). This interaction is proposed to mediate microtubule capture at specific sites such as kinetochores and leading edges of migrating cells. Similarly, Kar9 specifies Biml function in mediating attachment of bud proximal microtubules to cortical sites. However, while Kar9 interaction with microtubule strictly depends on Biml (see above), interaction of EB1 and APC with microtubule appears to be largely independent of each other and are also regulated differently. APC overexpression recruits EB1 to microtubule ends (Mimori-Kiyosue et al., 2000b). APC bears a microtubule binding domain that mediates direct interaction to microtubule (Bienz, 2002). In parallel to Kar9-Biml transport to microtubule plus ends, APC transport to microtubule plus ends also occurs via the kinesin motor activity (Jimbo et al., 2002; Mimori-Kiyosue et al., 2000a).

Like APC, Kar9 has also been shown to interact with itself via its coiled coil domain. Selfassembly could add to the mechanism that help keeping Kar9 on microtubule in a processive manner. APC localizes via specific other motives, so called Armadillo repeats, to actin rich cortical sites in a largely microtubule independent way (Bienz, 2002; Rosin-
Arbesfeld et al., 2001). By analogy, Kar9 also interacts with cortical actin cables via binding to yeast type V myosin Myo2, that allow direct interaction with the cortical (Yin et al., 2000).

**How is Kar9 localization regulated?**

**Kar9 localization during the cell cycle**

Two colour fluorescence microscopy indicated that Kar9 localization is regulated during the cell cycle. During G1, Kar9 resides mainly on the SPB from where it is also partially loaded onto subset of microtubule. However, given a reloading onto SPB from shrinking microtubule, the high incidence of SPB localization can also be due to high microtubule dynamics during G1. Upon SPB duplication and separation in S/G2, we observe asymmetric localization of Kar9 on the bud proximal SPB. From here it is loaded onto bud proximal microtubule. Differently, its interaction partner Bim1 localizes symmetrically on the spindle. During early stages of mitosis, where the spindle is very short, occasionally we observe symmetric localization. In anaphase we observe a strong tendency in symmetric localization on the spindle. Shortly before the spindle breaks down most of the spindles are symmetric in Kar9 localization (Appendix 3C). The expression levels of Kar9 do not vary during the cell cycle neither does the amount of Kar9 localized on microtubule/SPB (Liakopoulos et al., 2002) Chapter III Fig. 4A).

**Role of phosphorylation**

Restriction of Kar9 localization to the bud proximal half of the spindle relies on Kar9 phosphorylation by Cdc28/Clb4. (Liakopoulos et al., 2002) Chapter III Fig. 2, 3 and 4) Unphosphorylatable Kar9 mutants symmetrically localize to both sides of the spindle (Liakopoulos et al., 2002) Chapter III Fig. 4B and C). As phosphorylation occurs in the Bim1 interaction motif of Kar9 this modification is hypothesized to affect interaction of Kar9 with Bim1 at the distal SPB (Liakopoulos et al., 2002) Chapter III Fig. 4A). This is consistent with data with APC, which is phosphorylated at the EB1-interaction site to increase affinity for EB1 (Nakamura et al., 2001).

Similar to Kar9, also APC localizes asymmetrically on microtubule ends that specifically target specific cortical site in migrating cells. In contrast, its interaction partners EB1 localizes symmetrically to all microtubule in the aster. However, how asymmetric localization of APC is regulated, is not well understood. Recent publication by Etienne Manneville 2003 indicate that Cdc42 might be responsible for polarized activity of GSK3beta to specifically recruit APC to microtubule ends in these polar regions of the cell (Etienne-Manneville and Hall, 2003). This indicates that APC asymmetry on microtubule ends in certain cortical regions is
linked to Cdc42 regulated cortical polarity. In contrast, we demonstrated that Kar9 polarity is largely independent of cortical polarity in cells lacking either polarized actin cables or expressing mutant forms of Cdc42. This difference between Kar9 and APC indicates different recruitment routes that might be based on different context in different cell types.

**Guidance: a new mechanism for microtubule attachment in yeast**

Discovery of Kar9 as a strict microtubule associated protein gave rise to a new view on microtubule attachment in budding yeast. Together with results from previous reports, which indicated that Kar9 directly interacts with the myosin motor Myo2, our identification of Kar9 as a +TIP suggested that it may recruit Myo2 to microtubule ends. This would open the possibility that microtubule might be guided by Kar9 and Myo2 to attachment sites along actin cables. Time lapse analysis demonstrated that microtubules are indeed guided along actin calbes (Liakopoulos et al., 2002) Chapter III Fig.6). Analysis of different mutants indicated that guidance depended on Myo2, actin and Kar9. This was supported by the observation of Myo2 recruitment to microtubule ends (Hwang et al., 2003) Fig. 4).

**Guidance: a way to efficiently target microtubules to specific cortical sites in yeast**

In yeast, spindle positioning is achieved by only few cytoplasmic microtubules. Usually only one microtubule bundle can be observed that emanates from the SPB and attaches at specific cortical sites such as the bud neck and the bud cortex to pull the spindle towards the bud neck. A stochastic search and capture had been proposed as a mechanism for how microtubule could attach to specific sites (Kirschner and Mitchison, 1986). Strong microtubule dynamics during G1 had been proposed to facilitate the search for the emerging bud site that provides a microtubule capture site. (Beach et al., 2000; Schuyler and Pellman, 2001b; Tirnauer et al., 1999).

We propose that microtubule guidance along actin cables that transverse the bud neck from the mother into the bud provides a mechanism to efficiently target few yeast microtubule to specific sites such as bud neck and bud cortex (Liakopoulos et al., 2002) Chapter III Fig.8).

**Guidance: a way to target microtubules to different attachment sites**

Kar9 dependent microtubule recruitment to different cortical sites such as bud neck and bud cortex, exposes microtubule ends to different regulatory influences at various sites. In this way, Kar9 can be regarded as a general adaptor for microtubule to interact with different sites.
As described above, attachment to the bud neck induces microtubule shrinkage and spindle movement to the neck. Differently, microtubule attachment at the bud cortex is accompanied by stabilization of microtubule ends which are guided further towards bud tip. Time lapse analysis revealed that microtubule guidance along the bud cortex mediates spindle alignment with the mother bud axis. Repetitive microtubule transport towards the bud tip could reinforce proper spindle alignment within the polarity axis prior to anaphase. Complementary functions of different attachment sites or types are also discussed in (Kusch et al., 2002) Chapter II and represented in the model Fig. 7 (see also Figure 2 in this chapter).

Microtubules also appear to be stabilized while being guided along actin cables along the mother cortex accounting for strong spindle movements towards the bud neck (Appendix 4A). Thus, microtubule guidance serves itself in spindle positioning and alignment. Stabilization of microtubule ends during guidance in the mother cell could also serve to ensure that microtubule reach their attachment sites at bud neck and in the bud.

The molecular basis for regulation of microtubule dynamics at different sites is not clear. Kar9 deletion mutants exhibit long microtubule (Appendix 4B). However, this potential destabilizing activity of Kar9 appears to be modulated by other +TIPs such as Bim1 or Stu2 at the microtubule plus ends as well as by capture factors at the complex. It could be helpful to test in vitro properties of these purified proteins to assess their individual properties and complexity of interactions in vivo.

**Guidance: a way to coordinate different microtubule attachments in yeast?**

An actin guided microtubule usually first passes the neck before it can enter into the bud. Guidance could be a mechanism to coordinate bud neck attachment and bud cortex attachment. This would also imply, that bud neck and bud cortex attachment sites compete with each other for few microtubule. This idea is supported by our observation that SDK mutants stabilize microtubule at the bud neck on cost of bud attachment (Kusch et al., 2002) Chapter II Fig. 5B), microtubule appear to pause when they attach at the bud neck for up to (Kusch et al., 2002) Chapter II Fig 4B), followed by either microtubule shrinkage and spindle pulling, or by entry of the microtubule into the bud. Given also the observation, that microtubule attachment behaviour in the bud switches from microtubule guidance at their ends to lateral microtubule gliding at anaphase suggests, that microtubule attachment to different sites is regulated during the cell cycle. Could the bud neck work as a control station that regulates the decision how a microtubule would go on after neck attachment? Would SDK play a role in this process in response to cell cycle regulatory factors?

To test such a cell cycle regulation, it would be useful to analyse occurrence of different microtubule attachment events during the course of the cell cycle (Fig. 2).
**Chapter IV**

Figure 1 Model of spindle positioning in yeast. Usually one microtubule is observed to attach to different attachment sites and to mediate major spindle movements towards the bud neck and along the mother bud axis. Attachment to different sites is associated with different effects on microtubule behaviour and spindle movement. Different attachment events appear to preferentially occur at certain cell cycle stages (indicated by the position along the time axis, bud size and spindle length). Regulation of attachment during the cell cycle is unclear. Potentially, regulatory events at the bud neck decide upon the switch between “Shrinkage back to SPB” followed by another “Bud neck attachment” cycle and “Bud entry” and like that control cell cycle dependent spindle movements. Kar9 acts as a general adaptor that recruits microtubules to different sites. Different sites can be contacted by one microtubule during its life time. Alternatively the microtubule shrinks back to the SPB before a new cycle of outgrowth and attachment begins. Repeated attachment might be important to reinforce spindle position.

**Different ways to polarize the microtubule cytoskeleton**

In yeast

An efficient long distance targeting mechanism such as guidance along actin cables bears the danger, that too many microtubule are able to contact specific sites and exert the same function. In the case of budding yeast, this would also lead to targeting of microtubule from the distal pole at the bud neck and in the bud. This ultimately would result in spindle misalignment. How is the microtubule cytoskeleton polarized to exert specific local functions?

By central asymmetric loading of the guidance factor Kar9 from the proximal SPB onto on proximal microtubule yeast developed a system to restrict a certain function onto a subset of microtubule. Consistent with asymmetric distribution of Kar9 we found that guidance behaviour of microtubule was asymmetric too (Liakopoulos et al., 2002) Chapter III Fig.6 B and C). This ensured that only the proximal SPB was targeted into the bud. Expression of a dominant negative Kar9 variant, that localizes symmetrically onto both spindle poles resulted in targeting of both poles into the bud and in spindle misorientation (Liakopoulos et al., 2002)
Chapter III Fig.7 B, C and E). Synthetic lethality of dominant negative Kar9 mutation with deletion of dynein indicated that asymmetric distribution of microtubule attachment factors was essential for the cell (Liakopoulos et al., 2002) Chapter III Fig.7 B, C and E). Consistent with this, also components involved in microtubule attachment during anaphase spindle positioning need to be asymmetric (Sheeman et al., 2003).

In animal cells

Similar long distance guidance can be observed in migrating fibroblasts, where microtubule ends are also transported along stress induced actin fibres near polar cortical regions (Ishizaki et al., 2001; Kaverina et al., 2002; Salmon et al., 2002). However, the major mechanism of microtubule polarization in these cells relies on local stabilization of microtubule ends in certain peripheral regions of the cell. While all microtubule are able to grow out from the centrosome towards the cell periphery, presumably on account of generally distributed stabilizing +TIPs like EB1, only a subset of microtubule is specifically stabilized additionally in specific regions by asymmetrically localized factors like APC or CLASP. These stabilize microtubule ends synergistically with their binding partners EB1 and CLIP170 (Akhmanova et al., 2001; Carvalho et al., 2003; Mimori-Kiyosue and Tsukita, 2003; Nakamura et al., 2001; Schuyler and Pellman, 2001a). CLIP170 had recently been demonstrated to exert a rescue effect on microtubule ends, that shrink back from the periphery in NRK and CHO cells, a mechanism that could add to concentration of ends in specific regions in the cell periphery (Komarova et al., 2002a). Besides stabilizing microtubule ends in certain cortical regions, APC and CLIP170 were proposed to synergistically activate Rac GTPase directly by different modes (Rodriguez et al., 2003; Small and Kaverina, 2003). In turn, Rac promotes microtubule polymerization by activating PAK, an inhibitor of microtubule destabilizing factor Stathmin/Op18. Targetting microtubule ends at polar regions is enhanced by local polarity factors such as small GTPases Cdc42 that provides a binding platform for CLIP170 via its effector IQGAP and EB1 via the formin mDia (Gundersen, 2002; Rodriguez et al., 2003; Small and Kaverina, 2003). In this way, upstream regulators of actin polarization such as small GTPases Cdc42 and Rho couple polarization of actin cytoskeleton and cell morphology with polarization of microtubule function, to allow coordination of actin and microtubule dependent processes in response to various signals.

Thus, guidance and asymmetry are conserved principles of microtubule function that are finetuned in the respective cellular context in different organisms. This complex interplay between actin and microtubule reflects one conserved feature to polarize microtubule function.

While higher eukaryotes rely on abundant number of microtubule that allow stochastic mechanism to find attachment sites followed by a local polarization system to specify
microtubule function, low number of microtubule in yeast necessitates central polarization from SPB and long distance targeting to efficiently reach specific sites.

Yeast as a model for +TIP function and regulation

The different +TIP complexes in yeast

In parallel to the +TIP complex Kar9-Bim1 which is essential for early spindle positioning, also components of the anaphase spindle positioning pathway predominantly localize to microtubule plus ends. The CLIP170 homolog Bik1 localizes to microtubule ends and recruits dynein to plus ends via LIS1 homologue Pac1 (Sheeman et al., 2003). Interaction of dynein/dynactin with the cortical protein Num1 at the bud tip cortex is thought to induce processive movement of this motor towards the minus end (Sheeman et al., 2003). This then mediates lateral microtubule attachment and gliding along the cortex resulting in strong final spindle pulling inside the neck.

Localization of Bim1-Kar9 and dynein/dynactin-Pacl-Bik1 attachment complexes on the same site gives rise to the question, how activity of these complexes - mediating different types of attachment at different times - is regulated temporally. CDK dependent Kar9 localization to microtubule plus ends might account for activation of Kar9 at plus ends and could also be a way to inactivate Kar9 during later stages.

Conservation

The two +TIP complexes in budding yeast, Kar9-Bim1 and dynein-Pacl-Bik1, are reminiscent of +TIP complexes in higher eukaryotes, that are constituted of the respective homologues APC-EB1 and dynein/dynactin-Lis1-CLIP170-CLASP. These two complexes have been shown to act in a mutual exclusive way at kinetochores, where each complex fulfills a specific function. However, more and more cases of crossinteractions between individual members of different complexes (EB1-p150glued-CLIP170) suggest microtubule plus ends as platforms for synergistic or regulatory relationships. Versatile interaction properties with cortical components add to complications of this network and ultimately to the complication in elucidation of microtubule regulation. For instance Rho like small GTPase Cdc42 appears to interact with different +TIPs via different pathways and thereby could coordinate different complexes with each other in leading edges of migrating cells (Carvalho et al., 2003; Galjart and Perez, 2003; Gundersen and Bretscher, 2003; Mimori-Kiyosue and Tsukita, 2003; Small and Kaverina, 2003).

Interestingly, Num1 represents another interaction partner for Kar9 on the bud cortex. Num1 in budding yeast could be a cortical crossinteraction partner between the two +TIP complexes.
and coordinate their respective functions. Kar9 dependent microtubule guidance towards the bud cortex and interaction with Num1 capture sites might provide a potential switch from preanaphase to anaphase spindle positioning.

Not only is interaction of the +TIP homologues similar, also localization and regulation bear a high degree of conservation. Moreover, recent implications of the APC-EB1 complex in spindle orientation during asymmetric and symmetric division also point to a functional conservation (Carvalho et al., 2003; Galjart and Perez, 2003; Mimori-Kiyosue and Tsukita, 2003; Schuyler and Pellman, 2001a).

As still only little is known about recruitment, regulation of +TIP regulation, it will be interesting to use budding yeast as a system to elucidate regulatory mechanisms of its +TIP interactions among each other and with different cortical targets, possible cross interactions and physiological functionality of these. Yeast could serve as a model for +TIP function to elucidate basic principles in microtubule specification in asymmetric cell division, but also in other polar processes like cell migration and cell polarity.
References

References for Chapters I through IV


References


Drewes, G., and Nurse, P. (2003). The protein kinase kinl, the fission yeast orthologue of mammalian MARK/PAR-1, localises to new cell ends after mitosis and is important for bipolar growth. FEBS Lett 554, 45-49.


References


References


References


107
References


References


Chapter V

Spindle asymmetry: a compass for the cell

Justine Kusch, Dimitris Liakopoulos and Yves Barral*

Institute of Biochemistry
Swiss Federal Institute of Technology (ETH)
Building HPM, Room F13.1
ETH Hoenggerberg
8093-Zuerich
SWITZERLAND

* Tel: ++41-1-632 06 78
Fax: ++41-1-632 15 91
yves.barral@bc.biol.ethz.ch

This chapter was published in:
TRENDS in Cell Biology, Vol 13, 562-569, in November 2003
Abstract

Spatial coordination of the cell division axis with cellular polarity and/or the position of neighboring cells is crucial for embryonic development, organogenesis and tissue homeostasis. In most cell types, the position of the mitotic spindle at anaphase onset dictates the orientation of the division axis and, in unicellular organisms, plays an important role in chromosome segregation. Cortical factors play a key role in the orientation of the spindle. Recent data from yeast revealed that the spindle does not passively react to cortical signals but actively interprets them in order to find its correct position. We review the data leading to a "compass model" for spindle positioning and discuss its potential generality.

Introduction

During cell division, the position of the spindle within the cell dictates whether polarized factors become symmetrically or asymmetrically segregated between daughter cells (see Fig. 1). In many cell types, polarized factors comprise cell fate determinants. Asymmetric segregation of these factors is a major mechanism for the generation of cell diversity in multi- and uni-cellular organisms. Accordingly, spindle mis-orientation severely impairs the segregation of fate determinants and hence, development.

During spindle positioning, spindle rotation ensures spindle alignment with the polarity axis (Box 1). Other spindle movements include displacement towards a cell edge, leading to different sizes of the daughter cells. Here, we focus our attention on the mechanisms of spindle alignment. Using Drosophila and C. elegans as models, many laboratories identified a number of factors involved in this process. These factors are thought to provide cortical cues to orient the spindle. They participate in the cortical capture and/or pulling of astral microtubules, and govern the movements of spindle poles within the cell. How this achieves differential positioning of centrosomes to different cell ends is unclear. Data from budding yeast indicate that, depending on the spindle pole from which they emanate, astral microtubules have distinct abilities to interact with the cortex. These results suggest that asymmetry of the mitotic spindle is a prerequisite to the interpretation of cortical polarity by the spindle. Here we will review the yeast data implicating spindle asymmetry in spindle orientation. We will then discuss the possibility of related mechanisms being used in higher eukaryotes.
Spindle asymmetry

Spindle alignment vs spindle orientation
Spindle alignment and orientation should not be confused. (A) shows a symmetric spindle aligned with the polarity axis of the cell. In the case of an inherently asymmetric spindle like in (B), alignment can result in two orientations, (C) or (D), because the two poles are not equal. Anticlockwise rotation of the spindle in (B) by 90° results in orientation as in (C), clockwise rotation in orientation as in (D).

The yeast spindle pole body
The yeast SPB is the equivalent of the centrosome in higher eukaryotes. SPB remains embedded in the nuclear envelope during the entire cell cycle. It consists of a multilayered structure with an intranuclear inner, a membrane embedded central and an outer plaque facing the cytoplasm. These layers are associated with and are held together by specific proteins linking the different plaques. Different from animal cells, the nuclear envelope doesn’t break down during mitosis in yeast. Two classes of MT arrays are nucleated from the SPB: the intranuclear array is nucleated from the inner plaque, and assembles the mitotic spindle upon SPB duplication and separation. Cytoplasmic microtubules are nucleated from the outer plaque and ensure spindle positioning. A number of proteins with regulatory function in cellular processes have been shown to associate with SPB and centrosomes. They often have a coiled-coil domain in common. Thus these microtubule-organizing centers (MTOCs) can be regarded as important locations for protein interaction and sequestration and play a regulatory role in many cellular processes. The yeast spindle possesses its own polarity as SPB duplication results in the formation of a new SPB at the side of the older one. It is always the old SPB that enters the bud at anaphase.

Spindle positioning: the role of the cortex
Developmental model organisms were powerful to study the role of cortical polarity in spindle movements. During the first division of the C. elegans embryo, the mitotic spindle aligns with the antero-posterior axis of the oocyte. A rotation by 90° also occurs in the resulting posterior cell to reorient the spindle again along the antero-posterior axis of the embryo (1-3). Ground-breaking experiments by Tony Hyman established that interaction of astral microtubules with the cortex governs centrosome movements (13,76). Genetic approaches identified the PAR proteins (4) as factors that ensure correct partition of P granules to the germ line precursor cells. P granules are ribonucleoprotein particles containing germ line determinants. The PAR proteins are all cortical factors that control spindle positioning (4-8). While, PAR-4 and PAR-5 localize uniformly to the cortex, PAR-1 and PAR-2 accumulate at the posterior of the early embryo, and PAR-3 and PAR-6 at the anterior. Anterior and posterior PAR proteins maintain their polarity by excluding each other: In the absence of PAR-2, PAR-3 covers the entire cortex, while in absence of PAR-3,
PAR-2 localizes uniformly (7,9). PAR proteins exert their spindle positioning function by controlling microtubule/cortex interactions: Laser ablation of the central spindle in the one cell embryo demonstrated that forces applied on astral microtubules are stronger at the posterior cortex than at the anterior (11). This asymmetry is abolished in par-2 and par-3 mutants. Thus, spindle movements rely on asymmetric pulling forces applied on astral microtubules. Somehow the cortical PAR proteins are involved in establishing this asymmetry.

It is not yet completely clear how the PAR proteins impinge on microtubules. Recent studies suggest that heterotrimeric G-proteins are required for PAR-dependent spindle movements but not for PAR protein localization (58). Thus, G-proteins are thought to translate PAR spatial information into regulation of the forces applied on microtubules (10,12,58). Gß is involved in spindle displacement and Gß in spindle rotation. Both dynein and actin microfilaments are required for force generation at the cortex (12-16) and for spindle positioning (17,18). They are therefore good target candidates for G-protein signaling. It is still unclear though how PAR and G-proteins talk to each other and which are their direct effectors. Also, it is not clear how, in C. elegans, actin impinges on spindle positioning. However, time-lapse analysis indicates that astral microtubules are more dynamic at the posterior than at the anterior extremity of the one cell embryo (10). Thus, heterotrimeric G-proteins may somehow impinge on the regulation of microtubule stability.

Analysis of spindle rotation during the asymmetric division of Drosophila neuroblasts revealed a remarkable conservation of PAR proteins across multicellular organisms. The Drosophila PAR complex is composed of Bazooka (PAR-3), PAR-6 and atypical protein kinase C (1,8,19,20). The additional factors inscuteable85 (Ins) and the microtubule-associated protein (MAP) Cornetto (21), which have no obvious homology to other known proteins, appear to link PAR protein and cortical capture of microtubules. Inscuteable is recruited by PAR proteins to the apical domain of neuroblasts and is required for spindle orientation (22-24,85). Its ectopic expression overrides the planar polarity of epithelial cells and enforces apical-basal orientation of the spindle (85). Thus, Inscuteable is necessary and sufficient to orient the spindle along the PAR polarity axis. At the cortex, Inscuteable recruits Pins (86), a Ga-binding protein, and Cornetto. Thus, Inscuteable may links PAR proteins to both heterotrimeric G-proteins and microtubules.

In summary, these studies substantiated the idea that spindle positioning is governed by the interaction of astral microtubules with asymmetrically distributed cortical factors. Upon attachment to these factors, microtubule ends can be either stabilized or destabilized, leading to the formation of pulling or pushing forces on centrosomes.
Spindle asymmetry

Figure 1. Principle of asymmetric cell division

A polarized cell can produce identical progeny (symmetric cell division, A) or two different daughters (asymmetric cell division, B). The cleavage plane (arrowheads) is determined by the position of the mitotic spindle (red bar). Cellular factors such as cell fate determinants, localize asymmetrically in the cytoplasm and at the cell cortex, generating a polarity axis. During a symmetric cell division the mitotic spindle aligns perpendicular to this polarity axis. In contrast, in an asymmetric cell division the spindle orients parallel to the cell polarity axis and partitioning of cellular components is unequal.

Translation of cortical cues into spindle orientation

How cortical cues lead to proper spindle orientation is not as evident as it seems. Indeed, it is not obvious how attachment and pulling on astral microtubules at specific cortical sites ensures that spindle poles go to opposite ends of the cell. What keeps both poles from being attached and pulled to the same part of the cell? Two explanations were proposed. First, the geometry of the cell may be a determinant (16,28,30). Second, the different spindle poles may respond differently to cortical cues (29).

The role of cell geometry

Fission yeast is a good example, where cellular geometry helps spindle alignment with the division axis. Here, sliding of the spindle poles along the cortex during spindle elongation imposes its alignment with the long axis of the cell. Remarkably however, S. pombe cells dispose of a specialized pathway that aligns the spindle with the long axis of the cell already before spindle elongation starts (28,30). This pathway involves astral microtubules and the Slk19-related protein mia1 (28). Slk19 is a S. cerevisiae microtubule-associated protein.
Moreover, a specific cell cycle checkpoint appears to delay anaphase when the spindle fails to align (28,30). Thus, S. pombe cells do not rely only on their geometry for spindle alignment, suggesting that geometry-driven spindle rotation is not robust enough.

Geometric constraints have been proposed to ensure spindle rotation also in C. elegans (16). According to this model, cortical regions of lowest curvature should attract spindle poles. The function of PAR-3 and LET-99, another cortical protein involved in spindle rotation (31), may be to modulate geometry effects, such as to override them in specific cell types or at specific cortical sites. This model nicely accounts for force generation and differences observed between wild type and certain mutant embryos (16). However, it fails to explain why only one pole should move towards the region of lowest curvature and not both.

**Response to cortical cues in S. cerevisiae**

The view that spindle poles respond differently to cortical cues was suggested by yeast data. In budding yeast, chromosome segregation between mother and bud relies on the alignment of the spindle along the mother-bud axis, which defines the polarity axis of the cell. Astral microtubules, which are nucleated at the spindle pole bodies (SPBs, Box1), mediate spindle positioning (32-34).

Two overlapping pathways link astral microtubules to the yeast cortex. During metaphase, the first pathway depends on the MAPs Kar9 and Bim1 (homologous to EB1), the kinesin-related protein Kip3, type V myosin Myo2, actin cables and the formin Bni1 (35,36,39,40,42,43,45). The second pathway involves dynein, dynactin, the kinesin-related protein Kip2, the plus-end-tracking protein Bikl (homologous to CLIP170) and the cortical protein Num1 (35-39). This pathway is activated at anaphase, i.e., after the execution point of the first pathway (77), and pulls one spindle pole through the neck. Each pathway is sufficient for cell viability, but inactivation of both is lethal (39). The only obvious features of the Kar9 protein are a coiled-coil domain in its N-terminal half and a short, C-terminal EB1/Bim1-binding domain shared with the adenomatous polyposis coli protein (APC) (40,41,78).

**Kar9 localizes asymmetrically on spindle poles and microtubules**

Kar9 was originally proposed to form a microtubule capture site at the bud cortex (40,42), which microtubules reach by random search. Capture would be mediated by the interaction of microtubule-bound Bim1 with Kar9 (40,42-44,50). Recent data suggest an alternative model (29,45-48) (Fig. 2). Time-resolved colocalization studies and nocodazole experiments
showed that Kar9 expressed at endogenous level constantly localizes to microtubules and SPBs (29,46-48). FRAP studies indicate that endogenous Kar9 is loaded onto microtubules mainly at SPBs (29) (see Fig. 2A), rather than at the cortex. These experiments also indicate that Kar9 localizes processively to microtubules (see below and Fig. 3). This is in contrast to the conclusion drawn from earlier studies (40,42), where, upon overexpression, Kar9 was found at the cell cortex independently of microtubules. Whereas microtubule-independent localization of Kar9 to the cortex upon its overexpression revealed the ability of Kar9 to bind Myo2 and hence to cortical actin, the physiological localization of Kar9 appears to be to microtubule ends.

Time lapse analysis also revealed that microtubules decorated with Kar9 do not reach the bud by random but are actively guided (29,48). Many data indicate that microtubule ends are actively transported along actin cables by type V myosin (29,45,48) (Fig. 2B). First, Myo2 was visualized at the tip of microtubules and movement of microtubule ends towards the bud is slowed down in myosin mutants with reduced speed (48). Myo2 recruitment to microtubule ends depended on Kar9 (45,48). Second, when Myo2-Kar9 interaction is impaired, microtubules fail to orient towards the bud (45,29). Mutations that affect vesicle transport by Myo2 but neither Kar9 binding nor motor activity do not impair microtubule orientation. Third, Kar9 can be functionally replaced by a Bim1-Myo2 fusion protein, indicating that the major function of Kar9 is to link these two proteins (48). Thus, a new model for microtubule orientation is that it is ensured by Kar9-dependent recruitment of myosin V on microtubule tips followed by myosin-driven guidance along actin cables. Such a mechanism would be more efficient than random search. However, since disruption of KAR9 is not lethal (40) random search can support survival in normal growth conditions.

Strikingly, Kar9 localizes only to the SPB destined to the bud and to associated microtubules (29,46,47). Microtubules emanating from the Kar9-less pole do not recruit Myo2 (Kusch and Barral unpublished results) and do not orient towards the bud (29,47). In reverse, dominant forms of Kar9 that localize to both SPBs and associated microtubules cause microtubules from both poles to orient and both spindle poles to migrate towards the bud (29). In absence of Kar9, poles remain distant from the bud prior to anaphase and the spindle fails to orient. Thus, the presence of Kar9 on SPBs targets them to the bud. Furthermore, asymmetric localization of Kar9 achieves specific targeting of a single pole to the bud (Fig. 2).

Altogether, these results suggest that spindle asymmetry is required for spindle alignment. However, cells lacking Kar9, or mutants where Kar9 localizes symmetrically, are still able to align their spindle during anaphase (29,40), suggesting that asymmetry is required only during metaphase, or that dynein, which acts in anaphase, acts asymmetrically as well.
Chapter V

Figure 2 Model of spindle orientation mechanism in yeast
A. The loading of Kar9 onto microtubules is an active and spatially regulated process. Kar9 loading onto microtubules takes place at the spindle pole destined to the bud (45). Kar9 is loaded from the old spindle pole onto microtubules probably via its interaction with Bim1.

B. Microtubule-bound Kar9 physically interacts with the type V myosin Myo2 (42-43, 45), which guides microtubules along the polarized actin cables. Actin cables are nucleated at the bud cortex.

C. At the spindle pole destined to the mother, the Cdc28/Clb4 and Cdc28/Clb3 kinases phosphorylate Kar9 and repress its interaction with Bim1 (45). Therefore, Kar9 loading onto microtubules is prevented at the distal spindle pole, which does not orient towards the bud.

Remarkably, recent results indicate that dynein localizes to astral microtubule tips and spindle poles, much like Kar9 (51). In addition, dynein and the dynein-associated proteins Pacl and dynamitin localize asymmetrically on microtubules and SPBs (51-53, 79) (Grava, and Barral, unpublished). Thus, asymmetric distribution of microtubule-associated factors onto spindle poles and aster microtubules may be a general requirement for their function in spindle positioning.

Recently, Pereira et al. showed that the old SPB is inherited in about 90% of the cases by the bud (59). This observation suggests that the age of spindle poles influences which recruits Kar9. In cells treated with nocodazole prior to spindle pole separation and then released from the drug, this inheritance bias is abolished. In these cells, Kar9 localizes to the spindle pole oriented towards the bud, independently of its age (47). Thus, the inheritance bias is
Spindle asymmetry presumably not due to intrinsic differences between old and new SPBs. This suggests that positional information determines which of the two SPBs recruits Kar9. However, spindles forming in unpolarized cells, such as those forming in a cdc42-1 mutant, are still asymmetric regarding to Kar9 distribution (29). Thus, spindle asymmetry is not a mere reflection of SPB’s age or position relative to the bud. The mechanism directing Kar9 preferentially to the old SPB in unaffected cells still awaits elucidation.

The ability of Kar9 to maintain its association with specific microtubules while they grow and shrink, demonstrated by FRAP experiments (29), is remarkable. We propose to call processivity this property, by analogy to motor proteins and polymerases (Fig. 3A). In contrast, EB1 and its S. pombe homologue, mal3, go on and off microtubules rapidly, and decorate all microtubules (49) (D. Brunner, personal communication), indicating that they are not processive. Bim1 also localizes to all microtubules and by similarity is unlikely to be processive (80). Since Kar9 binds microtubules via EB1/Bim1 (50,43,81), the different processivity of Kar9 and Bim1 is paradoxical. However, Kar9 oligomerization (43,50) and/or association with a microtubule-dependent motor (47) may support its processivity independently of Bim1 exchange (see speculative model on figure 3B). Another apparent paradox is that EB1-related molecules localize only to growing microtubules (80), while Kar9 stays at the tip of growing and shrinking microtubules (29,46). A detailed analysis of Bim1 localization by time-lapse microscopy will be required to resolve this paradox. In summary, Kar9 has two molecular functions: it labels selected microtubules processively and links Bim1 to Myo2, and hence, microtubules to actin.

Figure 3 A. Model for “processive” binding of MAPs along microtubules. Possible movements of a microtubule-binding protein and microtubule-dependent motor are described. The microtubule-bound protein can either fall off the microtubule and become soluble (1), or move laterally to a new position of the microtubule (2). The soluble protein can come back to the same microtubule (3) or interact with another one (4). In principle (3) and (4) have the same probability, (3)≈(4). Microtubule-dependent motors are processive when they can walk long distances along a same microtubule. Thus (2)≈(1), or (3)≈(4). MAPs are thought to interact with microtubules in an on/off manner, i.e., (1)≈(2) and (3)≈(4). In order for a MAP to be able to track the same microtubule over time, some mechanism must ensure that (2)≈(1), or that (3)≈(4). B. Speculative model for Kar9 processivity. In the absence of Kar9 microtubule-bound Bim1 rapidly exchanges with the cytoplasmic pool (A). As a result Bim1 decorates microtubule (+)ends during microtubule growth, but dissociates from them upon microtubule shrinkage (37, 46, 48). Kar9 oligomerization is required for Kar9 to bind processively to the microtubule while Bim1 exchanges. Kar9 binding may reduce Bim1 exchange with the cytoplasm and ensure that Kar9-bound Bim1 remains associated with microtubules even during depolymerization.
How is Kar9 asymmetry established?

Spindle positioning in yeast is under the control of the cyclin-dependent kinase Cdk1/Cdc28. Specifically, Cdk1 is involved in the maintenance of spindle asymmetry, as shown by M. Segal, who found that in the cdc28-4 clb5μ double mutant microtubules frequently extend from both poles into the bud, leading to mis-positioning of metaphase spindles (53,54). The fact that in this mutant spindle poles are equivalent, indicates that Cdk1/Cdc28 is involved in establishment or maintenance of spindle asymmetry.

One step towards understanding the role of Cdc28 was the discovery that Cdc28 phosphorylates Kar9 in vivo and in vitro (29,47). Cdc28/Clb3 and Cdc28/Clb4 are the main kinase activities involved in Kar9 phosphorylation (29). Non-phosphorylatable forms of Kar9 are symmetrically distributed. Thus, phosphorylation of Kar9 controls its asymmetry (29) (Fig. 2C). These results apparently contradict Segal et al. that didn’t detect spindle asymmetry defects in cdc28-4 clb3μ clb4μ cells, but in the cdc28-4 clb5μ double mutant. Kar9 localization was not assayed in these studies. In the cdc28-4 background, Cdc28 activity is strongly reduced and Clb3- Clb4-dependent phosphorylation of Kar9 is almost abolished, even at permissive temperature (29). Accordingly, in this mutant Kar9 localizes symmetrically to spindle poles at all temperatures (Liakopoulos, unpublished result). The additional effect due to the clb5μ mutation may reflect Clb5 function in additional, Kar9-independent events. In any case, the analysis of Kar9 regulation suggests that Cdc28 may also act asymmetrically on spindle poles. Supporting this view, Cdk1/Cdc28 localizes to SPBs (47), and Clb3 and Clb4 seem to localize or act asymmetrically (29). Intriguingly, a GFP fusion of Cdc28 also travels in a Kar9-dependent manner along microtubules emanating from the proximal SPB (47). There, Cdc28 may regulate the interaction of microtubule ends with the cortex. Together these data indicate that intrinsic spindle asymmetry is required for spindle alignment in preanaphase S. cerevisiae cells. This asymmetry depends on Cdk1 activity and controls the asymmetric guidance of microtubules towards the bud.

Microtubule guidance: is it conserved?

Little is known about direct microtubule/actin interactions in organisms other than yeast. Although in higher eukaryotes actin and tubulin work together in many processes, such as cell migration and cytokinesis, movement of microtubule ends along actin cables remains speculative. However, analysis of microtubule paths towards focal adhesion complexes
indicates that it is a guided process and suggests the involvement of actin (55,56). Thus, guidance mechanisms may be conserved between yeast and mammals.

...and spindle asymmetry?

Cell cycle regulators involved in late stages of mitosis are asymmetrically distributed onto spindle poles of many organisms. This is for example the case for the components of the MEN and SIN pathways involved in exit of mitosis and cytokinesis in budding and fission yeast (71,72) and in mammalian cells (73). Importantly, these asymmetries are not restricted to polarized cell types, supporting the idea that spindles are intrinsically asymmetric. However, these factors have not been involved in spindle positioning so far.

Intrinsic centrosome asymmetry plays an obvious role in asymmetric cell divisions of the mollusk Ilyanassa obsolete (60) (Fig. 4). Here, mRNAs encoding patterning factors accumulate around the interphase centrosome, migrate to the cortex during mitosis, and segregate asymmetrically between newly formed blastomers. In di-centrosomal cells, resulting from a cytokinetic block, these mRNAs localize to only one centrosome, which orients towards the cellular end supposed to inherit the mRNAs. Thus, in this organism the two centrosomes are not identical and their differences appear to drive both spindle positioning and asymmetric distribution of fate determinants.

Spindle asymmetry is also apparent in Drosophila neuroblasts (27,61,62) (Fig. 4), where the apical centrosome is larger, contains more gamma-tubulin (61), nucleates more and longer astral microtubules and resides farther away from the cortex than the basal one. This spindle pole asymmetry is independent of apical factors Bazooka, Insuteable and Pins (27,61), suggesting that neuroblast spindles are intrinsically asymmetric. It is tempting to speculate that this asymmetry drives spindle alignment. Supporting this idea, the direction of spindle rotation, clockwise or counterclockwise, is predetermined by the position of the centrosome in the neuroblast prior to its duplication (61). This determination suggests that spindle poles do not migrate randomly, as a random search and capture model would predict, but that some mechanism targets centrosomes to specific cell ends.

In contrast, in C. elegans spindle asymmetry appears to not play a role in spindle rotation. In this organism, cortical influence accounts for differences in aster size (11) (Fig. 4) but the spindle seems to not be asymmetric on its own. Early studies by Anthony Hyman (13) demonstrated that both poles of the first posterior cell are equivalently able to migrate towards the site of previous cytokinesis. Thus, centrosomes are equivalent. However, nocodazole treatment in yeast randomizes the orientation of spindle poles towards the bud.
Microdissection experiments might have the same effect. Finding ways to differently label the two sister centrosomes could be useful to determine whether spindle poles are indeed equivalent in *C. elegans* or not.

![Images of C. elegans zygote, Drosophila neuroblast, and Ilyanassa obsoleta](image)

**Figure 4** Examples of polar spindles during asymmetric cell division. In the *C. elegans* zygote, the mitotic spindle is morphologically asymmetric. The posterior centrosome is flattened, due to strong pulling forces exerted on it by posterior astral microtubules. In *Drosophila* neuroblasts the apical spindle pole is bigger, contains higher amounts of γ-tubulin and nucleates more astral microtubules than the anterior pole. In *Ilyanassa obsoleta* asymmetric localization of mRNAs (purple dots) takes place not at the cortex but on one specific centrosome. These mRNAs, coding for morphogens, are then asymmetrically segregated and determine developmental fates of the cells that inherit them.

**Role of cell cycle regulators in asymmetric cell division**

Cell cycle regulators such as cyclin/Cdk1 complexes are obvious candidates to regulate many aspects of spindle function. Cdk1 is found on spindles, spindle poles and astral microtubules in many organisms (47,63,64) and the activity of Cdc2/cyclin-B appears to be associated with centrosomes (65,66). Cdc2 and other mitotic kinases also regulate the activities of microtubule-based motors and microtubule-associated proteins during spindle formation (67-70). Many of these targets, such as dynein and kinesins, are required for spindle positioning and microtubule-cortex interactions. Thus, Cdk1 may control spindle positioning not only in *S. cerevisiae*.

Only recently have cell cycle regulators indeed emerged as key factors of asymmetric cell division. In *Drosophila* neuroblasts for example, asymmetric distribution of cortical factors and spindle alignment are affected by a mutation in Ddc2 (cdc2E51Q)(74) that does not affect mitosis itself. In *C. elegans*, the anaphase promoting complex (APC/c) is also implicated in asymmetric division of the one and two cell embryo(75). Again, the mutants showing defects in asymmetric division were hypomorphs that do not interfere much with mitosis itself, but rather with the cortical polarity and spindle alignment. In both *Drosophila*
Spindle asymmetry

and C. elegans, the primary effects observed relied on the distribution of cortical cues, whereas misalignment of the spindle may rather be secondary defects. Nevertheless, due to the spindle pole association of Cdc2 and APC/c, these results suggest that cell cycle regulation and the spindle play a more important role in cellular asymmetry than originally assumed.

The most striking similarities between yeast and higher eukaryotes reside between Kar9 and APC. Like Kar9, APC is a +TIP protein hitchhiking (80) on microtubule ends with EB1 and motor molecules (78,80). Also like Kar9, APC interacts with a myosin (I. Nathke, personal communication). In addition, its interaction with β-catenin links it to adherence junctions and actin (82). Strikingly, the interaction of APC with EB1 is regulated by Cdc2-dependent phosphorylation, much like for Kar9 (83). Furthermore, APC and EB1 have been found to control spindle positioning in Drosophila epithelial cells (26). Thus, it is tempting to speculate that APC and Kar9 share some highly conserved functions, and that the role of Cdk1 in asymmetric cell division is conserved.

Figure 5 The compass theory Spindle orientation may work similar to a compass. Similarly to a magnetized needle the spindle can only orient if it possesses its own polarity.

Concluding remarks

Astral microtubules have long been thought to search the cortex in a random fashion. New data indicate that they can also be guided. This allows the targeting of distinct microtubules to different cortical sites depending on the spindle pole they emanate from. Such spindle asymmetry is essential for spindle positioning at least in S. cerevisiae and possibly in other organisms.
Chapter V

The yeast data led to the formulation of the compass model for spindle positioning (29). According to this model spindle asymmetry is absolutely required for the spindle to interpret cortical cues and align. This is similar to the manner a compass works (see Fig. 5). In this case magnetized needles follow the orientation of the surrounding magnetic field. In contrast, non-magnetized needles, which do not have a polarity, do not orient. This is not because the needle does not respond to the magnetic field, but because both ends of the needle react equivalently. Similarly, the spindle may only align if poles react differently to cortical cues. The overall theme that emerges throughout different systems is that spindle polarity, either inherent or resulting from spindle pole-cortex communication, is required for the spindle to interpret cortical information. In that sense, the spindle behaves like a chemotactic or "sensing" machine that is able to perceive, interpret and use the polarity of its environment to orient. Remarkably, the cell cycle machinery appears to directly control the establishment or maintenance of spindle asymmetry. Fascinating questions ahead of us concern the generality and the origin of spindle asymmetry as well as how it is maintained.

References

7 Boyd, L. et al. (1996) PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans. Development 122 (10), 3075-3084
Spindle asymmetry

38 Muhua, L. et al. (1994) A yeast actin-related protein homologous to that in vertebrate dynactin complex is important for spindle orientation and nuclear migration. Cell 78 (4), 669-679.
46 Kusch, J. et al. (2002) Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast. Genes Dev 16 (13), 1627-1639
47 Maekawa, H. et al. (2003) Yeast Cdk1 translocates to the plus end of cytoplasmic microtubules to regulate bud cortex interactions. Embo J 22 (3), 438-449
Chapter V


59 Pereira, G. et al. (2001) Modes of spindle pole body inheritance and segregation of the Bfa1p-Bub2p checkpoint protein complex. Embo J 20 (22), 6359-6370


Spindle asymmetry

Trzepacz, C. et al. (1997) Phosphorylation of the tumor suppressor adenomatous polyposis coli (APC) by the cyclin-dependent kinase p34. J Biol Chem 272 (35), 21681-21684
Appendix 1: Role of MARK-related kinases in regulation of microtubule attachment

Nuclear migration defect in SDK mutants is independent of their function in the septin checkpoint that arrests the cells in G2/M, as swel disruption does not suppress the nuclear migration defect.

Micrographs of DAPI labelled hydroxyurea arrested cells

Time lapse series of GFP-labelled microtubuli in WT (left, 7sec time interval) and SDK (right, 9sec time interval) mutants.

Microtubule attachment at the neck: in WT microtubule shrinkage is induced upon capture at neck followed by spindle pulling to the neck; in mutant, microtubule reside longer at neck and grow, resulting in pushing of the spindle away from the neck.

The micrograph of Kin2-GFP (left) shows localization of this MARK-related kinase to polar sites during the cell cycle and at cytokinesis at the neck. DAPI staining in kin2 mutants (micrograph in middle, hydroxyurea arrest) reveals a nuclear migration defect, quantified on the right.
Appendix 2: Kar9 is hyperphosphorylated in SDK mutants

Western blot analysis of Kar9-GFP in SDK mutants revealed a hyperphosphorylation, which has been shown to be dependent on Swel activity in these mutants by Dimitris Liakopoulos. He demonstrated further that Kar9 is phosphorylated by Cdk25/Cdc2/Clb4 (Liakopoulos et al., 2003) which gives presumably rise to the shift of Kar9 migration in this preliminary blot.
Appendix 3: Localization of Kar9

A Localization of overexpressed GFP-Kar9
Time lapse series of GAL-GFP-KAR9 induced for 1 h (left upper panel).
Colabelling of microtubules (GFP-TUB1) revealed a microtubule independent cortical Kar9 dot moving with different kinetics than microtubules. As microtubules are presumed to interact with the bud cortex via myosin Myo2, the question arises: how dynamic bud cortex localization of the Kar9 aggregate is mediated. Induction kinetics revealed that Kar9 preferentially localizes to microtubules before it can be visualized as a microtubule independent dot (not shown). Left lower panel: overexpressed Kar9 localizes to cortical sites in bni1Δ mutants and nocodazole treated cells. A faint cytoplasmic dot reveals microtubule and Bni1Δ independent localization onto SPB (demonstrated in lower right panel: SPC-42-CFP GAL-GFP-KAR9 in bni1Δ mutants, YFP channel: the GFP signal is weak, but XFP channel: GFP signal is strong and merge from left to right).

B Kar9-GFP (endogenous levels) localization on growing and shrinking microtubule ends in a time lapse series with coexpression of GFP-TUB1.

C Kar9-YFP distribution on CFP-TUB1 labelled microtubules during the cell cycle depicted are representative cases.
Appendix 4: Microtubule guidance

A and C are time lapse series that illustrate the effect of Kar9 localization on microtubule guidance in cells expressing Kar9-YFP in C, the symmetrically localizing mutant form Kar9-AAA-YFP (in A) and CFP-alpha-tubulin (taken from Liakopoulos et al., 2003). As discussed in Chapter IV, guidance itself leads to spindle movement, which is particularly apparent in movement of the distal pole along with a guided microtubule to the neck in the symmetrically localizing mutant (indicated by red arrow).

B GFP-labelled microtubules are long and stable in kar9 mutants. They meet the mother cortex, but they are not guided towards the bud neck and shrink back.
**Curriculum vitae**

### PERSONAL DATA

<table>
<thead>
<tr>
<th>Name:</th>
<th>Justine Kusch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of Birth:</td>
<td>18. September 1972</td>
</tr>
<tr>
<td>Place of Birth:</td>
<td>Gleiwitz (Poland)</td>
</tr>
<tr>
<td>Marital status:</td>
<td>single</td>
</tr>
<tr>
<td>Nationality:</td>
<td>German</td>
</tr>
</tbody>
</table>

### EDUCATION

| Since 02/00                | Ph. D. Thesis at the Institute of Biochemistry, ETH Zürich, (Supervisor Prof. Dr. Y. Barral).  
|                            | “Molecular Mechanisms of Spindle Positioning in Budding Yeast *Saccharomyces cerevisiae*”  |
| 05/98-05/99                | Diploma work at the Institute of Immunology, University of Konstanz, (Supervisor: Prof. Dr. U. Krawinkel).  
|                            | “Die biologische Funktion der MMP-19”  |
| 10/92-04/98                | Biology studies at the University of Konstanz                      |
| 09/83-08/92                | Gymnasium Paul-von-Denis-Schulzentrum Schifferstadt                |

### STUDIES ABROAD

| 08/97                      | IAESTE Practical at the Hydrometeorological Institute of Ljubljana, Slovenia.  |
| 10/95-05/96                | Biology studies within an exchange program at the University College Cork, Ireland.  |
Publication list


Acknowledgements

Thanks to my supervisor Yves Barral for giving me the opportunity to work on these exciting projects and to take part in setting up our microscope system. Thanks for being such a confident, enthusiastic, encouraging, understanding and -sometimes- also pushing supervisor!

Thanks to Michael Hall and Urs Greber, my thesis committee members, for interesting and helpful discussions.

Thanks to Matthias Peter and Monica Gotta for spontaneously agreeing to be my co-examiners.

Rolf, Roland and Toni, thanks for their great engagement, not always because they anyway had to!

Thanks Eva, for being there any time to find a way out of just any situation, for the wonderful marmelades, greek dreams and many nice, chatty breaks!

Thanks to the Barral group for a lively, sportive and very entertaining atmosphere in the lab: Jeroen and Cosima also for help in microscopical matters, Corinne for organizing soooo many trips, Christina for always being incredibly enthusiastic - even during my explanations, Zhanna, so young! so cool! Caren for her ability to think around corners and -ach!- for really taking things easy, Mahamadou for many exciting ideas, perspectives and wonderful music. Especially thanks to Stephanie (thank you!!!), Sandrine and Dimitris for correcting and discussing parts of the manuscript. Thanks Dimi also for the great teamwork during long, cold Christmas nights -besides for never getting tired to announce the weather forecast and for entrusting the secret recipe for the best coffee in the house to me!

Merci Sandrine, for all the diverting, "just short" distractions from work I wouldn’t want to miss!

My former flat mates, Erica, Lukas, Susanne, Michi and Carmen I thank for accompanying my whole PhD life with open ears, many great dinners and a lot of orientation help for life in Zürich. Thank you, Lala, for sparing me so much time!

Andreas, thanks for all the sportive, entertaining and comforting breaks from the lab.

Thanks the Italian connection, Raffa, Ale and Oleo for many funny and delicious dinners.

Merci, Céline for making life outside the lab so enjoyable - in just any season.

Thanks to family Willmann-Hornemann-Schnuffidu! Especially Raffa I thank for being there anytime! And for always being ready to spend long nights bringing clarity into things - like manuscripts.

Thanks, Uli for his support, patience, endurance, help, opinion… and and and!

Thanks to my family Claudia, Sylvia and Christian, Achim and the KIDs, and especially my parents, without whom I would not have been able to go this way.