Investigating spatial and temporal coordination of cytokinesis with spindle function

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Investigating spatial and temporal coordination of cytokinesis with spindle function

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Summary

Cytokinesis is a multistep process by which a cell physically divides into two. In higher eukaryotes it starts with actomyosin ring contraction at the cleavage plane, which is followed by the cleavage of the plasma membrane, a process called abscission. In yeast cells, which contain a cell wall, abscission is followed by septum degradation. In budding yeast the actomyosin ring is not essential. Cells can separate by mere septum formation. Chromosome segregation and cell division (cytokinesis) have to be tightly coordinated to ensure proper genetic segregation. We know little about how cells ensure that cytokinesis does not take place before all chromosome arms are pulled out of the cleavage plane.

To understand more about cytokinetic events and the underlying machinery we used two approaches. On the one hand we used an actin-overexpression assay. Actin is the key component of the contractile machinery located at the bud neck together with the type II myosin Myo1. As it was reported that actin overexpression is lethal, we wanted to investigate actin dynamics and turnover in overexpressing cells to study the reason for this lethality. Furthermore, initial experiments suggested that actin overexpression induces a new actin structure, an actin ring that is always in tight association with the nucleus. We wanted to investigate if this structure was related to a cytokinetic in vivo actin structure and if this association around the nucleus means that there is a spatial regulation between cortical/cytokinetic factors and the nucleus that regulate cytokinetic onset or execution.

We found that already a four-fold overexpression of actin is toxic. Furthermore, we were able to show that actin overexpression interferes differently with distinct aspects of actin function. Overexpression of actin did not affect the establishment of actin polarity, whereas it abrogated its maintenance.

The new ring-like actin structure was genetically distinguishable from the actomyosin contractile ring. Formation of the actin structure upon actin overexpression was dependent on the septin cytoskeleton, the cytokinetic protein Hof1 and the Arp2/3 complex. In contrast to the actomyosin ring, the ring formed upon actin overexpression required neither Myo1 nor formins to assemble. Hof1 might therefore act as a linker between actin and septins. Furthermore, we found that a Hof1-dependent actin belt is formed at the bud neck of anaphase cells in the absence of actin overexpression. The physiological role of this belt might be related to that of the similar structure observed in dividing fission yeast. In this organism a ring nucleating actin
structure is seen before cytokinetic actin ring formation, which constricts to a ring and depends on the Hof1 homologue cdc15.

As mentioned, an interesting feature of the ring was that it is always associated with the nucleus. We found that certain importins and exportins played a role in its formation. This was an indication that spatial communication between the cytokinetic machinery and the nucleus exists. However, a general necessity of the nucleus to pass into the daughter cell before cytokinesis could not be shown.

In a second approach we investigated how spindle components influence cytokinetic onset and progression. Cells with central spindle defects, like cells mutated for Ase1, the kinetochore and central spindle component Ndc10 as well as cells in which the spindle is completely destroyed like in \textit{mad2Δ} cells treated with nocodazole show a defect in cytokinesis although they start a new cycle.

We were able to pin down this cytokinetic defect to a defect in abscission because actomyosin contraction takes place with similar kinetics as in wild type cells, but the plasma membrane fails to separate.

We investigated the signalling pathway that underlies the abscission defects in cells with spindle midzone defects. We could show that abscission defects depend on the aurora kinase Ipl1 and the anillin-related proteins Boi1 and Boi2. Boi1 and Boi2 localize to the site of cleavage in an Ipl1-dependent manner, where they act as abscission inhibitors. Also Polo kinase Cdc5 and Separase Esp1 play a role in abscission inhibition.

The inactivation of Boi1/Boi2 leads to a slight increase in chromosome breakage by the cytokinetic machinery and is lethal in cells with spindle elongation defects. At this point we think that the signalling pathway we found monitors spindle elongation and chromosome separation to ensure that cytokinesis is completed only after that the cleavage plane is cleared of chromatin.
Zusammenfassung


Bevor es zur Zytokinese kommt müssen Chromosomaufteilung und der Beginn der Zellteilung eng koordiniert werden, damit das genetische Material korrekt verteilt ist.


Wir stellten fest, dass bereits eine vierfache Ueberexpression von Aktin toxisch auf die Hefezellen wirkt. Ausserdem konnten wir zeigen, dass sich die Aktinüeberexpression unterschiedlich auf verschiedene Aspekte von Aktin auswirkt. Die Ueberexpression hatte keinen Effekt auf die Entstehung der Aktinpolarität, beeinflusste allerdings deren Aufrechterhaltung.

Die neue, ringförmige Aktinstruktur, die wir bei Ueberexpression beobachteten, war genetisch vom Aktomyosinring unterscheidbar. Die Formation der bei der Ueberexpression beobachteten Struktur hing vom Vorhandensein von Septinen, dem Zytokinese Portein Hof1, sowie dem
Arp2/3 Komplex ab. Im Gegensatz zum Aktomoyisinring benötigte dieser Aktinring für seine Formation weder Myo1 noch Formine. Aus diesen Ergebnissen schlossen wir, dass Hof1 als Bindeglied zwischen Aktin und Septinen agieren könnte. Desweiteren endeknten wir in der Abwesenheit von Aktin Überexpression einen Hof1-abhängigen Aktingürtel am Knospenhals in Zellen, die die Anaphase durchliefen.

Ein weiteres interessantes Detail des Aktinringes war, dass er immer in enger Assoziation mit dem Zellkern auftrat. Wir fanden heraus, dass einige Importine und Exportine bei der Entstehung des Rings eine Rolle spielen was auf eine räumliche Koordination zwischen der Zytokinesemaschinerie und dem Zellkern hinweist. Wir konnten allerdings nicht zeigen, dass ein Durchschreiten des Zellkerns durch den Knospenhals für den Start der Zytokinese notwendig ist.

Ein anderer Ansatz, den wir verfolgten war herauszufinden, ob und wie Komponenten der mitotischen Spindel den Beginn und das Voranschreiten der Zytokinese beeinflussen. Zellen, die Defekte im Bereich der mittleren Spindel aufwiesen, wie zum Beispiel Zellen in denen das Zentralspindelprotein Ase1 mutiert war, Zellen, die eine Mutation im Kinetochore- und Spindelprotein Ndc10 haben, wie auch Zellen, in denen die gesamte Spindel mit Nokodazol zerstört ist und denen zusätzlich das Checkpoint Protein Mad2 fehlt, entwickeln einen Zytokinesedefekt obwohl sie einen neuen Zellzyklus starten.

Wir stellten fest, dass es sich bei diesem Zytokinesedefekt um einen Defekt in der finalen Abschnürung der Plasmamembran handelt. Die Kontraktion des Aktomyosinringes findet mit der gleichen Kinetik wie in Wildtypzellen statt, doch die Plasmamembran wird nicht geteilt.

Wir untersuchten als nächstes den Signalweg, der verantwortlich für den Abschnürungsdefekt in Zellen mit Fehlern in der zentralen Spindel ist. Wir konnten zeigen, dass dieser Defekt von der Aurora-Kinase Ipl1 sowie den Annillin verwandten Proteinen Boi1 und Boi2 abhängt. Boi1 und Boi2 werden Ipl1 abhängig an den Knospenhals und damit an die Stelle der Zellteilung geliefert, wo sie als Abschnürungsinhibitoren agieren. Ausserdem spielen noch die Polokinase Cdc5 und die Separase Esp1 eine Rolle in der Inhibition der Plasmamembrantrennung.

Werde Boi1 und Boi2 gleichzeitig inaktiviert, so führt dies zum Bruch von Chromosomen, die sich in der Nähe der Zellteilungsebene aufhalten. Zellen, die weder Boi1 noch Boi2 enthalten können ausserdem nicht mit Zellen gekreuzt werden, die Spindeldefekte aufweisen. Die Tripelmutanten sind nicht lebensfähig. Der jetzige Stand unserer Untersuchungen deutet darauf hin, dass die von uns gefundene Signalkaskade Spindelelongation und Chromosomenaufteilung
überwacht. Dadurch wird sichergestellt, dass die Zytokinese nur stattfindet, wenn alles genetische Material von der Zellteilungsebene wegbewegt wurde.
Introduction

Coordination of late cell cycle events
To ensure their survival and to create multicellular organisms, cells multiply by dividing. Cell division is a very complex process that involves every single substructure of the cell. Subcellular structures like DNA and centrosomes must be duplicated/replicated successfully before the cell divides to create two daughter cells out of one mother cell. Other structures like the ER and the Golgi must be transiently disorganized or have to be moved to a certain place at a certain time. All these events are precisely monitored and coordinated in space and time.

The process of cell division starts with DNA replication, which is followed by chromosome segregation and ends in two independent cells. Most of the mechanisms underlying these steps are fairly well understood and will be explained in detail below. The core component of cell division in almost all organisms is an actomyosin ring. The exception is the plant kingdom that developed a different although related mode of cell division.

The actomyosin-ring constricts the plasma membrane in the plane of cleavage and thereby brings the membranes into contact. Cleavage can then follow through abscission. The molecular details of actomyosin ring constriction have been subject of many studies during the last decade, while molecular details and surveillance mechanisms of abscission are less well understood.

The temporal controls through which cells ensure that they segregate chromosomes correctly before they exit mitosis and cytokinesis are intensely studied in yeast as well as in higher eukaryotes. The discovery of cell cycle checkpoints, which halt the cell cycle if a certain cell cycle event like DNA duplication or microtubule kinetochore attachment is not completed, deepened our understanding of cell cycle events. Taking into account the checkpoint concept, helped to understand how cells developed such a precise sequence of events to let cytokinesis happen with the right timing. It is not known, if further control mechanisms exist after a cell exited mitosis but before the actual event of cell cleavage.

Cytokinesis and spindle function has to be tightly coordinated with each other at a spatial and temporal level because the genetic material has to be fully segregated between the mother and the daughter cell before cytokinesis takes place. Otherwise genetic material might be distributed incorrectly or persist in the vicinity of the cleavage plane, which could lead to DNA
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damage if cytokinesis happens prematurely. In animal cells it is the mitotic spindle that induces cleavage plane formation. In yeast cells the mitotic spindle is not involved in cleavage plane formation per se but most probably still plays a role in cytokinesis.

In my thesis, I asked if the nucleus and or the spindle play a direct role in cytokinesis onset. A communication between the nucleus and the cleavage machinery via proteins that shuttle between these two areas could ensure that cytokinesis only takes place when chromosome segregation is complete and occurred in the right spatial and temporal manner. This would ensure that both cells always receive a complete set of the genome that is pulled away from the vicinity of the cytokinetic machinery.

I used Saccharomyces cerevisiae as a model organism for my studies. For the temporal control of cytokinesis it was already shown that S.cerevisiae shares a lot of similarities, especially in the cell cycle checkpoint system, with S.pombe and higher eukaryotes.

General principles in cytokinesis

At first glance, different cell types use very different mechanisms to undergo cytokinesis. However, the molecular components involved in cytokinesis are often conserved. This suggests that the molecular mechanisms that underlie cell cleavage are fairly similar among different sets of cells. Here I will review our knowledge on cytokinesis in different organisms answering the questions:
A) What are the common themes? B) In which situations do cells vary them? C) What evolutionary reasons could account for that?

Cytokinesis is the process through which two newly formed daughter cells become physically separated. Thereby, it is the final step of the cell cycle and the actual accomplishment of cell division.

Plants achieve cytokinesis by a microtubule driven process, which sends Golgi derived vesicles to the equatorial plane of the spindle. There, the vesicles fuse and form a disk called the phragmoplast. This disk grows outward and thereby forms a physical barrier that separates the cytoplasms of the two newly formed daughter cells (Fig 1-1 lower panel).
Thus, these organisms are different from fungi and the animal kingdom as they do not use an actomyosin ring and complete the physical separation of two cells by septum formation.
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In contrast, fungi and animal cells accomplish cytokinesis by the constriction of an actomyosin ring. Assembly and localization of this actomyosin ring must be actively coordinated with the position of the spindle (see figure 1-1 upper 3 panels). In animal cells the central spindle has the capacity to induce cleavage furrow formation when it is positioned in the vicinity of the cell cortex [5]. In the fungi fission yeast the division plane and thereby also the cleavage furrow is specified by the pre-dividing nucleus, which is centred by astral microtubules during interphase.

In the fungi budding yeast the site of actomyosin ring formation is predetermined because this organism features a bud neck, which is the predestined site of cell division. The spindle has to align along the mother bud axis to ensure that cleavage takes place between the two sets of genetic material. Whether the spindle itself plays a direct role in controlling the onset of actomyosin ring contraction or cell separation is not known (see figure 1-1 first panel).

In all organisms spindle positioning and actomyosin ring formation is followed by contraction of the ring.

After actomyosin ring constriction, cells are left with a narrow cytoplasmic bridge that still connects the two daughter cells. In higher eukaryotes, the midbody, which consists of bundled microtubules that are embedded in a proteinaceous matrix, lies within this cytoplasmic bridge. So far no midbody-like structure has been found in yeast, but also in yeast a narrow cytoplasmic bridge connects cells after actomyosin ring contraction. The molecular details of this bridge are unknown.

The final cleavage of the midbody, or the midbody-like structure in yeast, is called abscission. In animal cells, cytokinesis is finalized by the cleavage of the midbody. In yeast, abscission leads to the definite separation of the cytoplasms and plasma membranes of the two daughter cells. However, these cells remain attached to each other by the presence of a common cell wall called the septum. This septum consists of three layers: A) the primary septum lies in the middle and is composed of chitin. B) Secondary septa are localized to each side of the primary septum and have the same composition as the rest of the cell wall. As opposed to animal cells, yeast cells need to digest the chitin septum between the two separated plasma membranes before the cells become completely independent of each other (see figure 1-1 upper two panels).
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Figure 1-1
Comparison of general mechanisms of cytokinesis in different eukaryotic organisms. In budding yeast, the division plane is specified in G1. Afterwards, during mitosis, the actomyosin ring is formed within the septin scaffold. Upon telophase this ring contracts. Concomitantly, a cell wall is synthesized to separate the two cells. Fission yeast, similar to mammalian cells, establishes the cleavage furrow during mitosis. The position of the nucleus induces the position of the furrow. Like in budding yeast, septum formation coincides with actomyosin ring constriction. In animals, the spindle microtubules and the midbody determine the cleavage plane. The furrow constricts followed by abscission. In higher plants, microtubules guide vesicles to the equatorial region. These vesicles form the phragmoplast, which will finally contact the cell cortex and form the cell plate to separate the two daughters. Taken from thesis Jeroen Dobbelaeere ETH 2004.

In fungi and animal cells the understanding of the exact mechanisms that coordinate the position of the cleavage apparatus relative to the spindle are not fully understood. We know even less about the mechanisms ensuring that the different steps of the cytokinetic process are temporally coordinated with chromosome segregation. A possible regulation could occur at multiple levels: A) Actomyosin ring assembly, B) Onset of contraction or C) Abscission. Before to asking how these processes are regulated I will describe them in more detail in the following paragraphs.

Actomyosin ring assembly and constriction
Actomyosin rings have been shown to play an important role in cytokinesis in almost all organisms. This ring at the division plane is a very complex structure, which consists of more than 50 different molecules. In all organisms that feature an actomyosin ring, non-muscle
myosin II is the central force-generating machine in cytokinesis. This motor protein consists of a parallel dimer of heavy chains, each bound to an essential light chain and a regulatory light chain. These hexamers assemble into filaments that translocate actin filaments and drive constriction of the contractile ring.

Although the bud neck in yeast is very narrow (1µm in diameter) this organism features an actomyosin ring that acts in cytokinesis. It constricts upon exit of mitosis in a Myo1 (the myosin II of budding yeast) dependent manner. The actomyosin ring is not essential in budding yeast a situation that is otherwise only found in Dictyostelium.

In budding yeast, formation of the actomyosin ring starts already in late G1. At G1/S transition Myo1 assembles at the developing bud site. Interestingly, this recruitment of Myo1 to the bud site is septin dependent but actin independent. At the same time the myosin light chain Mlc2 reaches this site (Fig. 1-2 A). Mlc2 is a regulatory chain for Myo1 but is not essential for actomyosin ring formation. It only plays a role in Myo1 ring disassembly during and/or at the end of actomyosin ring contraction [6].

A second stage of assembly starts by the recruitment of actin filaments to the Myo1 ring in late anaphase (see figure 1-2 B). This recruitment is regulated by the IQGAP homologue Iqg1. Iqg1 is a multidomain protein with GTPase binding domain and a calponin-homology domain at its amino terminus that binds to F-actin in vitro and is required for actin ring formation [6].

This second step of recruitment also gets actin to the ring and thereby completes actomyosin ring formation. The actomyosin ring is now ready to constrict.

In fission yeast S.pombe, actomyosin ring formation starts in G2. The protein Mid1 plays a critical role in actomyosin ring assembly in S.pombe [7]. Mid1, which is weakly related to the metazoan protein anillin, is largely localized in the nuclei of interphase cells. It then assembles into a cortical band overlying the nucleus as the cells approach mitosis. Upon entry into
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mitosis, all Mid1 relocates to the cortex. It localizes to the cortex where it physically interacts with Myo2. This promotes the medial accumulation of actomyosin ring components in a band-like organization overlying the Mid1p band. When its assembly is completed the actomyosin ring constricts. Constriction and the following septum deposition require the activation of the Septation Initiation Network (see below). Taken together, Mid1 is responsible for cleavage site specification. In addition to Mid1, the nucleus itself is responsible for induction of the cleavage plane in \( S.pombe \) [7]. However, opposing the settings in higher eukaryotes the mitotic spindle and astral microtubules appear to play a minor role in actomyosin ring placement in fission yeast. It is more cytoplasmic microtubules that are important for medial positioning of the interphase nucleus, which in turn is important for actomyosin ring placement. To ensure that the division plane remains fixed, a post-anaphase array of microtubules forms underneath the actomyosin ring and retains the actomyosin ring at the initial site of assembly [8]. In higher eukaryotes, cleavage apparatus assembly starts at anaphase onset. What exactly induces furrow formation depends on the cell type. Both, astral microtubules and the central spindle (see below) have the potential to direct the position of the cleavage plane. Formation of a functional cleavage furrow also requires the GTPase RhoA. RhoA activation is an early event in furrow formation and is triggered by the exchange factor pebble in flies (ECT2 in mammals LET-21 in \( C.elegans \)). Pebble activity is required for furrow ingression and might be regulated by its BRCT domains [9]. In higher eukaryotes, upon completion of actomyosin ring formation, the ring contracts during anaphase. This contraction leads to the formation of a midbody that needs to be cleaved at a later step of cytokinesis.

Septins and their role in cytokinesis

In all model organisms in which septins have been found they localize to the site of cleavage. However, their role in cytokinesis is just starting to be understood. Septins are conserved GTPases and are found in all organisms except plants. They assemble into filamentous structures tightly associated with membranes. In budding yeast, where they were first discovered, septin filaments form a ring at the bud neck [10]. During vegetative growth five septins are expressed in \( S.cerevisiae \): Cdc3, Cdc10, Cdc11, Cdc12 and Shs1 [11]. Two other septins, Spr3 and Spr28, act specifically during sporulation and localize beneath the developing prospore wall.

The localization pattern of septins in vegetative growing cells is well understood and FRAP analysis studies unraveled that they switch between dynamic and frozen states during the cell cycle of both budding and fission yeast [12, 13] budding yeast, septins are mobile within the
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ring at bud emergence and telophase and are immobile during S, G2, and M phases and during cytokinesis. In G1 septins are observed as a ring underlying the plasma membrane around the site of future bud emergence. During bud growth the septin ring changes its shape and forms an hourglass-shaped collar at the bud neck. Concomitant with the exit of anaphase the septin ring splits into two equal rings by depolymerization of septin filaments in the middle [14]. Septin ring organization and splitting is similar in all fungi.

In mammalian cells, septins localize more broadly to the cleavage furrow [15]. During cytokinesis they are observed associated with the midbody [16]. In some cells however, septins are seen as a double ring enclosing the cleavage site as shown for Sept2 and Sept9 in NRK cells [17].

In budding yeast, both mother and daughter cells inherit one septin ring upon separation. These “old” septin rings serve as a spatial landmark to direct bud site selection in the next cycle. Although the septin ring is required for actomyosin ring assembly at the bud neck (septin ring assembly is actin independent in yeast) [18], no direct interaction between contractile ring components and the septins has been demonstrated. Septins do not contract with the actomyosin ring but rather mark the cleavage furrow and are important for the recruitment and/or maintenance of cytokinetic components to/at the site of cleavage.

It was shown that all bud-neck components investigated so far depend on a functional septin ring for their localization [18]. However, a direct interaction with septins was only shown for a minority of bud-neck components. Thus, the mechanisms of septin function remained obscure until recently. Progress came from the observation that septins act as a diffusion barrier to maintain membrane-associated proteins in the bud during bud growth (G2 and M phase) [19, 20] This barrier helps to maintain polarity proteins in the daughter by preventing their lateral diffusion along the plasma membrane at the bud neck. Examples for septin barrier-dependent proteins include Sec3 (a component of the exocyst), Spa2 (a component of the polarizome), Ist2 (an integral protein) and Lte1 (a regulator of the MEN), which exclusively localize to the daughter cell [19-21]. These proteins are either integral-membrane or membrane-associated proteins. Upon disassembly of the septin ring, they start to leak through the bud neck by lateral diffusion and loose their polarized distribution. The diffusion barrier model helped to understand septin function in G2 and M-phase and lead to new ideas for how septins might function during cytokinesis. Recent studies established that septins also serve as barriers to isolate the bud neck as a separate cortical compartment during cytokinesis. Upon septin ring splitting each of the two rings forms a diffusion barrier on either side of the bud neck. This allows cortical proteins to diffuse within this compartment without dispersing throughout
mother and daughter cells [22]. These diffusion barriers around the actomyosin ring are required for proper actomyosin ring contraction and essential for abscission.

Another indication that septins specifically regulate abscission comes from earlier studies. Cells depleted for Rts1, a PP2A phosphatase subunit are defective for abscission. In these cells actomyosin ring contraction takes place but the cells fail to separate their membranes. In these cells, septin localization is aberrant and septin rings do not disassemble, not even after a new bud emerges [12]. This means that abscission defects are accompanied by septin disassembly defects, which argues that abscission is a septin dependent process. If the role of the septins in abscission is conserved is not yet known. What is known however is that in mammalian cells septins are required late in cytokinesis. It was also proposed that septins facilitate the formation and stabilization of the intercellular cytoplasmic bridge and the midbody (reviewed in [23]. Because multiple groups could see various septins localizing to the cleavage furrow after actomyosin ring contraction [24-26], it was suggested that septins in mammals are required after cleavage furrow ingression to stabilize a connection between the two daughter cells.

**Molecular events during abscission and proteins involved in this mechanism**

As mentioned, abscission is the final and irreversible step in cytokinesis. Mistakes in chromosome segregation cannot be reversed afterwards. Thus, this step is a good candidate for being a target of checkpoints that would prevent division as long as not all conditions necessary are fulfilled. Surprisingly though, not much is known about abscission and its regulation. Some progress has been made the recent years in the understanding of the events of abscission and their order but not much was done to investigate whether abscission is the target of specific control mechanisms.

Most studies on abscission have been carried out in cultures cells. Three steps are important for the scission of two cells: stabilization of the midbody that spans between the two cytoplasms, disassembly of the contractile ring and membrane remodeling [23].

**Midbody components:**

The mammalian midbody consists of a compact dense matrix of proteins embedded in the region of microtubule overlap. The microtubules derive from the spindle midzone. A fruitful study shed light into the molecular composition of the midbody in CHO (Chinese hamster ovarian) cells [27]. Homologues of midbody proteins that were isolated from CHO cells were analyzed using RNAi in *C.elegans*. Interestingly most RNAi experiments with assumptive
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midbody components lead to a failure in some step of cytokinesis. This study revealed that in higher eukaryotes, ribosomes and mitochondria, secretory proteins, actin- and microtubule-associated proteins, proteins that are known to play a role in lipid raft formation, cell cycle regulator proteins (Cdc2 and mitotic exit proteins for example components of the APC) and ubiquitin ligase components are associated with the midbody. A hypothesis that derived from this study was that the midbody possesses functions that are analogous to the phragmoblast in plants. Like the phragmoblast also the midbody contains Golgi associated components that are required for cytokinesis. Furthermore, recent work in Drosophila and C.elegans has shown that BFA, a drug that affects vesicle formation from the Golgi, has an effect on cytokinesis [28, 29]. These results might argue that cytokinesis in animals and plants that have always been described as different processes are more similar than anticipated.

Abscission:

Already 20 years ago, Sanger et al. discovered in animal cells that even after the full ingression of the cleavage furrow the two daughter cells still remain physically connected, sometimes for hours [30]. This was the first study that showed that cytokinesis is a multistep process that is not complete after actomyosin ring contraction.

In fact, the completion of cytokinesis, called abscission, is an active process. Proteins required for completion of cytokinesis are involved in cytoskeletal remodeling, vesicular trafficking and membrane fusion (reviewed in [23]). Stabilization of the intracellular bridge that links the two daughter cells involves the kinesin related proteins of the MKLP1 family, RB6K and CHO1 in addition to septins (see above) [31-34]. RB6K (Rab6 Kinesin) localizes to the midzone in late anaphase and to the midbody in cytokinesis. Depletion of RB6K results in the formation of multinucleated cells, which results from the regression of the cleavage furrow. This RB6K localization to the midzone and/or the midbody is crucial for cytokinesis. CHO1 also localizes to the midbody after furrow ingression. Downregulation and mutations in the ATPase domain of this protein prevents cytokinetic completion. CHO1 is assumed to act in proper midbody formation. Thus, a stabilized midbody is essential for the cytokinetic completion and the loss of stabilizing proteins leads to cytokinetic defects.

Disassembly of the contractile ring involves the inactivation of the small GTPase Rho, which mediates actin polymerisation. Rho first accumulates at the cleavage furrow during mitosis
where it is essential for the assembly of the contractile ring. Down-regulation of Rho activity at the midbody is crucial for contractile ring disassembly upon cleavage and furrow ingression. Two proteins responsible for this downregulation might be MgcRacGAP (Cyk4 in *C. elegans*) and Nir2. MgcRacGAP is a Rho-family GTPase-activating protein (GAP) and localizes to the spindle midzone and midbody during telophase and cytokinesis. If Hela cells express a version of MgRac in which the Rho-GAP activity is inhibited these cells display a defect in late cytokinesis seen by a significant delay in completion of cytokinesis or even furrow regression [35].

Nir2 carries a Rho inhibitory domain (Rid) and is bound preferentially to Rho-GDP whereby it inhibits Rho-mediated stress fiber formation. Nir2 also localizes to the cleavage furrow and midbody during cytokinesis together with RhoA. Downregulation of Nir2 by antibody microinjection results in binucleated cells indicating that it plays a role in cytokinesis. Furthermore if cells are transfected with a Nir2 that lacks its Rho inhibitory domain cells exhibit late cytokinetic defects. For example, furrow regression takes place up to several hours after furrow ingression also leading to binucleated cells [36].

During abscission the most important events most likely affect the plasma membrane (reviewed in [23]. Supporting this view, SNARE proteins such as syntaxin are absolutely required for successful abscission. Thus, membrane fusion events are essential for cytokinesis. It is unclear though, which of these events involve vesicle to plasma membrane traffic and which might involve phenotypic fusion events between the two ingressing sides of the plasma membrane. Dynamin and the ARF6 GTPase were found to play a role in late cytokinetic events [23] suggesting that endocytosis is also involved in abscission. This suggests that proteins and lipids that are present in the plasma membrane of the cleavage furrow might act as abscission inhibitors and need to be removed.

An interesting finding in recent years was the fact that centrosomes might also play a role in abscission [37]. Centriolin for example is a centrosome component and is required for successful cleavage. This protein shares homology domains with Cdc11 in *S. pombe* and Nud1 in *S. cerevisiae* [38]. Both, Cdc11 and Nud1 are spindle pole body components and contribute to the regulation of cytokinesis in budding and fission yeast.

In animal cells, centriolin localizes specifically to the mother centriole. This centriole has been reported to move towards the midbody at the end of mitosis. Depletion of Centriolin by RNAi results in an increase of cells that are stuck in late mitotic stages often connected only by intracellular bridges which argues for a role of this protein in abscission [38].
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Not much is known so far about abscission in budding yeast. \textit{rts1}\textDelta is the only mutant strain in which an abscission defect has been reported. This study unraveled the role of the septins in abscission [12].

A subsequent study showed an involvement of the exocyst complex in the completion of cytokinesis. Genetic analysis revealed that some alleles of the exocyst component Sec3 are synthetic lethal with both \textit{myo1}\textDelta and \textit{hof1}\textDelta cells. Furthermore, 50\% of the Sec3 mutant cells shifted to the restrictive temperature during cytokinesis failed to complete abscission but not actomyosin ring contraction. This suggests that Sec3 acts in parallel to Myo1 and Hof1, maybe in the Cyk3/septin pathway of cytokinesis.

Cytokinesis in budding yeast can be achieved in different ways: Roles of Myo1, Hof1 and Cyk3

As indicated above, actomyosin ring contraction is not the only possible way to undergo cytokinesis (which is best demonstrated in plant cells that do not contain such a structure).

Also in budding yeast cytokinesis does not exclusively depend on type II myosin (called Myo1 in this organism) and thereby on actomyosin ring contraction. In multiple budding yeast strain backgrounds the \textit{myo1}\textDelta mutation is viable \cite{39, 40} at 24°C although the deletion becomes lethal at 37°C.

The strains lacking Myo1 show a slow growth rate but undergo cytokinesis successfully \cite{40, 41}. This demonstrates that other pathways must exist that ensure cytokinesis in cells that do not contain Myo1. Hof1/Cyk2 is a protein that plays a role in such a parallel pathway. Hof1 is homologues to cdc15 in \textit{S.pombe}. Both proteins contain a potential coiled-coil region and an SH3 domain at the COOH-terminus and localize to the actomyosin ring. cdc15 is essential for fission yeast cytokinesis \cite{42}. Cells lacking Hof1 exhibit a phenotype similar to what is observed for \textit{myo1}\textDelta cells. They have broad bud necks and aberrant cytokinesis, which often leads to the formation of cell chains. However, also \textit{hof1}\textDelta cells are viable at 24°C \cite{2, 43}. Indications that Myo1 and Hof1 can compensate for each other come from the fact that the \textit{hof1}\textDelta \textit{myo1}\textDelta double mutant is dead \cite{2}. This means that in cases where Myo1 or a member of this cytokinesis pathway is aberrant the Hof1 pathway compensates for their absence and vice versa. This ensures that cytokinesis can be carried out even in cells in which the actomyosin ring is not functional.

Hof1 localizes to the bud neck during bud growth namely in G1, G2, prophase and metaphase. It becomes integrated into the actomyosin ring during anaphase \cite{43, 44}. Vallen et al. suggest that Hof1 plays a role in septum formation/maturation and does not directly act in the
regulation of actomyosin ring dynamics (Figure 1-3 B). In favor of this model, chitin deposition is delocalized in $hof1\Delta$ mutants and Hof1 strongly co-localizes with chitinase, even before cytokinesis. Furthermore, time-lapse microscopy showed that $hof1\Delta$ cells do contract their actomyosin ring but then are defective for septum deposition and cell separation. Taken together this means that cytokinesis can be achieved via two distinct mechanisms: through actomyosin contraction (Myo1) or through septum formation (Hof1). Either one of these mechanisms is sufficient when cells are growing under optimal conditions. However, the fact that inactivation of either pathway leads to apparent phenotypes indicates that both pathways act together and support each other in normal mitosis (Figure 1-3 A).

A third protein that plays a role in cytokinesis in parallel to Hof1 and Myo1 is the SH3 domain protein Cyk3. The $cyk3\Delta$ cells are viable but their growth is slowed and 10% of them show cytokinetic defects. Cyk3, like Myo1 and Hof1 localizes to the bud neck starting in anaphase until cell separation (Harald Rauter personal communication). Furthermore, $cyk3\Delta \ myo1\Delta$ and $cyk3\Delta \ hof1\Delta$ double mutants are dead [3]. These observations suggest the existence of three cytokinetic pathways. Some speculations suggest that the Cyk3 pathway acts downstream of septins but this hypothesis needs further investigation (Harald Rauter personal communication).
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A) Three parallel pathways that ensure cytokinesis in *S. cerevisiae*. If one pathway is disrupted the other two can compensate this deficit. If two pathways are missing the cell cannot undergo cytokinesis and dies.

B) Adapted from Schmidt et al. 2002:
Schematic view of septation in wild-type (left side) and in myo1 mutants (right side).

WT: The membrane invaginates and chitin is laid down in the invagination. Continuation of this process leads to generation of the primary septum disk and to pinching off of the membrane. Next, secondary septa are built up from both the mother and daughter cell sides. A trilaminar septum results.

myo1∆: invagination of the plasma membrane in a small area and growth of a primary septum do not take place. Instead, inward growth of cell wall material over a large portion of the plasma membrane pushes the membrane toward the center of the channel finally closing the latter and generating a thick and uniform septum. This most probably represents growth of secondary septa, which would occur at 90° to the normal direction. The green spots designate chitin formed by the action of chitin synthase III that is required for the remedial septa but not for the normal secondary septa. A lacuna resulting from the uneven fusion of the advancing secondary septum is also shown.

To unravel which protein pathway plays a role in which step of cytokinesis, and which pathway consists of which proteins will be a major task to understand the process of cytokinesis in molecular detail.

The central spindle and its composition

After the metaphase-anaphase transition, the sister chromatids separate and the chromosomes segregate away from each other. This occurs in two separated steps: during anaphase-A sister chromosomes migrate towards opposing spindle poles; during anaphase-B these spindle poles move away from each other due to spindle elongation. Thus, anaphase-A depends on the shrinkage of kinetochore microtubules, while the elongation of the polar microtubules and their sliding along each other at the spindle midzone mediates anaphase-B.

The molecular details of this process are not known. What is clear though, is that upon anaphase onset the mitotic spindle shows dramatic rearrangements, which are essential for spindle elongation, stability and timely disassembly. The spindle midzone plays a major role in all these events and is therefore crucial for the onset and progression of anaphase.

The spindle midzone, or central spindle, forms during anaphase and mediates the interaction between the two half spindles generated by opposing spindle poles. This is thought to be a
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major site of force generation for spindle elongation besides the pulling forces that are generated by dynein.

In various organisms the spindle midzone organizes antiparallel microtubules into highly ordered geometrical arrays.

Central spindle formation depends on centralspindlin. Centralspindlin is a protein complex that consists of the kinesin MKLP1 (mammals) or ZEN-4 (C.elegans), the GAP MgcRac (mammals) or Cyk-4 (C.elegans) and the coiled coiled protein PRC1. Disruption of any factor disrupts microtubule bundling at the spindle midzone [9, 45].

PRC1 is homologues to budding yeast Ase1. Ase1 (anaphase spindle elongation) resembles a nonmotor microtubule binding protein, which localizes to the spindle midzone. It belongs to a conserved family of midzone specific microtubule-associated proteins (Maps). Ase1 acts as a dimer as shown by velocity centrifugation and size exclusion chromatography. The Ase1 homodimer is thought to crosslink and stabilize intercalating microtubules [46]. Nondegradable forms of Ase1 block spindle disassembly in telophase, while Ase1 overexpression is sufficient for the induction of spindle elongation even in metaphase. Furthermore, spindle microtubules collapse abruptly in anaphase in aselΔ cells. This means that Ase1 is required for the correct elongation of the spindle and therefore for the separation of spindle poles in anaphase-B. The protein is degraded by the APC at the end of mitosis [1]. Thus, Ase1 appears to link the two halves of the anaphase spindle and its degradation seems to be a prerequisite for spindle disassembly.

Homologues of Ase1 are PRC1 in human cells and Feo in Drosophila. Knock down and overexpression phenotypes of PRC1 resemble those of Ase1: Overexpression leads to bundling of interphase microtubules while lack of PRC1 causes a failure in microtubule interdigitation between half spindles. Studies of PRC1 establish that it associates with three midzone motor proteins: Kif4, MKLP1 and CENP-E but not with the chromosomal passenger complex [47, 48]. Feo in Drosophila stands for fascetto, which means small bundle. This name reflects the phenotype of Feo depletion: central spindles do not form as robust hourglass bundles but as thin and compact microtubule arrays. Feo probably interacts with antiparallel microtubules of the midzone and maintains their overlap during elongation and antiparallel sliding [49].

Slk19 is another component of the central spindle in S.cerevisiae. During metaphase, this protein localizes to kinetochores, but a fraction of Slk19 is shifted to the central spindle in late anaphase. Localization of Slk19 to the central spindle is enhanced but not dependent on cleavage by the Separase Esp1. However, cleavage of Slk19 by Esp1 alone is not sufficient to
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promote its function in stabilizing the central spindle as shown by Uhlmann et al using a non-cleavable form of Slk19. Other features of Slk19 must also account for spindle stabilization. In mutants in which Esp1 is replaced by an artificial TEV protease both, Slk19 as well as Ase1 fail to reach the spindle midzone [50]. Absence of Slk19 from the central spindle leads to frequent spindle breakage and prolonged mitosis. Although it is assumed that Slk19 and Ase1 are involved in protecting microtubule plus ends from shrinking and thereby stabilize the central spindle together, Slk19 depletion does not affect Ase1 localization to this site. If Slk19 has any homologues in higher eukaryotes is not known [50, 51]. A role of this protein in the FEAR pathway will be described below.

Another conserved component of the central spindle is the INCENP-AuroraB complex that I will describe in detail below. I should note, that the relocalization of this complex from kinetochores to the central spindle in budding yeast is triggered through dephosphorylation of the INCENP homologue Sli15 by the Cdc14 phosphatase. This event is necessary and sufficient for the localization of this complex to the central spindle. To enable this dephosphorylation Cdc14 has to be freed from the nucleolus by the FEAR network [52]. However, if the FEAR is not active, at least Sli15 can eventually get to the central spindle in a MEN dependent manner [53].

At the central spindle the chromosomal passenger complex plays a role in spindle stability. One report states that Ipl1 plays a role in spindle destabilization and thereby possibly regulates the timing of spindle disassembly [54]. Sli15 on the other hand is thought to play a role in spindle stabilization earlier in the cycle possibly together with Slk19 [52]. However, the exact mechanism of how the chromosomal passenger complex is interacting with the spindle is far from being fully understood.

Also some kinetochore proteins localize to the central spindle in anaphase of budding yeast cells. One example is the kinetochore protein Ndc10, which is a member of the CBF3 complex. Ndc10 associates with the spindle and translocates to the spindle midzone in late anaphase where it is thought to stabilize this structure in anaphase. Indeed, spindles fail to fully elongate and break down prematurely in an Ndc10 mutant [55]. Although Ndc10 is a substrate of Ipl1, this phosphorylation is not necessary for Ndc10 localization to the spindle midzone [55].

Another Ipl1 substrate and kinetochore component that is seen at central spindles is the kinetochore protein Dam1 and also Ndc80 has been shown to localize along the entire spindle during parts of the cell cycle [56].
Several microtubule motor proteins localize to the central spindle of yeast and animal cells. In budding yeast it is thought that antagonistic forces between Stu2 and Kip3, are important for spindle integrity and proper elongation. Stu2 belongs to the Dis1 family of microtubule-associated proteins and is homologues to *Xenopus* XMAP215. Kip3 belongs to the Kin1 superfamily of kinesins [57, 58]. In higher eukaryotes motor proteins like MKLP1, MKLP2, Kif4 and CENP-E play a role in spindle elongation [59]. How these proteins work during spindle elongation is still largely unknown.

The central spindle is thought to play a direct role in cytokinesis. In *Drosophila* for example, mutations in the central spindle complex lead to a failure in contractile ring formation [45]. The central spindle complex in this organism consists of the proteins Pavarotti, RacGAP50c, ZEN-4 and CYK-4. While loss of Pavarotti and RacGAP50c effect already central spindle assembly and no furrow formation takes place, in cells depleted for ZEN-4 or CYK-4 a furrow is formed but it regresses which leads to a cytokinetic failure [45]. According to these results, it is speculated that a cooperative interdependence exists between these midzone components and furrow formation/ingression. In *C.elegans*, actomyosin ring formation does not require the central spindle but astral microtubules induce actomyosin ring formation. The same is true for some human cell lines. As mentioned above, in *C.elegans* cells that lack central spindle components the actomyosin ring contracts almost to the end but then regresses suggesting that abscission rather than furrowing is defective [9]. Cases of furrow regression have also been described in cells with incomplete DNA segregation [60-62]. The exact relevance of these findings was one of the investigations of my thesis.

**The chromosomal passenger complex and its functions**

The so-called chromosomal passenger complex is highly conserved among species and consists of at least four members: AuroraB (Ip11 in budding yeast), INCENP (Sli15 in budding yeast), Survivin (Brl1 in budding yeast) and Borealin. In many studies it has been shown that these are involved in early mitosis processes like monitoring the generation of tension between two sister chromatids. Furthermore in higher eukaryotes they positively regulate cytokinesis.

This protein complex has a complex localization pattern during the cell cycle. In prophase, proteins of this complex accumulate in the nucleus to associate along the length of condensing chromosomes. Subsequently, they accumulate at the inner centromere of prometa- and metaphase chromosomes. At anaphase onset the complex concentrates to the central spindle [63].
AuroraB is a serine threonine kinase and INCENP is its activating and targeting sub-unit. The exact functions of Survivin and Borealin are not yet known. However, work of the Medema lab shows that Survivin is responsible to recruit all the other members of the chromosomal passenger complex to the spindle midzone in higher eukaryotes (René Medema personal communication).

Homologues of INCENP have been identified in all species from human to yeast. The hallmark of these proteins is the so-called IN-box, a 60-80 amino acid long region that binds to AuroraB in mitosis and is subsequently phosphorylated. It was shown that this phosphorylation enhances AuroraB kinase activity. In most species including yeast, the INCENP gene is essential. In higher eukaryotes the inactivation this gene leads to defects in chromosome congression and failure in cytokinesis. In budding yeast Sli15 defective cells clearly segregate chromosomes albeit in an asymmetric manner. Furthermore, in *S.cerevisiae*, DNA replication, SPB duplication, spindle formation and cytokinesis all appear unaffected in Sli15 mutants [51].

AuroraB is conserved from yeast to human. AuroraB substrates comprise histone H3, CENP-A, INCENP, Survivin and MCAK. The list of potential and real substrates is still expanding. AuroraB inactivation leads to severe phenotypes: Loss of kinetochore attachment to microtubules, exit from mitosis without the completion of anaphase, unattached chromosomes and defects in kinetochore assembly [51]. Remarkably, the *S.cerevisiae* Ipl1 mutants do not show any defect in cytokinesis. These cells finish the cell cycle and end up with two daughter cells with aberrant chromosome content [54].

The third member of the chromosomal passenger complex, Survivin (Bir1 in budding yeast) is proposed to be a butterfly shaped dimer with long α helices and a paired BIR motif. Its localization is strictly dependent on INCENP and vice versa. Survivin is an essential gene in most organisms although some yeast backgrounds exist, in which a Bir1 deletion is viable. In Hela cells mutations induce defects in chromosomal alignments, spindle assembly and cytokinesis. If this phenotype is conserved in yeast has not yet been investigated [51].

The current idea of how this complex is assembled is that AuroraB and Survivin both bind to INCENP, AuroraB to the C-terminal region and Survivin to the N-terminal region of the protein (Figure 1-4). This ternary complex is also present in *S.cerevisiae*. The kinase activity of AuroraB is activated by INCENP binding which stimulates AuroraB autophosphorylation. The activation of AuroraB then leads to INCENP phosphorylation, which is crucial for the activation of the whole complex [64].
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One role of the complex is the promotion and stabilization of protein recruitment to kinetochores. In higher eukaryotes, the passenger complex is required for the stable targeting of the checkpoint proteins BUBR1 and MAD2 (see below) to unattached kinetochores. In the presence of microtubules, AuroraB is also required for the recruitment of CENP-E, MCAK and dynein to centromeres.

The assumption that the passenger complex plays a role in chromosome condensation is still under debate. In animal cells, disruption of any passenger protein leads to the accumulation of chromosomes that are unable to congress to the metaphase plate. A possible mechanism for this phenotype was unraveled in budding yeast: neither Sli15 nor Ipl1 mutants can biorient their chromosomes which led to the hypothesis that Aurora kinase activity is required to release kinetochores from improper microtubule attachments [65]. A similar mechanism was found to also apply to Hela cells [66].

Monopolar attachment, which is the attachment both kinetochores to microtubules emanating from the same side, is a common cause of aneuploidy in cells. Experiments in yeast and higher eukaryotes suggested that one role of the chromosomal passenger complex is the correction of these events [65, 67]. It was shown that in this process Dam1 is a key Ipl1 target for the regulating kinetochore-microtubule attachments [68].

The current model is that the activity of the passenger complex responds to spindle tension. This model is supported by the observation that Ipl1 in yeast mediates spindle checkpoint activation (see below) in response to a loss of tension. In Ipl1 mutants the spindle checkpoint cannot be activated [69]. Furthermore, Ipl1 mediates the detachment of microtubules from kinetochores that are not under tension, while it does not interfere with microtubule attachment
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to kinetochores under tension. A physical separation between AuroraB and its substrates by a stretched bi-oriented centromere could be the trigger for the inactivation of the complex to allow progression in the cycle. In support of this theory Ipl1 is inactivated as soon as tension is established between two sister kinetochores [65, 67]. Alternatively, Ipl1 activity might be directly turned off by the response to tension. In either case, inactivation of Ipl1 function in metaphase appears necessary for the cell to progress into anaphase.

The exact role of passenger proteins in cytokinesis is debated. Whilst in higher eukaryotes inactivation of passenger proteins lead to cytokinesis defects, budding yeast cells lacking Ipl1 or Sli15 activity arrest in G1 only in the next cycle after successful cytokinesis. This arrest is thought to be due to the fact that these cells contain aberrant complements of chromosomes.

From genome duplication to cytokinesis

How cytokinetic steps happen in space and time, and which proteins are involved, either as regulators or directly, is more and more understood.

One aspect that is not understood though is whether and how cytokinesis is coordinated with the other events of mitosis and cell physiology. For example, are there specific steps that have to be completed successfully before actomyosin ring contraction and finally abscission can be triggered? What surveillance mechanisms aroused during evolution, which minimize the amount of mistakes that occur in this process? Indeed, many cytokinetic mistakes are fatal for the cell. For example, premature abscission can lead to uneven genetic distribution or cutting of the genetic material. On the other hand delayed cytokinesis can lead to multinucleated cells.

I will briefly review what we have learned over the recent years from model organisms such as yeast but also higher eukaryotes about how the onset of cytokinesis is controlled.

Molecular mechanisms of mitotic entry

The first step for the cell to enter a new cycle that leads to two independent cells is the replication of the genetic material. The initiation of this process is based on two distinct steps: first, prereplicative complexes at the future origin of replication assemble. For this to happen cyclin dependent kinase (CDK) activity must be downregulated. After the assembly of prereplicative complexes re-activation of CDK triggers the unwinding of origins of replication. The recruitment of enzymes responsible for replication is also triggered by CDK activation [70, 71]. This means that chromosome replication can only happen if a period of low CDK activity is followed by a period of high CDK activity. This on its own is a good surveillance
mechanism for the cell to not re-replicate DNA before all genetic material is doubled. Like this only one set of the chromosomes will be pulled into the daughter cell.

After the full replication of the genome, cells keep high CDK activity until metaphase. This mechanism further ensures that no re-replication is initiated.

The replicated sister chromatids are held together before their segregation to the opposite poles of the spindle. A multiprotein complex called cohesin provides sister cohesion. Cohesin forms a ring around sister chromatids, and consists of at least four subunits: Smc1, Smc3, Scc1 and Scc3 [72]. The cohesin ring forms during S-phase and stays to connect sister chromatids until metaphase. At the metaphase to anaphase transition the ring is dissolved allowing the separation of sister chromatids [73].

While sisters are still held together every kinetochore attaches to a kinetochore microtubule. Only when all sister chromatid kinetochores are connected to microtubules of opposing poles (amphitelic attachment) tension is created. Upon the generation of tension at all kinetochores and only then (see below) the cohesin connections of sisters are all simultaneously destroyed [74]. This destruction is achieved by the activation of a site-specific endopeptidase protease: Separase (Esp1 in budding yeast, Cut1 in fission yeast). Separase cleaves Scc1 and thereby opens the cohesin ring [75-78].

The timing of separase activation is due to securin (Pds1 in budding yeast, Cut2 in fission yeast), which keeps separase in an inactive state. Securin is a major target of the Ubiquitin ligase APC/C (anaphase promoting complex) and disappears from the cells as soon as the APC/C is no longer inhibited by the spindle checkpoint, i.e. as soon as all kinetochores are attached properly. Upon destruction of securin separase becomes active, sister chromatids are separated and anaphase can start [71].

**Cdc14 in early anaphase and the FEAR network**

Cdc14 was discovered already in 1974 as a cell cycle arrest gene [79]. So far all known functions of this gene depend on its serin threonin phosphatase activity. The main activity of this phosphatase is to revert Cdk activity by the dephosphorylation of its substrates. Like this the cell can move on in the cell cycle and exit mitosis.

Besides playing a role in the mitotic exit network (MEN see below) Cdc14 has recently been discovered to be the main target of an earlier signaling cascade, the Cdcfourteen early anaphase release network (FEAR) [80].

During most of the cell cycle, its binding partner and inhibitor Cfl1/Net1 traps Cdc14 in the nucleolus. The loss of association with this inhibitor results in release of Cdc14 from the...
nucleolus into the nucleus in the case FEAR activation and into the entire cell in the case of MEN activation. This stepwise release happens during anaphase and at mitotic exit. Later Cdc14 is also needed to dephosphorylate Cdc15, a member of the mitotic exit network (MEN) [81] (see figure 1-5). The factors that play a role in FEAR release are five positive and two negative regulators. Positive regulators are the budding yeast separase, Esp1, the kinetochore/spindle protein Slk19, Spo12 (and its homologue Bns1) and the Polo kinase Cdc5. Negative regulators are the separase inhibitor Pds1 (the yeast securin) and the nucleolar protein Fob1, which binds to Cfi1/Net1 and thus inhibits Cdc14 release [80] (see figure 1-5).

Our current understanding of the FEAR pathway is based on genetic studies [53] and indicates that Esp1 acts upstream of this pathway. The involvement of Esp1 in the FEAR network is independent of its protease activity and its role in cohesin cleavage. Slk19, which is thought to play an important role to target Esp1 to the spindle midzone (and vice versa) [50], is thought to act downstream of or together with Esp1. Slk19 binds directly to and is cleaved by Esp1. However, cleavage is not required for its function. Presumably Spo12 acts in parallel to Esp1. Spo12 is a direct interactor of Fob1, an inhibitor of the FEAR. Cdc5 appears to act on the Slk19/Esp1 branch, although its exact role in this pathway is not yet clear. One prediction is that it promotes the phosphorylation of Cfi1/Net1 as well as of Cdc14 itself (see figure 1-5).

The exact function of the FEAR is not completely clear yet. One model is that the FEAR network acts as a timer or a buffer between chromosome segregation and cytokinesis. One of the phenotypes of FEAR network mutants is that they delay progression through the cell cycle by 20 min. The reason for this delay in exit from mitosis is that Cdc14 released by the FEAR network has the ability to act in a feed-forward amplification loop to activate itself by activating the MEN. This means that the FEAR fires a positive feedback loop to activate the MEN and thereby exit of mitosis. On the other hand the FEAR also triggers anaphase events: Spindle midzone stability, as mentioned above, depends partly on the FEAR members Slk19 and Esp1. A further function of the FEAR therefore seems to be the stabilization of the anaphase spindle as it is also important to target the chromosomal passenger complex to this site [52].

Finally, he FEAR network not only regulates spindle stability, but also forces exerted by cytoplasmic microtubules during anaphase [82]. These forces play an important role in positioning the dividing nucleus so that the mother and daughter cells will each receive their proper half of the duplicated genome.
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No network analogues of the FEAR have yet been found in higher eukaryotes. Also, although the Cdc14 homologue in *S.pombe*, Clp1/Flp1, antagonizes mitotic CDK activity and the activity of Clp1/Flp1 is at least partly regulated by the septation-initiation network a signaling pathway homologous to the MEN (see below), no FEAR network could be found in this organism [83] and Susanne Trautmann, personal communication).

Orthologs of FEAR components have been found in other yeast and higher eukaryotes although none for Slk19 [53]. Although there might not be a parallel network in higher eukaryotes some aspects of the FEAR network seem to be conserved. Among them is the fact that Cdc14 has been implicated in spindle midzone formation in *C.elegans* [84] and chromosome separation in *S.pombe*. For now it is unclear if other FEAR components than Cdc14 are involved in mitotic exit in other organisms than budding yeast.

**Figure 1-5**
The Cdc14-activating networks. Schematic representation of the functional relationship among the members of the MEN and FEAR network. The ultimate function of these networks is to activate Cdc14 by releasing it from its inhibitor Cfi1/Net1. Lines ending with an arrowhead indicate the stimulation of the downstream effector, whereas lines ending with a perpendicular bar indicate the inhibition of the target protein.

*Mitotic exit network in S.cerevisiae and potential homologues in other organisms*

Exit of mitosis occurs after chromosome segregation and involves the disassembly of the mitotic spindle and the decondensation of chromosomes. The inactivation of mitotic dependent kinases (CDKs), which promoted entry into mitosis earlier in the cycle, is the crucial event for mitotic exit. This inactivation is primarily achieved by ubiquitin-dependent degradation of the cyclins through the anaphase promoting complex (APC/C), its specificity factors Cdc20 and Cdk1 and the proteasome. However, at least in budding yeast a pool of active cyclins persist
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until the end of spindle elongation. It is thought that this CDK activity is required for the maintenance of the mitotic spindle [85].

In budding yeast the mitotic exit network (MEN) is responsible for the inactivation of the mitotic CDKs. It acts after the FEAR network (see above) in late anaphase and telophase and resembles a Ras-like signaling cascade. A small GTPase and two downstream kinases form this signaling cascade.

Mitotic exit starts when cyclins are degraded. This degradation is triggered by the phosphatase Cdc14. The MEN is responsible for the release of Cdc14 into the cytoplasm at the end of anaphase. Dbf2 most probably achieves this release through phosphorylation of Net1. Dbf2 is the most downstream kinase in the MEN cascade.

The MEN consists of the following proteins (see figure 1-6): the GTPase Tem1, the protein kinases Cdc15, Dbf2 and Cdc5, the phosphatase Cdc14, the Dbf2 associated factor Mob1, the two component GAP Bub2-Bfa1, the GEF Lte1 and the scaffold protein Nud1. Interestingly, Bub2-Bfa1 and Lte1 are non-essential proteins that are not required to activate the MEN in an unperturbed cycle. Genetic dissection indicated that Tem1 is the most upstream component of the MEN pathway and that its activity is regulated by its GAP and its putative GEF, Lte1 (reviewed in [86]).

The MEN ensures is that mitotic exit only happens after that the spindle elongated between the mother and the daughter compartment pulling half of the genetic material into the daughter cell while the other half stays in the mother. This is achieved by the MEN because the GEF for Tem1, Lte1 is localizes specifically to the bud cortex, while Tem1 localizes to the cytoplasmic face of the spindle pole body (SPB) that migrates into the bud. This means that Tem1 and Lte1 get in contact with each other only when one spindle pole migrates into the bud. This setup enables the cell to guarantee that Tem1 activation takes place only when the spindle is properly aligned between mother and daughter cell.

The other MEN components control and propagate the MEN signal. Bub2 and Bfa1 localize to SPBs similarly to Tem1 and it is thought that pre-telophase localization of Tem1 to the SPB depends on these two proteins (reviewed in [86]. Nud1 serves as a scaffold for the Bub2-Bfa1-Tem1 complex at the SPB. After Tem1 activation by Lte1, Nud1 is thought to signal to the protein kinase Cdc15, which then activates the protein kinase complex Dbf2/Mob1.

The Cdc5 polo kinase in the MEN pathway acts in more than one step to regulate the MEN and thereby exit of mitosis. Cdc5 contributes positively to the MEN in two ways: First by negatively regulating the Tem1 inhibitor complex Bub2-Bfa1 [87] and by secondly by
stimulating Cdc15 kinase activity via the FEAR network [80]. In addition to the role of the MEN in mitotic exit components of the MEN pathway directly act in the onset of cytokinesis [88]. As mentioned above, silencing of the human protein centriolin, which shares some homology with Nud1 leads to cytokinetic failure [38]. This cytokinesis defect is in abscission, since cells arrest after actomyosin ring contraction with a cytoplasmic bridge. It would be interesting to investigate if Nud1 also plays a role in abscission in yeast. However, this will be hard to study, as Nud1 mutant cells like nud1-44 and nud1-52 arrest large budded cells unable to complete mitosis [89].

The Septation Initiation Network (SIN), a structurally very similar network to the MEN, was discovered in *S.pombe*, (reviewed in [86]). As its name suggests, this network is more important for septum formation, i.e. for cytokinesis, than for exit of mitosis.

Most of the components involved in the SIN share similarities to components of the MEN (see figure 1-6): The SIN pathway features a GTPase, spg1, at the top of the cascade. spg1 activates three protein kinases, which localize to the SPB and the cytokinetic ring. These are cdc7, sid1 and sid2, which carry the “contract the ring and make a septum” message to the cytokinesis area [86]. These kinases depend on the associated factors cdc14 and mob1. *S.pombe* cdc14 should not be mixed up with Cdc14 in *S.cerevisiae*, as it is not a phosphatase but the co-factor of the kinase sid1. Overexpression experiments showed that the polo kinase plo1, which is also essential for septation, is a positive regulator of the SIN as Cdc5 is for the MEN [90].

The main difference between MEN and SIN is that the MEN triggers mitotic CDK inactivation while the SIN does not but regulates the formation of the division septum after mitotic CDK inactivation.

In higher eukaryotes a real homologous pathway to the MEN/SIN has not yet been found. The GTPases Tem1 and spg1 are most similar to Rab GTPases but to date no such GTPase has been found to play a role in mitosis and/or cytokinesis. However, quite some members of the MEN/SIN pathway are also found in higher eukaryotes. These are for example the homologues to Mob1 (MOBKL2A in humans) and its interactor Dbf2 (NDR in humans). NDR is needed to activate human Mob1 and both proteins are suspected to play a role in cytokinesis although the concrete evidence of this is still lacking [91]. Another example is human Cdc14 (hCdc14A), which is related to Cdc14 of budding yeast and was shown to phosphorylate human Cdh1 and thereby might activates the APC and promote exit of mitosis [92].
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Another component of the yeast MEN/SIN pathway in other eukaryotes is polo kinase. In Xenopus egg extracts the polo kinase homologue Plx1 is required for exit of mitosis [93]. Furthermore, various kinds of polo kinase in different organisms have been found to localize to centrosomes and/or the cleavage furrow, which makes it likely that these homologues play a role in mitotic exit and cytokinesis [86].

Cell cycle checkpoints

Mistakes in genetic duplication and cell separation can have catastrophic consequences for the cells. Aneuploidy and polyploidy are major causes for cell death or uncontrolled cell proliferation. To circumvent this, cells contain several cell cycle checkpoints. These monitor key events of the cell cycle and provide time for error correction in cases in which one of these events is perturbed or delayed. However, cell cycle checkpoints do not lead to a final arrest of the cell cycle. If the cell cannot fix the error it will eventually move on in the cycle to not irrevocably arrest and thereby kill itself. Here, I will describe checkpoints that occur during mitosis. It is to note that also earlier in the cycle DNA replication is carefully regulated and checked by the DNA damage and DNA replication checkpoint that halts the cell cycle in case of DNA perturbations.
**I Introduction**

*The septin checkpoint:*

One event, which has to be monitored by the budding yeast cell, is that DNA segregation does not occur before bud emergence. If cells enter a new nuclear cycle without the possibility to segregate half of the genetic material into the bud this would lead to binucleated cells.

One event that could interfere with bud emergence is perturbation of the actin cytoskeleton because polarized actin is responsible for the polarized growth of the bud. It was shown that actin depolarization causes a significant delay in bud formation, which is accompanied by a delayed nuclear cycle. This led to the hypothesis that defects in bud formation are monitored by a “morphogenesis” checkpoint. This checkpoint depends on the kinase Swe1, the homologue of Wee1 in *S.pombe*, which causes inhibition of mitosis promoting CDKs by phosphorylation [94].

This so-called morphology checkpoint would be purely yeast specific, but another group found another regulatory mechanism that depends on Swe1.

Barral et al. showed in 1999 that there is a link between Swe1 degradation and the septin cortex in budding yeast. This link is triggered by the Nim1 related kinase Hsl1. Hsl1 negatively regulates Swe1 and thereby allows entry into mitosis. Barral found that cells that carry a septin mutation show a Swe1 dependent delay in metaphase which delays mitotic entry [95]. The kinases Gin4 and Kcc4 also play a role in this process.

This argues that cells monitor the organization of their septin cytoskeleton before the entry into mitosis. Septins are the core elements for the formation of the cleavage apparatus, which means that the septin-checkpoint ensures that the formation of a cleavage apparatus is triggered before entry into mitosis. If the septin checkpoint is conserved in fission yeast or higher eukaryotes is not clear yet. It was shown that in *S.pombe* the Gin4 related kinase cdr2 acts in a septin independent manner [96]. If the two Gin4 homologues in *C.elegans* aak-2 and Par2.3 play a role in septin organization is not known. Hsl1 and Gin4 are related to MARK kinases in higher eukaryotes. If these proteins are linked to septin function needs to be investigated.

Taken together, the function of the septin checkpoint in budding yeast is the coordination between the cytokinetic machinery and the mitotic spindle.

*Kinetochoore checkpoint/Spindle assembly checkpoint:*

The function of the spindle assembly checkpoint is to prevent sister chromatid separation until all chromosomes achieve bipolar attachment to the mitotic spindle [97]!

Bipolar attachment of sister chromatids to the mitotic spindle is essential to permit the accurate segregation of the genome between daughter cells. Spindle-chromatid attachment is
accomplished by connections between kinetochores and spindle microtubules. A single unattached chromosome is sufficient to delay entry into anaphase [97]. However, what is exactly sensed at the kinetochore level is still unclear. It has been proposed that both, a lack of tension [69, 98], which would normally be produced by the bipolar spindle, and a lack of proper microtubule occupancy at the kinetochore are the signals that each can activate the checkpoint [99, 100]. Complications are caused by the difficulty to distinguish between lack of tension and microtubule occupancy. What is clear though is that merotelic attachment of bi-oriented chromosomes (one or both sister kinetochores have microtubule attachment from both poles) in which chromosomes are under tension is not detected by the checkpoint.

AuroraB phosphorylates and thereby inactivates MCAK in higher eukaryotic cells. One role of MCAK is to correct merotelic attachment. As merotelic attachments persist following the establishment of spindle tension the spatial segregation of MCAK and the chromosomal passenger complex would lead to an MCAK activation and would allow this protein to resolve merotelic attachments that are not sensed by the chromosomal passenger complex [101].

The proteins involved in the spindle assembly checkpoint include Mad1, Mad2, Mad3 (BubR1 in higher eukaryotes), Bub1, Bub3 and Mps1. Mad1 is a coiled-coiled protein that binds to Mad2, a small protein with a HORMA domain. Mad3 is a kinase and binds to Bub3. Bub1 is also a kinase and phosphorylates Mad1 \textit{in vitro}. Bub3 contains a WD40 domain and binds to Mad3 and Bub1 [102].

Upon kinetochore microtubule defects, the spindle assembly checkpoint blocks APC/C activity and prevents separase liberation from securin and thereby cohesin cleavage (see above). The block of APC/C is accomplished by the inhibition of Cdc20, the substrate recognition unit of the APC/C. This inhibition is achieved by direct binding of Mad2 to Cdc20. In cells in which the Cdc20 binding site of Mad2 is mutated, anaphase onset takes place prematurely, even if Mad2 is overexpressed. [103, 104]. In normal cells that experience kinetochore defects, Mad2 moves from a Mad1/Mad2 complex to a Cdc20/Mad2 complex and blocks APC/c function for a number of substrates [102].

Another important feature of Mad2 and its checkpoint function is its ability to bind to unattached kinetochores. Kinetochore localization of Mad2 is not always essential for an active checkpoint in insect spermatocytes [105]. The same was observed in mammalian cells depleted by RNAi for the kinetochore protein Hec1 [106]. It was proposed that a high concentration of Mad2 at unattached kinetochores is required for the initial nucleation of Mad2-containing inhibitors.
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In cases in which the cell got enough time to fix the unattached kinetochore and completed proper kinetochore microtubule attachment the spindle checkpoint has to be silenced to allow the cell to move on in the cycle. Phosphorylation of Mad2 seems to be an important mechanism for checkpoint inactivation. It is not known yet which kinase is involved in this process [107].

An interesting finding by Cimini et al. is that merotelic attachment of chromosomes delays telophase and cytokinesis in MRC-5 and PtK1 cells without affecting the onset of anaphase [108]. Thus unlike other attachment errors, merotelic attachment is not sensed by the spindle checkpoint. However, cells that contained a lagging chromosome near the cleavage plane took longer times to trigger cytokinesis than control cells in which all chromosomes are aligned. That cells still exhibit a cell cycle delay might be due to an additional checkpoint mechanism later in the cycle. Unraveling if this hypothesis is true and which proteins are involved in this potential additional checkpoint/surveillance pathway was one goal of my thesis.

Spindle positioning checkpoint

Budding yeast offers the unique scenario that the site at which cytokinesis takes place, the bud neck, is determined before entry into mitosis. Due to this circumstance it is important for the cell to correctly orient its spindle along the mother-bud axis in order for spindle elongation to correctly segregate the genetic material between mother and daughter cell. According to these considerations, budding yeast cells contain a spindle-positioning checkpoint. When anaphase takes place in the mother cell, the cell delays its exit from mitosis until one SPB and its associated chromosomes enter into the daughter cell [97]. Misorientation of the spindle can be observed at low temperatures and in various mutants for example in cells that lack dynein. The spindle-positioning checkpoint does not monitor chromosome segregation but the entry of one SPB into the bud. Even when the SPB enters only transiently, mitotic exit is triggered [109].

The ultimate target of this control mechanism is the MEN (see above) specifically the activation of the GTPase Tem1 which triggers mitotic exit when it enters the bud together with the daughter bound SPB and is there activated by Lte1. The spindle-positioning checkpoint is presumably not conserved in higher eukaryotes. However, analogies seem to exist since it was shown that in cultured rat epithelial cells misoriented spindles cause a short but significant delay in anaphase spindle elongation.
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Remaining questions and aim of this work

Our understanding of the cell division and its control has evolved a lot in the recent years. Especially studies in model organisms like budding and fission yeast contributed many molecular and mechanistic details to our understanding of these cellular events. If all these pathway and control mechanisms are conserved in higher eukaryotes remains to be shown. However, from what is known today it is clear that a lot of parallels can be drawn.

What is known about spatial and temporal control of cell division? Although we understand quite well what prerequisites a cell must fulfill to enter and exit mitosis and several checkpoint mechanism that are associated with mitotic exit were found, our knowledge is most probably not complete. For example, budding yeast mutants exist that develop a bibudded phenotype without segregating DNA into the daughter cell [110]. It is possible that this phenotype relates to a cytokinetic defect of these cells. This would mean that in these cells the spindle checkpoint failed the cell cycle is not completed in these cells. Cytokinesis does not occur albeit cells start a new round of budding.

What would be the advantage for the cell to have such a monitoring mechanisms so late in the cycle? If such a mechanism exists what is exactly being monitored? These are two of the questions on which I will focus and to which I attempted to find an answer during my PhD. I was also focusing on spatial control of cell separation. In many organisms including fission yeast, the nucleus induces the site of cleavage and furrow formation. The nucleus is transported to the cell center where it does this via varying mechanisms depending on the cell type.

How about budding yeast? Obviously the striking particularity of this model organism is that the bud neck pre-establishes the site of cleavage. Furthermore, the spindle-positioning checkpoint at least partly ensures that no mitotic exit takes place before one set of chromosomes reaches the bud neck. But what about cytokinesis? Would it not make sense for the cell to ensure a communication between the nucleus and cleavage apparatus components to guarantee that cytokinesis only takes place when the nucleus has passed the bud neck and thereby the cleavage plane? Therefore, I started my thesis work by asking if there is such a communication between the nucleus and the cleavage machinery. I found that the position of the spindle does not play an essential role in this process but that there is a tight coordination between the nucleus and the cortex to ensure that the genetic material is well segregated before cytokinesis is allowed to go to completion.
II Results and Discussion

Results and Discussion

Introduction:
In higher eukaryotes anillin is required late in cytokinesis to localize the contractile ring, specifically the activity of myosin. It first localizes within the nucleus to then shuttle to the contractile ring and the cell cortex in *Drosophila* and human cells. Cells in which anillin is defective start to ingress cleavage furrows but regress these furrows later and accumulate binucleated cells [111, 112]. Thereby anillin is supposed to link the position of the nucleus to the site of cleavage. The same situation is found in fission yeast. In this organism the anillin related protein mid1, which also shuttles between the nucleus and the cell cortex, is responsible for coordination between cortical components and the nucleus to properly position the nucleus relative to the cleavage machinery [7]. *S.cerevisiae* features a different system to spatially regulate cell division, which is the predisposed site of the cleavage plane at the bud neck.

An initial question in my thesis was if those systems evolved independently or if these situations reflect different developments of the same underlying machinery. Also in *S.cerevisiae* there is a need for spatial and temporal control of cytokinesis and the question was if the nucleus provides some information in this process. Do the nucleus and the cortex communicate while the nucleus moves through the cell towards and finally through the bud neck? Does the nucleus provide information at the time and place it divides?

When I joined the lab preliminary data suggested that overexpression of actin led to the formation of an actin ring that strongly co-localized with the nucleus. I wanted to investigate if this structure has derived from an *in vivo* cytokinetic structure. And if yes what does the tight association around the nucleus tell us? Which factors are involved in ring formation and maturation? Does this structure mean that there are exchanges of factors between the nucleus and the bud neck to coordinate mitosis and cytokinesis spatially and temporarily? Concluding from our actin overexpression studies we found that the nucleus does play a role in certain aspects of cytokinesis. However, the biological significance of this connection in budding yeast is not clear to us yet. Finally those initial questions led us to investigate a role of the mitotic spindle, being a part of the nuclear machinery, in late cytokinetic events.
II Results and Discussion

Experiments:

1

Actin overexpression is lethal and affects bud formation

To investigate the effect of actin overexpression in yeast, we used a *GAL:ACT1* construct isolated from a cDNA library [113]. This construct, expressing wild-type actin under the control of the strong and regulatable *GAL1-10* promoter, allows actin overexpression in media containing galactose as the sole carbon source. Sequencing of the insert indicated that it contained the full coding sequence of a spliced variant of the wild-type *ACT1* gene [114]. Cells transformed with this plasmid grew normally on glucose but failed to form colonies on galactose (fig 2-1 A). The growth of transformants carrying the corresponding empty vector was not affected under either condition. Thus, our *GAL:ACT1* plasmid reproduced the toxic effect previously reported for actin overexpression [113, 115].

To characterize the expression levels that were achieved by yeast cells containing the *GAL:ACT1* plasmid in addition to the endogenous Act1 gene, were either grown on glucose or induced on galactose for 4 and 8 h. Equal amounts of protein were subjected to SDS-PAGE gel separation, and the relative amount of actin present in the different lysates was determined by immunoblotting. A clear increase in actin levels was obtained over the 8h of induction. However, quantification of the signal indicated that, in our experiment, actin concentration did not increase by more than a factor of 4 (fig2-1 B).

Using interference contrast microscopy, it was determined that cells overexpressing actin arrest as large swollen cells with multiple small bumps on their surface. Up to six bumps were
observed upon longer galactose induction times. Only very few cells (<14%) had formed normal-sized buds. The cells that overexpressed actin were much bigger than the control cells grown on sucrose (fig 2-2 A). A few of these cells contained two nuclei or more (fig2-2 A), which indicated that in these cells the nuclear cycle continued despite the bud formation defect. However, the low level of binucleated cells that was observed indicated that the progression of the nuclear cycle was slowed down relative to the cell volume expansion. This is consistent with the existence of the morphogenetic checkpoint that is thought to delay cell cycle progression in response to defects in bud formation. As described above, this checkpoint depends on the activity of the Swe1 kinase. Swe1 responds to actin perturbation and arrests the cells with a metaphase spindle by phosphorylating and thereby by inhibition of the cyclin-dependent kinase Cdc28.

In the swe1Δ GAL:ACT1 strain grown on galactose, the number of multinucleated cells increased greatly relative to the GAL:ACT1 cells in the same conditions (fig2-2 B). Therefore, cells overexpressing actin do indeed activate the morphogenetic checkpoint. The presence of some binucleated cells in the wild-type population suggests that actin-overexpressing cells adapt to the checkpoint arrest.

Thus, actin overexpression impairs proper bud formation and engages the morphogenetic checkpoint. Upon longer induction times, actin overexpression becomes lethal. Although many proteins can be overexpressed several fold without much effect on cell viability and morphology, especially cytoskeletal proteins like actin and tubulin become toxic upon a slight
increase in their intracellular level. This shows how important cytoskeletal elements are for cell viability and how much their levels are tightly regulated.

**The protrusions seen upon actin overexpression correspond to successive and abortive buds**

To determine which aspect of the budding process is affected by actin overexpression, we investigated whether the protrusions observed at the surface of the cells corresponded to buds. To address this question, we determined whether a bud-specific protein such as Spa2 was enriched in these bumps. Analysis of Spa2-GFP distribution in non-induced cells indicated that it localized to the tip of small buds, the entire surface of isotropically growing buds and to the bud neck of large budded cells as reported for wild-type Spa2 [116] (fig2-3 A: A,B). When cells expressing Spa2-GFP as the sole source of Spa2 were induced to overexpress actin, they formed swollen mothers with bud-like protrusions, just like wild type cells. Spa2-GFP localized to one of the protrusions in a large majority of the cells. In some cases, Spa2 localized diffusely throughout the cell (fig2-3 A:C-H). Only in extremely rare cases, it localized to more than one of the protrusions. These results indicate that the protrusions are similar to buds with regard to Spa2 distribution. That only one such bud contained Spa2 at each time suggested that only one bud was growing at a time.
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A) Localization of Spa2 in cells over-expressing actin. The cells express Spa2-GFP as the sole source of Spa2. Cells grown under non-inducing (A and B) and inducing (C-H) conditions. DIC is shown to visualize the presence of the multiple buds (C, E) (unbudded, small-budded, large-budded, bi-budded, tri-budded) in cells grown in non-inducing conditions.

B) A culture consisting of 95% unbudded cells was resuspended in fresh medium (sucrose) and samples were characterized at regular intervals over 14 hours. Lower panel: The same analysis was made with cells grown under inducing condition (galactose medium).

Two explanations can account for the formation of multiple small buds. Either the cell fails to limit the number of buds that it produces at a specific time point, and these buds emerge simultaneously, or the cell reiterates successive rounds of abortive budding events. To distinguish between these two possibilities, we analyzed the kinetics with which buds were formed. Unbudded, stationary phase cells that contained the $GAL:ACT1$ plasmid were diluted into fresh medium containing either galactose or sucrose as the sole carbon source. Samples were collected every 2 h for 14 h. The cells were then fixed and stained to visualize nuclei and actin. The cells grown in sucrose, in which the $GAL:ACT1$ construct was not induced, started to form small buds more or less synchronously, which developed into large buds. At the 6 h time point, the culture had reached a steady state and contained unbudded (32–44%), small- (16–18%) and large-budded (36–39%) cells. These proportions remained stable over the next 5 h. After 14 h, the proportion of budded cells dropped and unbudded cells re-accumulated, which indicated that the culture had reached saturation (fig2-3 B).
Results and Discussion

Like the cells grown in sucrose, the cells grown in galactose rapidly formed a first small bud. However, the portion of large budded cells failed to increase at the following time points. Instead, the fraction of cells with a small bud kept increasing over the first 8 h. Thus, the cells were able to form a single small bud at the same rate as cells growing on sucrose, but these first buds did not increase. After 8h, cells with more than one bud started to accumulate. The fraction of bibudded cells reached its maximum after 11 h when cells with three small buds appeared. None of the buds reached a normal size, which suggested that they were aborted. Hence, actin-overexpressing cells did not form multiple buds simultaneously but initiated successive abortive budding events. Therefore, actin overexpression does not impair bud emergence but specifically prevents bud growth (fig 2-3 B).

These results indicate that actin overexpression does not interfere with the polarization of actin during bud emergence but, rather, with the maintenance of actin polarity during bud growth. Bud formation can happen undisturbed but then the polarization of actin seems to become aberrant as the buds get aborted and the cell starts a new attempt to develop a bud. This phenotype could be explained with the fact that actin is involved in the directed transport of vesicles and organelles towards growth sites [117, 118]. Exocytic vesicles are required for the delivery of new cell wall material and cell wall-remodelling enzymes. Accordingly, actin polarity defects prevent localized cell wall expansion. Thus, the defect in actin polarity maintenance under actin overexpression is likely to explain the observed abortion of bud growth.

A multibudded phenotype with numerous small buds was already observed in a specific Cdc42 mutant [119]. However, the morphology of the buds observed is very different. Indeed, Cdc42 mutant cells form at least medium-sized buds and often arrest with elongated buds. In our case, very small buds already abort before starting isotropic growth. Still, an aberrant actin cytoskeleton may be responsible for both phenotypes as Cdc42 is a key regulator of polarization of the cytoskeleton to regions of growth.

Actin overexpression leads to the formation of a new ring-like structure in the cytoplasm

We also analyzed the effect of actin overexpression on actin organization. Rhodamine phalloidin staining showed that actin was unpolarized in unbudded cells and polarized towards the small buds of budding cells. However, cells rapidly depolarized actin, most probably concurrent with aborted budding.

In many cells with depolarized actin, a new actin structure formed in the shape of an intense polygonal or ring-like structure in the cytoplasm of the mother cell. This actin ring was
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composed of focal points, which were linked together by bundles of actin filaments and looked like a continuous structure from the en-face view. Inspected from the side, it looked like a straight bar, indicating that this structure was planar (fig2-4 violet star picture). This actin structure was never observed under non-inducing conditions. After 10–12h of actin overexpression, this ring was present in 30–45% of the cells. Careful analysis showed that a small proportion of the cells also contained a smaller, less intensely stained actin ring at the cell cortex throughout the experiment (fig2-4 light blue star picture). In all cases, these small rings localized to the vicinity of the small bud. As these small rings appeared already after 2h of induction and never co-existed with the big actin ring, we speculate that they may be a precursor of the larger actin ring. As the observed structures are strongly labelled with phalloidin, they are composed of F-actin.

![Actin overexpression](image)

**Figure 2-4**

Cells containing the GAL::ACT1 plasmid were fixed at the indicated times with formaldehyde stained with rhodamine phalloidin and actin localization was characterized. The different categories of cells observed upon actin over-expression.

To ensure that the observed actin structure does not result from cell death, we assayed cell viability during the course of the experiment. First, cells were stained after 10h and after 24h with Trypan blue, which is a dye that only dead and dying cells internalize. Second, we tested the ability of the cells to resume colony formation upon plating back on glucose-containing plates. Both tests consistently showed that, even after 10h of actin overexpression, the vast majority of cells remained viable. Internalization of Trypan blue was observed in only 16% of the cells after 10h in galactose medium compared to 42% after 24h (fig2-5 A). In wild type
II Results and Discussion

cells less than 5% of cells internalized the dye. After 10 h on galactose, these cells formed actin rings with the same frequency as in previous experiments. The dilution series shows a comparable survival rate for the cells grown in sucrose and in galactose for 10 h (fig2-5 B). Therefore, the actin structures that we observed upon actin overexpression are not the result of actin accumulation in dying cells. Rather, cell death might be attributed to the failure of the budding cycle.

Figure 2-5
A) To make sure that the observed structure is not only a result of death process of the cells the cells were treated with Trypan Blue to look at survival rate (left panel).
B) Furthermore cells of an OD of approximately 0,1 were plated in a 1:10 dilution series after the indicated overexpression times (right panel).

We therefore think that the structure that we observed corresponds to the exaggeration of a structure already present under physiological conditions.

The Boone lab [120] saw comparable actin structures in viable cells overexpressing the formin Bni1. These structures are nevertheless generally not ring-like and often tilted such that it is unclear whether they have the same origin as the actin ring we observed here.

Septins and Hof1 but not Myo1 influence ring formation

The observation of a potentially early form of the actin ring close to the site of bud emergence as seen above (light blue star) suggested that actin rings might initially assemble at the cell cortex and may depend on cortical proteins that are present at the bud site. To test whether this is the case, we investigated whether the ring still formed in cells lacking specific cortical factors.

The only other actin ring known in yeast is the actomyosin ring at the bud neck. Organization of actin into a ring most probably depends on the presence of a template to guide actin assembly into a closed circle. The septin ring, which does not depend on actin for assembly in yeast, is a good candidate to form such a template. We therefore investigated whether septins were required for ring formation upon actin overexpression.

The septin mutant cdc12-1 cells carrying the GAL:ACT1 construct were induced to express actin at either semi-permissive (30°C) or restrictive (37°C) temperature. Compared with the
wild type, ring formation upon actin overexpression was strongly impaired in septin-defective cells, although rings formed properly at the permissive temperature (22°C) (fig2-6 left graph). Thus, actin ring formation was temperature sensitive in the cdc12-1 strain.

Septin-defective mutants show a strong morphological phenotype characterized by the formation of elongated buds. Therefore, ring formation defects may simply be a secondary consequence of morphological problems. This possibility was excluded by assaying ring formation in the cdc34-2 mutant that also forms elongated buds. These cells showed an increase rather than a reduction in ring frequency (fig2-6 A). Consequently, the effect of the septin mutation on ring formation was not caused by cellular morphology defects. Septins therefore appear to play a specific role in actin ring formation.

Myo1 and Hof1 are two proteins that have been implicated in yeast cytokinesis, and both localize to the bud neck in a septin-dependent manner [2, 43, 121]. We tested whether these two proteins were required for actin ring formation upon actin overexpression. Myo1 was not needed for the formation of the actin ring. In contrast, cells lacking Hof1 only rarely formed a ring (< 8% compared to > 40% in wild type) (fig2-6 B). These rings were always much smaller and fainter than rings observed in wild type (fig2-6 C). Therefore, the Hof1 pathway of cytokinesis seems to play a role in actin ring formation upon actin overexpression.

This finding is insofar interesting, as it is the first time that an actin structure that might have evolved from the cytokinetic machinery is not connected to the motor protein Myo1 but to a member of another cytokinetic pathway, Hof1. The possibility that the ring structure observed
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upon overexpression is involved in cytokinesis could not be tested in the overexpression study. However, formation of the actin ring depends on proper function of two sets of proteins that play a role in cytokinesis, septins and Hof1. They localize to the bud neck upon or shortly after bud emergence [10, 18, 43]. Although septins are involved in non-cytokinetic processes as well [19], only cytokinetic phenotypes have been reported for cells lacking Hof1, which makes it more likely, that this actin ring derived from an *in vivo* cytokinetic structure.

We next wanted to show what impact actin overexpression has on the proteins that are involved in ring formation. For this we used GFP tagged proteins.

**Actin overexpression leads to the mislocalization of septins and Hof1 whereas Myo1 localization is not affected**

We next investigated whether actin overexpression had any effect on the localization of Myo1, Hof1 and/or the septins.

Septins localize to the bud neck already in G1 and stay at this site during the entire budding cycle. The *GAL:ACT1* construct was induced in a strain expressing a tagged version of the Cdc3 septin, 3xmyc-Cdc3 [95], and septin localization was assayed by indirect immunofluorescence. Cells were co-stained with rhodamine–phalloidin to visualize actin. At early time points of actin overexpression, septin rings formed properly, concomitant with actin polarization. Actin polarization and septin ring formation were restricted to the same edge of the cell, and actin and septins co-localized extensively. At later times, when cells started to swell and actin depolarized, septins were dispersed into patches and broken ring-like structures. In cells in which actin was completely depolarized, septin patches were randomly dispersed over the cell cortex (fig2-7 A). The same septin localization was observed using GFP-Cdc12 as a reporter in cells overexpressing actin (fig2-7 B) Hence, actin overexpression led to septin disorganization.

Hof1 is recruited to the bud neck in a septin-dependent manner early in the budding cycle and stays at this site until cytokinesis [2, 43]. In sucrose, *HOF1-GFP GAL:ACT1* cells showed a GFP distribution identical to that reported for Hof1. Before anaphase, as judged by DAPI staining, Hof1-GFP localized to the neck as a double ring. In late anaphase/telophase, these rings merged, and the resulting ring started to contract. On galactose, *HOF1-GFP GAL:ACT1* cells did not impair the swelling phenotype or the formation of bumps. In these cells, Hof1-GFP was observed at the neck of only a very few of the small buds (8%, *n* = 66). Instead, diffuse Hof1 structures were observed in the cytosol (fig2-6 B).
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In contrast to Hof1, Cdc3 and Cdc12, Myo1 localization was not affected by actin overexpression (fig2-6 B). In actin-overexpressing cells, Myo1-GFP only 30% of cells show the protein not properly at the neck, the rest has proper Myo1 localization ($n = 88$) (fig2-7 C). These cells showed the same morphological phenotypes as wild type overexpressing actin and formed actin rings to the same extent as wild type. Thus, actin overexpression does not affect Myo1 localization. These data suggest that, in contrast to its recruitment to the bud neck, Myo1 does not need the septins to remain at this site. This also means that actin-overexpressing cells do not have a general defect in the localization of bud neck proteins. Therefore, the mislocalization of septins and Hof1 upon actin overexpression was specific. These observations were in good agreement with the fact that $hof1^{\Delta}$ and septin mutants have defects in actin ring formation upon actin overexpression, whereas the $myo1^{\Delta}$ cells do not.

![Figure 2-7](image)

**Figure 2-7**

**A**) Effect of actin over-expression on the septin ring. Cells grown under non-inducing (A and B) or inducing conditions (C - J) were fixed after 0 h (A, B), 2 h (E, F) and 8 hours (C, D, G - J) and the septin 3Xmyc-Cdc3 was visualized by indirect immunofluorescence using anti-myc antibodies as primary antibodies (A – E, G, I). Rhodamine-Phalloidin of the same cells is shown (F, H, J).

**B**) Effect of actin over-expression on the localization of bud neck proteins. Cells GFP-tagged for the proteins Cdc12, Hof1 and Myo1 were transformed with the $GAL:ACT1$ plasmid and the GFP tagged proteins were visualized under non-inducing (left) and inducing (right) conditions.

**C**) Quantitative analysis of the mislocalization of the GFP tagged proteins.

Thus, ring formation depends on Hof1 and septins but in turn the same ring interferes with the localisation of the very proteins that permitted its formation. A likely explanation for this is that the ring is connected to these bud neck components. For example, they might serve as templates for actin organization. Upon actin overexpression, grows exuberantly until it disrupts the structures that it was first connected to. Hof1 is removed from the bud neck a dispersed in
the cytoplasm, while septin rings are dislocated. Only septin patches remained near the bud neck. These results also suggest that, in yeast as in animal cells [122], septins are able to recruit and organize actin.

**Deletion of Hof1 suppresses septin mislocalization upon actin overexpression**

We next wanted to investigate whether there was any causal correlation between actin ring formation and septin ring disruption upon actin overexpression. As the formation of the actin ring was Hof1 dependent, we asked whether the septin ring still disorganized upon actin overexpression in *hof1Δ* cells. Cells with the genotype *hof1ΔGAL:ACT1 GFP-CDC12* were induced in galactose for 15 h, and septin distribution was analyzed. In these cells, septins localized properly to the neck of the small buds (fig2-8 A). No additional septin structures were observed. In contrast, disruption of Myo1 did not rescue septin organization in actin-overexpressing cells (fig2-8 A). Since *hof1Δ* cells fail to form an actin ring upon actin overexpression, our data indicate that this actin ring is responsible for septin ring disruption. Furthermore, Hof1 was required for septin ring disorganization upon actin overexpression. These results suggest that Hof1 links actin and septins in our actin overexpression experiments. Interestingly, Hof1 interaction with either septins or actin does not depend on myosin, suggesting that Hof1 may in fact provide a direct physical link between actin and septins. As actin has been shown to be dispensable for the assembly of the septin ring in yeast, we do not believe that the actin–septin interactions that we describe here play any major role in the formation of the septin ring. It has also been shown that septins and Hof1 are not required for bud emergence and the polarization of actin. Thus, this actin–septin interaction is likely not to be required for bud growth and overall actin organization, but rather for cytokinesis only.

We then asked whether it is only the formation of the thick actin ring that is the cause of lethality resulting from actin overexpression. If this was true, *hof1Δ* cells should survive actin overexpression. However, this was not the case (fig2-8 B). The *hof1Δ* cells that overexpress actin accumulated small abortive buds, like the wild type. Thus, the lethality observed upon actin overexpression is not the result of actin ring formation and/or septin ring disruption.
Mini-screen for mutants that have an effect on ring formation

We next studied more systematically which other proteins were required for actin ring formation upon actin overexpression. It is known that the formation of the actomyosin ring at the bud neck during cytokinesis depends on the formins Bnr1 and Bni1 [123]. Remarkably, \textit{bnr1\Delta}, \textit{bni1\Delta} and \textit{bnr1\Delta bni1\Delta 185} (S1023\rightarrow G) single and double mutants all formed actin rings upon actin overexpression comparable to wild type levels. We also tested the roles of other proteins known to localize to the bud neck as we rationalized that a direct effect on ring formation does most likely happen at this place. Spa2 (see above), the bud site selection marker Bud6 and the APC homologue Kar9 which plays a role in nuclear migration [124], also did not have any effect on actin-ring formation upon overexpression. Hence, none of Bnr1, Bni1, Bud6, Spa2 and Kar9 played a role in ring formation.

In contrast, cells that lacked Boi1, Boi2, Rvs167, Rvs161, Bud4, Vrp1 or that carried an \textit{arp2-2} mutation were defective in formation of the actin ring upon actin overexpression. Boi1, Boi2 and, particularly, Bud4 share structural similarities with anillin [122, 125, 126], Rvs161 and Rvs167 are amphiphysin homologues [127, 128], and Vrp1 is a protein functionally and structurally related to WASP that interacts physically with Hof1 [4]. The Arp2/3 complex is known to nucleate and branch actin filaments. Furthermore, it is a target of WASP family proteins [129, 130]. These data (fig2-9) indicate that the actin ring formed upon actin
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overexpression is distinct from the actomyosin ring. Unlike the actomyosin contractile ring, the actin overexpression ring did not require either Myo1 or the formins for assembly, but instead the function of the Arp2/3 complex.

The outcome of this screen underlines, that the actin ring seen upon actin overexpression is independent of the Myo1 pathway of cytokinesis. On the other hand, an interactor of Hof1, Vrp1, is involved in ring formation upon overexpression. Furthermore the screen made clear that the molecular building blocks of the ring seem to be actin patches and not actin cables as only the Arp2/3 complex is involved in the formation but not the formins Bni1 and Bnr1.

Boi1, Boi2 and Bud4 have an effect on ring formation. They are all homologues of anilin, which is known to play a role in cytokinesis in higher eukaryotes. The exact analysis of this interaction between actin ring formation and anilin related homologues still needs to be done.

A Hof1-dependent actin belt forms in late anaphase cells

Altogether, these results suggest that an actin structure distinct from the actomyosin ring may assemble at the bud neck in a Hof1-dependent manner some time before cytokinesis. In other eukaryotes, actin ring assembly and furrowing takes place during anaphase [131]. Therefore, we tested whether yeast cells assemble some actin structure at the bud neck at that time. Our original studies in wild-type cells were complicated by the large number of actin patches and cables in the bud before anaphase. This prevented the visualization of potential bud neck-specific actin structures. To look specifically at actin structures that are assembled in late anaphase we used the cdc15-1 mutation. Cdc15 is an upstream component of the MEN and its mutation impairs cells to exit mitosis. Cells were arrested at the restrictive temperature (37°C)
for 4 h, fixed and stained to visualize actin and nuclei. Remarkably, around 20% of these cells assembled a broad band of actin at the bud neck, which we refer to as the actin belt (fig2-10 A). This structure is morphologically distinct from the actomyosin ring, which forms a tight string around the bud neck of dividing yeast cells [6, 132, 133]. This actin belt was also clearly distinct from the actin structure reported by Bi et al. [40], which forms only after ring contraction. In contrast to this post-cytokinetic structure, the actin belt that we report is less bright and forms only on one side of the bud neck.

The actomyosin ring is thought to form only after mitosis in a Myo1-dependent manner. In contrast, the actin belt observed in the cdc15-I cells forms before mitotic exit and did not require Myo1. However, its formation depended on the presence of Hof1. This means that Hof1 is required for the assembly of an actin belt at the bud neck during anaphase.

This belt satisfies several criteria for being a new actin structure, distinct from the actomyosin ring. First, although the hof1Δ mutation does not interfere with the formation and contraction of the actomyosin ring [2, 22], it affects the formation of the actin belt (fig2-10 B). Secondly, formation of this structure is not affected by the disruption of the type II myosin (fig2-10 A and B). Thirdly, the actin belt is morphologically distinct from the actomyosin ring. However, at this point, we do not know exactly what function this belt plays in wild-type cells. It might assemble an original pool of actin filaments at the bud neck that can subsequently be recruited into the actomyosin ring. Alternatively, the actin belt may play other roles, such as vesicle
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delivery to the site of cleavage, or it may contract independently of Myo1. Also a combination of these functions is possible. In favour of the first interpretation, we never saw the actin belt and the actomyosin ring co-exist in the same cell. Furthermore, the Hof1 protein becomes incorporated into the actomyosin ring upon actomyosin ring contraction [22, 43]. In support to this hypothesis, a similar structure is also observed in Schizosaccharomyces pombe. In that case, a faint, broad actin belt forms in an Arp2/3-dependent manner shortly before anaphase and sharpens as anaphase progresses. This belt is then compacted into the sharp actin contractile ring characteristic of cytokinesis [134]. Remarkably, the Hof1 homologue in S. pombe, Cdc15, is necessary for the assembly of the medial actin belt and required for the recruitment of formins and the Arp2/3 complex to the cytokinetic area [135]. Thus, our data suggest that Hof1 function is highly conserved between budding and fission yeast. Considering all these data, it is possible that the belt is a precursor of the actin ring observed, as both structures are Hof1 dependent. Future experiments like live cell imaging using an actin bound GFP marker protein or genetic studies could clarify this point.

Unravelling interconnections and involved proteins in the Hof1 and Myo1 dependent pathway of cytokinesis

Next, we attempted to sort cytokinetic genes into either the Hof1 or the Myo1 pathway of cytokinesis.

It was already shown that lqg1 as well as Vrp1 act in both pathways [3, 4]. A bni1 mutation causes co-lethality with a hof1 mutation while a bnr1 mutation does not [2]. We crossed rvs161Δ, rvs167Δ, bud4Δ and arp2-1 into both hof1Δ and mo1Δ mutants, and the viability of the resulting double mutants was assayed. This analysis indicated that Bud4 was required for Hof1 function. boi1Δ and boi2Δ showed no genetic interaction with either mo1Δ or hof1Δ such that it was not possible to sort these proteins in either of the cytokinetic pathways. It would be interesting to also cross the double mutant boi1Δ boi2Δ with mo1Δ and hof1Δ cells to see if these leads to any inviability phenotypes. arp2-1 was unviable in combination with either mo1Δ or hof1Δ. The Arp2/3 complex therefore presumably acts upstream of both pathways, or in the Cyk3-pathway.

The model in fig2-11 summarizes our understanding of the two cytokinetic pathways, the proteins and the structures involved as we draw it from our actin overexpression studies. A lot more studies have to be done to understand why yeast evolved different pathways of cytokinesis and kept them all during evolution. Also our studies did not deal with members of
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the Cyk3 pathway of cytokinesis. Detailed analysis and screens are necessary to unravel the molecular make-up of this pathway and set it in relation with the other two pathways. This will be discussed in more details in the Perspectives part.

Figure 2-11
The indicated mutants were crossed with myo1Δ and hof1Δ by us and others [2-4]. Co-lethality with solely myo1Δ indicated that the mutated gene acts in the HOF1 pathway. Vice versa, co-lethality with hof1Δ puts the gene in the MYO1 pathway. Co-lethality with both puts the gene in an upstream pathway. Formins were put in the MYO1 pathway because they do not affect ring formation upon actin overexpression. Boi1 and Boi2 are thought to regulate the HOF1 pathway because they are required for ring formation upon actin overexpression, and co-lethality tests put the anillin homologue Bud4 in the HOF1 pathway.

The actin ring observed always localizes around the nucleus in a dynactin-dependent manner

After investigating the molecular requirements for the formation of an actin ring upon actin overexpression we wanted to investigate its intracellular location in more detail. To do this, we co-stained cells that overexpressed actin with DAPI and rhodamine-phalloidin to visualize the actin-ring structure and the nucleus at the same time. Interestingly, we observed that the ring almost exclusively localized around the nucleus (86% WT cells, N=300; fig2-12). This remained true in all mutants in which an actin ring is formed (fig 2-12). At least 70% of rings were always tightly wrapped around the nucleus.
We have already shown that the ring is not a product of cell death but more likely an exaggerated native structure. Thus, this is the first study that raises the issue that nuclear positioning might be important for the formation and maintenance of some cytokinetic substructures also in budding yeast.

We next wanted to investigate the molecular mechanisms that underlie the tight association of the overexpressed actin ring with the nucleus. It is possible that in a wild type situation, in which actin is not overexpressed, the nucleus and the actin belt (the presumed precursor of the ring) come in contact with each other at the neck, when the nucleus passes through the bud neck. The observation of the ring around the nucleus might be the reverse situation, in which the ring travels towards the nucleus when bud growth is disrupted.

The fact that at earlier times of galactose induction a faint ring structure is seen in the vicinity of the bud neck which later seems to mature around the nucleus into a stronger structure made us investigate which structures could be responsible for this movement. To investigate a potential role of microtubules, we tested whether components of the dynactin complex participate in ring formation. The dynactin complex consists of, Jmn1, Act5 and Nip100 and is necessary for dynein-mediated movement in vivo [136, 137].
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Actin-overexpressing cells lacking dynactin components were scored for actin ring formation around the nucleus. Interestingly, none of the mutants formed rings like wild type. Only faint actin rings were formed in dynein deficient \( \text{dhc} \Delta \) cells (fig2-13 A and B). Criss-crossing actin structures but no matured rings were seen when actin was overexpressed in cells lacking Act5, Jnm1 or Nip100 (data not shown and fig2-13 A and B). In all cases the structures failed to localize around the nucleus.

Figure 2-13
A) Cells of the indicated phenotype containing the \( \text{GAL:ACT1} \) plasmid were fixed upon 10h induction in Galactose with formaldehyde and stained with rhodamine phalloidin for actin and DAPI for nuclei. Representative micrographs of cells are shown.
B) The listed strains were transformed with the \( \text{GAL:ACT1} \) plasmid and induced in Galactose for eight respectively ten hours. Fixed cells were stained with rhodamine phalloidin and inspected for the presence of an actin ring. In each case \( n=300 \).

Thus, the dynactin complex is required for the formation of the actin ring. One possibility is that dynein and microtubules are required for the recruitment of cortically assembled rings to the nuclear periphery. This step might be crucial for the thickening of the ring around the nucleus.

All studies presented here were done using fixed cells and the assumption that the ring performs a dynactin dependent movement from the neck to the nucleus is hypothetical. A nice way to test this assumption would be to follow the actin structure over time. To do this one would need an \( \text{in vivo} \) marker of filamentous actin and perform time-lapse microscopy. I tested an Abp-140-GFP construct that exclusively stains filamentous actin [138]. The ring that forms upon actin overexpression could be visualized with this construct in WT cells. Therefore it would be a nice tool to investigate the role and mechanism of the dynactin complex in ring formation and ring localization.
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The Importins Sxm1, Pse1 Srp1 and Nmd5 and the Exportin Xpo1 influence the formation of the ring structure upon actin overexpression

As mentioned above, one possible explanation for the tight association of the actin ring with the nucleus might be that there is a communication between these two structures. Candidate molecules for such a communication are nuclear importins and exportins, which act in the Ran pathway. Ran proteins form a subfamily of the Ras superfamily of GTPases. The Ran pathway consists of a Ran-GEF in the nucleus (Prp20 in yeast) and a Ran-GAP in the cytoplasm (Rna1 in yeast). This compartmentalization creates a Ran-GTP gradient that is important for exportin binding in the nucleus and release in the cytoplasm and the reverse for importins [139].

We first tested mutants in the Ran-GEF and GAP for their ability to form actin rings upon actin overexpression. As both proteins are essential we induced actin overexpression in galactose for 8h at the permissive temperature, and subsequently shifted cells for 1h to the restrictive temperature. In both strains ring formation was severely reduced. This suggested that nuclear import/export controlled by the Ran pathway had an effect on ring formation. To be more specific we screened mutants lacking various importins or exportins (all kind gifts of Markus Künzler Microbiology ETH-Zürich) to determine which ones are defective for ring formation. The importin mutants tested were səm1Δ, pse1-1, srp1-31, nmd5Δ, kap122Δ, kap114Δ, yrb4Δ and rsl1-4. Indeed some of these strains showed an effect on ring formation (fig2-14). These were səm1Δ, pse1-1, srp1-31 and nmd5Δ. As exportin mutants we tested xpo1-1, msn5Δ, cse1Δ and los1Δ. Only xpo1-1 cells formed a reduced number of rings upon actin overexpression (fig2-14). However, this reduction was not as severe as for the importin mutants that had an effect.

![Figure 2-14](https://example.com/figure2-14.png)

Screen for importin and exportin mutants that have an effect on actin ring formation. The listed strains were transformed with the GAL:ACT1 plasmid and induced in galactose for eight respectively ten hours. Fixed cells were stained with rhodamine phalloidin and inspected for the presence of an actin ring. In each case n=300. The grey bars indicate that the cells were induced eight hours at permissive temperature and shifted up to the restrictive temperature (37°C) for the following 2 hours, prior to fixation.
To test whether some of the identified importins could also be involved in cytokinesis, we crossed the corresponding mutants with the myo1Δ and hof1Δ strains and investigated whether co-lethality interactions were observed. This experiment suggested that Nmd5 acts in the Cyk3 pathway, as the nmd5Δ mutation is co-lethal with both, myo1Δ and hof1Δ. Sxm1 and Srp1 on the other hand can be seen in the Hof1 pathway of cytokinesis as they are synthetic lethal only with the myo1Δ but not with the hof1Δ mutation. It would be interesting to further investigate the exact roles of these proteins and of the Ran pathway in cytokinesis.

Our study for the first time links nuclear importins and exportins to cytokinetic pathways. The co-lethality studies suggest that there might be a link between some step in cytokinesis and nuclear transport. Furthermore, we could also show that in nmd5Δ cells, septins are misorganized like it was shown for abscission mutants (see also fig2-15).

This observation means that at least some importins and exportins may have a direct influence on cytokinesis under wild type conditions, not only when cells overexpress actin. A thorough analysis of the cytokinetic defect in nmd5Δ cells is still to be done. Furthermore it should be investigated if also other importin/exportin mutants play a role in cytokinesis.

Taken together, our studies strongly argue that cytokinesis is linked to nuclear factors. Some correlation between those two structures would make sense insofar, that it would be useful for the cell to sense that one nucleus has passed the bud neck before to undergo cytokinesis. This way it would be assured that both cells inherit a complete set of the genome.

The role of positioning of the nucleus for successful cytokinesis is not clear yet

The hypothesis that resulted from our observations was that there is a communication between the nucleus and the cytokinetic machinery that allows cytokinesis to happen only in cases in which one nucleus has passed the bud neck. To test this possibility moved away from the actin overexpression approach and investigated mutants which triggered mitotic exit although no nucleus entered the bud. To create such a situation we used cells that lacked the spindle pole components Cnm67 and Spc72. Both proteins localize to the outer plaque of the spindle pole body and deletion of the corresponding genes results in the uncoupling of Tem1, the GTPase acting at the top of the MEN, from the daughter bound SPB. This has for consequence that mitotic exit is triggered even in cases in which no SPB enters the daughter compartment. Additionally, the function of cytoplasmic microtubules is impaired in these cells and anaphase often takes place in the mother. Buds that do not receive a nucleus do not separate from their mother; the cells however exit mitosis, start a new cycle and form a new bud [140]. This
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phenotype suggests that in the absence of nuclear migration through the bud neck, completion of cytokinesis is impaired.

To investigate if the failure of cell separation is due to a cytokinetic defect, we treated \textit{cnm67Δ} cells and \textit{spc72Δ} cells with zymolyase. This enzyme is β-1,3-glucan laminaripentao-hydrolase, and hydrolyzes glucose polymers at β-1,3- glucan linkages. Therefore zymolyase degrades the septum but not the plasma membrane of cells. Cells that failed to complete cytokinesis should not be separated by zymolyase treatment, because the cytoplasm and the plasma membrane are still continuous between the two cells and there is no septum to be degraded. In contrast, cells that completed cytokinesis but are kept together by a common septum should separate from each other upon digestion of the cell wall.

Zymolyase treatments showed that neither \textit{cnm67Δ} nor \textit{spc72Δ} cells exhibit a cytokinetic defect under the conditions tested: Almost all cells separated upon zymolyase treatment in both mutants. The number of multibudded cells dropped from 27% before digestion to 5% after digestion in the \textit{cnm67Δ} cells and from 29% to 1% in the \textit{spc72Δ} mutants. This indicated that the separation failure of these mutant cells was due to a septum digestion defect and not to a cytokinesis failure. The reason for this defect will be discussed later.

Although we could not directly prove our hypothesis that interaction between the nucleus and the cytokinetic machinery influences the onset of cytokinesis or the assembly of the actomyosin ring, it is still possible that such a regulation exists. However, this regulation would clearly not be essential for the cells. Preliminary experiments show that a \textit{cnm67Δ} mutation is synthetic lethal with both, a \textit{myo1Δ} mutation and a \textit{hof1Δ} mutation. This means that \textit{cnm67Δ} cells are weakened for some aspects of cytokinesis and that the perturbation of members of either cytokinetic pathway, Hof1 related or Myo1 related leads to cell death.

II

Role of the midzone in cytokinesis

We showed that in budding yeast spatial coordination between the cortex/cleavage machinery and the nucleus is likely to exist but is not essential for cytokinetic onset. However, we found nuclear factors that influence specifically one pathway of cytokinesis. Nmd5 can be genetically sorted into the Cyk3/abscission pathway of cytokinesis (see above). Furthermore, preliminary results show that septins are disorganized in this mutant. Such a disorganization has before only been seen in abscission mutants (fig 2-15).
Rts1 was shown to be a member of the abscission pathway [12]. This protein shuttles in late anaphase from the kinetochore to the bud neck.

According to this data we hypothesized that it might be the regulation of abscission, which is influenced by nuclear components, rather than the assembly of the cytokinetic machinery. The central spindle seemed to be a good candidate for being such a regulator.

We anticipated this for the following reason: Unpublished data from the lab shows that Rts1 is seen at the spindle before it reaches the bud neck. This is reminiscent of what is seen for passenger proteins in higher eukaryotes that are known to play a role in late cytokinetic events. Subsequently we asked the following questions:

1. Rts1 localizes to the kinetochore before it goes to the neck. Is there a direct role of the kinetochore and its associated proteins in cytokinesis?

2. It was reported that in human cells a non-phosphorylatable form of the centromeric histone H3 homologue protein CENP-A induces cytokinetic defects [141]. Can cytokinesis also be impaired in *S. cerevisiae* kinetochore mutants?

3. Is there a role for the spindle midzone in cytokinesis as observed in higher eukaryotes?

We decided to test a variety of cells with defects in different spindle components or the kinetochore for the presence of cytokinetic defects.

**Mutations of specific kinetochore and spindle proteins lead to a cytokinetic defect**

In order to determine whether malfunction of the spindle affects cytokinesis in *S. cerevisiae*, we characterized the division of mutants that are defective for different aspects of spindle function. Yeast mitotic spindles organize around three major structures: the spindle pole body (SPB), the kinetochores and polar microtubules. Polar microtubules emanate from both poles, interdigitate in the spindle midzone and form the central spindle during anaphase.
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To investigate whether any of these different parts of the spindle affect cytokinesis we started by characterizing cell division in cells that carried mutations in different kinetochore substructures.

As a member of the core kinetochore we tested Ndc10 (ndc10-1), a component of the Cbf3 complex. To test mutants in parts of the central kinetochore we used Ndc80, of the Ndc80 complex, Nnf1 and Mtw1 (nnf1-17 and mtw1-1) of the Mtw1 complex, and Ame1 (ame1-1) from the Ctf19 complex [142]. Only in the ndc10-1 mutant the whole kinetochore is destroyed. The ndc80-1 mutation leads to complete detachment of kinetochores and microtubule while in the other kinetochore microtubule attachment is only partly disturbed.

Cells carrying the nnf1-17, mtw1-1, ndc80-1 and ame1-1 alleles fail to enter anaphase at the restrictive temperature because they activate the spindle checkpoint. This checkpoint is inactive in ndc10-1 cells. To circumvent this problem, the MAD2 gene was deleted in the cells carrying these mutations to permit cell cycle progression. As a consequence, all strains used in this experiment had the ability to enter anaphase and to exit mitosis.

Cells of the different strains were arrested in G1 with α-factor and synchronously released in fresh medium at the restrictive temperature, samples were taken at regular intervals, and their budding index was monitored by light microscopy. All cultures started to form a first bud with the same schedule, and restarted forming a second bud between 120min and 150min after the release, all with the same kinetics. This indicates that the cells had entered and exited a first mitosis normally to restart a new cycle. A careful analysis of the budding index in WT versus ndc10-1 cells revealed that the mutant cells did not separate from their first bud but restarted a new budding cycle (fig 2-16 B)

Remarkably, as seen for ndc10-1 all mutant cells failed to separate from their first buds, forming bibudded cells upon rebudding. Wild type cells separated from their first buds prior to rebud. Analysis of DNA distribution by DAPI staining showed that whereas wild type cells segregated two DNA masses of similar volume between mother and bud, the kinetochore mutants failed to segregate their chromosomes and kept most DNA in the mother cell (fig2-16 A). However, these cells exited mitosis normally, and restarted a new round of budding and DNA replication.

Thus, when the spindle assembly checkpoint is inactive, cells with kinetochore defects progressed through the cell cycle, exited mitosis and restarted budding and DNA replication, yet failed to separate from their buds.
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A) Representative micrographs of kinetochore defective cells allowed to progress through mitosis. Cells were released from G1 arrest at 37°C and images were acquired 240 min after the temperature shift. Phase and DAPI stainings (to visualize DNA) are shown. The genotypes of cells are indicated. Defects in the inner- (ndc10-1) and the outer-kinetochore (nnf1-17) prevent cell separation, without affecting the timing of the budding cycle.

B) WT, ndc10-1 and nnf1-17 mad2Δ cells were synchronized with α factor, released at 37°C and their budding index was scored at the indicated times (N>100). A scheme of the different budding categories is shown.

Figure 2-16

A) Representative micrographs of kinetochore defective cells allowed to progress through mitosis. Cells were released from G1 arrest at 37°C and images were acquired 240 min after the temperature shift. Phase and DAPI stainings (to visualize DNA) are shown. The genotypes of cells are indicated. Defects in the inner- (ndc10-1) and the outer-kinetochore (nnf1-17) prevent cell separation, without affecting the timing of the budding cycle.

B) WT, ndc10-1 and nnf1-17 mad2Δ cells were synchronized with α factor, released at 37°C and their budding index was scored at the indicated times (N>100). A scheme of the different budding categories is shown.

To be sure, that rebudding only starts after a successful re-replication of the genetic material we performed FACS analysis experiments for ndc10-1 cells and compared them to wild type cells. While the wild type culture oscillates between a 2N and 1N DNA state starting 100 min after the release, the ndc10-1 culture starts out with 1N DNA after the release but this population disappears during the experiment. At 100 min most of the cells have a 2N DNA content and at 200 min a 4N population (fig2-17). This shows that cells do not halt in the cell cycle although cytokinesis did not take place. The machinery that is responsible for the cytokinetic defect seems to be uncoupled from the continuation of the cell cycle.
The bibudded phenotype of the cells with defective kinetochores might be due either to the failure to complete cytokinesis or to separate daughter from mother upon completion of cytokinesis. Indeed, the final separation of yeast daughter cells requires the digestion of the chitin-rich primary septum that otherwise keeps mother and bud together [143]. This septum is digested by the chitinase Cts1. Cts1 is under the control of the bud specific transcription factor Ace2 that enters the nucleus, which is inherited into the bud after exit of mitosis. Thereby it causes that Cts1 is only expressed in the daughter cell [144, 145]. Since all kinetochore-defective cells extensively failed to put DNA into the bud, we rationalized that the lack of cell separation might be due to a failure in Cts1 expression and primary septum digestion. To test this possibility, we used zymolyase. The population of bibudded cells decreased immensely in the case of the mtwl-1 mad2Δ, nnf1-17 mad2Δ and amel-1 mad2Δ strains (fig2-18 A) meaning that they merely have a defect in cell separation but consist two autonomous cytoplasmics. However, zymolyase treatment only marginally reduced the number of ndc10-1 and ndc80 mad2Δ bibudded cells (fig2-18 A). This means that these cells have a cytokinetic defect and did not form a septum yet.

To confirm this result we used a different approach: we crossed the dominant ACE2-1 mutation into our different kinetochore mutants. This mutation is close to the NES of ACE2. It results in continual localization of ACE2-1 in the nucleus during the whole cell cycle, which leads to expression of the chitinase Cts1 also in the mother cell so that septa are degraded even if the daughter cell does not receive a nucleus.

For the following experiments we used the nnf1-17 mad2Δ mutant as a control. These cells have a kinetochore defect but do undergo cytokinesis.
II Results and Discussion

We inspected that \( \text{nuf1-17 mad2A ACE2-1} \) triple mutant cells separated as well as wild type upon completion of mitosis (9% of bibudded cell in \( \text{nuf1-17 mad2A ACE2-1} \), compared to 23% in \( \text{nuf1-17 mad2A} \)). In contrast \( \text{ndc10-1 ACE2-1} \) double mutants formed bi-budded cells to the same extent as \( \text{ndc10-1} \) (49% of bibudded cells in the double mutant and 40% in the single mutant) (fig2-18 B). These results are in agreement with the zymolyase results seen before and argue that mutations in the central kinetochore Mtw1 complex lead to a separation defect possibly due to an inactive chitinase transcription while mutation in the core component Ndc10 leads to a failure in cytokinesis. The same might be true for the Ndc80 mutant but the \( \text{ACE2-1} \) mutation was not tested here.

To determine whether the \( \text{nuf1-17} \) mutation is dominant over the \( \text{ndc10-1} \) mutation and can bypass the cytokinetic defect we performed a time course experiment using double mutant cells.

Upon release of the cells from \( \alpha \)-factor arrest at the restrictive temperature, bibudded cells accumulated, which could not be separated by zymolyase treatment indicating that \( \text{ndc10-1} \) was epistatic over \( \text{nuf1-17} \).

Disturbed central spindle integrity is the cause for the cytokinetic defect

We concluded from the experiments above that Ndc10, and probably also Ndc80, exhibit some function required for the completion of cytokinesis that is not shared with the other kinetochore mutants. In contrast to other kinetochore mutants Ndc10 an Ndc80 both localize to the spindle additionally to their kinetochore localization. It was shown that Ndc10 localizes to the spindle midzone during anaphase and is required for central spindle stability [54, 55]. Ndc80 is seen
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along the whole spindle [56]. Taking this into account we postulated that the mutants establish a spindle defect in addition to their kinetochore defect.

To test this we decided to investigate central spindle composition in more detail in ndc10-1 cells. Analysis of spindle morphology in life ndc10-1 cells undergoing anaphase using CFP-tubulin as a reporter established that spindles form and elongate but they are very fragile and often break (fig 2-19). We could not see frequent spindle breakage in the wild type control cells.

![Figure 2-19](image)

**Figure 2-19**
Morphologies of anaphase spindles in WT and ndc10-1 cells. Spindles were visualized using Tub1-CFP as a reporter.

From this result we concluded that the cytokinetic defect observed in ndc10-1 cells, might be due to spindle midzone defects. To test this possibility, we monitored the formation and stability of the midzone in the some of the kinetochore mutants mentioned above using the spindle midzone proteins Slk19-GFP and Ase1-GFP as markers. Slk19 is a non-essential kinetochore protein that translocates to the midzone during anaphase [50]. Ase1 is a microtubule bundling protein homologous to human PRC1 [146].

To investigate Slk19-GFP localization we arrested WT, ndc10-1, ndc80-1 mad2Δ and nnf1-17 mad2Δ cells in α-factor and observed Slk19 distribution after the release. In WT and nnf1-17 mad2Δ cells does the protein reach the central spindle in anaphase (fig 2-20 A a,b and C). In the two other mutants it stays as two dots mainly in the mother cell during the whole cell cycle (fig 2-10 A d, d’, e and C). To confirm this result we also performed a statistic analysis counting only anaphase cells for Slk19-GFP localization in which we also included mtw1-1 mad2Δ cells. We got the same result as with the time course experiment, namely that only the mutants that showed a cytokinetic defect have problems to localize Slk19 to the central spindle in anaphase (fig 2-20 B).
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A) Localization of the midzone marker Slk19-GFP in kinetochore mutants undergoing anaphase. Two time points are shown for ndc10-1.

B) Quantification of Slk19 localization to the spindle midzone in wild type and mutant strains is shown. N>100.

C) Cell cycle dependent localization of Slk19-GFP in WT and ndc10-1 cells after release from α-factor arrest. Drawings indicate the different categories of cells.

The Ase1-GFP localized to the spindle midzone in wild type cells (fig2-21 A). In ndc10-1 cells this localization is abolished (fig2-21 B). In these cells Ase1 localizes either along the whole spindle or concentrates at the spindle ends of presumably broken spindles. This localization pattern was never observed in wild-type cells. Together the localization analyses of the central spindle components show that central spindle integrity is abolished in ndc10-1 cells.

Figure 2-20
A) Localization of the midzone marker Slk19-GFP in kinetochore mutants undergoing anaphase. Two time points are shown for ndc10-1.
B) Quantification of Slk19 localization to the spindle midzone in wild type and mutant strains is shown. N>100.
C) Cell cycle dependent localization of Slk19-GFP in WT and ndc10-1 cells after release from α-factor arrest. Drawings indicate the different categories of cells.

Figure 2-21
Localization of the midzone component Ase1 in WT and in ndc10-1 cells. Cells expressing Ase1-GFP were released from α-factor at 37°C pictures were taken after 120min.
Taken together the results propose a role of the central spindle in cytokinesis. To confirm the role of the midzone, we investigated whether cells that enter anaphase without microtubules, such as mad2Δ cells treated with nocodazole, complete cytokinesis. Because of the experiments with the kinetochore mutants other than ndc10-1 and ndc80-1 we reasoned that if mad2Δ cells show a cytokinetic defect upon nocodazole treatment, this could not be due to defects in kinetochore-microtubule interactions and chromosome segregation. When synchronized in G1 with α-factor and released into fresh medium containing nocodazole, mad2Δ cells progressed through the cell cycle, exited mitosis and entered a new cycle, which was indicated by the formation of a second bud and reduplication of DNA (measured by FACS analysis). These bibudded cells were not resolved by zymolyase, which suggested that they failed to complete cytokinesis (fig 2-22 graph). Thus, the spindle midzone and/or cytoplasmic microtubules are required for cytokinesis.

To distinguish between these possibilities, we investigated whether defects in cytoplasmic microtubules affect cell cleavage. Cnm67 is a component of the inner-plaque of the spindle pole body and is required for anchorage of cytoplasmic microtubules to the SPB. Cells lacking Cnm67 fail to properly position their spindle yet exit mitosis [147] and seen above). Consequently, a large proportion of the cnm67Δ cells is multi-nucleated and these cells fail to separate from their anucleated buds. Repeating the experiments from part I, zymolyase treatment and ACE2-1 expression indicated that this separation defect was due to a lack of Ace2-dependent digestion of the primary septum (fig 2-22 A and B). In these cells, Slk19-GFP localizes to the spindle midzone normally (fig 2-22 C). We therefore concluded that microtubule-kinetochore attachment and the function of the cytoplasmic microtubules are dispensable for proper cytokinesis. In contrast, cells lacking the spindle midzone fail to complete cytokinesis.
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Together this shows that cells that misposition the spindle midzone relative to the cleavage apparatus, such as the \textit{cnm67}\Delta cells, do not fail to complete cytokinesis. Thus, the role of the midzone in yeast cytokinesis is independent of its position, excluding the possibility that it acts by providing spatial information. This finding is opposite to what has been found in \textit{S.pombe} and animal cells.

**Deletion of the central spindle component Ase1 leads to a cytokinetic defect**

Next we wanted to directly show that the central spindle plays a role in cytokinesis. For this we chose a mutant that exclusively functions in assuring central spindle stability.

The microtubule-binding protein Ase1, the homologue of human PRC1, is the only yeast protein known to localize exclusively to the spindle midzone. Yeast cells that lack Ase1 form a highly unstable spindle, which breaks down prematurely during anaphase [1]. We therefore investigated whether cells lacking Ase1 complete cytokinesis normally. \textit{ase1}\Delta cells are viable, form normal sized colonies and segregate chromosomes fairly normally [146]. To determine if \textit{ase1}\Delta cells exhibit any kind of cytokinetic defect, we characterized their arrest in G1 upon \textit{α}-factor treatment. We reasoned that cells impaired or delayed for cytokinesis would arrest as connected cells. After 2 hours of \textit{α}-factor treatment, 98% of the wild type cells arrested as separated G1 cells with a mating projection, as expected. In contrast, 42% of the \textit{ase1}\Delta cells remained attached in pairs (fig 2-23 A). These cells had exited mitosis and properly arrested in G1 since they also formed mating projections and resumed budding upon release from the \textit{α}-factor arrest with the same kinetics as the control cells. All cells resumed budding with similar
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kinetics upon release from α-factor arrest. The bibudded cells that formed under these conditions were not resolved by zymolyase treatment, indicating that the cells had started a new division cycle without completing the previous cytokinesis. Remarkably, at the end of the next cycle most of these cells separated from both of their new and old buds, indicating that they had kept the ability to complete cytokinesis at both bud necks. Together, these data suggested that cells with a fragile anaphase spindle, such as \( ase1\Delta \) cells delay the completion of cytokinesis.

However, it was puzzling to us that asynchronous cultures of \( ase1\Delta \) cells did not contain a high fraction of multibudded cells. This finding suggested that the cytokinetic defect might be less penetrant in actively cycling cells. Alternatively, the cytokinetic defect might be suppressed after several passages of the constitutively defective \( ase1\Delta \) mutant strain or α-factor induces a cytokinetic delay under these special conditions. To differentiate between these possibilities, we created a conditional \( ASE1 \) allele. The endogenous promoter was replaced by a weak version of the regulatable \( GAL \) promoter (GALS) [148], which allowed Ase1 synthesis to be turned off in glucose media. Accordingly, \( GALS:HA-ASE1 \) cells did not contain detectable levels of HA-Ase1 protein when arrested in G1 or grown on glucose medium, similar to the situation in \( ase1\Delta \) cells [1] (fig2-24 B). Also, as in \( ase1\Delta \) cells spindles of the \( GALS:HA-ASE1 \) cells grown in glucose failed to elongate and broke down prematurely compared to wild type cells (fig2-24 A).

Thus, the \( GALS:HA-ASE1 \) construct allowed efficient Ase1 depletion within a single cell cycle and was therefore used to determine whether Ase1 inactivation affects cytokinesis.
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Within the first two cycles of growth in repressing media the \textit{GALS:HA-ASE1} cells started to form chains of unseparated and bibudded cells. This bibudded phenotype could be due to defects either in cytokinesis or in subsequent cell separation [143]. Neither \textit{GALS:HA-ASE1} cells grown in the inducing medium nor wild type cells grown in glucose showed such defects in cell separation. Post-cytokinetically separated wild type cells is achieved through digestion of the primary septum by the chitinase Cts1.

Cell wall digestion with zymolyase failed to dissociate the chains of \textit{GALS:HA-ASE1} cells (fig2-25 A). Thus, their separation defect was due to a failure of cytokinesis and not of primary septum digestion. Accordingly, staining of chitin with calcoflour indicated that primary septa failed to form properly between mother and daughter cells (fig2-25 B). This indicated that cytokinesis did not take place.

The chain phenotype of the \textit{GALS:HA-ASE1} cells was less obvious after a few passages on repressing media, which suggested the appearance of suppressor mutations. In support of this possibility, the phenotype was recovered in a fraction of the \textit{GALS:HA-ASE1} spores upon backcrossing. Together, these data indicate that wild type cells depleted for the spindle midzone component Ase1 fail to complete cell cleavage upon exit of mitosis. This result
combined with the results in the other spindle mutants we conclude that the integrity of the spindle midzone is required for cytokinesis but that at least *ase1Δ* cells adapt, leading to the consequence that only mild defects are observed after several passages.

**The observed cytokinetic defect is in abscission**

As a next step we wanted to analyze the exact nature of the observed cytokinetic defect. As explained in the introduction, cytokinesis is a multistep process and we wondered which of the steps is influenced, actomyosin ring contraction or abscission, in cases when central spindle formation and/or integrity is perturbed.

To get a first indication if cells that are not dissolvable by zymolyase treatment still shared a cytoplasm we stained WT, *nnf1-17 mad2Δ, cnm67Δ, ndc10-1* and nocodazole treated *mad2Δ* cells with calcofluor (a widely used chitin dye) to visualize primary septum formation.

In WT, *nnf1-17mad2Δ* and *cnm67Δ* cells, the primary septum fully closed the bud neck of postmitotic cells (fig2-26 a,b,c). In binned *cnm67Δ* and *nnf1-17 mad2Δ* cells a chitin wall always closed at least one of the two bud necks. In contrast, no septum ever closed any of the bud necks of *ndc10-1* and nocodazole treated *mad2Δ* cells (fig2-26 d,e). This confirmed our assumption that these cells were impaired for some cytokinetic process.

![Figure 2-26](image)

*Figure 2-26*  
Cells of the indicated phenotype were stained with calcofluor white to visualize septa. Arrows indicate closed septa while arrowheads point at open bud necks.

In yeast cells cytokinesis starts with the assembly and contraction of the actomyosin ring. Upon contraction, this ring disassembles and the small cytoplasmic bridge left between the two daughter cells is resolved during abscission (reviewed in [23]). We first assayed if actomyosin ring contraction is influenced mutants with central spindle defects. To perform this, endogenous type II myosin, Myo1, was tagged with GFP and actomyosin ring contraction and disassembly was monitored. Time-lapse fluorescence microscopy indicated that the Myo1-GFP ring contracted and disassembled with similar kinetics in wild type, *ndc10-1* and nocodazole treated *mad2Δ* cells (fig2-27). This showed that actomyosin ring contraction was not defective in cells with midzone defects. Since actomyosin ring contraction is directly under the control
of the mitotic exit network in yeast these data additionally confirmed that cells with midzone defects properly exited mitosis before starting to rebud.

These observations suggested that spindle midzone defects affect cytokinesis at a step after actomyosin ring contraction. The next step in cytokinesis would be abscission. To investigate the possibility that abscission is impaired in central spindle mutants, we expressed a GFP-tagged version of the plasma membrane protein Ras2 and used this reporter to visualize the plasma membrane at the bud neck. Tagged cells were imaged in three dimensions by spinning disk confocal microscopy. Images were taken every 150 nanometers throughout the bud neck. Analysis of cnm67Δ and untreated mad2Δ mutant cells showed that the plasma membrane at bud necks was either fully open (in premitotic cells) or cleaved (in postmitotic cells) (fig2-28 upper panels). In the latter case, the two plasma membranes facing each other were clearly separated by the un-labeled septum. Nocodazole treated mad2Δ cells that had formed a second bud without separating from the previous one presented a different phenotype. The bud neck of the smaller bud was generally fully open, as expected for a pre-cytokinetic situation. However, between the mother and the larger bud it could be observed that the plasma membrane formed a continuous narrow bridge through the bud neck (fig2-28 lower panel). This is consistent with the assumption that the plasma membrane was pinged by the contraction of the actomyosin ring but remained unresolved. These data are consistent with our hypothesis that cells with a defect in the spindle midzone fail to complete abscission upon actomyosin ring contraction.
It was recently shown that septins play a crucial role in abscission, and that defects in septin organization can lead to an abscission defect without affecting actomyosin ring contraction [12]. We next checked if this abscission related septin phenotype could also be observed in our central spindle abscission mutants. To accomplish this, we visualized septin rings in WT, \textit{cnm}67\textDelta, \textit{ndc}10-1 and nocodazole-treated \textit{mad}2\textDelta cells using plasmid derived GFP-Cdc12 as a reporter.

In wild type cells the septin ring splits into two continuous rings on each side of the bud neck at the onset of cytokinesis. After cell separation and prior the emergence of a new bud, septin rings disassemble (fig2-29 a). In contrast to the wild type situation, the midzone defective mutants \textit{ndc}10-1 and nocodazole treated \textit{mad}2\textDelta cells revealed abnormalities in septin ring constitution. In \textit{ndc}10-1 and nocodazole treated \textit{mad}2\textDelta cells, the split rings appeared discontinuous as in abscission mutants [12] (fig2-29 c,d). Moreover, these misshapen rings remained at the neck of the uncleaved bud despite the formation of new septin structures at the neck of the new bud.

In \textit{cnm}67\textDelta cells that did not encounter any problems in cytokinesis or abscission the septin rings remained continuous upon splitting. The mother cell correctly disassembled their first split ring before the assembly of a new one (fig2-29 b). Only the presumably anucleated daughter cells maintained a septin ring at the former division site.
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Taken together, these results indicate that cells with spindle midzone defects have a severe septin localization defect as seen for other abscission mutants. It is well possible that it is (maybe among other things) this septin organization defect that leads to failure in abscission.

Together, our results consistently prove that cells that exhibit a midzone defect do not succeed in abscission. Cells do not form a primary septum although actomyosin ring contraction proceeds successfully. The Ras-GFP reporter showed that the plasma membrane is pinched but not resolved. Furthermore, by using a combination of vortexing and zymolyase treatment it was possible to separate a high fraction of the postmitotic buds, which would not have been the case for cell with problems in actomyosin ring contraction. It also indicated that only a narrow plasma membrane bridge kept mother and bud together. This is one of the first reports of defects specifically in abscission in budding yeast. This phenomenon is not very well studied and almost no factors are known so far that exclusively interfere with this late step of cytokinesis. The involvement of the central spindle in this process shows that it not just happens upon actomyosin ring contraction but that there is need for regulation.

Previous studies reported that the spindle midzone of animal cells is required for proper abscission [47, 149-151]. However, we show that in contrast to what has been proposed for animal cells, proper positioning of the spindle midzone is not relevant in budding yeast. Thus, the midzone plays a signalling role in the abscission process independently of its position.

**Positive or negative signalling of the central spindle in abscission?**

Two scenarios can account for the cytokinesis defect observed in cells with midzone defects. It is possible that the spindle midzone provides a positive signal to trigger abscission. This would mean that cells with a midzone defect do not undergo abscission because the positive trigger is not activated.
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Alternatively, the cytokinetic defect might be due to an active inhibition of abscission as a response to the midzone defect. In this case, mutants defective in the inhibitory pathway should complete abscission even in the absence of a properly formed central spindle.

The phosphatase PP2A regulatory subunit Rts1 is a possible candidate to positively regulate abscission. First because of its localization pattern: it localizes to kinetochores first and is also seen at the spindle (Dobbelaeere unpublished results) before it goes to the neck region.

Second, Dobbelaeere et al. showed that rts1Δ mutants failed to complete abscission specifically. Furthermore, the same authors showed that Rts1 reaches the neck just after spindle breakdown, which would be the timing one would expect for a positive trigger. Our hypothesis was that Rts1 carries a signal to the bud neck to initiate completion of cytokinesis after spindle breakdown. To test this hypothesis we studied Rts1 localization in one of the abscission mutants, ndc10-1. Cells were synchronized with α-factor and Rts1-GFP localization was observed after the release.

Indeed, in this mutant Rts1 localizes to the neck only in a very small fraction of cells compared to WT (fig2-30 A and B). It mainly stays as two dots in the mother cell, even when the cell rebuds. This result indicates a correlation between the bud neck localization of Rts1 and the completion of cytokinesis.

![Figure 2-30](image)

A) Localization of Rts1-GFP in WT ndc10-1 cells.
B) Cell cycle dependent localization of Rts1-GFP in WT and ndc10-1 cells after release from α-factor arrest. Drawings indicate the different categories of cells.
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In ndc10-1 mutant cells, the core component of the kinetochore is not formed and no kinetochore develops at all. We also know from the Tub1-CFP experiments that spindles elongate in ndc10-1 cells. Thus, the Rts1 dots seen in the cytoplasm of ndc10-1 cells can neither be kinetochores nor SPBs. We verified that kinetochores really disassembled in the ndc10-1 mutant by imaging another kinetochore marker, CFP tagged Ndc80, over time in synchronized cells. No Ndc80-CFP staining can be observed in ndc10-1 cells as soon as cells enter a new cycle. Thus, Rts1 most likely localizes to centromeric DNA rather than to kinetochores as assumed before. This point would need further investigation. In S.pombe it was reported that Shugoshin sgo1 recruits the Rts1 homologue to centromeric chromatin, which argues that the localization pattern we observe for Rts1 might be conserved (Nasmyth unpublished data).

Ipl1 is a chromosomal passenger protein and localizes to the central spindle in late anaphase. Ipl1 mutant cells complete cytokinesis although chromosome segregation is aberrant. If our hypothesis was right and Rts1 was needed for successful cytokinesis it should shuttle to the neck of Ipl1 mutant cells. We used an ipl1-321 strain to investigate Rts1 localization. Surprisingly, Rts1 is not seen at the neck region in these cells. Upon cell synchronization and release we almost never observe Rts1 at the neck. However, these cells undergo cytokinesis without problems.

This result left us with two possible conclusions: Either the data from our lab that suggested a positive role of Rts1 in abscission was wrongly interpreted or incompletely understood or Ipl1 itself was a negative regulator of abscission that was epistatic over Rts1. To investigate the latter possibility, we constructed an ipl1-321 rts1Δ strain and investigated if these cells fail cytokinesis. We compared the ability of rts1Δ and rts1Δ ndc10-1 mutants to undergo abscission at the restrictive temperature.

In rts1Δ ipl1-321 cells the number of bibudded cells was highly reduced compared to rts1Δ single mutant cells at 37°C (fig2-31 A). Furthermore, the misshapen septin phenotype was suppressed in the double mutant: Only 5% of the double mutant cells formed misshapen rings compared to 30% of single mutants (fig2-31 B).
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Thus, despite the fact that Rts1 is needed for abscission in wild type cells, Rts1 localization to the neck was not needed for the successful abscission of the ipl1-321 mutant cells. This seemingly contradiction can be solved with the following model (fig2-32):

Rts1 is a phosphatase subunit (PP2A). Thereby it might dephosphorylate and thereby inactivate a bud neck protein that was sent to this site to inhibit abscission. In this scenario Rts1 would have a positive influence on abscission. Ipl1 or other components of the central spindle might send the protein Rts1 dephosphorylates to the neck. Such a scenario would explain why rts1Δ cells have an abscission defect (no dephosphorylation of target X, abscission stays inhibited) and why the rts1Δ ipl1-321 mutant does not (No target X at the neck, no dephosphorylation is needed).

Figure 2-31
A) rts1Δ single and rts1Δ ipl1-321 double mutants were shifted to 37°C for the indicated times and the percentage of binned cells was evaluated.
B) GFP-Cdc12 septin localization was analyzed in both strains. The double mutants showed a major decrease of misshapen septin rings.

Figure 2-32
Model explaining how abscission inhibition and activation might work taking the information we got so far. Details are explained in the text.
The Aurora Kinase Ipl1 Inhibits Abscission in Cells with Spindle Midzone Defects

Our data indicated that spindle midzone defects are associated with abscission defects. Ipl1 cells do not have any noticeable problem to undergo cytokinesis [54, 69] but are able to suppress the abscission defect of rts1Δ cells. This indicates that Ipl1 activity might inhibit abscission.

We wanted to know whether Ipl1 also inhibits abscission in cells with central spindle defects. Thus, we compared the ability of ndc10-1 and ndc10-1 ipl1-321 mutants to undergo abscission at the restrictive temperature.

The result was the same as for the rts1Δ cells.

As discussed before, ndc10-1 cells do not form kinetochores. Therefore we did not expect a major decrease in bibudded cells in the ndc10-1 ipl1-321 double mutant because no chromosomes are pulled into the daughter cell and there is no Ace2-dependent transcription of the chitinase gene in these cells. Thus, we needed to determine whether these double mutant cells remained bibudded due to a cytokinetic or a cell separation defect. We tested whether they remained attached by their primary septum using the zymolyase assay described above.

The experiment demonstrated that the ipl1-321 allele suppressed the cytokinetic defect of ndc10-1 cells. Almost all bibudded cells could be separated by digestion of the cell wall (Fig 2-33 A). Furthermore calcofluor staining showed that the double mutant properly formed a primary septum before to rebud (fig2-33 B).

![Figure 2-33](image)

**A** Fractions of bibudded cells in cultures of the indicated phenotypes are shown before and after zymolyase treatment. Synchronized cells were analyzed 240 min after release of the G1 block at 37°C.

**B** Calcofluor staining of cells of the indicated genotype, 240 min after release as in the digestion experiment.

To exclude the possibility that the ipl1-321 mutation restored abscission due to suppression of the central spindle defects caused by the ndc10-1 mutation we visualized Slk19-GFP in the
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ndc10-1 ipl1-321 double mutants. No suppression of the spindle midzone defects was observed in the ndc10-1 ipl1-321 cells as no Slk19 can be seen at central spindles of these cells (fig2-34).

**Figure 2-34**
The localization of Slk19-GFP to the central spindle is not restored in ndc10-1 ipl1-321 cells. Cells were treated as in the figure before.

Here we established that Ipl1 actively inhibits abscission in cells with spindle defects. This shows that the central spindle does not act positively in abscission but rather that abscission is delayed when the midzone is defective.

The next questions were: a) How does Ipl1 signal to the cell cortex to inhibit abscission? b) Does Ipl1 inhibit abscission directly or indirectly? In WT cells, Ipl1 localizes to kinetochores early in the cycle and shifts to the central spindle in late anaphase (fig2-35 A). We first investigated whether Ipl1 itself translocates to the bud neck in cells with spindle midzone defects, which would suggest that it plays direct role in the inhibition of abscission.

In ndc10-1 cells Ipl1-3GFP localized along the polar microtubules of the broken spindles. No staining was observed at the central spindle or at the bud neck (fig2-35 B). In nocodazole treated mad2Δ cells, no spindle structures were illuminated by Ipl1-3GFP at all. The whole spindle was destroyed by nocodazole. In contrast Ipl1 was seen as a nuclear punctuate staining (fig2-35 C). Again no Ipl1 was seen at the bud neck. Thus, we postulated that Ipl1 signals through some other protein, which is able to shuttle between the nucleus and the bud neck.

**Figure 2-35**
A,B) Localization of Ipl1-3GFP during anaphase of WT and ndc10-1 cells grown at the restrictive temperature. Both Ipl1 (Ipl1-3GFP, red) and Tub1 (Tub1-CFP, green) are shown.
C) As there are no microtubules in nocodazole treated mad2Δ cells, only the Ipl1-3GFP channel is shown.

Taken together our data show that spindle defects lead to Ipl1 dependent inhibition of abscission. This explains why the ipl1 mutant alone does not show a cytokinetic defect.
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**Ipl1 inhibits abscission through the bud neck proteins Boi1 and Boi2**

Knowing that Ipl1 itself cannot accomplish abscission inhibition at the bud neck, we wanted to investigate, which proteins could possibly mediate this function.

Boi1 and Boi2 are highly related proteins involved in polarized growth. They are structurally related to anillin, a family of proteins characterized by the presence of a plekstrin-homology (PH) domain at their C-terminus, and by the ability to shuttle between the nucleus and the site of cell division at the cell cortex. Indeed, Boi1 and Boi2 carry a PH domain at their C-termini and localize to the bud cortex during bud emergence and to the bud neck during mitosis [152]. Furthermore, they are occasionally observed in the nucleus of G1 cells (Eric Bailly personal communication). Finally, boi1Δ boi2Δ double mutant cells do not evolve a significant fraction of bibudded cells (8% of the population that can be digested with zymolyase, which means they have a separation problem) this indicates that they do not play a positive role in cytokinesis. Thus, we asked whether these proteins act instead as cytokinetic inhibitors.

From the literature, we know that the localization pattern of Boi1 and Boi2 is compatible with a function in abscission inhibition [152]. We also verified the localization of these two proteins ourselves. In wild type cells, endogenous Boi1 tagged with GFP occasionally accumulated in the nucleus. Prior to anaphase, it localized to the bud cortex and it reached the bud neck during anaphase. In 41% of cells with an elongated spindle, Boi1-GFP localized to the bud neck as a crisp line (n=44). After spindle breakdown, Boi1-GFP was faintly seen on both sides of the neck and it disappeared from the bud neck prior to cell separation and the beginning of a new cycle (fig2-36 A). Similar results were obtained for Boi2-GFP expressed from a multi-copy plasmid, in which case the accumulation of Boi2-GFP to the nucleus of G1 cells was more obvious. Thus, in wild type cells Boi1 and Boi2 localized to the bud neck during anaphase and disappeared from the site of cytokinesis upon spindle breakdown.

In contrast, Boi1 remained at the bud neck after spindle breakdown and the emergence of a new bud in 77% of ndc10-1 cells that failed abscission (fig2-36 B). Thus, the localization pattern of Boi proteins was compatible with them playing an inhibitory role on cytokinesis.
II Results and Discussion

Figure 2-36
Localization of Boi1-GFP expressed from the endogenous locus in anaphase and telophase wild type and ndc10-1 cells. Tub1-CFP was used to monitor the status of the spindle. Arrows mark bud neck localization of Boi1-GFP.

If we assume that Boi1 and Boi2 indeed act downstream of Ipl1, the deletion of these proteins should also result in a suppression of abscission defects. Boi1 and Boi2 act as a complex but the deletion of one of the two proteins shows only very mild defects [153]. Therefore, we used double deletions for our experiments.

We investigated whether boi1Δ boi2Δ ndc10-1 cells are able to complete cytokinesis. After releasing cells from α-factor at the restrictive temperature 26% of the triple mutant cells were bi-budded (versus 36% in ndc10-1) but 70% of this population could be resolved to un-budded and single-budded cells by zymolyase treatment (versus 14% in ndc10-1) (fig2-37 A). Consistent with these data the triple mutant cells formed their primary septum properly at cytokinesis. Thus, Boi1 and Boi2 are required for the inhibition of cytokinesis in response to spindle midzone defects. However, as seen in the ndc10-1 ipi1-321 double mutant the midzone defect of the ndc10-1 cells, was not suppressed in the boi1Δ boi2Δ ndc10-1 triple mutant which was determined using Slk19-GFP staining (fig2-37 B).

Figure 2-37
A) The fraction of bi-budded cells in cultures of the indicated phenotypes are shown before and after zymolyase treatment. Synchronized cells were analyzed 240min after release of the G1 block at 37°C.

B) The localization of Slk19-GFP to the central spindle is not restored in boi1Δ boi2Δ ndc10-1 cells.
II Results and Discussion

To determine understand if Boi1 and Boi2 acted downstream of Ipl1, we investigated their localization in cells lacking Ipl1 function versus wild type cells. The *ipl1-321* cells expressing Boi1-GFP were synchronized with α-factor and released into fresh medium at the restrictive temperature. In these cells Boi1-GFP remained nuclear for the entire cell cycle and localized neither to the cortex of growing buds nor to the bud neck of anaphase cells (fig2-38 A). The same results were obtained with Boi2-GFP (fig2-38 B). Thus, Ipl1 activity was required for proper localization of Boi1 and Boi2.

![Figure 2-38](image)

**Figure 2-38**

A) Time course of Boi1-GFP localization in wild type and *ipl1-321* cells progressing through the cell cycle.

B) Cortical localization of Boi2-GFP requires Ipl1 activity. Micrographs of wild type and *ipl1-321* cells expressing Boi2-GFP are shown. Arrows (at bud necks) and arrowheads (at the bud surface) indicate cortical Boi2-GFP while asterisks indicate nuclear staining.

Our data suggest that the Boi proteins transduce an Ipl1-dependent signal from the nucleoplasm to the cell cortex to repress abscission. Boi1 and Boi2 contain several Ipl1 consensus phosphorylation sites. However, simultaneous mutation of all sites in both Boi1 and Boi2 did not alter their localization. This indicates that either some other Ipl1 substrate might function in the control of Boi1 and Boi2 localization, or that there are additional unidentified Ipl1 sites on these proteins.
Live cell imaging further proves the existence of a checkpoint-like signalling cascade

To get a visual proof for the existence of the signalling cascade that inhibits abscission we performed live cell imaging experiments to determine the timing of abscission in different strains using the plasma membrane marker Ras2-GFP (described above). Additionally, we crossed an Spc42-CFP marker into all our strains to observe spindle elongation and breakdown. Inspected strains were wild type, \textit{boi1\Delta boi2\Delta}, \textit{ase1\Delta}, \textit{GAL:HA-ASE1} and \textit{GAL:HA-ASE1 boi1\Delta boi2\Delta}. If our hypothesis about the signalling cascade that inhibits abscission in cases of spindle damage was right, we would expect an accelerated abscission in a \textit{boi1\Delta boi2\Delta} strain because no sensor for central spindle damage would be present in these cells. On the other hand abscission should be delayed in strains with midzone defects like \textit{ase1\Delta} and \textit{GAL:HA-ASE1} because a central spindle defect should be sensed which would activate the checkpoint. This delay should be fully compensated in a \textit{GAL:HA-ASE1 boi1\Delta boi2\Delta} strain in which there is a defect but no checkpoint to delay cell cycle progression.

Spindle elongation and breakdown proceeded fairly normally in wild type and \textit{boi1\Delta boi2\Delta} cells. In wild type and \textit{boi1\Delta boi2\Delta} cells the time between plasma membrane constriction and separation was measured. As seen in the upper graphs in fig2-39 spindle lengths have the tendency to plateau before spindle breakdown, a situation that is not observed in wild type strains. This delay is most probably due to the Ras2 marker. Ras2 is expressed on a 2\mu plasmid and therefore slightly overexpressed. It has been shown, that Ras2 influences mitotic exit [154], which might be the reason for a slightly longer time with an elongated spindle in our experiments than what was shown in wild type cells that do not have extra copies of Ras2.

For the \textit{ase1\Delta} and \textit{GAL:HA-ASE1} strain we observe, like others, that the spindle breaks down almost immediately after start of elongation (fig2-29 \textit{ase1\Delta} graph and [1]). In these strains we only scored the time between the onset of plasma membrane constriction and separation. An example movie is shown in fig2-39.

The box and whiskers graph shows that indeed \textit{boi1\Delta boi2\Delta} cells need less time between plasma membrane invagination and separation than the wild type cells. The mean for the double mutant is 11.87min (n=9) while wild type cells need 15.24min in average (n=13). Also the assumption that \textit{ase1\Delta} and \textit{GAL:HA-ASE1} should have an abscission delay could be verified. In these two strains the average between onset of membrane contraction and abscission is 24.21min for \textit{ase1\Delta} cells and 22.16min for \textit{GAL:HA-ASE1} cells (n=10 for \textit{ase1\Delta};

80
n=11 for GAL:HA-ASE1). Only the assumption that GAL:HA-ASE1 boi1Δ boi2Δ cells revert this delay could not be exhaustively shown. One would expect that lacking the checkpoint proteins Boi1 and Boi2, timing of abscission should be the same as in the boi1Δ boi2Δ cells. In contrary to that assumption in GAL:HA-ASE1 boi1Δ boi2Δ cells timing of abscission goes back only to wild type levels. What these results show is that the abscission delay of GAL:HA-ASE1 cells does partly depend on Boi1 and Boi2. However, that the timing is not the same as in the boi1Δ boi2Δ strain suggests that there are additional factors involved that do not depend on Boi1 and Boi2. This point needs further investigation.

Altogether the life cell imaging experiments offer further support that Boi1 and Boi2 play a role in abscission inhibition. It would be interesting to test other mutants that are likely to play a role in the checkpoint and see if also other mutations lead to an advanced abscission.
Figure 2-39
A) Example for a Ras2-GFP Spc42-CFP wild type movie. The asterisk marks open septa, red arrows mark spindle elongation, arrowheads mark pinched membranes, white arrows mark resolved membranes.
B) Representative spindle measurements for the strains tested. Times of spindle breakdown, start of actomyosin contraction and separation are indicated. Note that spindles break prematurely in ase1Δ strains as published [1]. The same was observed for the GAL::HA-ASE1 strain.
C) Boxes mark the times between the onset of membrane contraction and separation for 50% of the data points. Whiskers mark the most extreme data points. The bar within the box marks the median of the data point. N= 13 for wild type, 9 for boi1Δ boi2Δ, 10 for ase1Δ, 11 for GAL::HA-ASE1 and 10 for GAL::HA-ASE1 boi1Δ boi2Δ.
II Results and Discussion

Failure to inhibit abscission causes chromosome breakage by the cytokinetic machinery

In summary, we found that cells developed a pathway to inhibit abscission in cases of central spindle defects. We next wondered about the biological relevance of such a pathway. Cells that lack a functional midzone frequently fail to elongate their spindle properly and, hence, might fail to separate the sister chromatids along their entire length and thereby to clear the center of the spindle from chromatin. We rationalized this might be the reason why cells prevented the completion of cytokinesis in cases of midzone defects. In such situations, abscission might have the potential to damage the chromosomes that are still engaged in the cleavage plane. To test this possibility, we checked whether Boi1 and Boi2 protected chromosomes from being broken by the cleavage machinery upon spindle damage. We investigated whether inactivation of Boi1 and Boi2 in cells lacking Ase1 would lead to chromosome breakage. To this end, we sought to characterize the phenotype of the \textit{ase1}Δ \textit{boi1}Δ \textit{boi2}Δ cells. While any combination of the single and double mutants was viable, the triple mutant \textit{ase1}Δ \textit{boi1}Δ \textit{boi2}Δ spores were unable to form colonies. Upon germination, micro-colonies started to form but within a few divisions the cells died. We wanted to know if this phenotype is common also for other abscission mutants and constructed the \textit{ndc10-1 boi1}Δ \textit{boi2}Δ triple mutant. Remarkably, also the triple mutant \textit{ndc10-1 boi1}Δ \textit{boi2}Δ grew extremely slowly at 24°C, a temperature at which \textit{ndc10-1} and the \textit{boi1}Δ \textit{boi2}Δ double mutant were not affected for growth.

Thus Boi1 and Boi2 are required for the survival of cells with fragile anaphase spindles. To permit the phenotypical analysis of the \textit{boi1}Δ \textit{boi2}Δ cells depleted from Ase1 we constructed the \textit{boi1}Δ \textit{boi2}Δ \textit{GALS:HA-ASE1} triple mutant, which was viable on galactose. Upon shift of these cells to the repressive condition, chromosome segregation was strongly slowed down, as seen by DAPI staining (fig2-40 B). However, the number of bibudded cells present in the \textit{GALS:HA-ASE1} single mutant population dropped significantly upon growth in glucose containing medium in the triple mutant. The anaphase nuclei failed to fully elongate and DNA bridges remained in the bud neck of many cells, as expected for cells that undergo premature spindle breakdown (fig2-39 C).
II Results and Discussion

To monitor the effect of Boi inactivation on chromosome breakage, a Ddc1-GFP reporter construct was introduced in the cells. Ddc1 is a protein involved in the recognition and repair of double strand breaks (DSB), and Ddc1-GFP accumulates into foci at sites of DNA damage, which allows the visualization of DBS.

*DDC1-GFP* and *GALS:HA-ASE1 DDC1-GFP* cells were synchronized in G1 with α-factor and released in glucose to repress the *GALS:HA-ASE1* construct. A low fraction of cells with Ddc1-GFP foci appeared in both strains at early time points, probably corresponding to S phase (fig 2-41 A). The frequency of cells with Ddc1-GFP foci then dropped to about 4% as the cells progressed through mitosis into the next G1 (fig2-41 B). Thus, Ase1 depletion did not increase the frequency of double strand breaks compared to wild type despite the frequent presence of DNA bridges in the plane of cleavage. In contrast, cells lacking Boi1 and Boi2 showed a higher frequency of Ddc1-GFP foci. The frequency of these foci further doubled (up to 27%) in cells that were depleted for Ase1. The increase in Ddc1 foci frequency was obvious at later times of the experiment, when cells went through mitosis. Thus, Boi1 and Boi2 seem to have some DNA-protecting activity particularly in cells with a fragile midzone. To test whether DNA damage was caused by cytokinesis, we tested whether the frequency of Ddc1-GFP foci decreases in cells that were defective for cytokinesis. We crossed the *boi1Δ boi2Δ GALS:HA-ASE1* into an cdc12-6 septin mutant background, which causes an abscission defect at the restrictive temperature [12]. Asynchronous cultures were shifted from galactose to glucose at
37°C and Ddc1-GFP foci were scored after four hours. Again, repression of Asc1 did not strongly increase the frequency of Ddc1 foci relative to wild type. In contrast, disruption of the BOI genes did. Depletion of Asc1 in the boi1Δ boi2Δ double mutant, further doubled the frequency of Ddc1 foci formation. Most boi1Δ boi2Δ and GAL:HA-ASE1 boi1Δ boi2Δ cells with Ddc1-GFP dots were undergoing anaphase, and the dot frequently localized to the bud neck. Such a correlation between mitosis and Ddc1-GFP foci formation was not evident in BOI+ cells. In accordance with the idea that DNA damage was due to cytokinesis, the frequency of Ddc1 foci was reduced to wild-type levels in the cdc12-6 boi1Δ boi2Δ and in the cdc12-6 GAL:HA-ASE1 boi1Δ boi2Δ strains.

These results are consistent with the idea that Boi1 and Boi2 activity in the repression of abscission serves to protect chromosomes from breakage by the cleavage machinery. Together, these data indicate that the inhibition of cytokinesis by Boi1 and Boi2 played a key role in protecting the integrity of the chromosomes in cells with central spindle defects, and thereby in maintaining them alive. Thus, Boi1 and Boi2 have the potential to protect chromosomes from damage by the cytokinetic, machinery when spindle elongation has not yet pulled all chromosomes out of the cleavage plane. The importance of this function is underlined by the observation that the triple mutant ase1Δ boi1Δ boi2Δ is inviable and the triple mutant ndc10-1 boi1Δ boi2Δ is shows extremely slow growth. The Ddc1-GFP experiments rule out the possibility that chromosomes lying in the cleavage plane sterically inhibit cytokinesis as it has been assumed before. The fact that cytokinesis resumes upon Ipl1 inactivation, and the
II Results and Discussion

demonstration that such cytokinesis events cut through the nucleus and chromatids lying in the bud neck demonstrate that neither the nuclear envelope nor chromosomes represent a hindrance for abscission.

Roles of Cdc5 and Esp1 in abscission inhibition

The fact that we could not identify a direct interaction between Ipl1 and the Boi proteins made us investigate if there are other components that could play a role in the NoCut pathway. The Polo kinase Cdc5 has been shown to play a role in cytokinesis in various organisms including budding yeast. Previous data showed that overexpression of the COOH-terminal domain of Cdc5 (cdc5ΔN), but not the corresponding polo-box mutant, resulted in a cytokinetic defect [155]. This cytokinetic defect suggested that Cdc5 inhibits cytokinesis. Thus, we asked whether Cdc5 interferes with abscission.

First we investigated whether overexpression of full length Cdc5 resulted in a cytokinetic defect and if yes what the exact nature of this defect was. To accomplish this, we used an inducible GAL:CDC5 construct (kind gift from the Kim Nasmyth lab). We found that indeed, 27% of cells induced for 15h in Galactose become bibudded (fig2-42). The difference to the bibudded cells observed that carry mutations in the kinetochore is that these daughters contain DNA, which was expected since Cdc5 plays no known role in DNA segregation. We rationalized that since the daughter cells contained a nucleus the ACE2 program is most likely not influenced in these cells. We concluded that the bibudded phenotype is due to a cytokinetic rather than a cell separation defects.

![Figure 2-42](image_url)

Cells were transformed with the GAL:CDC5 plasmid and induced in Galactose for 15h. Representative micrographs are shown. DAPI staining was used to stain the nuclei.
II Results and Discussion

To investigate the nature of this defect septins were visualized in cells overexpressing Cdc5. Like the abscission mutants described above also the bibudded \textit{GAL:}\textit{CDC5} cells accumulated misshapen septins at both necks upon 15h in Galactose (fig2-43 A). Furthermore, we analyzed whether cells that overexpress Cdc5 contract their actomyosin ring properly. The endogenously tagged Myo1-GFP described above was used as a reporter. \textit{GAL:}\textit{CDC5} cells show similar kinetics for actomyosin ring contraction relative to wild type and the other abscission mutants (fig2-43 B). Thus, actomyosin ring contraction is not impaired upon Cdc5 overexpression. Furthermore, as shown for nocodazole treated \textit{mad2}^\Delta cells, cells overexpressing Cdc5 still share a plasma membrane between the mother and both buds of bibudded cells, which was visualized using Ras2-GFP as a marker (fig2-43 C). Thus, the cytoplasm is continuous and no septum is formed between mother and either bud. We concluded that Cdc5 overexpressing cells fail to complete abscission specifically.

This being, we asked how Cdc5 inhibits abscission. We first analyzed the Cdc5 localization during the cell cycle. As already reported, Cdc5 localizes to SPBs during the whole cell cycle and to the mother bud neck in mitosis [156]. To analyze this localization pattern more precisely

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\textbf{Figure 2-43}

\textbf{A)} Septin rings were visualized using the septin Cdc12-GFP as a reporter. Arrowheads point at misshapen, split rings.

\textbf{B)} Kymographs showing actomyosin ring contraction in \textit{GAL:}\textit{CDC5} cells. Myo1-GFP was used as a reporter to visualize the actomyosin ring. The time is indicated in the x-axis.

\textbf{C)} The status of the plasma membrane was investigated for \textit{GAL:}\textit{CDC5} cells over night in Galactose expressing the plasma membrane marker Ras2-GFP as a reporter. Cells were inspected upon 12h Galactose induction. Pictures were taken at 150nm intervals along the z-axis using a spinning disk confocal microscope. Asterisks mark premitotic, open bud necks.
Results and Discussion

We filmed dividing wild type cells in which Cdc5 was tagged with GFP (YCplac111-EGFP-CDC5 with LEU2 marker kind gift of Kyong Lee) and Tub1 with CFP. In these movies and also in still pictures of these cells we observed that Cdc5 reached the bud neck at the onset of spindle elongation stayed at this site until spindle breakdown and rapidly disappeared from the bud neck afterwards (fig2-44).

Because our construct turned out to be temperature sensitive (no Cdc5 signal could be observed any more when cells were shifted to 34°C and higher) and because we wanted to investigate Cdc5 localization under endogenous levels we constructed a Cdc5-Venus fusion protein expressed from the endogenous promoter at the endogenous locus and did all following localization studies using this YFP construct.

We analyzed if any of the genes that we found to inhibit abscission interfered with Cdc5 localization to the bud neck. Interestingly, we observed only mild effects. While in WT 93% of cells with an elongating spindle Cdc5 localizes to the bud neck this number is reduced to 50% in ipl1-321 cells (fig2-45 A, B). Similarly, 45% of the boi1Δ boi2Δ double mutant cells still localize Cdc5 to the bud neck upon spindle elongation. Why this reduction is only partial is not clear to us. Clearly, Ipl1 is not crucial for Cdc5 neck-localization. It is possible that Cdc5 acts upstream of Ipl1 but that there is a feedback loop from Ipl1 to Cdc5. This would also explain the result for the other members of the abscission inhibition pathway Boi1 and Boi2.
II Results and Discussion

Figure 2-45
A) Localization of the Cdc5-venus in the indicated mutants in cells with an elongated spindle. Quantification of Cdc5 localization to the bud neck in wild type and mutant strains is shown. B) Representative micrographs of Cdc5 localization in cells of the indicated phenotype.

This result further indicated that the NoCut pathway is not a linear pathway but that there are other proteins that act in parallel branches involved. We wanted to understand which proteins might act together with Cdc5, as the abscission defect seen upon overexpression of this protein does not depend on Ipl1 or the Boi proteins.

In this perspective, a first candidate to investigate was Esp1, the yeast homologue of separase, which is responsible for cohesin cleavage. Esp1 is also a member of the newly discovered signaling network called FEAR, which modulates the organization, dynamics and stability of the spindle midzone during anaphase [53, 80, 157, 158]. This network, which comprises the non-essential proteins Slk19, Spo12 and Bns1, but also Cdc5, is activated by Esp1 upon anaphase onset and is also responsible for sending Ipl1 and Sli15 to the midzone [52].

Remarkably, despite possessing a fragile midzone, none of the FEAR mutants show noticeable cytokinetic defects. Particularly, cells lacking Esp1 activity fail to elongate their spindle but still complete cytokinesis, including abscission, with normal kinetics and efficiency [159]. Thus, we characterized the epitasis relationships between ndc10-1 and esp1-1 mutants. While zymolyase treatment resolved only a minority (14%) of the bi-budded ndc10-1 cells (see above), it resolved almost all of the bi-budded ndc10-1 esp1-1 mutant cells (96%) (fig2-45 B).

In addition, in contrast to the ndc10-1 cells, ndc10-1 esp1-1 cells formed a primary septum before to rebud (fig2-46 A).
II Results and Discussion

Thus, the activity of Esp1, like that of Ipl1, is required for the cytokinesis failure observed in ndc10-1 cells.

To investigate if Esp1 functions in the same pathway as Ipl1 and Boi1/2 for abscission inhibition, we characterized Boi1/2 localization in the esp1-1 mutant. The esp1-1 cells were arrested in G1 with α-factor and released into a new cycle at the restrictive temperature. Although Boi1-GFP first localized to the bud cortex and reached the neck in anaphase like in wild type it seemed to accumulate in the nucleus in the next cycle, starting at 120min after the release (fig2-47). What exactly this means we do not understand yet, but this result shows that Esp1 is not required for Boi1/2 localization in the same manner as Ipl1. Esp1 could act downstream of Ipl1 and Boi1 Boi2 and thereby influence the two Boi proteins only in the second cycle. Alternatively, esp1-1 cells might fail to restart a new cycle upon the end of cytokinesis.

We next investigated the localization of Cdc5 in esp1-1 cells. Using the Cdc5-Venus construct, we observed that only 9% of esp1-1 cells that move the nucleus through the bud neck do succeed to localize Cdc5 to the neck region (fig2-48).
Thus, Cdc5 and Esp1 appeared to function together, with Cdc5 activity depending on Esp1 function. Together, our data suggest that NoCut is most probably not a pathway but a network of proteins that inhibits abscission in cases of central spindle defects. For now we think that at least two pathways act in parallel to each other to control abscission. The first pathway relies on the function of Ipl1 and Boi1/2 while the second one requires Esp1 and Cdc5 activity. Inactivation of either pathway is sufficient to restore abscission. *ipl1-321* and *esp1-1* on their own have the ability to suppress the abscission failure of the *ndc10-1* cells.
Conclusions and Perspectives

General conclusions NoCut

Although it was assumed for some time that the central spindle plays a role in cytokinesis in higher eukaryotes the exact nature of this role remained elusive. One possibility was that the spindle midzone provided spatial information and/or structural support for the assembly of the abscission machinery, i.e., that it was an intrinsic player of cytokinesis. Alternatively, the cell might respond to midzone defects by preventing abscission, similar to a checkpoint response. In this second model, the midzone does not have an instructive role in cytokinesis. Rather, mutations that inactivate the sensing machinery, which detects midzone defects, would render abscission independently of midzone integrity. Supporting this model, we show that cells lacking aurora activity, or the anillin-related molecules Boi1 and Boi2, such as ndc10-1 ipl1-321, ndc10-1 boi1Δ boi2Δ and boi1Δ boi2Δ cells depleted for Ase1, do complete cytokinesis despite the absence of a functional spindle midzone. The same is also true for esp1-1 ndc10-1 cells, which means that also the separase Esp1 plays a role in abscission inhibition. Thus, the spindle midzone is not intrinsically required for cytokinesis. Instead, our results indicate that Ipl1 and the Boi proteins form an inhibitory network (which we call NoCut) that inhibits abscission in cells with midzone defects.

One branch of the NoCut pathway involves the FEAR pathway. Esp1 and Cdc5 play a role in abscission inhibition since mutations in Esp1 suppress the abscission defect in cells with spindle defect and overexpression of Cdc5 prevents abscission even in wild type cells. Manuel Mendoza in the lab also showed that inactivation of any other member of the FEAR suppresses the abscission defect of the ndc10-1 cells. Thus, our data indicate that Esp1 and Cdc5 act in abscission inhibition through the FEAR network.

The exact genetic relationship of all components shown to be involved in abscission inhibition is not clear to us yet, partly because Cdc5 localization is not completely abolished in Ipl1 mutants, although Cdc5 overexpression leads to an abscission defect. It would be interesting, to look if Ipl1 and Boi1,2 localization are changed in cells lacking Cdc5. The genetic relationships of the different NoCut components will be an interesting point to study. Which proteins act upstream or downstream will tell us a lot about NoCut development and conservation.
III Conclusions and Perspectives

What is clear already is that it is unlikely that the Ipl1 and the Esp1 branches of the NoCut simply act in parallel because deletion of a component of only one pathway is sufficient to bypass abscission inhibition. This indicates, that the two pathways converge into a single one at the level of abscission inhibition. A simple model is that both pathways converge on the same target, some abscission inhibitor at the bud neck. This target could be the Boi1/Boi2 complex, maybe together with additional factors. The exact relations and interplay between branches of the NoCut need further investigation.

Also how Boi1 and Boi2 exactly inhibit abscission on a mechanistic basis is not clear to us yet. It is possible that these two proteins play a role in membrane fusion events as they are shown to interact with Bem1, which is a protein that interacts with Cdc24 and Cdc42 and plays a role in membrane fusion [160]. Alternatively Boi1 and Boi2 could be responsible to remove proteins or specific lipids from the cleavage site, which inhibit successful membrane fusion events. boi1Δ boi2Δ cells are sick and have problems in polarized growth. However, on their own they do not show any cytokinetic defects. This fact makes it more likely that Boi1 and Boi2 rather inhibit membrane fusion when they are located to the bud neck but not, when they are not transferred to this site. One possibility is that they bind one or multiple factors involved in successful membrane fission and fusion events and that binding inhibits this factor. As a first candidate Bem2 will be tested in the lab if it plays a Boi1/Boi2 dependent role in the process of abscission.

The molecular details of how NoCut represses abscission in response to midzone defects are not clear to us yet. At this point we can only draw a rough map of the NoCut pathway.

In wild-type cells, Ipl1 localizes to kinetochores during metaphase and to midzone microtubules during anaphase, whereas in ndc10-1 cells, it localizes over the entire length of intra-nuclear microtubules. We have not found evidence that Ipl1 exits the nucleus or translocates to the site of cleavage to directly repress abscission. It most likely acts upstream in the pathway, while downstream molecules must transduce the inhibitory signal to the cell cortex. Boi1 and Boi2 appear to function in this transduction step. They localize to the cell cortex when Ipl1 is active but accumulate in the nucleus when Ipl1 is inactive, i.e., in ipl1-321 cells shifted to the restrictive condition. Thus, Boi1 and Boi2 respond to Ipl1 activity by translocating from the nucleus to the cortex, where they might directly act as abscission inhibitors, or help recruit or activate more direct inhibitor(s). Since we could not show that Ipl1 directly phosphorylates Boi1 and Boi2, there must be other kinases and/or mediator proteins.
involved. It will be interesting to investigate how Cdc5 is involved in this signalling cascade and what are its potential targets.

At this point we would like to propose the following model for NoCut function: We propose that Ipl1 activity is stimulated by the proximity of chromatin. It is possible that Ran-GTP, which in animal cells forms a gradient around chromatin, mediates this activation as we have seen in the actin overexpression studies, that some importins and exportins are likely to play a role in cytokinesis. The prediction would be that as long as high RAN levels stimulate Ipl1, the anaphase activity of Ipl1 remains high. This would be the case as long as chromatin surrounds the spindle midzone, i.e., as long as the spindle is not fully elongated. Active Ipl1 then ensures that Boi1 and Boi2 localize to the bud neck, where they inhibit abscission. Upon spindle elongation, the midzone and hence Ipl1 are segregated away from chromatin and therefore Ipl1 activity drops. Boi1 and Boi2 no longer localize to the bud neck and abscission can occur.

Whether the inactivation of Boi1 and Boi2 during telophase is triggered by dephosphorylation by Rts1/PP2A needs to be tested.

In the presence of midzone defects, Ipl1 cannot be segregated away from chromatin and therefore cannot be inactivated. This leads to inhibition of abscission. Alternatively, NoCut might simply sense defects in midzone assembly. One approach to distinguish between these models would be to artificially maintain chromatin in the vicinity of the spindle midzone despite proper spindle elongation (see more in the perspectives part).

Remarkably, midzone-defective cells also developed septin organization phenotypes very similar to those observed in *rts1A*, a known abscission mutant [12]. It was recently shown that the two septin rings present on each side of the cleavage site serve as diffusion barriers to maintain abscission factors to their site of action [22]. Thus, the abscission failure of cells with midzone defects is likely to be due to disorganization of the septin rings.
Figure 3-1
Model of the NoCut pathway
During early anaphase surrounding chromatin activates Ipl1 at the central spindle. Active Ipl1 causes Boi1 and Boi2 to translocate to the cortex, where they inhibit septin function. Abscission is repressed.

Upon segregation of the chromosomes away from the central spindle, Ipl1 is no longer kept active by chromatin. Boi1 and Boi2 leave the bud neck. Abscission can take place.

In cells with midzone defects Ipl1 stays close to chromatin even after chromosome segregation. Boi1 and Boi2 are not removed

Whether the inactivation of Boi1 and Boi2 during telophase is triggered by dephosphorylation by Rts1/PP2A needs to be tested.

In the presence of midzone defects, Ipl1 cannot be segregated away from chromatin and therefore cannot be inactivated. This leads to inhibition of abscission. Alternatively, NoCut might simply sense defects in midzone assembly. One approach to distinguish between these models would be to artificially maintain chromatin in the vicinity of the spindle midzone despite proper spindle elongation.

Biological relevance of NoCut:
Our results suggest that the NoCut pathway exists to warrant genetic stability by coordinating anaphase and cytokinesis.

The \textit{MAD2} and the \textit{BUB2} checkpoints already ensure faithful segregation of chromosomes between mother and daughter cells [97] at earlier steps in the cell cycle. Therefore, an obvious question is what is the biological function of the NoCut pathway?

A possible explanation might be that the cleavage plane must be cleared from all genetic material before cytokinesis takes place. Experiments with Ddc1-GFP indicate that a major function of the NoCut pathway is the prevention of cytokinesis in case chromatin is still engaged in the cleavage plane. In line with that, Manuel Mendoza in the lab could show that
also in Esp1 mutants the number of Ddc1 foci is increased. In these cells the spindle does not elongate because cohesin is not cleaved but still moves to the neck region. Thus, there is no inhibition of abscission in esp1-1 cells despite the presence of genetic material in the cleavage plane. The consistent role of in the two branches of NoCut in protecting chromatin from the cleavage machinery underlines the importance of the NoCut pathway in this process.

NoCut as we see it now shows many similarities to cell cycle checkpoints: it represses a cell cycle event, cytokinesis, in response to defects in a previous step, anaphase. This repression prevents a catastrophic division and helps the cell to remain viable. However, unlike classical checkpoints, NoCut does not lead to a cell cycle arrest.

Conservation of NoCut:
One of the most important questions is of course whether this pathway is conserved among species or whether it is a special feature of yeast cells.
We think that at least among yeast conservation is likely:
It is known for a long time that so-called cut mutants in the fission yeast S. pombe undergo cytokinesis despite of the nucleus still being entangled in the cleavage plane [161]. The conclusion classically drawn from these results is that cytokinesis and chromosome segregation are not coordinated with each other. The results presented in this study offer a potentially different interpretation. Namely, that the pombe “cut” mutants disrupt both, spindle function/elongation and the NoCut pathway. esp1-1 and pds1A mutants are examples for such a situation in S.cerevisiae. Remarkably, the S.pombe orthologues of ESP1 and PDS1 are cut1 and cut2. Similarly, cut17 encodes a regulatory subunit of aurora kinase Ark1 and Cut17 mutants display a cut phenotype. Thus, re-examining the function of other cut-genes in S.pombe might identify more members of the NoCut pathway also in S.cerevisiae and shed light into how NoCut monitors spindle defects in anaphase.

At least the function of Separase in the prevention of chromosomal damage seems to be further conserved beyond budding and fission yeast. Separase mutants in Drosophila and C. elegans do not undergo spindle elongation yet complete cytokinesis normally. As a result, the cleavage machinery cuts through the unsegregated chromosomes and mutant cells display DNA “cuts” upon cytokinesis [162, 163]. Further, the role of central spindle components in abscission inhibition might be evolutionary conserved. Multiple studies have shown that the spindle midzone is required for cytokinesis in animal cells, yet the exact reason for this requirement
remained unclear [84, 164-166]. These findings underline the possibility that the NoCut pathway is conserved through evolution although molecular details might differ.

There are hints that a NoCut pathway exists also in animal cells, which prevents cytokinesis to not damage any genetic material that erroneously remains in the cleavage plane. It has been shown that PtK1 cells with merotelic attachment stay longer in telophase and have a delayed cytokinesis [108]. Similarly, several studies have already established that DNA bridges, i.e., incomplete separation of sister chromatids, cause abscission to fail and the furrow to regress in animal cells [60, 61, 112, 167]. This suggests that also animal cells possess a mechanism to inhibit cytokinesis in order to protect the chromosomes from being damaged by the cleavage machinery. It also suggests that the NoCut pathway might monitor the presence of DNA in the midzone, rather than midzone integrity itself.

It is established that Aurora-B homologues are required for cytokinesis in higher eukaryotes [168-170]. In contrast we and others show that Ipl1 is not essential for yeast cytokinesis [54] but instead is required for the inhibition of abscission. It is possible that these differences are due to different functions of aurora kinases in evolutionarily distant organisms. However, we believe that present evidence does not rule out a role of Aurora kinases in NoCut pathway in higher eukaryotes. It is possible that a role of Aurora B in the inhibition of abscission is masked by its earlier role in promoting furrow ingression. Indeed, abscission cannot take place in animal cells if the furrow does not first ingress. Alternatively, a different Aurora protein might take over the NoCut function. A possible candidate for this would be Aurora A. Aurora A is not required for cytokinesis but its overexpression leads to the failure of abscission, the regression of the furrow and multinucleate cells (as reviewed in [171]. This suggests that Aurora A might act in the repression of abscission.

To differentiate between these two possibilities one could test Sli15 mutants in S.cerevisiae. Sli15 is the S.cerevisiae homologue of INCENP and the activating unit of Ipl1. If Sli15 mutants were able to suppress inhibition of abscission, this would suggest that an AuroraB like function of Ipl1 is responsible for abscission inhibition. If there is no repression of abscission inhibition in Sli15 mutants the abscission inhibition could be more an AuroraA feature of Ipl1. Preliminary experiments suggest that also sli15-3 mutants are able to suppress the inhibition of abscission in ndc10-1 cells. Abscission inhibition therefore seems to depend on an AuroraB-like function of Ipl1. However, a role of AuroraA-like functions in abscission inhibition cannot be excluded by this experiment.
III Conclusions and Perspectives

A conservation of abscission inhibition functions of AuroraB or AuroraA need to be shown in higher eukaryotes to find out if this function is also relevant in other organisms than yeast.

Perspectives

In this thesis we addressed the question of how cytokinesis is spatially and temporally regulated in the budding yeast *S. cerevisiae*. We found that spatial regulation through the position of the spindle is not essential for cytokinesis onset. However, some control might still be provided by the position of the nucleus, although we do not understand yet how this works and what the function might be in a regular cell cycle. Our main progress resides in the understanding of the temporal control of the last step of cytokinesis, abscission, and which factors are involved in its control. For the first time, our study shows how the central spindle regulates cytokinesis (or more precisely abscission). However, we are far away from a complete understanding of the NoCut pathway. More studies have to be done to fully grasp the complexity of cytokinesis and its regulation and to evaluate whether NoCut is conserved in higher eukaryotes. As a first step towards a better understanding of cytokinesis and abscission we need a more elaborate understanding of the molecular components of the three cytokinetic pathways in budding yeast. Extensive synthetic lethal screens using *myo1Δ*, *hof1Δ* and *cyk3Δ* strains as bait strains are ongoing in the lab. They will lead to a more complete list of the proteins involved in cytokinesis in budding yeast and will probably also give us hints for homologies with higher eukaryotes. It will be interesting to see which proteins act in which pathway and are only co-lethal with two of the bait mutation and which proteins act upstream in cytokinesis and are co-lethal with mutations from all three pathways. The ideal outcome would be to draw a complete map of all three pathways and their connections. This could also answer the questions of whether the Cyk3 pathway is really linked to septin function, as assumed but not tested yet, and whether this pathway does specifically act in abscission. Another interesting question that will be solved with such screens is whether more importins and exportins do play a role in cytokinesis and in which pathway they act. This could give a totally new perspective to our understanding of nuclear-cytokinetic communication. As I mentioned briefly in the actin overexpression results part, I could show that *nmd5Δ* cells, which influence actin ring formation upon overexpression, have a defect in septin localization. This septin phenotype looks a lot like the septin phenotype seen in abscission mutants. One obvious experimental approach resulting from this would be to investigate if RAN-pathway mutants that can be sorted specifically in the Cyk3 pathway of cytokinesis have a defect in abscission. The identification of importins and exportins acting in the Cyk3 pathway could provide
elements to test the idea according to which Ran-GTP stimulates Ipl1 to prevent abscission when chromatin still lies in the vicinity of the midzone.

One caveat of a synthetic lethal screen is that it cannot address the role of essential genes. However, the complete output of the screens will probably also give us indications about which essential genes we should test.

For a more complete understanding of the NoCut pathway the open questions are the following:

1. What are the exact molecular mechanisms of abscission inhibition?
2. What is the nature of the defect that is sensed?
3. How is it sensed, what is the mechanism?
4. How is the signal transduced from the midzone to the cleavage machinery?
5. What is the timing of this process?

Our hypothesis so far is, that genetic material that is left near the cleavage plane is what triggers the activation of NoCut. If we want to test this hypothesis in a more direct manner we have to artificially create a situation in which genetic material is left in the vicinity of the cleavage plane even after full spindle elongation.

One possibility to do this would be to create a hyperlong chromosome. The experimental setup would be the following: we would use the Cre-Lox system to fuse parts of chromosome XII with parts of chromosome IV. These two chromosomes are the longest found in yeast and this would generate a chromosome much longer than any of the wild type chromosomes (fig3-2 left graph). The first question would be how the cell deals with such a long chromosome. A possible solution could be that the cell elongates its spindle further to move the artificial chromosome away from the cleavage plane. Alternatively, the cell might condensate the chromosome more tightly to ensure that normal spindle elongation would be sufficient to pull it to the poles. If both attempts fail the cell would be left with a very long chromosome that cannot be pulled away from the cleavage plane. In this case, it will be interesting to see if this leads to a cytokinetic defect and, more specifically, to a failure of abscission (fig3-2 right graph). If this were the case, one would predict that a $boi1\Delta boi2\Delta$ double disruption would lead to specific cleavage of this long chromosome arm by the cytokinetic machinery, and might lead to cell death. Even if cells do not die the Ddc1-GFP essay should tell us if there is an increase in chromosome breaks compared to cells that do not carry the long chromosome in the $boi1\Delta boi2\Delta$ background.
Another experiment that would strengthen our argument would be to show that NoCut mutants have an increased rate of genomic instability. It is possible to place markers in subtelomeric regions of different chromosomes. When grown under regular conditions the boi1Δ boi2Δ strain should have a higher rate of marker loss than WT strains. In this line it will be interesting to see if this frequency of marker loss also correlates to the lengths of the tagged chromosomes. Longer chromosomes should become cut more frequently than shorter ones. Additionally one would of course have to show that these cuts are due to the cytokinetic machinery. This could be shown using the cdc12-6 mutation as done before in the Ddc1-GFP essay.

An encouraging hint in this direction is that boi1Δboi2Δ cells tend to lose their knockout markers. This phenomenon is also seen for mad2Δ cells for which it is known that they feature genetic instability due to increased non-disjunction.

If the boi1Δ boi2Δ strain shows to be genetically unstable, this would further underline the biological relevance of the NoCut pathway. Furthermore, it would show that there is a need for the cells to coordinate cytokinetic events with mechanical chromosome segregation to prevent the genome to be damaged by the cleavage machinery.

We have shown that Rts1 plays a role in abscission. However, how exactly it regulates or positively influences this event we could not answer yet. Rts1 is a subunit of the PP2A phosphatase, which raises the possibility that Rts1 dephosphorylates Boi1 and Boi2 at the neck right after spindle breakdown and thereby inactivates it. The localization pattern of the three proteins fits this assumption. Rts1 gets to the neck at the same time at which Boi1 and Boi2 start to disappear from this site. It will be interesting to test by 1D and if necessary also 2D gel electrophoresis if there is really a difference of the Boi1/2 phosphorylation pattern in an rts1Δ strain. This lack of dephosphorylation would, following our hypothesis, prevent the
deactivation of Boi1/Boi2 and cause a prolonged inhibition of abscission. This might explain why $\textit{rts1} \Delta$ cells fail to complete abscission. Also the exact role of Cdc5 in abscission needs to be investigated. It should be investigated if Cdc5 shows any Rts1-dependent changes in its phosphorylation state.

If a dephosphorylation of Boi1/2 or Cdc5 by Rts1 could be shown this would get us one step further in the understanding of NoCut regulation.

An interesting observation was that the abscission defect in $\textit{ndc10-1}$ cells is much more severe than in $\textit{ase1} \Delta$ cells. As shown by the time-lapse experiments, $\textit{ase1} \Delta$ cells show only a delay in abscission while $\textit{ndc10-1}$ cell do not undergo abscission at all at the restrictive temperature. We and others showed that the spindle defect in $\textit{ndc10-1}$ cells is not more severe than in $\textit{ase1} \Delta$ cells [55]. Thus, the only difference between the two strains is that $\textit{ndc10-1}$ cells also exhibit severe kinetochore defects. The kinetochore is most probably intact in $\textit{ase1} \Delta$ cells.

Another observation we made was that the $\textit{GAL:HA-ASE1}$ cells show a rapid adaptation to the abscission defect within a few passages. Following these observations we hypothesized that kinetochores are responsible for adaptation to the NoCut pathway. If this were correct, adaptation would not work any more in $\textit{ndc10-1}$ and $\textit{ndc80-1}$ cells. This is a possible reason why abscission is not delayed but abrogated in these mutants.

Two pieces of data strengthen this hypothesis: A) $\textit{rts1} \Delta$ cells - which have an abscission defect on their own - are derepressed in an $\textit{ipl1-321}$ background. The double mutant has no abscission defect because there is no NoCut induction, which would need Ipl1. Therefore Rts1 is no longer needed for the reversion of NoCut, as there is no need for adaptation. Furthermore, Rts1 is regulated from the kinetochore, which means that the possible Rst1-dependent adaptation to NoCut is a probably a kinetochore-dependent pathway.

B) An $\textit{ase1} \Delta$ synthetic lethal screen done in the lab shows that the Ase1 deletion is co-lethal with the Rts1 deletion (Harald Rauter personal communication). A preliminary assessment of the phenotype revealed that indeed a high fraction of the cell dies bibudded, which resembles the $\textit{ndc10-1}$ phenotype. A detailed analysis of a $\textit{GAL:HA-ASE1} \textit{rts1} \Delta$ strain should be conducted to verify this first observation. It needs to be shown that these cells really exhibit a non-repressed abscission defect. If this assumption were correct the NoCut pathway would contain an additional branch (connected to the kinetochore) responsible for the adaptation mechanism to this checkpoint.
Several approaches could be taken to screen for more members of the NoCut pathway. One of them would be to look at homologues of *S. pombe* Cut-genes in *S. cerevisiae*. We already found a couple of pombe Cut genes in the cerevisiae NoCut pathway like Esp1 (cut1) and Pds1 (cut2). Also the Bir1 homologue in *S. pombe* resembles a Cut gene, cut17. This indicates that NoCut is conserved at least between *S. cerevisiae* and *S. pombe*. Further inspection of other *S. pombe* cut mutant homologues in *S. cerevisiae* might be a convenient tool to identify more members of this pathway. The study of homologues of *S. pombe* Cut genes might help to uncover missing links to understand how a lack of spindle elongation or central spindle perturbation is detected and how exactly this leads to abscission inhibition on a mechanistic level. If some of these genes are members of a NoCut pathway in fission yeast and their function is conserved, these mutants should repress the abscission inhibition phenotype in, *ndc10-1* cells as seen for *ipl1-321* and *boi1Δ boi2Δ* mutants. Genes that can be confirmed to play a role in NoCut can then be analyzed for Boi1 and Cdc5 localization to sort them in one of the two NoCut branches. Furthermore, if the long chromosome assay works this would be a good tool to see if other NoCut mutants induce chromosome breakage. This has to be tested first for the known members, like Ipl1 and the Boi proteins but afterwards this assay could also serve as readout for other NoCut mutants.

Another approach to find more members of the NoCut pathway will be to carefully inspect the genes that were found in the *ase1Δ* synthetic lethal screen. The case of the *boi1Δ boi2Δ* mutant strongly indicated that mutations in proteins that are needed for the NoCut pathway are synthetic lethal with Ase1. A careful analysis of gene candidates that were found in the screen in the *GAL:HA-ASE1* background could lead to a more complete understanding of the NoCut pathway. The Ddc1-GFP assay could be used as a first read-out to evaluate if the mutants lead to an increase in double strand breaks which is a prerequisite to be a pathway member.

As discussed in the conclusions part, there are indications that also higher eukaryotes contain at least parts of the NoCut pathway. A) Multiple studies have shown that the spindle midzone is required for cytokinesis yet it remained unclear what the exact reason for this is [172]. We think that what is observed in these cells could be a secondary effect of the checkpoint response. B) Studies in *Drosophila* and *C. elegans* have shown that mutations in the respective separase mutants lead to DNA cuts as shown in this study for *S. cerevisiae* [162, 163]. It is very likely that these cuts have the same origin as in our study, namely a perturbation in the NoCut pathway. A further report by Hauf et al. shows that when noncleavable cohesin is expressed in human cells, separase is most probably activated but cannot cleave cohesin subunits. One
consequence of this is that the cytokinetic machinery frequently cuts the genetic material. One consequence of this is a high percentage of aneuploid cells. Some cells however, did not complete cytokinesis but induced furrow regression, which would speak for a NoCut pathway also in higher eukaryotic cells [173]. Also of relevance in this context is the recent work of Shi et al. [174]. These authors show that chromosome nondisjunction in diploid human keratinocytes immortalized with telomerase as well as in Hela cells leads to the formation of tetraploid cells. This is due to furrow regression upon the onset of cytokinesis. However, the authors claim that this furrow regression is not only due to genetic material lying in the vicinity of the cleavage machinery because only in 50% of cells that regressed their furrow genetic material was seen at the cleavage site. Furthermore, 15% of cells that do contain genetic material at the cleavage plane undergo cytokinesis correctly. One has to note though that these are studies that were carried out in cultured cells. These results do not exclude the possibility that genetic material at the site of cleavage activates a NoCut pathway also in these cells as seen in the majority of cells that contain genetic material at the cleavage plane. It is well possible that the 15% of cells that cleave through the genetic material accumulated additional mutations. Another explanation could be that it is indeed not genetic material per se that gives the signal for NoCut activation but that also the kinetochore is involved in the signalling.

A further investigation of homologies of NoCut in other yeast and higher eukaryotes will tell us how conserved the NoCut pathway really is and what impact it has on the cells. To find out about the conservation and homologies of NoCut in higher eukaryotes collaborations with labs that work in other organisms would be desirable.

Already the situation in *S. cerevisiae* shows that the cytokinetic machinery itself can be a danger to genetic material. If the last step of cytokinesis is not well controlled this can lead to genetic instability and in higher eukaryotes probably also to cancer development. If the NoCut really is conserved this would change our view of the cytokinetic apparatus and provide a new target in cancer detection and therapy. Inherited defects in NoCut genes could be a susceptibility for the development of certain cancers but might be easier to detect and treat if we know about the molecular details of the NoCut pathway from yeast to human.
**Material and Methods**

**Strains, plasmids and growth conditions:**

Strains were derived mainly from W303 or S288C parental stocks as a wild type served YYB384 (S288C) (MATa his3-A200 ura3-52 trp1-A63 leu2 lys2-801 ade2-101).

The *bmr1::HIS3* disruption as well as the *cdc12-1* and *cdc34-2* mutations were described [124, 175, 176]. The same is true for the 3Xmyc-CDC3 strain [95].

Also the *ndc10-1*, *mad2Δ*, *cnm67Δ*, *ACE2-1*, *nnf1-17*, *ASE1-GFP*, *SLK19-GFP*, *ipl1-321*, *DDC1-GFP*, *cdc12-6*, *esp1-1* strains have been described [19, 50, 69, 140, 144, 177-179].

*hof1::HIS3*, *dhc1::HIS* and *myo1::HIS3* were generated using the gene disruption cassette [180].

The *SPA2::GFP*, *HOF1::GFP*, *MYO1::GFP*, *BOI1::GFP* and *SPC42-CFP* insertions were generated by inserting the GFP- respectively CFP-cassette [180] on the chromosomal locus using PCR-tagging techniques and homologous recombination [181, 182]. Homologous sequences for recombination were contained in the primers used for amplification of the insertion cassette, as described. Insertion replaced the stop codon of the *SPA2*, *HOF1*, *RTSI* or *MYO1* genes by the coding sequence of GFP, which contained its own stop.

The overexpression *GAL-CDC5-HA:URA* strain was a kind gift of the Nasmyth lab and is published [183]. The Cdc5-GFP was tagged on YCplac111-EGFP-CDC5 with a LEU2 marker (kind gift from Kyong Lee). The Cdc5-Venus YFP strain was created using the plasmid pBES2 and the protocol published in [184].

The importin and exportin mutants were a kind gift of Marcus Künzler, ETH Zurich [185-187].

Strains disrupted for *KAR9*, *BUD6*, *BOI1*, *BOI2*, *RVS161*, *RVS167*, *VRP1*, *NIP100* and *JMN1*, *RTSI* were obtained from Euroscarf.

The Ipl1-GFP plasmid is a 3’ GFP fusion pSB706. Boi2-GFP is integrated on a YEp24 2µ plasmid (kind gift of Eric Bailly). The GFP-CDC12 plasmid is published [12]. The RAS2-GFP fusion protein under control of the *GPD* promoter on a 2µ *TRP1* marked plasmid is described (pJW192 [188]. The CFP-Tub1 integrative plasmid has been described [12]. CFP-Ndc80 was a kind gift from the Sorger lab. This is an integrative plasmid.

YPD (yeast extract, peptone, glucose) and SD (synthetic dextrose) media were prepared as described. YPGal (yeast extract, peptone, galactose) and SGal (synthetic galactose) contain 2% galactose instead of glucose. Ssuc (synthetic sucrose) contains 2% sucrose instead of glucose.
Standard genetic methods were used for crosses and tetrad analysis. Yeast transformations were done using a short version of the lithium acetate procedure. Other yeast media and standard genetic techniques were performed as described (Guthrie and Fink 1991). All strains where grown at room temperature (24°C) unless stated otherwise.

**Actin over-expression**
The plasmid pYB49 was isolated from the cDNA library constructed by Liu et al. [113]. This plasmid is derived from the pRS series [189] and contains ARS and CEN sequences to ensure replication and segregation in yeast and the URA3 gene for selection. The ACT1 cDNA is placed under the control of the GAL1-10 promoter, allowing over-expression in galactose medium and repression by glucose. The insert was sequenced on both strands to ensure its identity to the ACT1 genomic sequence. Sequencing revealed that the cDNA cloned corresponded to a spliced variant of the ACT1 coding sequence.

Induction of actin over-expression was achieved as follows: Yeast strains were first grown overnight in sucrose medium to terminate glucose repression. Cells of saturated sucrose cultures, which were at least 90% unbudded, were subsequently centrifuged down and re-suspended in galactose medium for induction. In the case of the temperature sensitive strains, the sucrose culture was grown at room temperature (permissive temperature), while the induction of actin over-expression was achieved at permissive, semi-restrictive (30°C), and restrictive (37°C) temperatures, as indicated in the text and figure legends. For the survival test cells were grown overnight in sucrose then diluted and grown additional 10h in sucrose respectively galactose. After 10h cells were diluted to an OD of about 0.1 (0.128 for sucrose and 0.137 for galactose) and plated as a dilution series of 1, 1:10, 1:100 and 1:1000.

**Visualization of actin, staining procedures, nocodazole and zymolyase treatment**
For actin staining and zymolyase treatments cells were fixed in 3.7% formaldehyde final in the medium for 20min-45min. Cells were then washed twice with PBS to remove remaining formaldehyde before the staining procedure/the digest. For all other fixations cells were resuspended in 70% ethanol for 10min-30min. Afterwards cells were washed in PBS. Zymolyase was added at a final concentration of 20 µg/ml in 1M Sorbitol to cells for 30min at room temperature to digest the cell wall. Afterwards the percentage of separable cells was inspected by light microscopy.
For all experiments that used synchronizes cultures cells were arrested with α factor at a final concentration of 10µg/ml for 2h at room temperature. The cells were then washed twice in YPD and released at the restrictive temperature (for temperature sensitive mutants) or at 24°C. To stain nuclei we performed DAPI staining. Cells were fixed for 30min in 70% ethanol, washed in PBS, and resuspended in PBS containing 1 µg/ml DAPI.

Actin staining was carried out using a 1:100 dilution of rhodamine phalloidin (suspended in methanol) in PBS for 30min to 45min to inspect actin cable and using a 1:10 dilution in PBS over night to inspect all actin structures. After the staining procedure cells were washed twice in PBS.

For microtubule disruption, nocodazole was added to the cultures at a concentration of 50µg/ml. The nocodazole was left in the cultures during the whole time of the experiments. For chitin staining, 0.01 mg/ml Calcofluor Fluorescence Brightener 28 (SIGMA) was added to the cells, which were then incubated 5min on ice and washed 3 to 5 times in PBS before microscopic inspection.

**FACS measurements**

To analyze DNA distribution by flow cytometry cells were fixed in 70% ethanol over night at 4°C, washed once with 0.2M Tril-HCL (pH7.5) containing 20mM EDTA, and resuspended in the same buffer. Afterwards cells were treated with RNAsel (1mg/ml) for 4h at 37°C, washed once with PBS, resuspended in 0.1ml of propidium iodide solution (50µg/ml in PBS), and incubated over night at 4°C. The cell suspension was diluted ten times with PBS and sonicated before analysis by flow cytometry. To do these experiments a BD Bioscience FACS Calibre device was used.

**Western Blots**

*Quantification of actin expression level*

Wild type cells carrying the plasmid pYB49 were induced for actin over-expression on galactose medium. Time points were taken at 4 and 8 hours. The cells were broken in sample buffer using glass beads. Protein samples were separated by SDS-PAGE and the gels were subsequently stained with comassie blue to assess the total amount of protein present in each lysate. New gels were run to have equal amount of protein extract in each lane, transferred onto PVDF membrane and the relative amount of actin monomers was estimated by immunoblotting. As a primary antibody against actin we used AC40, a monoclonal anti-actin
mouse ascites fluid (Sigma). The secondary antibody was an HRP-coupled anti mouse antibody (Santa Cruz Biotechnology). The immunoblot was detected by chemiluminescence using the ECL kit (Amersham).

**GALS:ASE1 Time course experiments:**
Samples for extract preparation were collected at the indicated times after the release of cells into fresh media containing glucose or galactose. Extracts were prepared using alkaline lysis and TCA precipitation as described [190]. Protein concentration was measured by the Bio-Rad protein assay and equal amounts were loaded on SDS gels. Gels were transferred on to polyvinylidene difluoride (PVDF) membrane, and the relative amount of Ase1 was estimated by immunoblotting using α-HA antibodies (SIGMA). Detection was carried out by chemiluminescence using the ECL kit (Amersham).

**Microscopy techniques**
Cells containing tagged proteins were grown overnight on YPD plates at room temperature. YPD plates were used because the signal to noise ratio was high compared to selective medium.

To visualize labeled proteins and inspect their localization *in vivo* we used an Olympus BX50 fluorescence microscope, a monochromator Polychrom IV as light source and a high-speed CCD camera (Imago, TillPhotonics). Additionally, a piezo motor, and the TILLVision software (TILL photonics, Martinsried, Germany) were used. For the localization of GFP-, YFP-, and CFP-labeled proteins, Z stacks of 6–11 layers (step size 0.3–0.4μm) were taken with appropriate exposure times and projected into a single plane.

The Myo1-GFP movies were taken using a Chroma long pass GFP filter set. For the Ras2-GFP Spc42-CFP movies a Chroma long pass CFP filter was used. The Ras2-GFP signal was strong enough to leak through into this filter set.

Two color movies were performed using a Chroma CFP/YFP dual band filter. In both cases, Z stacks of varying distances and acquisitions between 500ms and 1000ms for each channel were taken at 30s or 45s intervals.

For time-lapse acquisition, yeast cells were mounted on 1.6 - 2% high quality agarose pads. A mixture of agarose with non-fluorescent medium (NFM) [191] was boiled and hot agarose solution was put on pre-heated microscope slides. These slides were always prepared the day of the experiment with new solutions.
IV Material and Methods

For time lapse imaging of temperature sensitive mutants a heating device consisting of a lens-heater and a heating-table was used.

Acquisition of Z-stacks for visualization of the plasma membrane at the bud neck was carried out on a Zeiss 200M spinning disk confocal microscope. Pictures were taken at 150nm intervals throughout the cells.

After acquiring the data all images were converted to TIFF-format. Image editing was done using Adobe Photoshop or ImageJ.
References

References

proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. Mol Biol Cell 16, 3187-3199.


V References


References

Appendices

Appendix A: Abbreviation list

APC: anaphase promoting complex
CDK: cyclin dependent kinases
CFP: cyan fluorescent protein
CHO: Chinese hamster ovary
DAPI: 4',6-Diamidino-2-phenylindole
DSB: double strand break
FEAR: (cdc)-fourteen early release
Fig: figure
GAP: GTP activating factor
GEF: GDP exchange factor
GFP: green fluorescent protein
MEN: mitotic exit network
NES: nuclear export signal
PH-domain: pleckstrin homology domain
PP2A: Protein Phosphatase 2A
RNAi: RNA interference
SIN: septation initiation network
SPB: spindle pole body
WT: wild type
Appendix B: Kinetochores

Kinetochores
Some central spindle components are also kinetochore proteins (like Ndc10 and Ndc80) or localize to kinetochores (like Slk19). On the other hand, the chromosomal passenger complex localizes to kinetochores before it reaches the central spindle. This means that the kinetochore plays a role in cytokinesis although this role seems to be indirect. It serves more as an attachment site for various cytokinetic proteins during the cell cycle. Because this attachment is often essential for cytokinetic proteins to fulfill their function I will describe this structure here in brief.

Kinetochores structure:
The kinetochore is a proteinaceous structure that assembles on centromeric DNA. Kinetochores are the cellular substructures that link chromosomes and microtubules. Some kinetochore proteins also localize to the central spindle at some points of the cell cycle as described above.

The general assembly of this structure is conserved from yeast to human although their exact molecular components differ between organisms. I will concentrate on the yeast kinetochore.

The chromatin at the kinetochore region contains a special histone H3 variant called CENP-A in vertebrates and Cse4 in budding yeast. Although it is known that this H3 variant is not sufficient to initiate kinetochore assembly the possibility that this specialized centromeric H3 epigenetically marks the centromere is still discussed.

In S. cerevisiae centromeric DNA has three conserved elements: CDE I, II and III.

To the centromeric DNA binds the kinetochore. This structure is subdivided into three main substructures, the inner kinetochore, the central kinetochore and the outer kinetochore.

The inner kinetochore consists of the Cbf3 and the Cbf1 complex. Cbf1 itself is not essential although deletion of this gene leads to some chromosome loss. The CBF3 complex on the other hand consists of four essential proteins: Ndc10, Ctf13, Cep3 and Skp1. This complex is required for the assembly of all other kinetochore proteins and disruption of the Cbf3 complex leads to a complete lack of microtubule kinetochore attachment.

The central kinetochore contains the Ctf19 complex, the Ctf3 complex, the Mtw1 complex and the Ndc80 complex. Furthermore, some proteins that are less defined in terms of their physical
interaction and neither bind microtubules nor centromeric DNA are also assigned to the central kinetochore. These are Mcm19, Slk19, Bir1 and Chl4.

Mutations in any component of the Ndc80 complex result in complete detachment of chromosomes from microtubules. It was suggested that this complex plays a role in bridging DNA and microtubule-binding components.

The outer kinetochore is consists of the Dam1 complex, the microtubule associated motors Cin8, Kar3 and Kip3, the XMAP215 homologue and microtubule destabilizer Stu2, the plus end tracking MAP Bik1 and the chromosomal passenger proteins Ipl1 and Sli15. Except for the Dam1 complex the exact formation of this part of the kinetochore is not clear. All members of the Dam1 complex are essential. It binds to microtubules \textit{in vitro} and localizes to kinetochores and spindle microtubules. It is assumed that the Dam1 complex is required for the maintenance of bipolar microtubule attachment but not for microtubule capture.

Suppl. Fig1

Current model of the yeast kinetochore sub-complexes divided in inner, central and outer kinetochore. Adapted from Westermann et al. JCB 2003

\textbf{Kinetochore microtubule attachment:}

The most critical function of the kinetochore obviously is to connect chromosomes to microtubules.

One difference between higher eukaryotes and yeast is that in \textit{S. cerevisiae} the outer plate of the kinetochore only attaches to a single kinetochore microtubule whilst a vertebrate kinetochore has up to 20 end-on attachment sites for kinetochore microtubules. The reason for this difference is probably that \textit{S. cerevisiae} undergoes a closed mitosis and it is thought that kinetochores are bound to the spindle throughout the whole cycle. Capture of microtubules in
this organism is probably only necessary after centromere duplication when new kinetochores are formed.

Kinetochoore microtubule attachment is described by a “search and capture” mechanism. Microtubules show dynamic instability and establishments of kinetochore microtubule bindings are stochastic events. When kinetochore and microtubule reached a stable attachment the mono-oriented chromosome oscillates to achieve bi-polar attachment by binding to microtubules emanating from the opposite spindle pole. Successful bi-orientation then generates tension across sister chromatids. Once all chromosomes underwent proper microtubule attachment the segregation of chromosomes proceeds and cells enter anaphase.

The process that leads to mature attachment is rather complex. Multiple proteins are involved in kinetochore microtubule attachment and mutations in different microtubule attachment factors give rise to different phenotypes. These phenotypes are generally divided into three classes: Class I mutants like ndc10-1 and ndc80-1 completely lack kinetochore microtubule attachment. Class II mutants like dam1-1 and ipl1-321 lead to an enhancement of monopolar (only one kinetochore is bound to microtubules) or synthetic (both sister chromatids attach to the same spindle pole) attachment. Class III mutants like stu2-10 are able to create bipolar attachment but fail to present tension across sister chromatids.

If not all chromosomes succeeded to form bipolar attachment the cell can present a mechanism to correct this mistake: the spindle assembly checkpoint, which is described in the introduction. AuroraB kinase and INCENP play a role in targeting checkpoint proteins to the kinetochore. Furthermore, at least in yeast, these proteins play a role in delaying the cycle in cases when tension across sister chromatids is not achieved.
Another protein that affects spindle stability in budding yeast is Stu2. This protein is an XMAP215 homologue and effects microtubule stability. Other than its homologues in higher eukaryotes in budding yeast this protein is a plus end bound microtubule destabilizer.

A first observation of Marc van Breugel in the Hyman lab showed that stu2-10 cells accumulate as unbudded cells with a non-elongated spindle in the mother cell. In these cells all DNA stays in the mother (suppl. Fig 2). To differentiate between a separation and a cytokinetic defect we used the described zymolyase assay. Also after zymolyase digestion unbudded cells can be observed. This argues for a cytokinetic defect (suppl. Fig 2 right pict)

We next wondered if the observed defect was in abscission as observed with the other spindle mutants. Cells were tagged with Myo1-GFP and ring contraction was visualized. In stu2-10 cells the actomyosin ring contracted with normal kinetics (suppl. Fig 3 lower panel).

We furthermore studied septin localization in the stu2-10 mutant. Interestingly, although septins do stay at both mother-bud necks and are not degraded as they are in wild type cells, they are not as misshapen as in the other abscission mutants (suppl. Fig3 top panel). What exactly this impairs is not clear to us yet.

To visualize the abscission defect directly we scored Ras2-GFP in stu2-10 cells. Preliminary data without using the spinning disk confocal microscope suggest that no septum forms between mother and daughters in these cells (suppl. Fig3 middle panel). This data together suggests that also stu2-10 exhibit an abscission defect upon longer times at the restrictive temperature.
Septin rings were visualized using the septin Cdc12-GFP as a reporter.

The status of the plasma membrane was investigated for *stu2-10* cells expressing the plasma membrane marker Ras2-GFP as a reporter. Cells were inspected upon 4h at 37°C. Pictures were at the Olympus Kymographs showing actomyosin ring contraction in *stu2-10* cells. Myo1-GFP was used as a reporter to visualize the actomyosin ring. The time is indicated in the x-axis.

An interesting observation was that Stu2 seems to act in the Cyk3/Abscission pathway of cytokinesis. The *stu2-10* mutation is co-lethal with Myo1 deletion as well as with Hof1 deletion. In contrary it is not co-lethal with a Cyk3 deletion. An exact rule of Stu2 in abscission needs to be investigated.

Our results using the *stu2-10* cells raise further evidence that central spindle defects lead to an aberrant abscission. It will be interesting to test if the abscission defect in *stu2-10* cells can also be bypassed by mutations in NoCut components and to check if the *stu2-10* mutation is co-lethal or sick with the *boi1Δ boi2Δ* double mutant.
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**Extra Curricular Activities**

01/2002-01/2004  PhD-representative in the Departementskonferenz of the Department of Biology (ETH)

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11/2002-11/2003  Student representative in the steering committee of the joined PhD program of the University and the ETH in Zürich
Publications

**Norden, C.**, Mendoza M., Dobbelaere, J., Kotwaliwale, C, Biggins, S. and Y. Barral,
"The NoCut pathway links abscission to proper function of the spindle midzone and prevents chromosome breakage by the cytokinetic machinery", Cell (In Press)

Chang, MC, Wisco D, Ewers H, **Norden C**, Winckler B
"Inhibition of sphingolipid synthesis affects kinetics but not fidelity of L1/NgCAM transport along direct but not transcytotic axonal pathways", Mol Cell Neurosci. 2005


Meetings and Conferences

2005  
Cell and Molecular Biology of Cancer, Lausanne, Switzerland, January  
*Abstract title: A novel cell cycle checkpoint prevents abscission and cytokinesis in response to spindle damage in budding yeast*

USGEB Meeting: Molecules to Mind, Zurich, Switzerland, February  
*Datablitz and Abstract title: A novel cell cycle checkpoint prevents abscission and cytokinesis in response to spindle damage in budding yeast*

2004  
FEBS Special Meeting on Cytoskeletal Dynamics, Helsinki, Finland, June  
*Abstract title: Studies of late cytokinetic events in S.cerevisiae*

ASCB 2004 Summer Meeting on Cytokinesis, Vermont, USA, July  
*Abstract title: Studies of late cytokinetic events in S.cerevisiae*

2003  
21rst International Conference on Yeast Genetics and Molecular Biology, Gothenburg, Sweden, July  
*Abstract title: Is there a role for communication between the nucleus and the cleavage apparatus in the assembly, maturation and/or function of the cytokinetic machinery?*

3rd PhD Summer School, Department of Biology, Les Cotes, Switzerland, August
VII Curriculum vitae

Abstract title: Is there a role for communication between the nucleus and the cleavage apparatus in the assembly, maturation and/or function of the cytokinetic machinery?

2002 2nd PhD Summer School of the Department of Biology, Les Cotes, Switzerland, September

Abstract title: Potential signaling pathways between the cleavage machinery at the bud neck and the nucleus in budding yeast

Awards/ Memberships

2005 ASCB Predoctoral Student Travel Award
2005 Best Poster Award USGEB Meeting Zurich
2004 Youth Travel Fellowship for FEBS Special Meeting on Cytoskeletal Dynamics in Helsinki
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Dissection of septin actin interactions using actin overexpression in *Saccharomyces cerevisiae*

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Summary

Although many proteins can be overexpressed several fold without much effect on cell viability and morphology, some become toxic upon a slight increase in their intracellular level. This is particularly true for cytoskeletal proteins and has proven useful in the past for studying the cytoskeleton. In yeast, actin and tubulin are examples of proteins that cannot be overexpressed without affecting cell viability. Here, we have analysed the effect of actin overexpression in *Saccharomyces cerevisiae*. We show that actin overexpression interferes differently with distinct aspects of actin function. For example, two- to fourfold overexpression of actin did not affect the establishment of actin polarity, whereas it abrogated its maintenance. Also, actin structures that are barely visible in wild-type cells could be observed upon actin overexpression. This allowed us to identify a new ring-like actin structure genetically distinguishable from the actomyosin contractile ring. Formation of this actin structure upon actin overexpression was dependent on the septin cytoskeleton, the poorly understood cytokinetic protein Hof1 and the Arp2/3 complex. In contrast to the actomyosin ring, the ring formed upon actin overexpression required neither Myo1 nor formins for assembly. Therefore, we propose that Hof1 acts as a linker between actin and septins. Furthermore, we found that, in the absence of actin overexpression, a novel, Hof1-dependent actin belt is formed at the bud neck of anaphase cells. The physiological role of this belt might be related to that of the similar structure observed in dividing fission yeast.

Introduction

Cell shape, cell movements as well as the transport of organelles within eukaryotic cells depend on cytoskeletal functions. The cytoskeleton comprises microtubules, intermediate filaments and actin. A key feature of microtubules and actin filaments is their rapid dynamics. The ability to grow and shrink is an important factor for the generation of forces and to allow the rapid reorganization of the cytoskeleton in response to intra- and extracellular signals. Although microtubules are involved in the movement of large structures within the cytoplasm, cortical events such as cell polarization and cell cleavage at cytokinesis generally depend on actin. Actin acts through the formation of localized and specialized structures, rather than through a global control of its dynamic properties. Hence, actin is able to respond differentially to various local signals to fulfill diverse functions.

In the budding yeast *Saccharomyces cerevisiae*, actin assembles into cortical actin patches, actin cables (Botstein *et al*., 1997; Amberg, 1998; Karpova *et al*., 1998) and an actomyosin contractile ring (Epp and Chant, 1997; Bi *et al*., 1998; Lippincott and Li, 1998a; Bi, 2001). Each of these structures fulfills distinct functions.

Actin patches are believed to be associated with plasma membrane invaginations (Mulholland *et al*., 1994) and contain numerous proteins involved in endocytosis (Schott *et al*., 2002a). Therefore, actin patches are thought to mediate the internalization of plasma membrane domains and extracellular material. As actin patches are no longer detectable in cells lacking either Arp2 or Arp3, their assembly is thought to depend on the function of the Arp2/3 complex (Moreau *et al*., 1997; Winter *et al*., 1997).

Actin cables play a crucial role in intracellular movements such as the transport of exocytic vesicles to the growing bud and spindle positioning during metaphase (Pruyne *et al*., 1998; Bretscher, 2003; Hwang *et al*., 2003; Liakopoulos *et al*., 2003). They serve as tracks for the myosin-dependent movement of cargo within the cell. In polarized cells, the formins Bni1 and Bnr1 are localized to the bud cortex or the shmoo tip and are involved in both actin cable nucleation and overall polarization of actin cables (Evangelista *et al*., 1997; 2002; Sagot *et al*., 2002). This in turn directs the proper transport of vesicles and organelles towards the growing bud ( *et al*., 1999).

The actomyosin ring assembles at the bud neck and plays a crucial role in cell cleavage during cytokinesis. Its formation requires F-actin, type II myosin, IQGAP and the formins (Pruyne *et al*., 2002; Tolliday *et al*., 2002). Contraction is triggered upon completion of anaphase and leads to cytokinesis (Lippincott *et al*., 2001; Luca *et al*., 2004). We show that actin overexpression required neither Myo1 nor formins to assemble. Therefore, we propose that Hof1 acts as a linker between actin and septins. Furthermore, we found that, in the absence of actin overexpression, a novel, Hof1-dependent actin belt is formed at the bud neck of anaphase cells. The physiological role of this belt might be related to that of the similar structure observed in dividing fission yeast.
In budding yeast, localization of the actomyosin ring to the bud neck depends on septins (Roh et al., 2002). Septins form a filamentous ring around the site of bud emergence. During bud growth, the septin ring remains localized at the bud neck where it forms a stiff scaffold for the recruitment of additional factors (Gladfelter et al., 2002; Dobbelaere et al., 2003) such as Hof1 and Myo1 (Bi et al., 1998; Lippincott and Li, 1998b). In higher eukaryotes, septins have been shown to recruit actin filaments, together with anilin (Kinoshita et al., 2002).

Formation of the various actin structure in yeast cells is tightly controlled by intra- and extracellular signals and is co-ordinated with cell cycle progression. In early G1, actin patches are distributed homogeneously over the entire cortex of unpolarized cells. In contrast, they accumulate almost exclusively to the bud and shmoo tip of G2 mating cells (Adams and Pringle, 1984). Actin cables also reorganize and align along the mother bud axis upon polarization of the cell. Upon exit of mitosis, actin patches relocalize to both mother and bud, and the actomyosin ring assembles at the bud neck. Activation of the Dbf2 kinase by the mitotic exit network then triggers actin ring contraction and completion of cytokinesis (Lippincott et al., 2001; Luca et al., 2001; Menssen et al., 2001).

As in higher organisms, overexpression studies in yeast have proved useful in gathering insights into the function and regulation of a variety of cytoskeletal proteins. Remarkably, overexpression of actin or β-tubulin is lethal in yeast (Liu et al., 1992; Magdolen et al., 1993). The reason for this sensitivity is unknown. As the dynamics of cytoskeletal structures in vitro depend on the concentration of cytoskeletal monomers, it is possible that actin and tubulin overexpression affects the assembly and disassembly of specialized structures in vivo. Here, we have investigated the effect of actin overexpression in yeast. The results of this study indicate that increased actin monomer concentration differentially affects the distinct actin structures. They also demonstrate that overexpression of cytoskeletal proteins may be a useful approach for identifying novel cytoskeletal assemblies and/or interactions.

Results

Actin overexpression is lethal and affects bud formation

To investigate the effect of actin overexpression in yeast, we used a GAL:ACT1 construct isolated from a cDNA library (Liu et al., 1992). This construct, expressing wild-type actin under the control of the strong and regulatable GAL1-10 promoter, allows actin overexpression in media containing galactose as the sole carbon source. Sequencing of the insert indicated that it contained the full coding sequence of a spliced variant of the wild-type ACT1 gene (Goffeau et al., 1996). Cells transformed with this plasmid grew normally on glucose but failed to form colonies on galactose (Fig. 1A). The growth of transformants carrying the corresponding empty vector was not affected under either condition. Our GAL:ACT1 plasmid thus reproduced the toxic effect reported previously for actin overexpression (Liu et al., 1992; Magdolen et al., 1993).

To characterize the expression levels achieved, yeast cells containing the GAL:ACT1 plasmid in addition to the endogenous Act1 gene were either grown on glucose or induced on galactose for 4 and 8 h. Equal amounts of protein were subjected to SDS-PAGE gel separation, and the relative amount of actin present in the different lysates was determined by immunoblotting. A clear increase in actin levels was obtained over the 8 h of induction (Fig. 1B). However, quantification of the signal indicated that, in our experiment, actin concentration did not increase by more than a factor of 4. Using interference contrast microscopy, it was determined that cells overexpressing actin arrest as large swollen cells with multiple small bumps on their surface (Fig. 1C). Up to six bumps were observed with longer galactose induction times. Only very few cells (<14%) had formed normal-sized buds. Cells overexpressing actin were much larger than the control cells grown on sucrose (Fig. 1C). A few of these cells contained two nuclei or more, indicating that in these cells the nuclear cycle continued despite the bud formation defect. However, the low level of binucleated cells observed indicated that the progression of the nuclear cycle was slowed down relative to the cell volume expansion. This is consistent with the existence of the morphogenetic checkpoint that is thought to delay cell cycle progression in response to defects in bud formation (Lew, 2000). This checkpoint depends on the activity of the Swe1 kinase. Swe1 (S. cerevisiae homologue of Wee1) responds to actin perturbation and arrests the cells in G2 by phosphorylating and thereby inhibiting the cyclin-dependent kinase Cdc28. This regulatory loop is thought to link mitosis to bud growth.

In the swe1Δ GAL:ACT1 strain grown on galactose, the number of multinucleated cells increased greatly relative to the GAL:ACT1 cells in the same conditions (Fig. 1D). Therefore, cells overexpressing actin do indeed activate the morphogenetic checkpoint. The presence of some binucleated cells in the wild-type population suggests that actin overexpressing cells adapt to the checkpoint arrest. A similar situation is also observed in cells defective for Cdc42 function.

Altogether, these data indicate that actin overexpression interferes with bud formation.

The protrusions seen upon actin overexpression correspond to successive and abortive buds

To determine which aspect of the budding process is
affected by actin overexpression, we investigated whether the protrusions observed at the surface of the cells corresponded to buds. To address this question, we determined whether a bud-specific protein such as Spa2 was enriched in these bumps. Analysis of Spa2-GFP distribution in non-induced cells indicated that it localized to the tip of small buds, the entire surface of isotropically growing buds and to the bud neck of large budded cells (Fig. 2A, a and b) as reported previously for wild-type Spa2 (Snyder, 1989). When cells expressing Spa2-GFP as the sole source of Spa2 were induced to overexpress actin, they formed swollen mothers with bud-like protrusions, just like wild type (Fig. 2A, c, e and g). Spa2-GFP localized to one of the protrusions in a large majority of the cells (Fig. 2A, d,
In some cases, Spa2 localized diffusely throughout the cell (data not shown). In extremely rare cases, it localized to more than one of the protrusions (data not shown). These results indicate that the protrusions are similar to buds with regard to Spa2 distribution. That only one such bud contains Spa2 at a given time suggested that only one bud was growing at a certain time.

Two explanations can account for the formation of multiple small buds. Either the cell fails to limit the number of buds that it produces at a specific time point, and these buds emerge simultaneously, or the cell reiterates successive rounds of abortive budding events. To distinguish between these two possibilities, we analysed the kinetics with which buds were formed. Unbudded, stationary phase cells containing the GAL:ACT1 plasmid were diluted into fresh medium containing either galactose or sucrose as the sole carbon source. Samples were collected at 2 h intervals for 14 h. The cells were then fixed and stained to visualize nuclei and actin. The cells grown in sucrose, where the GAL:ACT1 construct was not induced, started to form small buds more or less synchronously (see Fig. 2B), which developed into large buds. At the 6 h time point, the culture had reached a steady state and contained unbudded (32–44%), small- (16–18%) and large-
budded (36–39%) cells. These proportions remained stable over the next 5 h. After 14 h, the proportion of budded cells dropped and unbudded cells reaccumulated, indicating that the culture had reached saturation.

Like the cells grown in sucrose, the cells grown in galactose rapidly formed a first small bud (Fig. 2B). However, the portion of large budded cells failed to increase at the following time points. Instead, the fraction of cells with a small bud kept increasing over the first 8 h. Thus, the cells were able to form a single small bud at the same rate as cells growing on sucrose, but these first buds did not grow any further. After 8 h, cells with more than one bud started to accumulate. The fraction of dibudded cells reached its maximum after 11 h when cells with three small buds appeared. None of the buds reached a normal size, suggesting that they were aborted. Hence, actin-overexpressing cells did not form multiple buds simultaneously but initiated successive abortive budding events. Therefore, actin overexpression does not impair bud emergence but specifically prevents bud growth.

Actin overexpression leads to the formation of a new ring-like structure in the cytoplasm

We also analysed the effect of actin overexpression on actin organization. Rhodamine–phalloidin staining showed that actin was unpolarized in unbudded cells (Fig. 3B) and polarized towards the small buds of budding cells (Fig. 3B). However, cells rapidly depolarized actin, most probably concurrent with aborted budding. In many cells with depolarized actin, a new actin structure formed in the shape of an intense polygonal or ring-like structure in the cytoplasm of the mother cell (Fig. 3B). We will refer to it as the actin ring in the rest of the paper. This actin ring was composed of a few focal points, which were linked together by bundles of actin filaments and looked like a continuous structure from the face-on view. Inspected from the side, it looked like a straight bar, indicating that this structure was planar. This actin structure was never observed in non-induced conditions. After 10–12 h of actin overexpression, this ring was present in 30–
45% of the cells (Fig. 3A). Careful analysis showed that a small proportion of the cells also contained a smaller, less intensely stained actin ring at the cell cortex throughout the experiment (Fig. 3B, second to last picture). In all cases, these small rings localized to the vicinity of the small bud. As these small rings appeared already after 2 h of induction and never co-existed with the big actin ring, we speculate that they may be a precursor of the larger actin ring.

To ensure that the observed actin structure does not result from cell death, we assayed cell viability during the course of the experiment. First, cells were stained after 10 h and after 24 h with Trypan blue, which only dead and dying cells internalize. Secondly, we tested the ability of the cells to resume colony formation upon plating back on glucose-containing plates. Both tests consistently showed that, even after 10 h of actin overexpression, the vast majority of cells remained viable (Fig. 3C). Internalization of Trypan blue was observed in only 16% of the cells after 10 h in galactose medium compared with 42% after 24 h. After 10 h on galactose, these cells formed actin rings with the same frequency as in previous experiments (data not shown). The dilution series shows a comparable survival rate for the cells grown in sucrose and in galactose for 10 h. Therefore, the actin structures that we observed upon actin overexpression are not the result of actin accumulation in dying cells. It is more likely that the structure that we observed corresponds to the exaggeration of a structure already present under physiological conditions.

**Septins and Hof1 but not Myo1 influence ring formation**

The observation of a potentially early form of the actin ring close to the site of bud emergence as seen in Fig. 3B (grey star) suggested that actin rings might initially assemble at the cell cortex and may depend on cortical proteins present at the budding site. To test whether this is the case, we investigated whether the ring still formed in cells lacking specific cortical factors. The only other actin ring known in yeast is the actomyosin ring at the bud neck. Formation of this ring depends on septins, the type II myosin Myo1, formins and the IQGAP homologue Iqg1 (Epp and Chant, 1997; Lippincott and Li, 1998b; Bi, 2001). Furthermore, organization of actin into a ring most probably depends on the presence of a template to guide actin assembly into a closed circle. The septin ring, which does not depend on actin for assembly in yeast, is a good candidate for such template function. We therefore investigated whether septins were required for ring formation upon actin overexpression. The septin mutant cdc12-1 carrying the GAL:ACT1 construct was induced to express actin at either a semi-permissive (30°C) or the restrictive (37°C) temperature (Fig. 4A). Compared with the wild type, ring formation upon actin overexpression was

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**Fig. 4.** Actin ring formation upon actin overexpression in different mutants.

A. Ring formation is impaired in septin-defective cells. Wild-type, cdc12-1 and cdc34-2 cells overexpressing actin for 10 h at the indicated temperatures were fixed, stained with rhodamine–phalloidin, and the percentage of cells with an actin ring was determined.

B. Ring formation in myo1Δ and hof1Δ cells. Cells of the indicated genotype were transformed with the GAL:ACT1 plasmid pYB49, and actin overexpression was induced for the indicated time. The cells were fixed, stained with rhodamine–phalloidin and the percentage of cells with an actin ring was determined.

C. Actin localization in fixed hof1Δ and myo1Δ cells after 10 h of actin overexpression. Actin is visualized by rhodamine–phalloidin staining. For all counts, n = 300 cells.
strongly impaired in septin-defective cells, although rings formed properly at the permissive temperature (22°C; data not shown). This means that, consistent with actin ring formation depending on septin function, actin ring formation was temperature sensitive in the cdc12-1 strain.

Septin-defective mutants show a strong morphological phenotype characterized by the formation of elongated buds. Therefore, ring formation defects may simply be a secondary consequence of morphological problems. This possibility was excluded by assaying ring formation in the cdc34-2 mutant that also forms elongated buds. These cells showed an increase rather than a reduction in ring frequency. Consequently, the effect of the septin mutation on ring formation was not caused by cellular morphology defects. Septins therefore appear to play a specific role in actin ring formation.

Myo1 and Hof1 are two proteins that have been implicated in yeast cytokinesis, and both localize to the bud neck in a septin-dependent manner (Lippincott and Li, 1998a,b; Vallen et al., 2000). We tested whether these two proteins were required for actin ring formation upon actin overexpression. As shown in Fig. 4B and C, Myo1 was not needed for the formation of the actin ring. In contrast, cells lacking Hof1 only rarely formed a ring (<8% compared with >40% in wild type; Fig. 4B), which were always much smaller and fainter than rings observed in wild type (Fig. 3C). Therefore, the Hof1 pathway of cytokinesis seems to play a role in actin ring formation upon actin overexpression.

**Actin overexpression leads to the mislocalization of septins and Hof1 whereas Myo1 localization is not affected**

We then investigated whether actin overexpression had any effect on the localization of Myo1, Hof1 and septins. Septins localize to the bud neck already in G1 and stay at the bud neck during the entire budding cycle (Fig. 5B).

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**Fig. 5.** A. Effect of actin overexpression on the septin ring. Cells grown under non-inducing (A and B) or inducing conditions (C–J) were fixed after 0 h (A and B), 2 h (E and F) and 8 h (C, D and G–J), and the septin 3Xmyc-Cdc3 was visualized by indirect immunofluorescence using anti-myc antibodies as primary antibodies (A–E, G and I). Rhodamine–phalloidin staining of the same cells is shown (F, H and J). B. Effect of actin overexpression on the localization of bud neck proteins. Cells GFP-tagged for the proteins Cdc12, Hof1 and Myo1 were transformed with the GAL:ACT1 plasmid, and the GFP-tagged proteins were visualized under non-inducing (left) and inducing (right) conditions. C. Quantitative analysis of the mislocalization of the GFP-tagged proteins.

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The GAL:ACT1 construct was induced in a strain expressing a tagged version of the Cdc3 septin, 3xmyc-Cdc3 (Barral et al., 1999), and septin localization was assayed by indirect immunofluorescence (Fig. 5A, A, C, E, G and I). Cells were co-stained with rhodamine–phalloidin to visualize actin (Fig. 5A, B, D, F, H and J). At early time points of actin overexpression, septin rings formed properly, concomitant with actin polarization. Actin polarization and septin ring formation were restricted to the same edge of the cell, and actin and septins co-localized extensively. At later times, when cells started to swell and actin depolarized, septins were dispersed into patches and broken ring-like structures (Fig. 5A, I and J). In cells in which actin was completely depolarized, septin patches were randomly dispersed over the cell cortex (Fig. 5A, G and H). The same septin localization was observed using GFP-Cdc12 as a reporter in cells overexpressing actin (Fig. 5B). Hence, actin overexpression led to septin disorganization.

Hof1 is recruited to the bud neck in a septin-dependent manner early in the budding cycle and stays at this site until cytokinesis (Lippincott and Li, 1998b; Vallen et al., 2000). In sucrose, HOF1-GFP GAL:ACT1 cells showed a GFP distribution identical to that reported for Hof1 (Fig. 5B, left). Before anaphase, as judged by DAPI staining, Hof1-GFP localized to the neck as a double ring. In late anaphase/telophase, these rings merged, and the resulting ring started to contract (data not shown). On galactose, HOF1-GFP GAL:ACT1 cells developed the same phenotype as wild-type cells carrying the same construct. They swelled and formed small abortive buds. In these cells, Hof1-GFP was observed at the neck of only a very few of the small buds (Fig. 5C; 8%, n = 66). Instead, diffuse Hof1 structures were observed in the cytosol.

In contrast to Hof1, Cdc3 and Cdc12, Myo1 localization was not affected by actin overexpression. In actin-overexpressing cells, Myo1-GFP localized to the necks of 70% of the small buds (Fig. 5C, n = 88). These cells showed the same morphological phenotypes as wild type overexpressing actin (Fig. 5B) and formed actin rings to the same extent as wild type (data not shown). Thus, actin overexpression does not affect Myo1 localization. These data suggest that, in contrast to its recruitment to the bud neck, Myo1 does not need the septins to remain at this site. This also means that actin-overexpressing cells do not have a general defect in the localization of bud neck proteins. Therefore, the mislocalization of septins and Hof1 upon actin overexpression was specific. These observations were in good agreement with the fact that hof1Δ and septin mutants have defects in actin ring formation upon actin overexpression, whereas the myo1Δ cells do not.

Deletion of Hof1 suppresses septin mislocalization upon actin overexpression

We next wanted to know whether there was any causal correlation between actin ring formation and septin ring disruption upon actin overexpression. As the formation of the actin ring was Hof1 dependent, we asked whether the septin ring still disorganized upon actin overexpression in hof1Δ cells. Cells with the genotype hof1Δ GAL:ACT1 GFP-CDC12 were induced in galactose for 15 h, and septin distribution was analysed. In these cells, septins localized properly to the neck of the small buds (Fig. 6A). No
additional septin structures were observed. In contrast, disruption of Myo1 did not rescue septin organization in actin-overexpressing cells (Fig. 6A). Thus, the actin ring formed upon actin overexpression is responsible for septin phenotype ring disruption. Furthermore, Hof1 was required for septin ring disorganization upon actin overexpression. These results suggest that Hof1 links actin and septins in our actin overexpression experiments.

We then asked whether it is only the formation of the thick actin ring that is the cause of lethality resulting from actin overexpression. If this was true, hof1Δ should survive actin overexpression. However, this was not the case (Fig. 6B). The hof1Δ cells overexpressing actin still accumulated small abortive buds, like the wild type (Fig. 6A). Thus, the lethality observed upon actin overexpression is not exclusively the result of actin ring formation and septin ring disruption.

**Systematic screen for mutants with an effect on ring formation**

We also studied more systematically which cytokinetic proteins were required for actin ring formation upon actin overexpression. Formation of the actomyosin ring at the bud neck during cytokinesis depends on the formins Bnr1 and Bni1 (Imamura, Tanaka et al., 1997). Remarkably, bnr1Δ, bni1Δ and bnr1Δbni1–185 (S1023→G, kindly provided by Charlie Boone) single and double mutants all formed actin rings upon actin overexpression comparable to wild type (Fig. 7). The same was true for cells lacking Spa2, Bud6/Aip3 or Kar9. Hence, none of Bnr1, Bni1, Bud6, Spa2 and Kar9 played a role in ring formation.

In contrast, cells lacking Boi1, Boi2, Rvs167, Rvs161 and Vrp1 or carrying an arp2-2 mutation were all defective in formation of the actin ring upon actin overexpression (Fig. 7). Boi1, Boi2 and, particularly, Bud4 share structural similarities with anilin (Matsui et al., 1998; Sanders and Herskowitz, 1996; Kinoshita et al., 2002), Rvs161 and Rvs167 are amphipathins homologues (Crouzet et al., 1991; David et al., 1994), and Vrp1 is a protein functionally and structurally related to WASP that interacts physically with Hof1 (Naqvi et al., 2001). The Arp2/3 complex is known to nucleate and branch actin filaments. Furthermore, it is a target of WASP family proteins (Taunton et al., 2000; Peterson et al., 2001). These data indicate that the actin ring formed upon actin overexpression is distinct from the actomyosin ring. Unlike the actomyosin contractile ring, the actin overexpression ring did not require either Myo1 or the formins for assembly, but instead the function of the Arp2/3 complex.

**A Hof1-dependent actin belt forms in late anaphase cells**

Altogether, these results suggest that an actin structure distinct from the actomyosin ring may assemble at the bud neck in a Hof1-dependent manner some time before cytokinesis. In other eukaryotes, actin ring assembly and furrowing takes place during anaphase (Watanabe et al., 1990; Mabuchi, 1994). Therefore, we tested whether yeast cells assemble some actin structure at the bud neck during anaphase. To do this, cdc15-1 cells were arrested at the restrictive temperature (37°C) for 4 h, fixed and stained to visualize actin and nuclei. Remarkably, around 20% of these cells assembled a broad band of actin at the bud neck, which we refer to as the actin belt. This structure is morphologically distinct from the actomyosin ring, which forms a tight string around the bud neck of dividing yeast cells (Bi, 2001; Tolliday et al., 2001; Schmidt et al., 2002). This actin belt was also clearly distinct from the actin structure reported by Bi et al. (1998), which forms only after ring contraction. In contrast to this post-cytokineti structure, the actin belt that we report is much less bright and forms only on one side of the bud neck.

The actomyosin ring is thought to form only after mitosis in a Myo1-dependent manner. In contrast, the actin belt observed in our experiment in the cdc15-1 cells forms before mitotic exit and did not require Myo1 (Fig. 8A and B). However, its formation depended on the presence of Hof1 (Fig. 8B). This means that Hof1 is required for the assembly of an actin belt at the bud neck during anaphase.

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**Fig. 7.** Screen for mutants that have an effect on actin ring formation. The listed strains were transformed with the GAL:ACT1 plasmid and induced in galactose for 8 or 10 h. Fixed cells were stained with rhodamine–phalloidin and inspected for the presence of an actin ring.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cells with actin ring (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0</td>
</tr>
<tr>
<td>bni1Δ</td>
<td>0</td>
</tr>
<tr>
<td>bnr1Δ</td>
<td>0</td>
</tr>
<tr>
<td>bnr1Δbni1–185</td>
<td>0.24</td>
</tr>
<tr>
<td>boi1Δ</td>
<td>0.19</td>
</tr>
<tr>
<td>boi2Δ</td>
<td>0.07</td>
</tr>
<tr>
<td>vrp1Δ</td>
<td>0.02</td>
</tr>
<tr>
<td>boi1Δboi2Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bni1Δboi1Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi1Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi2Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi1Δboi2Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi1Δvrp1Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi2Δvrp1Δ</td>
<td>0.01</td>
</tr>
<tr>
<td>bnr1Δboi1Δboi2Δ vrp1Δ</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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Cytokinesis in yeast has been shown to depend on two overlapping pathways, which depend on Hof1 and Myo1 to fulfil their function (Lippincott and Li, 1998b; Vallen et al., 2000). Mutants in which only one of these pathways is affected survive, whereas double mutants affecting both pathways die. Thus, we attempted to sort cytokinetic genes into either the Hof1 or the Myo1 pathway of cytokinesis.

As shown already, Iqg1 as well as Vrp1 act in both pathways of cytokinesis (Korinek et al., 2000; Naqvi et al., 2001). Bni1 and Bnr1 may act in either pathway (Vallen et al., 2000). We crossed rvs161Δ, rvs167Δ, bud4Δ and arp2-1 into both hof1Δ and myo1Δ mutants, and the viability of the resulting double mutants was assayed. This analysis indicated that Bud4 was required for Hof1 function. boi1Δ and boi2Δ alone as well as in combination showed no genetic interaction with either myo1Δ or hof1Δ such that it was not possible to sort these proteins in either of the cytokinetic pathways. arp2-1 was unviable in combination with either myo1Δ or hof1Δ. The Arp2/3 complex therefore presumably acts upstream of both pathways (Fig. 9). These data suggest that the anilin homologue Bud4 acts together with Hof1 in cytokinesis. Our results are consistent with the assumption that Bud4 might act like anilin in the septin-dependent assembly of actin structures at the site of cytokinesis.

Discussion

**Actin overexpression affects the maintenance of cell polarity in yeast**

In this study, we have investigated the nature of the cellular defects caused by actin overexpression in budding yeast. We show that a two- to fourfold overexpression of actin leads to the formation of large swollen cells. This arrest is accompanied by a Swe1-dependent delay of the

![Fig. 8. Actin distribution in late anaphase. cdc15-1 cells were arrested for 4 h at 37°C, fixed and stained with rhodamine-phalloidin. The presence of a broad actin belt is observed at the bud neck in about 20% of the cells. Formation of this belt does not require type II myosin as its formation is not affected in the cdc15-1 myo1Δ strain. However, belt formation was reduced in the hof1Δ cdc15-1 double mutant cells (graph).](image)

![Fig. 9. The indicated mutants were crossed with myo1Δ and hof1Δ by us and others (Korinek et al., 2000; Vallen et al., 2000; Naqvi et al., 2001). Co-lethality with solely myo1Δ indicated that the mutated gene acts in the HOF1 pathway. Vice versa, co-lethality with hof1Δ puts the gene in the MYO1 pathway. Co-lethality with both puts the gene in an upstream pathway. Formins were put in the MYO1 pathway because they do not affect ring formation upon actin overexpression. Boi1 and Boi2 are thought to regulate the HOF1 pathway because they are required for ring formation upon actin overexpression, and co-lethality tests put the anilin homologue Bud4 in the HOF1 pathway.](image)
nuclear cycle and the successive formation of several abortive buds. Thus, actin overexpression impairs proper bud formation and engages the morphogenetic checkpoint. Upon longer induction times, actin overexpression becomes lethal.

Analysis of actin organization indicates that actin overexpression does not interfere with the polarization of actin during bud emergence but, rather, with the maintenance of actin polarity during bud growth. Actin is involved in the directed transport of vesicles and organelles towards growth sites (Schott et al., 2002a,b). Exocytic vesicles are required for the delivery of new cell wall material and cell wall-remodelling enzymes. Accordingly, actin polarity defects prevent localized cell wall expansion. Thus, the defect in actin polarity maintenance under actin overexpression is likely to explain the observed abortion of bud growth. A multibudded phenotype with numerous small buds was already observed in a specific Cdc42 mutant (Richman and Johnson, 2000). However, the morphology of the buds observed is very different. Indeed, cdc42 cells form at least medium-sized buds and often arrest with elongated buds. In our case, very small buds already abort before starting isotropic growth. Still, an aberrant actin cytoskeleton may be responsible for both phenotypes as Cdc42 is a key regulator of polarization of the cytoskeleton to regions of growth.

Previous studies have already distinguished two steps in bud formation. During bud emergence, polarization of actin and vesicle delivery depends upon the activity of the Rho-like small GTPase Cdc42 (Richman et al., 1999; Jaquenoud and Peter, 2000). Cdc42 loss-of-function mutants show no obvious defects after that point, indicating that Cdc42 acts in the establishment but not the maintenance of cell polarity (Adams et al., 1990; Bose et al., 2001). Maintenance of actin polarity is thought to depend on Rho GTPases and the septin-dependent diffusion barrier at the bud neck (Hall, 1992; Imamura et al., 1997; Cabib et al., 1998; Johnson, 1999; Barral et al., 2000; Faty et al., 2002). The observation that actin overexpression also leads to septin disorganization prompted us to test whether septin ring defects account for the loss of actin polarity. However, this is not the case as the hof1Δ strain, which does not show septin ring defects upon actin overexpression, still fails to maintain actin polarity and to form normal buds under this condition. Consequently, the loss of septin structures and of actin polarity is independent of the effects of increased actin concentration.

Actin-overexpressing cells form a new ring-like actin structure

Analysis of actin localization in actin-overexpressing cells revealed the formation of a new actin-containing structure. This structure is strongly labelled with phalloidin, supporting the idea that it is composed of F-actin. We tested the viability of the cells containing this new structure and found that they can be rescued if actin overexpression is stopped. It is unlikely that this ring structure is responsible for cell death upon actin overexpression, as cells that had formed rings maintained viability upon termination of actin overexpression. Rather, cell death might be attributed to the failure of the budding cycle. Furthermore, Pruyne et al. (2002) saw comparable actin structures in viable cells overexpressing the formin Bni1. These structures are nevertheless generally not ring-like and often tilted so that we do not believe that they have the same origin as the actin ring we observed here.

We propose that the ring formed by actin-overexpressing cells derives from some cytokinesis structure and originally forms at the bud neck. This hypothesis is supported by the following observations. First, formation of the actin ring depends on proper septin and Hof1 function. Septins and Hof1 are proteins involved in cytokinesis in S. cerevisiae and localize to the bud neck upon or shortly after bud emergence (Longtine et al., 1996; Lippincott and Li, 1998b; Gladfelter et al., 2002). Although septins are involved in non-cytokinetic processes as well (Barral et al., 2000), only cytokinetic phenotypes have been reported for cells lacking Hof1. Secondly, not only did septin and hof1Δ mutations impair the formation of the actin ring, but the reverse was also true. Actin overexpression disrupted septin and Hof1 localization. Therefore, we propose that the actin ring formed upon actin overexpression uses the septin ring as a template to form. These results suggest that, in yeast as in animal cells (Kinoshita et al., 2002), septins are able to recruit and organize actin.

Remarkably, actin ring formation upon actin overexpression did not require type II myosin or formins. These molecules have been shown to be required for the formation of the yeast actomyosin contractile ring involved in cytokinesis (Bi et al., 1998; Lippincott and Li, 1998a,b). Moreover, formation of an actin ring upon actin overexpression required the presence of Hof1 and the function of the Arp2/3 complex, which are dispensable for actomyosin ring assembly. Hof1 shares 23% identity and 46% similarity with the actin polymerization protein Bzz1 that binds to the Arp2/3 regulator Las17.

Thus, our results suggest that the actin ring observed upon actin overexpression may derive from an endogenous structure potentially involved in cytokinesis but distinct from the actomyosin ring already described. As actin overexpression has no effect on septin ring organization in the hof1Δ cells, we propose that Hof1 physically links actin and septins during actin ring formation. Lippincott and Li (1998b) have already suggested that Hof1 might functionally link actin and septins. Our results indicate that Hof1 interaction with either septins or actin does not depend on myosin, suggesting that
Hof1 may in fact provide a direct physical link between actin and septins.

These results prompted us to investigate the possibility that actin structures other than the actomyosin ring may exist at the bud neck of wild-type yeast cells in the absence of actin overexpression. Our original studies in wild-type cells were complicated by the large number of actin patches and cables in the bud before anaphase. This prevented the visualization of potential bud neck-specific actin structures. However, in cells arrested in late anaphase, actin patches redistribute between mother and bud, and actin cables are shorter and no longer run along the mother–bud axis. In these arrested cells, phalloidin staining revealed the presence of an actin belt at the bud neck. This belt satisfies several criteria for being a new actin structure, distinct from the actomyosin ring. First, although the hof1Δ mutation does not interfere with the formation and contraction of the actomyosin ring (Vallen et al., 2000), J. Dobbelare and Y. Barral, unpublished data), it affects the formation of the actin belt. Secondly, formation of this structure is not affected by the disruption of the type II myosin. Thirdly, the actin belt is morphologically distinct from the actomyosin ring. However, at this point, we do not know exactly what function this belt assumes in wild-type cells. It might assemble an original pool of actin filaments at the bud neck that can subsequently be recruited into the actomyosin ring. Alternatively, the actin belt may play other roles, such as vesicle delivery to the site of cleavage, or it may contract independent of Myo1. In favour of the first interpretation, we never saw the actin belt and the actomyosin ring coexist in the same cell. Furthermore, the Hof1 protein becomes incorporated into the actomyosin ring upon actomyosin ring contraction (J. Dobbelare and Y. Barral, unpublished data; Lippincott and Li, 1998b). In support to this hypothesis, a similar structure is also observed in Schizosaccharomyces pombe. In that case, a faint and broad actin belt forms in an Arp2/3-dependent manner shortly before anaphase and sharpens as anaphase progresses. This belt is then compartmented into the sharp actin contractile ring characteristic of cytokinesis (Arai and Mabuchi, 2002). Remarkably, the Hof1 homologue in S. pombe, Cdc15, is necessary for the assembly of the medial actin belt and required for the recruitment of formins and the Arp2/3 complex to the cytokinetic area (Carnahan and Gould, 2003). Thus, our data suggest that Hof1 function is highly conserved between budding and fission yeast. Considering all these data, it is possible that the belt is a precursor of the actin ring observed, as both structures are Hof1 dependent. Future experiments will clarify this point.

As actin has been shown to be dispensable for the assembly of the septin ring in yeast, we do not believe that the actin–septin interactions that we describe here play any major role in the formation of the septin ring. It has also been shown that septins and Hof1 are not required for bud emergence and the polarization of actin. Thus, this actin–septin interaction is likely not to be required for bud growth and overall actin organization, but rather for cytokinesis only.

In summary, we have shown here that actin overexpression leads to a variety of independent cellular defects. Furthermore, careful analysis of actin overexpression phenotypes identified a new actin structure of yeast cells and was useful for the molecular characterization of this structure.

Experimental procedures

**Media, strains and growth conditions**

YPD (yeast extract, peptone, dextrose) and SD (synthetic dextrose) media were prepared as described. YPGal (yeast extract, peptone, galactose) and SGal (synthetic galactose) contain 2% galactose instead of dextrose. Ssuc (synthetic sucrose) contains 2% sucrose instead of dextrose. Standard genetic methods were used for crosses and tetrad analysis. Yeast transformations were done using a short version of the lithium acetate procedure.

The SPA2::GFP, HOF1::GFP and MYO1::GFP insertions were generated by inserting the green fluorescent protein (GFP) cassette (Longtine et al., 1998) on the chromosomal locus using polymerase chain reaction (PCR) tagging techniques and homologous recombination (Baudin et al., 1993; Schneider et al., 1995). Homologous sequences for recombination were contained in the primers used for amplification of the insertion cassette, as described previously. Insertion replaced the stop codon of the SPA2, HOF1 or MYO1 genes by the coding sequence of GFP, which contained its own stop.

The bnr1::HIS3 disruption as well as the cdc12-1 and cdc34-2 mutations were described previously (Hartwell et al., 1974; Adams et al., 1990; Kusch et al., 2002). The same is true for the 3Xmyc-CDC3 strain (Barral et al., 1999). hof1::HIS3 and myo1::HIS3 were generated using the gene disruption cassette (Longtine et al., 1998). All strains were backcrossed at least four times to the wild-type YYP384, an S288c derivative (MATa his3-Δ200 ura3-52 trp1-Δ63 leu2 lys2-801 ade2-101) to ensure isogenicity. Strains disrupted for KAR9, BUD6, BOI1, BOI2, RVS161, RVS167 and VRP1 were obtained from Euroscarf. All strains were grown at room temperature (22°C) unless stated otherwise.

**Actin overexpression**

The plasmid pYB49 was isolated from the cDNA library constructed by Liu et al. (1992). This plasmid is derived from the pRS series (Sikorski and Hieter, 1989) and contains ARS and CEN sequences to ensure replication and segregation in yeast, and the URA3 gene for selection. The ACT1 CDNA is placed under the control of the GAL1-10 promoter, allowing overexpression in galactose medium and repression by glucose. The insert was sequenced on
both strands to ensure its identity to the ACT1 genomic sequence. Sequencing revealed that the cDNA cloned corresponded to a spliced variant of the ACT1 coding sequence.

Induction of actin overexpression was achieved as follows. Yeast strains were first grown overnight in sucrose medium to terminate glucose repression. Cells of saturated sucrose cultures, which were at least 90% un budded, were subsequently centrifuged down and resuspended in galactose medium for induction. In the case of the temperature-sensitive strains, the sucrose culture was grown at room temperature (permissive temperature), while the induction of actin overexpression was achieved at permissive, semi-restrictive (30°C) and restrictive (37°C) temperatures, as indicated in the text and figure legends.

For the survival test, cells were grown overnight in sucrose, then diluted and grown for an additional 10 h in sucrose and galactose. After 10 h, cells were diluted to an OD of about 0.1 (0.128 for sucrose and 0.137 for galactose) and plated as a dilution series of 1, 1:10, 1:100 and 1:1000.

Visualization of actin, GFP-tagged proteins and immunofluorescence

Cells were fixed in 3.7% formaldehyde (final, in the medium), washed twice with PBS and stained. Rhodamine–phalloidin staining was carried out as described previously (Adams and Pringle, 1991). The GFP fusion proteins were visualized in living cells by fluorescent microscopy using a Chroma long-pass GFP filter set.

Visualization of the septin ring was also achieved by immunofluorescence staining of the 3Xmyc-tagged version of the Cdc3 septin, using anti-myc antibodies on fixed cells. This procedure was carried out as described previously. Visualization of the different structures was achieved using the appropriate filter sets.

For the survival test, a sample of cells grown for 10 h or 24 h in galactose was resuspended in the same volume of Trypan blue, and cells that incorporated the dye were counted under the light microscope.

Quantification of actin expression level

Wild-type cells carrying the plasmid pYB49 were induced for actin overexpression on galactose medium. Time points were taken at 4 and 8 h. The cells were broken in sample buffer using glass beads. Protein samples were separated by SDS-PAGE, and the gels were subsequently stained with Coomassie blue to assess the total amount of protein present in each lysate. New gels were run to have equal amount of protein extract in each lane, transferred on to polyvinylidene difluoride (PVDF) membrane, and the relative amount of actin monomers was estimated by immunoblotting. As a primary antibody against actin, we used AC40, a monoclonal anti-actin mouse ascites fluid (Sigma). The secondary antibody was a horseradish peroxidase (HRP)-coupled anti-mouse antibody (Santa Cruz Biotechnology). The immunoblot was detected by chemiluminescence using the ECL kit (Amersham).

Acknowledgements

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References


The NoCut pathway links abscission to proper function of the spindle midzone and prevents chromosome breakage by the cytokinetic machinery

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**Summary:**

During anaphase, spindle elongation pulls sister chromatids apart until each pair is fully separated. In turn, cytokinesis cleaves the cell between the separated chromosomes. What ensures that cytokinesis proceeds only after that all chromosome arms are pulled out of the cleavage plane was unknown. Here, we show that a signalling pathway, which we call NoCut, delays the completion of cytokinesis in cells with spindle midzone defects. NoCut depends on the Aurora kinase Ipl1 and the anillin-related proteins Boi1 and Boi2, which localize to the site of cleavage in an Ipl1-dependent manner and act as abscission inhibitors. Inactivation of NoCut leads to premature abscission and chromosome breakage by the cytokinetic machinery, and is lethal in cells with spindle elongation defects. We propose that NoCut monitors clearance of chromatin from the midzone to ensure that cytokinesis completes only after all chromosomes have migrated to the poles.
**Introduction:**

Mitosis is a highly coordinated process in which the two copies of each chromosome (sister chromatids) are segregated away from each other to opposite poles of the cell. Subsequently, the cell is cleaved between the two newly formed nuclei, leading to two independent cells. To ensure that each daughter cell inherits a single and complete copy of the genome, chromosome segregation and cell division are tightly coordinated. The fidelity of these events is ensured by complex surveillance mechanisms that detect and correct errors (for review, see Hartwell and Weinert, 1989).

The spindle checkpoint, together with kinetochore proteins and the kinase Aurora B (Ipl1 in yeast), ensures that chromosome segregation begins only after each pair of sister-chromatids achieves bipolar attachment to the mitotic spindle (Musacchio and Hardwick, 2002). Chromosome segregation is then fulfilled by elongation of the anaphase spindle. Elongation is supported by the spindle midzone, which bundles anti-parallel non-kinetochore spindle microtubules (Glotzer, 2005; McCollum, 2004). Numerous molecules localize to the spindle midzone. The microtubule-bundling protein PRC1 (Ase1 in budding yeast) is required for spindle stability. In addition, a number of yeast kinetochore components such as Ndc10, Ndc80 and Slk19, and the chromosomal passenger proteins Aurora B, INCENP and survivin (Ipl1, Sli15 and Bir1 in budding yeast) also localize to the midzone (Adams et al., 2001; Bouck and Bloom, 2005; Buvelot et al., 2003; Glotzer, 2005; Sullivan et al., 2001). When the spindle becomes longer than twice the longest chromosome arm, chromosome segregation is achieved.
Cytokinesis, the final step of cell division, physically dissociates the two daughter cells from each other (Glotzer, 2005; Guertin et al., 2002). In animal cells, cytokinesis starts with contraction of the equatorial actomyosin ring, leading to membrane furrowing. As furrow ingression completes, the cells remain linked by a cytoplasmic bridge containing the remnant of the spindle midzone, the midbody. The actomyosin ring then disassembles while resolution of the plasma membrane, called abscission, completes cytokinesis.

In animal cells, spindle midzone defects generally cause the furrow to regress, leading to the formation of binucleated cells (Glotzer, 2005; Guertin et al., 2002; McCollum, 2004). Cases of furrow regression have also been described in cells with incomplete DNA segregation (Meraldi et al., 2004a; Mullins and Biesele, 1977). However, it is not clear how and why defects in DNA segregation lead to cytokinesis failure.

Like in animal cells, yeast cytokinesis is a multistep process that also starts with the assembly and contraction of an actomyosin ring. Contraction depends on activation of the mitotic exit network (MEN) and Cdk1 inactivation (for review, see Tolliday et al., 2001). After contraction, the ring disassembles and the narrow cytoplasmic bridge left between the two daughter cells is resolved by abscission (Dobbelaere and Barral, 2004). Three independent molecular pathways fulfil these events. Type II myosin, Myo1, ensures actomyosin ring contraction. Hof1, homologous to *Schizosaccharomyces pombe* Cdc15p, defines a second pathway required for proper actin organization and septation. The third and least understood pathway depends on the protein Cyk3, and acts downstream of actomyosin ring contraction in septation and perhaps abscission. Inactivation of either of the MYO1, HOF1 or CYK3 genes
affects cytokinesis only mildly, whereas simultaneous disruption of any two of them abolishes cytokinesis and is lethal (Korinek et al., 2000; Tolliday et al., 2001).

Here, we investigate how cytokinesis is coordinated with anaphase. We show that similar to animal cells, yeast spindle midzone defects prevent the completion of cytokinesis. Abscission inhibition depended on NoCut, a signalling pathway that involved the Aurora kinase, Ipl1, and the anillin-related proteins Boi1 and Boi2. Inactivation of this pathway causes chromosome breakage due to precocious abscission. Thus, our results provide a model for how the timing of cytokinesis is coordinated with chromosome segregation.
Results

Ase1 is required for timely cytokinesis

In animal cells, spindle midzone defects lead to cytokinesis failure. We therefore investigated whether the midzone is also required for cytokinesis in budding yeast. The microtubule-binding protein Ase1, the homologue of human PRC1, is the only yeast protein known to localize exclusively to the spindle midzone. Yeast cells lacking Ase1 are viable and form highly unstable spindles that break down prematurely during anaphase (Schuyler et al., 2003). Tetrad analysis demonstrated that cells lacking Myo1 and Hof1 depend on Ase1 for survival, indicating that Ase1 is required for cytokinesis, and suggesting that it may function in the Cyk3 pathway (data not shown). In support of this conclusion, the ase1Δ cyk3Δ double mutant was fully viable. These data indicate that Ase1 or the spindle midzone participates in yeast cytokinesis.

To characterize the role of Ase1 in cytokinesis, we visualized the plasma membrane at the bud neck of dividing ase1Δ cells using Ras2-GFP as a marker (Whistler and Rine, 1997). Cells that had initiated anaphase were identified by the fact that they had one spindle pole body (SPB) in the mother cell and the other in the bud. SPBs were visualized using Spc42-CFP as a marker. In ana/telophase cells, the plasma membrane at the bud neck was in one of three states (Fig 1A): continuous ("open" neck, in which the furrow has not ingressed), contracted (the furrow had ingressed) or resolved into two, indicating that abscission was completed but mother cells had not yet separated from their buds. To determine which stage of cytokinesis was affected in ase1Δ mutants, we quantified the frequency of these different classes in
asynchronous wild type and ase1∆ cultures (Fig 1B). In wild type, 45% of the ana-
and telophase cells had not yet undergone contraction, while in 52% of the cells the membrane was clearly resolved into two. Only about 3% of the cells showed a "pinched" neck, in which the membrane had ingressed. The small fraction of cells with pinched bud necks reflects the short interval between furrow ingression and membrane resolution in wild type cells. This category of cells was specifically and strongly increased (8-fold) in the ase1∆ population (Fig 1B). Thus, furrow ingression progressed properly but membrane resolution was delayed in the ase1∆ cells. These data are consistent with Ase1 acting in cytokinesis posterior to actomyosin ring contraction, most likely in abscission.

To further analyze the role of Ase1, we created a conditional ASE1 shut-off allele. The Ase1 promoter was replaced by a weak version of the regulatable GAL promoter (GALS) (Janke et al., 2004), which allowed Ase1 synthesis to be turned on to appropriate levels in galactose and turned off in glucose media. Accordingly, GALS:HA-ASE1 cells did not contain detectable levels of HA-Ase1 protein when grown in glucose medium (Fig 1C). In these cells, the spindle failed to elongate and broke down prematurely compared to wild type cells (see Fig 6B). Thus, the GALS:HA-ASE1 allele allowed efficient depletion of Ase1 within a single cell cycle, consistent with Ase1 being unstable in G1 (Juang et al., 1997). Similar to the ase1∆ mutant cells, the GALS:HA-ASE1 cells delayed abscission upon shifting to the repressing medium (Fig 1B). In addition, a high fraction of GALS:HA-ASE1 cells formed chains that failed to separate, where primary septa failed to form properly, as indicated by chitin staining (Fig 1E). These cells were not merely kept together by cell wall remnants but rather by a continuous plasma membrane, since digestion of the cell wall using zymolyase failed to dissociate the chains (Fig 1D). Together,
these data indicate that Ase1 function is required for efficient resolution of the plasma membrane after furrow ingression, and hence for proper abscission.

**Mutations in kinetochore proteins that affect the spindle midzone also prevent normal cytokinesis**

The cytokinesis defect observed in cells lacking Ase1 suggested that the spindle midzone is involved in the control of abscission. Alternatively, cytokinesis might have been repressed until completion of chromosome segregation, which is delayed in these cells (Fig 7A). To distinguish between these possibilities, we tested whether mutations that impair chromosome segregation also impair cell cleavage. Kinetochore components fall into two classes relative to chromosome separation and spindle function. Ndc10, an inner kinetochore component, also localizes to the spindle midzone during anaphase and is required for both kinetochore assembly and midzone stability (Bouck and Bloom, 2005; Buvelot et al., 2003; Supplementary Fig S1A-B). Using the midzone marker Slk19, we found that the central kinetochore component Ndc80 falls in the same category (Fig. 2A). In contrast, the central kinetochore proteins Nnf1 and Mtw1 were required only for chromosome attachment to the spindle and not for midzone formation (Fig 2A and 2B). Progression of the ndc80-1, nnf1-17 and mtw1-1 mutants through anaphase was obtained by deleting the MAD2 gene. Analysis of bud formation indicated that all strains entered and exited mitosis with similar kinetics as wild type (Supplementary Fig. S1C, and data not shown).

Inactivation of kinetochore function in nnf1-17 mad2Δ and mtw1-1 mad2Δ double mutants resulted in little or no DNA being pulled into the bud upon anaphase, and led
to an increased number of cells that rebudded without separating from their first bud (Fig. 2B). However these mutants were not defective in cytokinesis because the bibudded cells were efficiently resolved by zymolyase treatment. Thus, the *nnf1-17 mad2Δ* and *mtw1-1 mad2Δ* double mutants completed cytokinesis but were defective in the subsequent degradation of the primary septum. In contrast, the *ndc10-1* single and *ndc80Δ mad2Δ* double mutants formed chains of cells that could not be separated by zymolyase treatment, and were therefore defective in cytokinesis (Fig. 2B). Thus, chromosome segregation was not required for proper completion of cytokinesis, and instead the cytokinesis failure correlated with midzone defects.

Accordingly, microtubules were found to be required for cytokinesis. When synchronized in G1 with α-factor and released into fresh medium containing nocodazole, *mad2Δ* cells progressed through the cell cycle, exited mitosis and entered a new cycle, indicated by the formation of a second bud (Li and Murray, 1991) (Fig 2C). These bibudded cells were not resolved by zymolyase (Fig 2D) indicating that they failed to complete cytokinesis. Our analysis of the kinetochore mutants *nnf1-17* and *mtw1-1* indicated that kinetochore microtubule-interactions were not involved in this process. To test whether cytoplasmic microtubules were involved, we analyzed the division of the *cnm67Δ* cells. Cnm67 is a component of the outer-plaque of the SPB that anchors cytoplasmic microtubules to the SPB. Cells lacking Cnm67 fail to properly position their spindle but exit mitosis on schedule, due to defective organization of the SPB and the spindle orientation checkpoint (Hoepfner et al., 2000). We found that *cnm67Δ* cells completed cytokinesis properly (Fig. 2C-E). In these cells, Slk19-GFP localized to the spindle midzone normally (Fig 2E). Thus, neither microtubule-kinetochore attachment nor cytoplasmic microtubules are required for cytokinesis. We concluded that the microtubules required for cytokinesis
Corresponded to those of the midzone. It is important to note that cytokinetic defects did not correlate with a failure in mitotic exit, since *ndc10-1* and nocodazole-treated *mad2Δ* cells went on to re-replicate DNA, degrade Pds1 and Clb2, and accumulate Sic1 with kinetics similar to wild type (Fraschini et al., 2001; Fig 3C, and data not shown). It is also noteworthy that *cnm67Δ* cells misposition the spindle midzone relative to the bud neck but complete cytokinesis normally. Therefore, the midzone position is irrelevant for yeast cytokinesis.

**Cells with damaged spindles are defective in abscission**

Because Ase1-depleted cells delay abscission, we tested whether *ndc10-1, ndc80-1 mad2Δ* and nocodazole-treated *mad2Δ* cells also suffer of abscission defects. Calcofluor staining showed that these cells failed to complete septation (Fig 3A). Moreover, time-lapse microscopy showed that the actomyosin ring contracted and disassembled with wild type kinetics in *ndc10-1* and nocodazole-treated *mad2Δ* cells (Fig 3B). Thus, in these cells, cytokinesis was impaired after actomyosin ring contraction.

To investigate whether abscission failed, we monitored the plasma membrane at the bud neck. Cells containing Ras2-GFP were imaged in three dimensions by spinning disk confocal microscopy. In *cnm67Δ* and untreated *mad2Δ* mutant cells, the plasma membrane was continuous through the bud neck of mitotic cells. In postmitotic cells, two plasma membranes were observed that were clearly separated by the unlabeled septum (Fig 3D), indicating that these cells completed abscission. In contrast, the plasma membrane of the postmitotic *mad2Δ* cells treated with nocodazole had ingressed but was not resolved, indicating that abscission failed (Fig 3D). Consistent
with this, *ndc10-1* and nocodazole treated *mad2Δ* cells displayed disorganized septin rings, a phenotype frequently observed in abscission mutants (Dobbelaere and Barral, 2004; Dobbelaere et al., 2003; Fig 3E). By comparison, abscission-competent *cnm67Δ* cells showed septin rings indistinguishable from wild-type. Taken together, these results indicated that yeast cells with spindle midzone defects display septin disorganization and delay or fail abscission.

**The Aurora kinase Ipl1 inhibits abscission in cells with spindle midzone defects**

Two scenarios can account for the cytokinesis defect of midzone-defective cells. The spindle midzone might provide a positive signal necessary for abscission. Alternatively, active inhibition of abscission might delay cytokinesis as a secondary response to midzone defects. In the latter case, cells that are defective in inhibition should complete abscission even in the absence of a midzone. The yeast aurora kinase Ipl1 and its associated protein Sli15/INCENP localize to the spindle midzone during anaphase and might be required for midzone stability (Pereira and Schiebel, 2003). However, *ipl1-321* mutants do not exhibit a cytokinesis defect (Fig 4A; (Biggins and Murray, 2001; Buvelot et al., 2003). We therefore tested whether the cytokinesis defect in midzone mutants requires Ipl1 kinase activity and asked whether the *ipl1-321* and *sli15-1* mutations rescued cytokinesis in midzone-defective cells. As shown in Fig 4A, inactivation of Ipl1 or Sli15 did not prevent the formation of bibudded cells. However, all bibudded cells formed a septum at postmitotic necks (Fig4B) and were efficiently separated by zymolyase (Fig4A). Spindle midzone defects were not rescued in these cells (Fig 4C), indicating that inactivation of *ipl1* and Sli15 directly restored abscission. Thus, the spindle midzone is not required per
se for cytokinesis. Instead, midzone defects cause Aurora/Ipl1- and INCENP/Sli15-dependent inhibition of abscission.

**Ipl1-dependent inhibition of abscission requires the bud neck proteins Boi1 and Boi2**

To elucidate how Ipl1 inhibits abscission, we investigated whether it translocates from the closed nucleus to the bud neck in response to midzone defects. In *ndc10-1* cells, Ipl1-3GFP localized along the broken spindles (Fig 4D). In nocodazole treated *mad2*Δ cells, no spindle assembled and Ipl1 localized to nuclear dots (data not shown). Ipl1 was never observed at the bud neck. Thus, we postulated that Ipl1 inhibits abscission through proteins that shuttle between the nucleus and the bud neck.

Boi1 and Boi2 are highly related proteins involved in polarized growth. They are structurally related to anillins, a family of cytokinesis proteins characterized by the presence of a pleckstrin-homology (PH) domain at their C-terminus, and by the ability to shuttle between the nucleus and the cell cortex. Accordingly, Boi1 and Boi2 carry a PH domain at their C-termini, localize to the bud cortex during bud emergence and to the bud neck during mitosis (Hallett et al., 2002). Furthermore, they are occasionally observed in the nucleus of G1 cells (Eric Bailly, personal communication and Fig 5C). Finally, *boi1Δ boi2Δ* double mutant cells separate normally (Fig 4A), indicating that they might not act positively in cytokinesis. Thus, we asked whether they acted as cytokinetic inhibitors.
Therefore, we constructed the ndc10-1 boi1Δ boi2Δ triple mutant and analyzed its phenotype. Four hours after α-factor release, 26% of the boi1Δ boi2Δ ndc10-1 triple mutant cells were bi-budded (versus 36% in ndc10-1). Most of these cells formed proper septa (Fig 4B) and were resolved to unbudded and single-budded cells by zymolyase treatment (Fig 4A). Furthermore, as in the ndc10-1 ipl1-321 double mutants, midzone defects were not suppressed in these cells (Fig 4C). Thus, Boi1 and Boi2 functioned in the inhibition of abscission in midzone defective cells.

We next carried out a detailed analysis of Boi1 and Boi2 localization. Co-expression of Boi1-GFP and CFP-Tub1 revealed that Boi1 translocated from the nucleus, where it was detected in a fraction of G1 cells, to the cortex of the emerging bud in G2 and to the bud neck of anaphase cells (Fig 5A (arrows) and Fig 5B). After spindle breakdown, low levels of Boi1-GFP were briefly present on both sides of the neck (Fig 5A, arrowheads), and faded away prior to cell separation. Similar results were obtained for Boi2-GFP expressed from a multi-copy plasmid, in which case the accumulation of Boi2-GFP to G1 nuclei was more obvious (Fig 5C). In contrast to wild-type cells, most ndc10-1 cells that failed abscission maintained Boi1 at the bud neck even after the emergence of a new bud (Fig 5A). Thus, the localization of Boi1 and Boi2 was consistent with a role in the inhibition of abscission.

Remarkably, in the ipl1-321 mutant shifted to the restrictive temperature, Boi1-GFP failed to localize to the cortex of growing buds and to the bud neck of anaphase cells. Instead, it remained nuclear for the whole cell cycle (Fig 5B). Similar results were obtained for plasmid borne Boi2-GFP (Fig 5C). Thus, Ipl1 activity was required for the proper localization of Boi1 and Boi2, establishing that Boi1 and Boi2 act downstream of Ipl1. Boi1 and Boi2 contain several Ipl1 consensus phosphorylation sites.
However, simultaneous mutation of all sites in both Boi1 and Boi2 did not alter their localization (data not shown). Thus, some other Ipl1 substrate might function in the control of Boi1 and Boi2 localization. Alternatively, additional Ipl1 sites might exist on these proteins.

**Boi1 and Boi2 prevent premature abscission in wild type cells**

To test whether Boi1/Boi2 are required to regulate abscission in all cells or only in cells with midzone defects, we analyzed the progression of cytokinesis in wild-type and *boi1Δ boi2Δ* double mutant cells using time-lapse microscopy. As in Fig 1A, we monitored mitotic progression and abscission using the reporters Spc42-CFP and Ras2-GFP. In all movies, the bud neck membrane contracted shortly after spindle breakdown, visualized by the sudden decrease in SPB-to-SPB distance (Fig. 6A, t=25 min; upper graph in Fig. 6B; wild-type: 1.6 ± 1.2 min after spindle breakdown; *boi1Δ boi2Δ*: 1.3 ± 1.1 min; N=13 and 9, respectively). In wild type cells, the membrane remained "pinched" for an average of 15 min (± 1.05 min, N=13) before being resolved by abscission (Fig 6A, t=43 min). Remarkably, the interval between membrane contraction and resolution was significantly reduced in the *boi1Δ boi2Δ* double mutant cells (11.9 ± 0.9 min, N=9, p<0.05). Anaphase proceeded normally in these cells (Fig. 6B). Thus, abscission was advanced in the *boi1Δ boi2Δ* double mutant as compared to wild-type, demonstrating that Boi1 and Boi2 control the timing of abscission in wild type cells.

**Boi1 and Boi2 delay abscission in Ase1-depleted cells**
Consistent with our original observation (Fig. 1B), time-lapse analysis showed that membrane resolution in ase1Δ mutant cells occurs approximately 24 min after contraction, 9 min later than wild type (Fig. 6B, C; mean ase1Δ: 24.2 ± 2.3 min, N=10, p<0.005). Similar results were obtained with the GALS:HA-ASE1 cells grown in glucose (22.2 ± 2.3 min, N=11, p<0.05). This delay depended on Boi1 and Boi2, since the boi1Δ boi2Δ GALS:HA-ASE1 triple mutant completed cytokinesis with wild type kinetics (15.2 ± 1.3 min, N=10). In these cells, spindle dynamics were identical to that of the GALS:HA-ASE1 and ase1Δ cells, indicating that the midzone defect was not suppressed (fig 6B). The timing of cytokinesis could not be determined for the boi1Δ boi2Δ ase1Δ triple mutant cells, because this strain was not viable. Micromanipulation of boi1Δ boi2Δ ase1Δ spores indicated that they germinated properly and underwent a few rounds of division, completing cytokinesis and cell separation before arrest (data not shown). Together, these data establish that Boi1 and Boi2 delay abscission and are required for cell viability in response to spindle midzone defects.

**Failure to delay abscission causes chromosome breakage by the cytokinetic machinery**

Together, our data indicate that spindle midzone defects lead to Ipl1- and Boi1/Boi2-dependent inhibition of abscission. We next wondered about the biological function of this inhibition. Central spindle collapse during anaphase delays the clearance of the cleavage plane from chromatin. Indeed, upon shift to the repressive condition, chromosome segregation was strongly impaired in GALS:HA-ASE1 cells, as assayed by DAPI staining (Fig 7A and 7B). Therefore, we rationalized that inhibition of
abscission might prevent the chromosome arms still lagging in the cleavage plane from being broken by the cytokinetic machinery.

To test this possibility, we monitored the effect of Boi1 and Boi2 inactivation on chromosome integrity, using Ddc1-GFP as a reporter of DNA damage. Ddc1 is involved in the recognition and repair of double strand breaks (DSB), and Ddc1-GFP foci accumulate at sites of DNA damage (Melo et al., 2001). When wild type and \textit{GALS:HA-ASE1} cells were released from $\alpha$-factor arrest into repressing conditions, a small fraction of cells with Ddc1-GFP foci appeared in both strains at early time points, likely corresponding to S phase (Fig 7D). In both strains, the frequency of cells with Ddc1-GFP foci then dropped to about 4% as the cells progressed through mitosis and into the next G1. Thus, Ase1 depletion did not increase the frequency of double strand breaks compared to wild type cells, despite the frequent presence of chromatin in the cleavage plane. In contrast, cells lacking Boi1 and Boi2 showed Ddc1-GFP foci at a higher frequency of up to 8%. The frequency of these foci increased further (up to 27%) in the Ase1-depleted, \textit{boi1$\Delta$ boi2$\Delta$} cells. The increased frequency of Ddc1 foci was most obvious at later times in the experiment, when cells went through mitosis (Fig 7D). In the \textit{boi1$\Delta$ boi2$\Delta$} double and \textit{boi1$\Delta$ boi2$\Delta$ GALS:HA-ASE1} triple mutant cells, most Ddc1-GFP dots were found in anaphase cells (see Fig 7E) and frequently localized to the bud neck (Fig 7C). No such correlation was observed in \textit{BOI$^+$} cells. Thus, Boi1 and Boi2 have DNA-protecting activity in a late stage of the cell cycle.

To test whether DNA damage was caused by premature cytokinesis in \textit{boi1$\Delta$ boi2$\Delta$} cells, we tested whether the frequency of Ddc1-GFP foci was suppressed when we
prevented cytokinesis using the \textit{cdc12-6} septin mutation. Asynchronous cultures were shifted from galactose to glucose at the restrictive temperature for \textit{cdc12-6} and Ddc1-GFP foci were scored after four hours. Consistent with cytokinesis causing DNA damage, the frequency of Ddc1 foci was reduced to wild-type levels in the \textit{boi1}\textdagger \textit{boi2}\textdagger \textit{cdc12-6} triple and \textit{GALS:HA-ASE1 boi1}\textdagger \textit{boi2}\textdagger \textit{cdc12-6} quadruple mutants (Fig 7F).

Double-strand breaks can be repaired by two alternative mechanisms. Homologous recombination (HR) uses the sequence of the homologous chromosome to repair the break, whereas non-homologous end joining (NHEJ) joins the ends of broken chromosomes irrespective of sequence homology (Krogh and Symington, 2004). We reasoned that if haploid \textit{boi1}\textdagger \textit{boi2}\textdagger double mutant cells experience an increased incidence of chromosome breaks during abscission, these cells should strongly rely on NHEJ for survival. Indeed, HR would not be able to process such breaks, since the damage would occur after segregation of homologous chromatids to opposite daughter cells. In agreement with this, \textit{boi1}\textdagger \textit{boi2}\textdagger cells carrying mutations in the NHEJ genes \textit{YKU70} and \textit{MRE11} showed impaired growth compared to the parental strains (Fig. 7G). Disruption of the HR gene \textit{RAD52} had no such effect. Thus, the \textit{boi1}\textdagger \textit{boi2}\textdagger cells experienced an increased incidence of chromosome breaks specifically after sister chromatid disjunction. Altogether, our results establish that Boi1 and Boi2 function in a pathway that protects chromosomes from being broken by premature cytokinesis.
Discussion

In this study, we show that spindle midzone defects lead to inhibition of abscission in yeast. Abscission inhibition depends on Ipl1/Aurora and the anillin-related proteins Boi1 and Boi2. Failure to inhibit abscission leads to an increased frequency of chromosome breakage during cell division, and loss of viability, indicating that cells possess a mechanism to delay cytokinesis when chromosome segregation is impaired.

Cells with spindle midzone defects fail to complete abscission

Here we report that spindle midzone defects impair cytokinesis in yeast. Failure of cytokinesis was not due to defects in mitotic exit and/or cell cycle progression, since cytokinesis-defective cells started a new round of budding and DNA replication on schedule. In addition, mutants that affected aspects of spindle function other than the spindle midzone (like *nnf1-1 mad2Δ*, *mtw1-1 mad2Δ* and *cnm67Δ*) often caused cell separation defects, but did not impair cytokinesis. Cell separation failed in these mutants probably because DNA did not segregate into the bud, preventing the bud-specific transcription factor Ace2 from initiating primary septum degradation (Colman-Lerner et al., 2001; Racki et al., 2000). In contrast, mutations that affected the spindle midzone, such as *ase1Δ*, *ndc10-1* and *ndc80-1*, delayed or completely abrogated cytokinesis. Cell wall digestion assays, as well as imaging of the cell wall, plasma membrane and actomyosin ring dynamics, indicated that abscission failed. Accordingly, genetic data placed Ase1 in the Cyk3 pathway, which is likely to act in abscission. Although some of the mutants that affected the midzone and cytokinesis
also affected microtubule-kinetochore interactions or cytoplasmic microtubules, these defects alone did not cause failure of cytokinesis. Thus, we conclude that similar to animal cells, the spindle midzone is required for proper completion of cell cleavage in budding yeast.

**How direct is the role of the spindle midzone during abscission?**

How spindle midzone defects interfere with cytokinesis in animal cells has remained elusive. It has been assumed that the spindle midzone provides spatial information and/or structural support for the assembly of the abscission machinery, i.e., that it is an intrinsic player of cytokinesis. Alternatively, the cell might respond to midzone defects by preventing abscission, similar to a checkpoint response. In the latter model, the midzone does not play a role in cytokinesis. Rather, a sensing mechanism would detect midzone defects or their consequence(s) and respond by inhibiting abscission. Inactivation of this machinery should therefore render abscission independent of midzone integrity. Strikingly, we show here that midzone-defective cells lacking Aurora kinase activity or the anillin-related molecules Boi1 and Boi2 restore cytokinesis without suppressing the midzone defects. Thus, our results indicate that Ipl1 and the Boi proteins are part of an inhibitory pathway, which we call NoCut, that represses abscission in cells with midzone defects. Ipl1 localizes exclusively to the nucleus and most likely acts upstream in NoCut. In turn, Boi1 and Boi2 translocate from the nucleus to the cortex in response to Ipl1/Sli15 activity. There, they repress abscission, possibly directly or indirectly by recruiting or activating other inhibitor(s). Thus, the spindle midzone is not intrinsically required for abscission in yeast.
The NoCut pathway prevents chromosome damage during cytokinesis

The spindle assembly and orientation checkpoints ensure the faithful segregation of chromosomes between mother and daughter cells (Bardin and Amon, 2001; Musacchio and Hardwick, 2002). What is the role of yet another surveillance mechanism during mitosis? Visualization of DNA damage sites using Ddc1-GFP indicated that boi1Δ boi2Δ cells suffer increased levels of DNA damage during anaphase. The frequency of DNA damage further increased when the boi1Δ boi2Δ cells failed to elongate their spindle properly. Preventing cytokinesis in the boi1Δ boi2Δ cdc12-6 and GALS:ASE1 boi1Δ boi2Δ cdc12-6 cells was sufficient to prevent this damage. Thus, a major conclusion from our study is that the cytokinesis machinery can cause DNA damage if not properly regulated. A main function of NoCut therefore appears to be the protection of chromosomes while they are still engaged in the cleavage plane. Our finding that an ase1Δ boi1Δ boi2Δ triple mutant is inviable underscores the importance of this control.

Remarkably, our data also show that NoCut delays abscission during normal mitosis in addition to midzone-defective cells. The increased frequency of DNA damage in the boi1Δ boi2Δ double mutants indicates that, even when spindle elongation is normal, some chromatin lingers at the bud neck in a significant fraction of telophase cells. Thus, clearance of chromatin from the cleavage plane is error prone and requires the existence of a surveillance mechanism. Shielding genetic material from the cytokinesis machinery is probably a common theme in cell division because a NoCut-like system also exists in bacteria (Wu and Errington, 2004) although the molecular mechanisms differ. In mammalian cells, merotely attached chromatids, which are pulled towards both poles of the spindle and therefore remain in the
cleavage plane, cause an abscission delay until they segregate to one side of the
midzone (Cimini et al., 2002). Similarly, studies in animal cells have established that
DNA bridges due to incomplete separation of sister chromatids also cause abscission
to fail and the cleavage furrow to regress (Meraldi et al., 2004a; Mullins and Biesele,
1977). Furrow regression does not occur in midzone-defective budding yeast cells,
probably due to cell wall deposition during actomyosin ring contraction. Thus, NoCut-
like mechanisms might exist in most cell types.

In summary, NoCut appears to be a previously unidentified cell cycle checkpoint: it
represses a cell cycle event, cytokinesis, in response to defects in a previous step,
anaphase. This repression prevents a catastrophic division and helps the cell to
remain viable. However, unlike classical checkpoints, NoCut does not lead to cell
cycle arrest.

A model for the NoCut pathway

It is not clear what primary signal is being monitored by NoCut. Similarly, it is
unknown how NoCut inactivation allows abscission. Because midzone defects cause
abscission inhibition, it is possible that NoCut directly monitors midzone integrity.
Three observations argue against this idea: first, Boi1 and Boi2 localize to the bud
neck in anaphase, suggesting that NoCut is already active at this stage, although the
midzone is intact. Second, Boi1/Boi2 control the timing of abscission in all cells, not
only in those with damaged spindles. Third, in ase1Δ, NoCut delays abscission but
does not prevent it. The first two observations indicate that NoCut is already active
during anaphase in all cells. Thus, an attractive, although speculative, model is that
the spindle midzone is required for NoCut inactivation upon chromosome separation.
We propose that NoCut monitors the clearance of chromatin from the cleavage plane, and that the spindle midzone functions as a sensor in this process (Fig 8). For example, the proximity of chromatin might activate Ipl1, perhaps through a mechanism similar to the activation of Aurora-A by Ran-GTP (Gruss and Vernos, 2004). Ipl1 activity would then remain high as long as chromatin surrounds the midzone, and Boi1 and Boi2 would stay at the bud neck and hold abscission in check (Fig 8A). During spindle elongation, the spindle midzone would sequester Ipl1 away from chromatin, allowing its inactivation and permitting abscission to be relieved from Boi1- and Boi2-dependent repression (Fig 8B). On the other hand, in midzone-defective cells, Ipl1, which remains around chromatin, maintains its inhibition activity (fig 8C), explaining the abscission defect of midzone mutants. In the kinetochore mutants mtw1 and nnf1, chromosomes are successfully segregated from the midzone, in that they remain around the spindle pole in the mother cell. This would explain why defects in chromosome separation do not lead to a cytokinesis defect in these cells. Thus our observations are consistent with the incomplete clearance of chromatin from the spindle midzone triggering NoCut.

The “cut” phenotype has been most clearly described in S. pombe (Yanagida, 1998). In this organism, a number of mutations lead to completion of cytokinesis in the absence of chromosome segregation, and hence, to cutting of the nucleus that is still lying on the cleavage plane. Although this could indicate that the coordination between chromosome segregation and abscission is not conserved, other interpretations could reconcile the different findings. For example, the S. pombe cut mutants could in fact be components of both anaphase initiation and the fission yeast NoCut pathway. Alternatively, since NoCut causes a delay and not a complete block in cytokinesis, DNA cuts could still occur in the absence of chromosome segregation.
after a prolonged time; in fact, "adaptation" is known to occur with other surveillance mechanisms, like the spindle and DNA replication checkpoints.

In higher eukaryotes, Aurora-B proteins are required for furrowing and cytokinesis (Adams et al., 2001; Meraldi et al., 2004b), and might therefore not function in NoCut. However, a role for Aurora-B in abscission inhibition cannot be excluded at this point, since such a role would have been masked by its earlier role in furrow ingression. In this perspective, it is worth noting that overexpression of Aurora-A causes abscission failure in animal cells (Marumoto et al., 2005). It will be interesting to investigate whether this phenomenon is related to Aurora-A acting in a mammalian NoCut pathway.
Material and methods

Strains, Plasmids, and Growth Conditions

Strains are derivatives of W303 or S288C. All strains were grown in rich medium (YPD) at room temperature, unless indicated otherwise. Gene deletion, promoter replacement and tagging was performed using a single-step PCR based approach as described (Janke et al., 2004; Longtine et al., 1998). The ndc10-1, nrf1-17, mtw-1, ndc80-1, ipl1-321, sli15-1 and cdc12-6 mutations have been described (Barral et al., 2000; Biggins et al., 1999; Euskirchen, 2002; Goh and Kilmartin, 1993; Goshima and Yanagida, 2000; Kim et al., 1999; Wigge and Kilmartin, 2001). The cnm67Δ (Hoepfner et al., 2000) and boi1Δ boi2Δ and Boi2-GFP strains were kind gifts from P. Philippson (Biozentrum, Basel) and Eric Bailly (CNRS, Marseille). The RAS2-GFP construct has been described (Whistler and Rine, 1997).

Cell synchronization, staining procedures, and nocodazole and zymolyase treatment

Cells arrested with α factor (10 μg/ml, 2h at 22°C) were washed twice in YPD and released at the restrictive temperature (for temperature sensitive mutants) or at 22°C. For DAPI staining, cells were fixed for 30 min in 70% ethanol, washed in PBS, and resuspended in PBS containing 1 μg/ml DAPI. Nocodazole was used at a concentration of 50 μg/ml. For septum digestion, cells were fixed with 3.7% formaldehyde for 30 min, washed twice with PBS and resuspended in 1M sorbitol. Cell separation was evaluated by light microscopy after 30 min Zymolyase digestion (2 mg/ml) at 22°C. Calcofluor (SIGMA) was used at 0.01 mg/ml. FACS measurements were carried out as described (Lim et al., 1996).
**Western Blotting**

Samples for extract preparation were collected at the indicated times after release in fresh media containing glucose or galactose. Extracts were prepared using alkaline lysis and TCA precipitation as described (Janke et al., 2004). Total protein concentration was measured by a colorimetric assay (Bio-Rad) and equal amounts were loaded on SDS gels. Upon transfer to PVDF membranes, the relative amount of Ase1 was estimated by immunoblotting using α-HA antibodies (SIGMA) detected with an ECL kit (Amersham).

**Microscopy**

Acquisition of Z-stacks for visualization of the plasma membrane at the bud neck was carried out on a Zeiss 200M spinning disk confocal microscope (Fig 3) or on an Olympus BX50 fluorescence microscope equipped with a piezo motor (Fig 1 and 6) essentially as described (Kusch et al., 2002). Unpaired t-tests were used to assess the statistical significance of the data in Fig 6 (Prism software, GraphPad).
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Figure legends

**Figure 1:** Ase1 defective cells have a cytokinetic delay.

A) Visualization of the plasma membrane, labeled with Ras2-GFP, during cytokinesis (projections of non-confocal planes along the Z-axis). Segregation of one SPB into the bud (red arrow) indicates that the cells have initiated anaphase. White symbols mark open bud necks (asterisk), and necks with contracted (arrowhead) or resolved membranes (arrow).

B) Fraction of anaphase and post-anaphase cells in the cytokinesis stages defined in panel A. Resolution of the plasma membrane is delayed in the ase1Δ and GAL:HA-ASE1 strains.

C) Western blots of cell extracts from GAL:S:HA-ASE1 cells probed with anti-HA and anti-Bud27 antibodies (loading control). Ase1 fails to accumulate in cells grown in glucose medium. The carbon source (Glu = glucose, Gal = galactose) and the time (h) after release from G1 are indicated.

D) Fraction of GAL:S:HA-ASE1 bibudded cells grown under inducing and non-inducing conditions (4 hours), prior and after zymolyase treatment.

E) Morphology of wild type and GAL:S:HA-ASE1 cells grown for 4 hours in Glucose medium. Staining of the cell wall with calcofluor white is shown. Arrowheads mark open bud necks, while arrows point at closed septa.

**Figure 2:** Role of kinetochore components in spindle midzone assembly and cytokinesis.
A) Localization of Slk19-GFP in kinetochore mutants undergoing anaphase. Cells (N>100) were arrested in G1 and released at the restrictive temperature. The fraction of cells with spindle midzones is shown, as indicated by Slk19 localization in wild type and mutant strains. Arrows indicate the spindle midzones.

B) Phase micrographs and DAPI staining (to visualize DNA) of kinetochore mutants allowed to progress through mitosis. Cells were synchronized as in panel A and examined 240 min after the temperature shift. The graph shows the fraction of bibudded cells before and after digestion of the cell wall with zymolyase.

C) Morphology and DNA distribution (DAPI staining) of bibudded cnm67Δ and nocodazole-treated mad2Δ cells.

D) Fraction of bibudded cnm67Δ and nocodazole-treated mad2Δ cells before and after zymolyase treatment.

E) Localization of Slk19-GFP in cnm67Δ cells, showing that these cells fail to orient their spindles but assemble normal midzones (arrows).

**Figure 3**: Abscission is impaired in cells with midzone defects.

A) Cells were stained with calcofluor white to visualize septa. Arrows indicate closed septa while arrowheads indicate open bud necks.

B) Kymographs showing actomyosin ring contraction (Myo1-GFP) in cells of the indicated genotype. Asterisks mark the start of actomyosin ring contraction. The time in seconds is indicated in the x-axis.

C) ndc10-1 cells accumulate 4N DNA content after 200 min at the restrictive temperature. Aliquots from synchronized cultures were stained with propidium iodide and their DNA content was analyzed by FACS. DNA content is indicated.
D) The status of the plasma membrane was examined using Ras2-GFP as a marker. Cells were inspected 180 min after release from G1 block. Images were acquired at 150nm intervals along the z-axis using a spinning disk confocal microscope. Arrowheads indicate unresolved plasma membrane bridges, arrows point at resolved plasma membranes and asterisks mark open bud necks.

E) Analysis of septin ring morphology, using the septin Cdc12-GFP as a reporter. Normal double rings are indicated with an asterisk. Arrowheads point to abnormal discontinuous rings. An arrow points at a single ring lying on the bud side of a cnm67Δ mutant.

Figure 4: Aurora/Ipl1, INCENP/Sli15 and the anillin-like Boi1 and Boi2 proteins inhibit abscission in cells with midzone defects.

A) Fraction of bibudded cells before and after zymolyase treatment. Cells were synchronized with α-factor and analyzed 240 min after release from G1 block at 37°C.

B) Calcofluor staining of bibudded cells 240 min after release as in fig 4A. Arrows indicate completed septa while arrowheads mark open bud necks.

C) Formation of the central spindle, as assayed by localization of Slk19-GFP as a reporter, is not restored in ndc10-1 ipl1-321 and ndc10-1 boi1Δ boi2Δ cells. Cells were treated as in Fig 2A.

D) Localization of Ipl1-3GFP (red) and Tub1-CFP (green) during anaphase of wild type and ndc10-1 cells grown at the restrictive temperature.

Figure 5: Boi1 and Boi2 localization depends on Ipl1 function.
A) Localization of endogenously expressed Boi1-GFP in wild type and ndc10-1 cells during anaphase and telophase. CFP-Tub1 was used to monitor the status of the spindle. Arrows mark bud neck localization of Boi1-GFP.

B) Time course of Boi1-GFP localization in wild type and ipl1-321 cells progressing through the cell cycle.

C) Localization of plasmid-borne Boi2-GFP in wild type and ipl1-321 cells. Arrows (at bud necks) and arrowheads (at the bud surface) indicate cortical Boi2-GFP while asterisks indicate nuclear localization.

Figure 6: Boi1 and Boi2 prevent premature abscission in wild type and delay abscission in Ase1-depleted cells.

A) Selected frames (projections of three focal planes spaced 0.3 µm) from a movie of a wild type cell during mitosis and cytokinesis. The signals of Ras2-GFP and Spc42-CFP were acquired simultaneously in the CFP channel to visualize the plasma membrane and the SPBs, respectively. Time 0 indicates anaphase onset. Red arrows indicate the position of the SPBs. Symbols and letters indicate whether membranes at the bud neck are Open (O, asterisks), Contracted (C, arrowheads) or Resolved (R, arrows).

B) Quantification of the distance between SPBs during mitosis and cytokinesis in representative cells of the indicated strains expressing Ras2-GFP and Spc42-CFP. The timing of membrane contraction (C) and resolution (R) is indicated. The upper graph corresponds to the wild type cell shown in panel A.

C) "Box-and-whiskers" graph summarizing the time elapsed between membrane contraction and separation for all cells examined of the indicated strains. Boxes extend from the 25th to the 75th percentile, with a line at the median; whiskers extend to the most extreme values.
**Figure 7**: Boi1 and Boi2 protect chromosomes from breakage by the cytokinesis machinery.

**A and B)** DAPI staining (A) and quantification of DNA separation (B) of wild type and Ase1-depleted cells undergoing anaphase. Cells of the indicated strains were grown for 4 hours in glucose medium and the shape of anaphase nuclei was scored. ("short": unsegregated chromosomes; "elongated": chromosomes stretched through the bud neck).

**C)** Ddc1-GFP localization to double-strand break foci (arrows). Note that in boi1Δ boi2Δ and GALS:HA-ASE1 boi1Δ boi2Δ cells these foci frequently localize to the vicinity of the bud neck.

**D)** Time-course of Ddc1-GFP focus formation in cells released from G1 into glucose medium.

**E)** Fraction of anaphase cells with Ddc1-GFP foci, treated as in (D). In this and the next panel, data are represented as mean ± SD.

**F)** Ddc1-GFP focus formation in asynchronous cultures after 4 hours in glucose media at 37°C.

**G)** Genetic interactions between boi1Δ boi2Δ mutations and mutations in non-homologues end joining and homologous recombination genes. Cells were grown to log phase in liquid media. 5-fold dilutions were plated and inspected after 3 days.

**Figure 8**: Model of the NoCut pathway.

**A)** During early anaphase, Ipl1 at the central spindle is activated by surrounding chromatin. Active Ipl1 causes Boi1 and Boi2 to translocate to the cortex, where they inhibit septin function. Abscission is repressed.
**B)** Upon segregation of the chromosomes away from the central spindle, Ipl1 is no longer kept active by chromatin. Boi1 and Boi2 leave the bud neck. Abscission can take place.

**C)** In cells with midzone defects Ipl1 stays close to chromatin even after chromosome segregation. Boi1 and Boi2 are not removed from the bud neck. Abscission remains inhibited.
Norden et al. Figure 1

A

open  contracted  resolved

RAS2-GFP SPC42-CFP

B

% ana- and post-anaphase cells

WT  ase1Δ  GALS:HA-ASE1

C

log +Gal
+Glu
+Gal

Bud27
HA-Ase1

D

% of multibudded
GALS:HA-ASE1

untreated  +zymolyase

GAL  GLU 4h

E

WT  GALS:HA-ASE1 4h in glucose

Phase  Calcofluor  Phase  Calcofluor

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Figure

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Norden et al. Figure 2

(A) % of anaphase cells with a spindle midzone (Slk19-GFP)

(B) % of bibudded cells

(C) % of bibudded cells

(D) % of bibudded cells

(E) % of bibudded cells
Figure

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A

B

C

D

Calcofluor

Slk19-GFP

WT

ipl1-321

boi1Δ boi2Δ

ndc10-1 sli15-1

ndc10-1 ipl1-321

ndc10-1 boi1Δ boi2Δ

ndc80-1 mad2Δ ipl1-321

bibudded cells (% of total)

untreated

+zymolyase

D

Ipl1-GFP

Tub1-CFP

Merge

Slk19-GFP

D

Ipl1-GFP

Tub1-CFP

Merge
Norden et al., Figure 6

(A) RAS2-GFP
SPC42-CFP

(B) wt
boi1Δ boi2Δ
ase1Δ
GALS:HA-ASE1
boi1Δ boi2Δ

(C) WT
boi1Δ boi2Δ
ase1Δ
GALS:HA-ASE1
GALS:HA-ASE1
boi1Δ boi2Δ

Figure
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Norden et al., Figure 7

A

WT

DAPI ("elongated")

GALS:HA-ASE1 + glucose

DAPI ("short")

B

% anaphase nuclei

WT GALS:HA-ASE1

short elongated

% of cells with DDC1-GFP foci

0 100

D

% of cells with DDC1-GFP foci

WT GALS:HA-ASE1 boi1 boi2 WT

DDC1-GFP, + glucose

GALS:HA-ASE1 boi1 boi2

C

WT

boi1 boi2

DDC1-GFP, + glucose

GALS:HA-ASE1 boi1 boi2

D

% of cells with DDC1-GFP foci

time in min

WT GALS:HA-ASE1 boi1 boi2

boi1 boi2

GALS:HA-ASE1 boi1 boi2

yku70 yku70 boi1 boi2 boi1 boi2 mre11 mre11 boi1 boi2 boi1 boi2 rad52 rad52 boi1 boi2

E

% anaphase cells with DDC1-GFP foci

WT GALS:HA-ASE1 boi1 boi2 GALS:HA-ASE1 boi1 boi2

F

% cells with DDC1-GFP foci

boi1 boi2 boi1 boi2 boi1 boi2 yku70 yku70 boi1 boi2 boi1 boi2 mre11 mre11 boi1 boi2 boi1 boi2 rad52 rad52 boi1 boi2

G

Click here to download Figure: Norden7.pdf
Figure
Norden et al. Figure 8

Anaphase
No Abscission

Telophase
Abscission

Midzone defect
No Abscission
Norden et al., Suppl. Fig 1

A

WT Slk19-GFP

% of cells

0 60 120 180

time in min

ndc10-1 Slk19-GFP

% of cells

0 60 120 180

time in min

B

GFP-Ase1

WT

ndc10-1

C

WT 37°C

% of cells

0 50 100

time in min

ndc10-1 37°C

% of cells

0 50 100

time in min

rrn1-17 madΔ 37°C

% of cells

0 50 100

time in min

D

WT

GALs:HA-ASE1

boΔ1, boΔ2Δ

Phase, + glucose

GALs:HA-ASE1

boΔ1, boΔ2Δ
Supplementary Figure 1:
A and B: Role of kinetochore components in spindle midzone assembly and stability

A) Cell cycle-dependent localization of Slk19-GFP in WT and ndc10-1 cells after release from α factor arrest. Drawings indicate the different categories of cells.

B) Localization of the midzone component Ase1 in WT and ndc10-1 cells. Cells expressing GFP-Ase1 were released from α-factor at 37°C. Images were acquired after 120 min.

C) Defects in the inner- (ndc10-1) and the outer-kinetochore (nnf1-17) prevent cell separation, without affecting the timing of the budding cycle. WT, ndc10-1 and nnf1-17 mad2Δ cells were synchronized with α factor, released at 37°C and their budding index was scored at the indicated times (N>100). A scheme of the different cell categories is shown.

D) Representative images showing accumulation of budded cells in GALS:HA-ASE1 but not in GALS:HA-ASE1 boi1Δ boi2Δ. Cells were shifted from galactose to glucose for 4 hours.