Mitotic Checkpoint Inactivation
at Anaphase Onset

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DECLARATION

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

ABSTRACT

The mitotic checkpoint prevents chromosome segregation until all chromosomes have reached bi-polar orientation and come under tension on the mitotic spindle. Once this is achieved, the protease separase is activated to cleave the chromosomal cohesin complex and trigger anaphase. Cohesin cleavage releases tension between sister chromatids, however the mitotic checkpoint fails to respond to this apparent tension defect. The aim of this study was to understand why the mitotic checkpoint remains silent when sisters lose tension due to cohesin cleavage in anaphase.

We showed in budding yeast that loss of sister chromatid cohesion at anaphase onset could re-activate the mitotic checkpoint. This is normally prevented by separase-dependent activation of the Cdc14 phosphatase. Cdc14 in turn downregulates the mitotic checkpoint by dephosphorylation of Sli15/INCENP, part of the conserved Aurora B kinase complex and proposed tension sensor at the kinetochores. Consequent relocation of Sli15/INCENP from centromeres to the central spindle during anaphase is a distinctive feature of the Aurora B kinase complex. Our results imply the existence of a conserved mechanism of mitotic checkpoint inactivation in anaphase. Dephosphorylation of Sli15/INCENP and its spatial separation from kinetochores prevent the checkpoint from re-engaging when tension between sister chromatids is lost in anaphase.

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PUBLICATIONS ARISING FROM THIS THESIS

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ABBREVIATIONS

ATP   adenosine triphosphate

APC/C   anaphase promoting complex/cyclosome

BSA   bovine serum albumin

bp   base pairs

Bub   budding uninhibited by benzamidazole

Cdk   Cyclin-dependent kinase

CPC   Chromosome Passenger Complex

D   Dalton (kDa, kilodalton; mDa, megadalton)

DAPI   4'-6'-diamidino-2-phenylindole

DMSO   dimethyl sulfoxide

DNAse   deoxyribonuclease

DTT   dithiothreitol

ECL   enhanced chemiluminescence

EM   electron microscopy

EDTA   ethylenediamine tetraacetic acid

FACS   fluorescence activated cell sorting

FRAP   fluorescence recovery after photobleaching

FEAR   Cdc14 (Fourteen) Early Anaphase Release

FRET   fluorescence resonance energy transfer

GAL1   galactose inducible promoter 1

GFP   green fluorescent protein

HA   hemagglutinin

HEPES   4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP   horseradish peroxidase

Ig	immunoglobulin
Mad	mitotic arrest deficient
MAT	mating type
MCC	mitotic checkpoint complex
MEN	Mitotic Exit Network
min	minute
Noc	nocodazole
O.D.	optical density
ORF	open reading frame
PEG	polyethylene glycol
PBS	phosphate buffered saline
rDNA	ribosomal deoxyribonucleic acid
RNAi	RNA interference
RNAse	ribonuclease
rpm	revolutions per minute
SAC	spindle assembly checkpoint
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide elecrophoresis
SPB	spindle pole body
TEV	tobacco etch virus
TCA	trichloroacetic acid
ts	temperature sensitive
Tris	2-amino-2-hydroxymethyl-1,3- propanediol
UTR	untranscribed region
WT	wild type

1 INTRODUCTION

The goal of mitosis is to equally distribute the genome into newly formed cells. This distribution is performed by the mitotic spindle, which captures replicated chromosomes at their kinetochores and aligns them on the cell's equatorial plane. The spindle microtubules generate force that pull sister chromatids in opposite directions before the cell cleaves in between the separated chromosomes. The accuracy of chromosome segregation is governed by two closely connected mechanisms: 1) the mitotic checkpoint detects incorrect spindle attachments to kinetochores and delays anaphase providing enough time for their correction; 2) the error correction mechanism is thought to facilitate disattachment of non-bioriented chromosomes.

1.1 MITOTIC CHECKPOINT IN THE CELL DIVISION

Cellular control mechanisms, called checkpoints, establish the dependence of one process upon completion of another (Hartwell and Weinert, 1989). The fidelity and control of chromosome segregation is governed by the mitotic checkpoint, also known as spindle assembly checkpoint (SAC). This checkpoint delays chromosome segregation until all chromosomes reach stable bi-polar attachments to the spindle microtubules.

1.1.1 A historical perspective

As a cellular control, mitotic checkpoint was probably first observed in time-lapse movies of plant cell mitosis by Andrew Bajer. He noticed that anaphase seems to "wait" until the last chromosome reaches the equatorial plate of the cell (Bajer, 1956). From other observations it

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was known that before metaphase alignment chromosomes keep dynamic connections to microtubules. This happens until all chromosomes bi-orient with chromatids of each chromosome pair attached to opposite poles of the cell. But how does the cell discriminate between incorrect and correct attachments of chromosomes to spindle microtubules? The answer came from the elegant micromanipulation studies performed by Nicklas and colleagues (Nicklas and Koch, 1969). It was suggested that tension between two bivalents is generated only in the bi-oriented state; this tension might be the reason why mono-oriented chromosomes are not stably attached and come off the spindle. Using grasshopper spermatocytes, Nicklas applied tension to a mono-oriented chromosome by pulling it with a glass microneedle. As a result, this incorrect attachment was stabilized and the chromosome stayed attached to one pole of the cell for many hours. In contrast, in the absence of tension, the same sister chromatids pair re-orients into the correct configuration within several minutes (Nicklas and Koch, 1969).

Two decades later, in 1991 two groups define a cellular mechanism, the spindle checkpoint, responsible for such anaphase delay. Spindle checkpoint, was shown to restrain the initiation of anaphase until proper spindle formation (Hoyt et al., 1991; Li and Murray, 1991). These groups performed two independent genetic screens in budding yeast (*Saccharomyces cerevisiae*) and identified components of the mitotic checkpoint pathway based on the phenotype of continued cell division in the absence of a spindle. The genes found in these screens included the MAD (mitotic arrest deficient) genes Mad1, Mad2, Mad3 (BubR1 in humans) and the BUB (budding uninhibited by

benzimidazole) genes Bub1 and Bub3. These genes are conserved through the eukaryotes and are essential for checkpoint functioning. Besides Mad and Bub proteins, other components of the mitotic checkpoint include kinases Mps1 (multipolar spindle-1) and Aurora B (Ipl1 in budding yeast). They are involved in the initial recruitment of checkpoint proteins to the kinetochore and in the amplification of the checkpoint signal.

After the discovery of microtubules and their chromosomal attachment site, the kinetochore, Conley Rieder and colleagues were able to determine the mitotic checkpoint as a cellular response that monitors chromosome attachment and orientation, and prevents the initiation of anaphase until the last chromosome congresses to the metaphase plate (Rieder et al., 1995; Rieder et al., 1994). The term "spindle assembly checkpoint" (SAC) is a partial misnomer, derived from Murray and Kirschner's hypothesis that proper assembly of the mitotic spindle is "checked" before further progression through mitosis occurs (Murray and Kirschner, 1989). Spindle assembly itself is a relatively fast process, comparing to the chromosome alignment and bi-orientation. Thus, the main focus of the checkpoint is in monitoring chromosome bi-polar attachments at metaphase to ensure accurate transition into anaphase. In this thesis I will use the other term of this mechanism in mitosis, the mitotic checkpoint, which is becoming widely accepted among scientific community. However, as the same mechanism also exists in meiosis, a new, more suitable name for this checkpoint should be considered in the future. The name should reflect the main focus of this control mechanism – the metaphase-to-anaphase transition.

Therefore we propose a term "metaphase-to-anaphase transition checkpoint" and its abbreviation "MATCh" for future consideration.

In the majority of higher eukaryotes the spindle checkpoint is essential for viability and requires additional proteins CENP-E, p31, ZW10, ROD and Zwilch that were not found in yeast (Murray and Kirschner, 1989; Musacchio and Salmon, 2007). As mentioned above, the mitotic checkpoint couples anaphase with the stochastic process of kinetochore attachment to the spindle, which takes variable times for completion from cell to cell. Thus, it seems reasonable that the checkpoint is essential and that mice knockout mutants, homozygous for the checkpoint genes are early embryonic lethal (reviewed in (Baker et al., 2005)). However, yeast lacking checkpoint genes are viable. Another example, Drosophila lacking Mad2, is viable and fertile (Buffin et al., 2007). Checkpoint-deficient yeast and flies manage to survive under otherwise normal conditions. In these cases either chromosome capture or error correction mechanisms must be efficient enough to allow proper bi-orientation of all chromosomes before the cell initiates anaphase. The fidelity of chromosome segregation in unperturbed mitosis of these organisms relies on timing and error correction rather than checkpoint control.

The studies of genes required for mitotic checkpoint function allowed a fast transition from the observations in live cells to the discovery that the kinetochore functioned as a site where the status of chromosome-microtubule attachments was monitored and this information was transformed into a diffusible checkpoint signal.

1.1.2 Checkpoint basics

A general prerequisite for precise distribution of the genome is chromosome bi-orientation, whereby each sister kinetochore is attached to the plus-ends of microtubules that emanate from opposing spindle poles. Cells deploy a highly sensitive surveillance mechanism, the mitotic checkpoint that locally monitors the state of the kinetochore attachments. If defects are apparent, the checkpoint generates and spreads the signal that brings about a delay in cell-cycle progression, not only at the site of the defect, but globally throughout the cell. Our current knowledge about how these actions are performed is described below.

1.1.2.1   Errors in kinetochore-microtubule attachment

The process of kinetochore-microtubule attachment is crucial for accurate chromosome segregation during cell division. This is relatively flawless in budding yeast, in which each kinetochore binds only one kinetochore microtubule (Winey et al., 1995). In contrast, vertebrate kinetochores possess multiple attachment sites for the microtubules and they are more prone to the kinetochore-microtubule attachment errors. Three types of such mis-attachments usually occur at early prometaphase (**Figure 1.1**):

(1) *Monotelic* attachment, in which only one sister kinetochore becomes attached to microtubules from one pole, leaving its sister kinetochore unattached. It is very common attachment in early mitosis and it is usually converted into amphitelic (or bi-polar) orientation before anaphase onset.

A. *Syntelic*

no tension
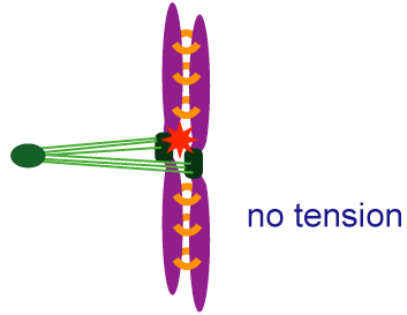
Checkpoint **ON**

B. *Monotelic*
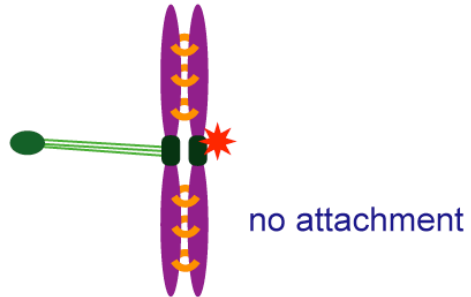
no attachment

Checkpoint **ON**

C. *Merotelic*
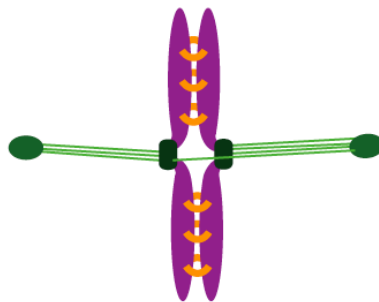
Checkpoint **OFF**

D. *Amphitelic*
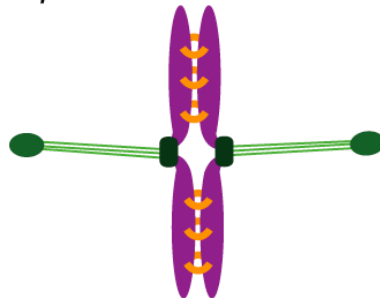
Checkpoint **OFF**

Figure 1.1   Schematic representation of kinetochore attachment and chromosome orientation before anaphase onset. *Microtubules of the mitotic spindle are represented as light green lines, kinetochores are in dark green. Sister chromatids (purple) are kept together with cohesin complex (orange). (A–C) Mal-orientations of chromosomes during pro(meta)phase. Lack of tension and/or attachment at kinetochore in syntelic and monotelic orientations, is recognized by the mitotic checkpoint. Merotelic kinetochore orientation, however does not trigger checkpoint engagement, thus providing potential risk of lagging chromosomes in anaphase. (D) Correct chromosome segregation is achieved through amphitelic orientation.*

(2) *Syntelic* attachment, in which both sister kinetochores face the same pole and attach to microtubules emanating from that pole. This orientation is rarely observed in untreated mitotic cells (Hauf et al., 2003). Syntelic attachment produces reduced tension between sister chromatids, and like monotelic attachment, is detected by the mitotic checkpoint.

(3) *Merotelic* attachments can only occur on vertebrates' kinetochores with multiple attachment sites. Sister kinetochores in this situation face opposite poles but one (or both) kinetochore(s) interact with microtubules from both poles. Merotelic kinetochore orientation does not interfere with chromosome alignment and is not recognized by the spindle checkpoint. Hence, this type of error attachments creates the potential risk of chromosome mis-segregation and aneuploidy. Cell employs another mechanism to detect and ensure correction of merotelic attachments. The crucial player of this mechanism is Aurora B kinase complex (see chapter 1.2.1).

1.1.2.2   Tension vs. attachment checkpoint

The first very important question in the checkpoint mechanism was: how the checkpoint monitors chromosome bi-orientation? Does it respond to absence of microtubule attachment or to the tension defects? Pioneering experiments in mitotic rat kangaroo PtK cells and meiotic mantid spermatocytes have laid the foundation for the attachment versus tension question by providing strong evidence that the mitotic checkpoint responds to both the lack of attachment and the absence of tension (see chapter 1.1.1). However, subsequent attempts to separate these signals and identify the primary defect sensed by the checkpoint

are complicated due to the intimate relationship between attachment and tension. Because unattached kinetochores are not under tension, distinguishing between potential activators requires analysis of attached, tension-defective kinetochores. However, microtubule attachments are also affected by tension. The application of tension both stabilizes and increases the number of kinetochore–microtubule attachments (King and Nicklas, 2000; Nicklas and Ward, 1994). This has been shown in a set of micromanipulation experiments, in which the number of microtubule attachments on an attached kinetochore was halved when tension was relieved by detaching its sister kinetochore. The subsequent reapplication of tension to the relaxed kinetochore restored attachment to its original level (King and Nicklas, 2000). Therefore, microtubule attachment and tension are coupled. This leads to the question of whether the absence of tension engages the checkpoint directly, by regulating a tension-sensitive component of the checkpoint, or indirectly, by altering kinetochore-microtubule occupancy.

The best evidence for the interdependence of tension and attachment signals comes from the grasshopper spermatocyte system in which kinetochores with "weak" attachments have been created using micromanipulation (Nicklas et al., 2001). These kinetochores completely lack tension and have only a few attached microtubules. In this situation, molecular checkpoint markers (such as kinetochore 3F3/2 phosphoepitopes, see also chapter 1.1.2.3) decrease despite the lack of tension. This indicates that the weak attachments are sufficient to regulate the checkpoint. However, the checkpoint is not silenced completely until the weakly attached kinetochores obtain full occupancy

and come under tension. Therefore, in this system, the checkpoint appears to monitor attachment and the role of tension is to promote the stabilization of these microtubule attachments.

In budding yeast, the relationship between tension and attachment is simplified. Budding yeast kinetochores attach to a single microtubule (Winey et al., 1995). The budding yeast kinetochore is either attached or unattached and cannot generate a "weak" attachment because of partial microtubule occupancy. Based on this, experiments performed in budding yeast provide the most compelling evidence that the mitotic checkpoint recognizes the absence of tension. To test the role of tension in both mitotic and meiotic progression, tension defects have been manufactured by preventing the chromosome pairing that is necessary to generate bi-polar force (Shonn et al., 2000; Stern and Murray, 2001). This has been achieved in mitosis, by inhibiting either replication (cells depleted for Cdc6) or sister chromatid cohesion (*mcd1-1* mutant); and in meiosis by preventing the recombination that holds homologous chromosomes together during meiosis I (*spo11* mutant). The absence of tension in these situations causes a delay in cell-cycle progression that is dependent on the mitotic checkpoint. In these experiments, the chromosomes were pulled to the poles, indicating that the tensionless kinetochores make microtubule attachments. Because kinetochores in budding yeast cannot be visualized by electron microscopy, it is difficult to determine the precise status of attachments. Although these experiments are not complicated by questions of partial microtubule occupancy, it is not clear if these kinetochore–microtubule interactions differ from the bi-oriented (or amphitelic) state. For example, the

tensionless kinetochore might bind to the side of the microtubule instead of interacting properly with the microtubule end (Pinsky and Biggins, 2005). Therefore, it is possible that the absence of tension on budding yeast kinetochores also affects microtubule attachment. It will be important to determine the type of yeast kinetochore attachments that occur in the absence of tension and how these budding yeast experiments translate to more complex kinetochores with multiple microtubule-binding sites. In conclusion, tension and attachment tightly regulate one another to communicate bi-orientation of chromosomes to the checkpoint.

1.1.2.3 *Kinetochore as a source of mitotic checkpoint activity*

Because the activity sensed by the mitotic checkpoint is the bi-polar attachment of sister kinetochores to the spindle, it is not surprising that the spindle checkpoint signaling cascade starts at the kinetochore. Many if not all checkpoint proteins are recruited to kinetochores that are unattached or not properly attached by spindle microtubules early in mitosis.

A striking discovery revealed that destroying the last remaining unattached kinetochore by laser inactivated the engaged checkpoint and allowed anaphase to begin in time (Rieder et al., 1995). Genetic studies in yeast showed that mutations affecting kinetochore function or its attachment to the spindle, led to the checkpoint-dependent mitotic arrest. Together these data suggest that kinetochore-microtubule attachments are monitored and serve as the sites from which checkpoint signal is generated (Pangilinan and Spencer, 1996; Wang and Burke, 1995).

1.1.2.4   Generation of the checkpoint signal

Depending on the mistake sensed by the mitotic checkpoint, different checkpoint proteins are recruited to the kinetochore. Localization studies show that Mad2 binds to unattached kinetochores in prometaphase of the unperturbed cell cycle and is lost from kinetochores upon microtubule attachment and chromosome bi-orientation (Chen et al., 1996; Li and Benezra, 1996). Tension loss at kinetochores, which is generated by either treatment with taxol or micromanipulation, was shown to be insufficient to induce Mad2 accumulation on kinetochores. Thus, it has been proposed that Mad2 specifically marks unattached kinetochores (Waters et al., 1998). In addition, in budding yeast, Mad2 is not observed at the kinetochores during the checkpoint delay induced by mutations that create tension defects (Gillett et al., 2004). However, it seems that Mad2 continues to inhibit the APC/C even after its displacement from kinetochores (Lens et al., 2003). This suggestion was supported by the study in Hec1/Ndc80-depleted cells, which maintained mitotic arrest in the Mad2-dependent manner, although Mad2 was not detected at the kinetochores (Martin-Lluesma et al., 2002). These observations suggest that either low, undetectable Mad2 levels are still present at kinetochores after taxol treatment, or that Mad2 regulates the checkpoint independently of kinetochores.

On the other hand, several checkpoint proteins are recruited specifically to tension-defective but attached kinetochores. The conserved checkpoint component Bub1 is reported to accumulate on kinetochores in the absence of tension (Shannon et al., 2002; Skoufias et al., 2001; Taylor et al., 2001). However, the kinetochore localization of

Bub1 also appears to be sensitive to defects in attachment (Logarinho et al., 2004; Taylor et al., 2001). Bub1 is also required for the kinetochore localization of a subset of checkpoint proteins that varies depending on the experimental system and conditions. These differences might explain why loss of Bub1 function causes varying degrees of checkpoint impairment (Johnson et al., 2004; Sharp-Baker and Chen, 2001). Additionally, the role of Bub1 in the checkpoint is difficult to assess due to its essential requirement for centromeric cohesion in meiosis and mitosis (Bernard et al., 2001; Perera et al., 2007; Tang et al., 2004b).

The conserved checkpoint component BubR1 accumulates on kinetochores in the absence of tension produced by treatment with taxol, incubation at low temperatures, and treatment with the microtubule inhibitor vinblastine (Logarinho et al., 2004; Shannon et al., 2002; Skoufias et al., 2001). However, these treatments might also affect microtubule occupancy. In addition, BubR1 function is required for the checkpoint in response to the lack of attachment, which indicates that BubR1 does not play a tension-specific role in the mitotic checkpoint (Chan et al., 1999; Johnson et al., 2004; Meraldi et al., 2004). In contrast, Mad3, the budding yeast homolog of BubR1, appears to be required for the checkpoint arrest in response to attachment defects (Lee and Spencer, 2004).

Another change in the kinetochore is observed in response to a tension defect in different systems: 3F3/2 kinetochore staining. The 3F3/2 antibodies were developed originally against thiophosphorylated substrates in Xenopus egg extracts and shown subsequently to recognize phosphoepitopes on kinetochores and spindle poles in many cell types

(Cyert et al., 1988; Gorbsky and Ricketts, 1993). When the mono-oriented kinetochore is put under tension in micromanipulation experiment, its 3F3/2 staining is significantly reduced compared with the unmanipulated control (Nicklas et al., 1995).

Therefore, irrespective of whether the checkpoint is indeed regulated by tension independently of occupancy or not, kinetochore localization of checkpoint components is in part differentially regulated by occupancy and tension.

1.1.2.5   *Mitotic checkpoint complex formation and APC/C inhibition*

Given the essential role of APC/C^{Cdc20} in triggering chromosome segregation, it is not surprising that APC/C^{Cdc20} is a key molecular target of the mitotic checkpoint.

Based on FRAP experiments, Mad1, Bub1 and about 50% of Mad2 appear to be stably associated with unattached kinetochores, whereas the remaining Mad2, as well as BubR1, Mps1 and Cdc20, exchange rapidly with their cytosolic pools (Howell et al., 2004; Shah et al., 2004). Furthermore, structural and biochemical analysis of Mad1 and Mad2 suggest that the Mad1-bound form of Mad2 at kinetochore could induce a second Mad2 molecule in the cytoplasm to acquire an "active" conformation (Mapelli et al., 2007; Yang et al., 2008). This activation of Mad2 primers its efficient binding to (and probably inhibition of) Cdc20. These findings led to the idea about the existence of a checkpoint scaffold at the kinetochore, which generates an APC/C-inhibitory checkpoint complex that is released into the cytosol to inhibit anaphase (**Figure 1.2**).
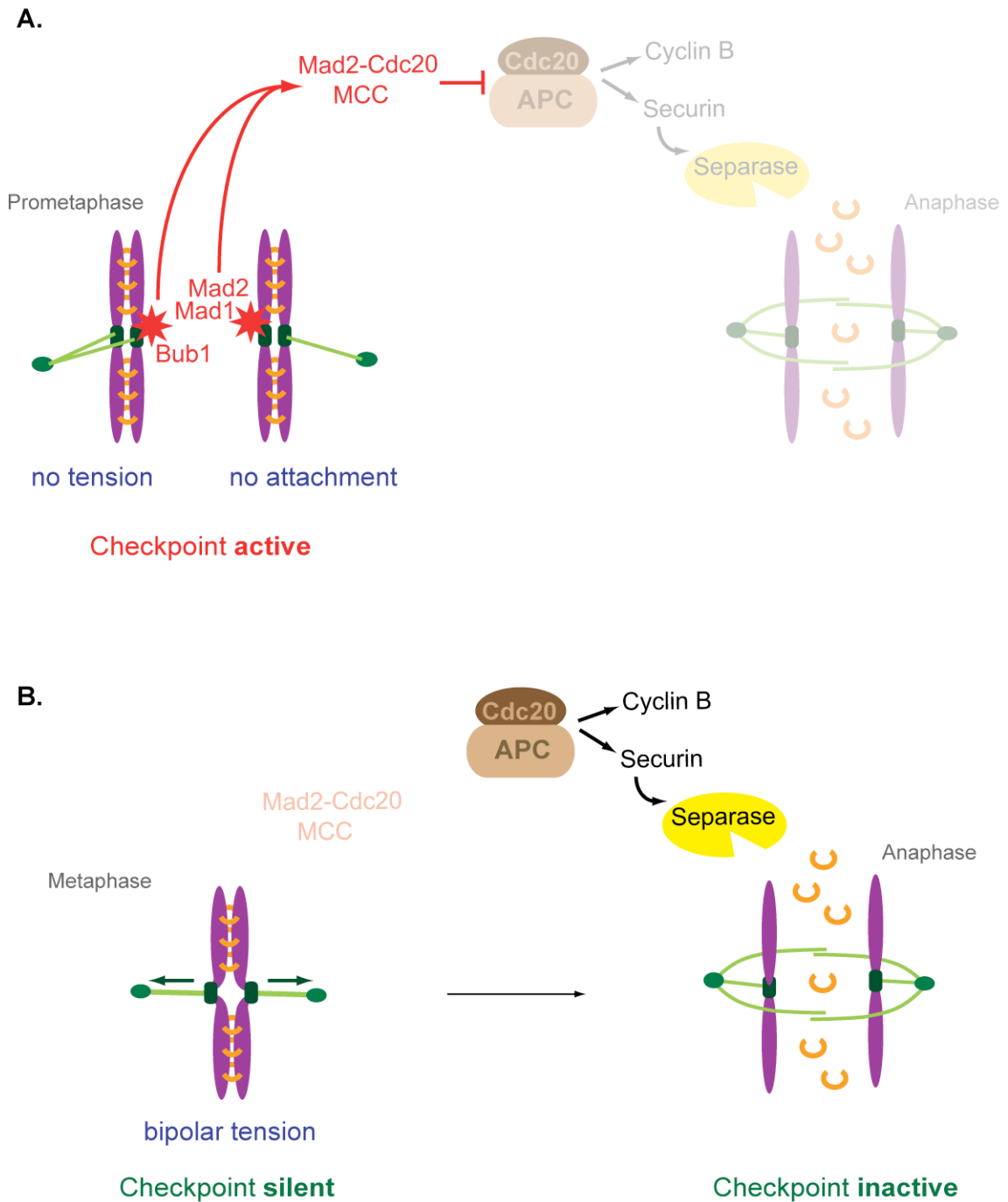
Figure 1.2   Mitotic checkpoint during cell division. *(A) Lack of tension and attachment engage the checkpoint in prometaphase. Checkpoint proteins localize to the sites of attachment mistakes (asterisk) – to kinetochores and produce diffusible inhibitory signals in the form of MCC and Mad2-Cdc20 complexes. This, in turn, inhibits APC/C and restrains anaphase onset. (B) Chromosome bi-orientation leads to checkpoint silencing and subsequent activation of APC/C. Securin degradation by APC/C releases separase to cleave cohesin and induce anaphase. (See text for details).*

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This diffusible inhibitory complex was named as Mitotic Checkpoint Complex (MCC) and found to be composed of the checkpoint proteins Mad2, BubR1 and Bub3 bound to Cdc20 (Hardwick et al., 2000; Sudakin et al., 2001). MCC binding to the APC/CCdc20 inhibits its ubiquitin-ligase activity and therefore prevents mitotic progression by restraining proteolysis of anaphase substrates (Peters, 2006; Yu, 2002).

The structural study from the Peters and Stark groups revealed that MCC associates with the Cdc20 binding site on APC/C, locks the otherwise flexible APC/C in a "closed" state, and prevents binding and ubiquitylation of a wide range of different APC/C substrates (Herzog et al., 2009).

Checkpoint proteins apparently use multiple strategies to inhibit APC/C, apart from MCC formation. For example, in yeast, Mad2 and Mad3 binding to Cdc20 triggers the ubiquitination and reduces the protein level of Cdc20, and thus facilitate further inhibition of APC/C (Pan and Chen, 2004). A recent study in human cells showed that Cdc20 ubiquitination by APC/C leads to Cdc20 degradation in cells with the checkpoint being engaged by nocodazole treatment (Nilsson et al., 2008). They also imply that Cdc20 degradation is itself a prerequisite for the maintenance of checkpoint-mediated arrest.

A number of checkpoint kinases (including Mps1, Bub1, Aurora B) are also involved in the checkpoint function. In some cases, they are required for assembly of the catalytic platform itself. However, it is also possible that these proteins have a more direct function in APC/C inhibition, or its relief. As an example, the checkpoint kinase Bub1, has a

key function in the recruitment of checkpoint proteins to kinetochores (Johnson et al., 2004; Meraldi and Sorger, 2005) but it can also phosphorylate Cdc20 to prevent it from interacting with APC/C (Tang et al., 2004a). The role of Cdc20 phosphorylation in the checkpoint was also reported from studies in Xenopus egg extracts, where it is required for efficient Mad2 binding to Cdc20. A recent study suggests a role for another checkpoint kinase, Mps1 in Cdc20 inhibition during interphase and mitosis (Maciejowski et al.).

1.1.2.6   *Manipulation and detection of the checkpoint response*

Several experimental approaches are used to engage the mitotic checkpoint by creating either unattached or tension-defective kinetochores. Although the use of chromosome micromanipulation to control the status of kinetochore attachment and tension is powerful, it is technically challenging and is not amenable to most cell types. More commonly, attachment and tension are manipulated in cells in culture by chemical inhibition of spindle function. To generate unattached kinetochores, cells are exposed to either nocodazole or benomyl, which prevent microtubule polymerization and, therefore, result in detachment from kinetochores. In contrast, defects in tension are produced typically by treatment with the microtubule-stabilizing drug taxol. The sister kinetochores in taxol-treated cells are closer together, indicating loss of kinetochore tension, and electron microscopy confirms that these tensionless kinetochores remain bound to microtubules (McEwen et al., 1997).

As a result of mitotic checkpoint engagement, APC/C became inhibited and anaphase substrates securin and cyclin B stabilized. The

localization of checkpoint proteins Mad2, Bub1, BubR1, Mps1 as well as modification some of them (like Mad1 phosphorylation) serves as the markers of checkpoint engagement (Gillett et al., 2004; Hardwick and Murray, 1995). Checkpoint-induced metaphase arrest at this stage can be relieved by deletion of Mad2 or BubR1 checkpoint proteins, as cells cannot sustain the checkpoint function without these crucial components.

1.1.2.7   *Mitotic delay as a result of checkpoint signaling*

As described above, treatment with nocodazole or taxol both causes mitotic arrest due to checkpoint engagement. However loss of microtubules in response to nocodazole, is considered to produce stronger checkpoint signal that is able to efficiently arrest cells in metaphase. In contrast, cells treated with taxol, especially at higher concentrations, manage to exit mitosis even with mono-oriented (syntelic) kinetochore attachments (Yang et al., 2009). This is due to the ability of taxol to stabilize microtubules and their attachments (even mono-polar) to kinetochores. Establishment of stable kinetochore-microtubule attachments leads to the checkpoint satisfaction and thus, to the release from mitotic block.

The different view suggests both these drugs are able to engage the checkpoint efficiently (Jordan et al., 1993). At lower concentrations taxol reduces tension at kinetochores, thus triggering Aurora B to disconnect microtubules from kinetochores. This results in unattached microtubules, similarly to nocodazole treatment, and hence, efficient mitotic delay.

1.2 ERROR-CORRECTION MECHANISMS

To ensure accurate segregation, every pair of sister kinetochores must attach to microtubules from opposite poles of the spindle. How this configuration is achieved for every pair of replicated chromosomes is a long-standing question in the field. The formation of kinetochore-microtubule attachments relies on the dynamic instability of spindle microtubules, which allows them to explore the cell volume and to bind to unattached kinetochores. Given the stochastic nature of this process, it is not surprising that some sister kinetochores fail to establish correct bi-oriented attachments, and instead form erroneous attachments, with both kinetochores bound by microtubules from the same spindle pole. Cells, however, have developed error correction mechanisms that detach improper attachments to ensure that ultimately all chromosomes become bi-oriented. The key regulator of the error correction pathways is the chromosome passenger complex (reviewed in (Kelly and Funabiki, 2009)).

1.2.1 Chromosome passenger complex

Aurora B (Ipl1 in budding yeast) is the catalytic component of the complex that also includes three non-enzymatic subunits: the inner centromere protein – INCENP (Sli15 in budding yeast), Survivin (Bir1), and Borealin (Nbl1) (Ruchaud et al., 2007) (Nakajima et al., 2009). These proteins comprise the chromosomal passenger complex (CPC), which is named because of its dynamic localization pattern (Earnshaw and Bernat, 1991). Upon entry into mitosis, the CPC is first localized to both chromosome arms and the inner centromere. As the cell cycle progresses to metaphase, the amount of CPC localized to the

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chromosome arms decreases and it is mainly detected at the centromere. When sister chromatids separate in anaphase, the CPC dissociates from centromeres and relocalizes to the spindle midzone. The Aurora B complex regulates numerous mitotic events at each of these locations, including bi-polar kinetochore attachment and mitotic checkpoint function, spindle assembly and disassembly, anaphase chromosome condensation, and cytokinesis.

The non-enzymatic members of the complex control the targeting, activity and stability of Aurora B kinase (Lens et al., 2006). Knockdown by RNA interference of any member of the complex delocalizes the others, disrupts mitotic progression and destabilizes the whole complex (Vagnarelli and Earnshaw, 2004).

1.2.2 Regulation of microtubule attachments by Aurora B kinase

1.2.2.1 *Detection of erroneous attachments by the Aurora B complex*

Analysis of temperature-sensitive, loss-of-function mutations in the *IPL1* gene revealed cells with massive chromosome missegregation caused by the persistence of incorrect kinetochore attachments to the spindle (Biggins et al., 1999; Chan and Botstein, 1993; Tanaka et al., 2002). Further analysis suggested that this segregation pattern reflects an underlying role for Ipl1 in allowing sister chromatids to bi-orient. In an elegant study, Tanaka and co-workers provided evidence that ipl1 mutants fail in bi-orientation because they cannot disconnect incorrect kinetochore-microtubule attachments (Tanaka et al., 2002).

Experiments in many model systems are consistent with the yeast experiments and indicate that attachment errors can be artificially

stabilized in high numbers if the activity of Aurora B kinase is inhibited (Cimini et al., 2006; Ditchfield et al., 2003; Hauf et al., 2003). Re-activation of Aurora B results in the correction of improper attachments (Lampson et al., 2004). These studies implicate Ipl1/Aurora B as an essential component of the error correction mechanism required to prevent the stabilization of improper attachments.

1.2.2.2   *Elimination of improperly oriented chromosomes is driven by Aurora B mediated phosphorylation*

The mechanisms by which Aurora B kinase activity destabilizes improper microtubule attachments are not completely understood. However, several key substrates have been elucidated. The Ndc80/Hec1 complex is a major attachment module for microtubules at the outer kinetochore. In the absence of tension, it was proposed that Aurora B phosphorylates the N-terminal tail of Ndc80, neutralizing the positive charge and decreasing the affinity of Ndc80 for microtubules (Cheeseman et al., 2006; Ciferri et al., 2008; DeLuca et al., 2006). In addition, Dam1, a protein that allows kinetochores to track depolymerizing plus ends of microtubules in budding yeast, is negatively regulated by Ipl1-mediated phosphorylation (Gestaut et al., 2008).

Aurora B also regulates the activity of MCAK and Kif2a, two kinesin-13 family members that are implicated in the regulation of the kinetochore microtubules stability (Ohi et al., 2003) (Andrews et al., 2004) (Lan et al., 2004) (Knowlton et al., 2006). Overall, these interactions may modulate the binding affinity of kinetochores for microtubules, as well as the dynamics of the microtubule plus end.

1.2.3 Aurora B complex as tension sensor at kinetochores

How does the cell distinguish correct from incorrect attachments? Tension generated between sister kinetochores is widely accepted to be a potential signaling mechanism (Gorbsky and Ricketts, 1993) (Nicklas et al., 1995). Moreover, tension is thought to regulate the activity of Aurora B and thus, stability of kinetochore-microtubule interactions. A study in budding yeast has revealed a possible role for other CPC components apart from Ipl1/Aurora B, in tension detection by kinetochores, (Sandall et al., 2006). They used an assay, which measures the ability of the core yeast kinetochore (centromeric DNA with the CBF3 complex bound) to attach to microtubules. CBF3–DNA complexes could bind to microtubules if they were pre-incubated in yeast cell extracts, but the crucial factor contributed by the extract was not known. This factor appeared to be a Sli15–Bir1 (INCENP–survivin) complex, and was proposed to bridge centromeres to microtubules. The authors suggested also that the Aurora kinase-activating domain of INCENP is the tension sensor that relays the mechanical state of centromere-microtubule attachments into local control of Ipl1 kinase activity.

The other models for sensing the kinetochore tension by Aurora B and their experimental conformation are described below.

1.2.3.1 Tension-regulated separation of Aurora B from its substrates

It has been proposed that it is the physical distance between Aurora B and its kinetochore substrates that determines whether microtubule-kinetochore connections are maintained (Tanaka et al., 2002). When sister chromatids are under tension, the distance between pairs of their kinetochores is increased relative to a relaxed state. Aurora

B remains at the inner centromere, and therefore its kinetochore substrates are no longer co-localized with the kinase (Andrews et al., 2004). Under this model, this leads to a situation in which phosphorylation of key kinetochore substrates (e.g. Ndc80) is low and microtubule-kinetochore interactions are stabilized. In turn, PP1, localized to kinetochores in metaphase, dephosphorylates kinetochore substrates to maintain correctly attached microtubules (Trinkle-Mulcahy et al., 2003). Conversely, when there is little or no tension, Aurora B is physically closer to its substrates and phosphorylation is high, thus leading to destabilization of attachments (**Figure 1.3**).

This model assumes that tension-dependent changes in distance between Aurora B and its substrates are enough to prevent their interaction. In mammalian cells, differences of roughly 1–3 µm are seen between kinetochores under tension and those in a relaxed state (Waters et al., 1998). As the CPC has been shown to be a highly elongated complex with maximum lengths of up to ~40–50 nm (Bolton et al., 2002), it is possible that tension can physically separate Aurora B from its substrates.

Recently, this hypothesis was corroborated by elegant experiments, in which an Aurora B substrate docked within the kinetochore at a sufficiently large distance from the centromere became dephosphorylated as microtubule attachment ensued (Liu et al., 2009). Substrates closer to the centromere, on the other hand, were constitutively phosphorylated with or without microtubule attachment.

No tension

Bipolar tension
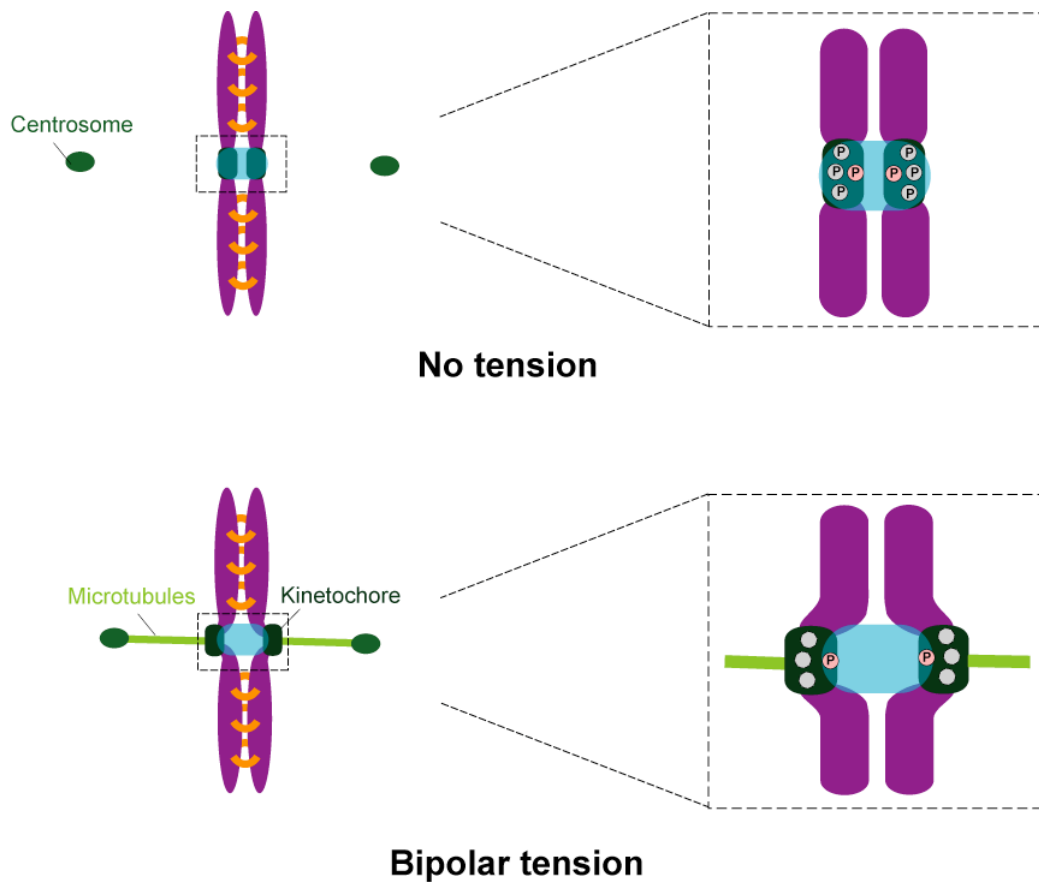
- area of Aurora B kinase activity
- Ⓟ - sites of constitutive phosphorylation by Aurora B kinase, regardless whether tension is applied or not
- Ⓟ - sites of substrate phosphorylation by Aurora B kinase only in the absence of tension

Figure 1.3   Tension-regulated stabilization of kinetochore-microtubule interactions depends on Aurora B.
Distance between Aurora B kinase and its kinetochore substrates determines the stability of microtubule attachment. The spatial separation of outer kinetochore substrates from Aurora B in bi-oriented state reduces phosphorylation, which results in firm kinetochore-microtubule attachments. In the absence of tension, outer kinetochore comes in close proximity to Aurora B, leading to Aurora B substrates phosphorylation and weakening of interaction with microtubules.

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The spatial separation model explains how mechanical forces at the centromere may regulate kinetochore–microtubule stability. Forces exerted by spindle microtubules pull bi-oriented sister kinetochores in opposite directions, which increases the distance between the inner centromere, where Aurora B localizes, and the outer kinetochore, where microtubules bind. In this configuration the kinase does not efficiently phosphorylate substrates at the outer kinetochore, such as the Ndc80 complex, and hence, attachments are stabilized. In the absence of such forces, kinetochore substrates are phosphorylated, as they are in close proximity to Aurora B at the inner centromere. This, in turn, leads to the destabilization of attachments.

Destabilization provides a fresh opportunity to bi-orient, which may be facilitated by a mechanism that transports mono-oriented chromosomes to the spindle equator, which increases the likelihood of a free kinetochore capturing a microtubule from the opposite pole (Kapoor et al., 2006). Selective destabilization in the absence of tension is therefore an integral component of a trial-and-error mechanism that ultimately promotes bi-orientation of all chromosomes.

1.2.3.2 *Regulation of Aurora B activity through structural changes in centromeric chromatin*

Other mechanisms may also contribute to regulation of Aurora B activity. Tension generated by microtubules pulling on kinetochores can provide enough force to potentially unwind nucleosomes at the centromere (Bloom, 2008). Therefore, tension might affect the distribution of CPC molecules at the centromere. It was proposed that under low tension, chromatin remains in the compact state resulting in a

high concentration of the CPC at the centromere (Kelly and Funabiki, 2009). This may increase the likelihood that one CPC molecule phosphorylates another, which has been shown to lead to sustained activation of Aurora B kinase (Kelly et al., 2007). When the centromere is under tension, this mechanism may be suppressed due to a decreased local concentration of the CPC or physical disruption of its oligomerisation state. Monitoring the dynamics of CPC inter-molecular interactions under conditions of both high and low tension will aid in the validation of this model (Kelly and Funabiki, 2009).

1.2.4 Aurora B as a part of mitotic checkpoint machinery

A role for Aurora B/Ipl1 in the checkpoint was first indicated by the analysis of temperature-sensitive *ipl1* mutant cells, which, despite existing mono-oriented chromosomes, proceed through the cell cycle without detectable checkpoint delay (Biggins et al., 1999). It was shown later that Ipl1 function is required for the mitotic checkpoint in response to the conditions that interfere with tension generation at the kinetochores (Biggins and Murray, 2001). By contrast, the loss of attachment that is induced by nocodazole treatment maintains the checkpoint-dependent arrest in an Ipl1-independent manner. This indicates a specific requirement for Ipl1 to allow the absence of tension to trigger the checkpoint response. Similarly, in cell-culture systems, the Aurora B kinase complex is required for checkpoint signalling due to defects in tension but not to lack of attachment (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003).

The mechanism by which Aurora B/Ipl1 regulates the mitotic checkpoint in response to the absence of tension is not known. The

simplest explanation is that Aurora B/Ipl1 facilitates both amphitelic attachment and mitotic checkpoint activation by promoting the turnover of kinetochore–microtubule interactions. This hypothesis suggests that Aurora B/ Ipl1 regulates checkpoint indirectly by creating unattached kinetochores and, essentially, amplifying the checkpoint signal. Consistent with this hypothesis, impairing Ipl1 function in a budding yeast kinetochore mutant *mtw1-1* both restores attachment and satisfies the checkpoint (Pinsky et al., 2003).

In contrast to this hypothesis, other studies imply that Aurora B/Ipl1 has a direct role in the mitotic checkpoint, which is independent of its role in kinetochore detachment or error correction. For example, in fission yeast and extracts of Xenopus eggs, Aurora B is required for the checkpoint, in a manner different from creating unattached kinetochores (Kallio et al., 2002) (Petersen and Hagan, 2003). For example, introduction of INCENP mutant allele that lacks its coiled-coil domain results in an overt defect in the checkpoint response to taxol treatment. This indicates that this domain is critical for CPC function in spindle checkpoint control. Surprisingly, this mutant is proficient in chromosome alignment and cytokinesis during unperturbed cell divisions and was capable of resolving syntelic attachments. Also, Aurora-B kinase was localized and activated normally on centromeres in these cells, ruling out a role for the coiled-coil domain in general Aurora-B activation. Thus, mere microtubule destabilization of non-bipolar attachments by the CPC is insufficient to install a checkpoint-dependent mitotic arrest, and additional, microtubule destabilization-independent CPC signalling

toward the mitotic checkpoint is required for this arrest (Vader et al., 2007).

Whether Aurora B regulates the tension checkpoint independently or whether the loss of tension results in microtubule release from kinetochores remains under intense debate. Whatever the detailed mechanism, the CPC is required for mitotic checkpoint function when tension is lost, but not in response to agents that disassemble microtubules. Using RNAi, survivin and INCENP were shown to be essential for checkpoint function in the presence of taxol (which perturbs spindle tension by dampening microtubule dynamics), and for the recruitment of the checkpoint protein BubR1 to the kinetochore (Carvalho et al., 2003; Lens et al., 2003). Aurora B also appears to cooperate with the checkpoint kinase Bub1 in maintaining the spindle checkpoint by promoting the association of BubR1 with the APC/C (Morrow et al., 2005). Aurora B is also required for the localization of checkpoint proteins to kinetochore (Vigneron et al., 2004) (Ditchfield et al., 2003).

1.3   CHECKPOINT SILENCING

1.3.1 Checkpoint silencing in metaphase

Checkpoint silencing is directly linked to the correct attachment of kinetochores to microtubules, and it is widely accepted that the signals inactivating the checkpoint emanate from the centromeres.

1.3.1.1 *Checkpoint silencing upon attachment and tension*

Certain SAC proteins are immediately removed after the attachment of the microtubule plus-ends and formation of kinetochore microtubules. For example, Mad1 and Mad2 localize to unattached kinetochores in prometaphase or in cells treated with nocodazole to prevent microtubule polymerization and formation of microtubule-kinetochore attachments. Conversely, the amount of Mad2 becomes highly reduced at metaphase kinetochores (50–100-fold compared with unattached prometaphase kinetochores). Therefore, Mad2 kinetochore localization strongly decreases as kinetochores become more occupied with microtubules at their attachment sites. In metazoans, Mad2 reduction at kinetochores depends not only on microtubule attachment but also on dynein motility along microtubules. Inhibiting dynein at metaphase kinetochores results in the increase of Mad2 to 25% of the level at unattached kinetochores, even without a loss in kinetochore-microtubule number (Howell et al., 2001; Wojcik et al., 2001). This indicates that kinetochore-microtubule formation promotes Mad1 and Mad2 dissociation by providing high local concentrations of microtubules to promote the ATP-dependent motility of dynein along the microtubule. Furthermore, the inhibition of BubR1 activity depends on CENP-E binding to microtubules (Mao et al., 2005). There is no nuclear dynein or CENP-E homologue in *S. cerevisiae*. Mutants for the minus-end directed motor protein Kar3 are synthetically lethal with mitotic checkpoint protein mutants in budding yeast. Therefore, Kar3 might have the equivalent function to dynein in checkpoint inactivation (Tong et al., 2004).

Metazoans present an additional mechanism to downregulate the checkpoint, based on inhibition of Mad2 catalysis by p31^{comet}. This inhibitor is activated upon kinetochore occupancy by microtubules and diminishes formation of the active Mad2 configuration (Vink et al., 2006).

Tension and centromere stretching also seem to have a significant role in the downregulation of checkpoint activity. Applied tension to mono-oriented chromosome inhibits the rate of checkpoint proteins association at kinetochores (see above, 1.1.2.5). However, distinguishing the relative contributions of tension and attachment when manipulating spindles is difficult, because creation of tension in this way probably affects attachment (Zhou et al., 2002). Tension instead might provide a fundamental criterion to discriminate against incorrect attachments and thus contribute to checkpoint down-regulation. In addition, two recent studies provide evidence that kinetochore stretching promotes silencing of the checkpoint signal (Maresca and Salmon, 2009; Uchida et al., 2009). They demonstrate that intra-kinetochore stretching, but not the increased distance between sister kinetochores, is necessary and sufficient for release of the checkpoint.

1.3.1.2   PP1 silences the checkpoint upon chromosome bi-orientation

The checkpoint functioning as well as its silencing are intimately linked to the attachment of kinetochores to microtubules. Thus, it is likely that certain players will regulate both processes simultaneously. To specifically study the role of such components in checkpoint inactivation requires separation-of-function alleles. Recently, such an allele was described for the kinetochore component Ndc80 in budding yeast

(Kemmler et al., 2009). Ndc80 is critical for kinetochore–microtubule attachments and kinetochore recruitment of the checkpoint components. Ndc80 is phosphorylated by the checkpoint kinase Mps1, and a phospho-mimic mutant fails to inactivate the checkpoint without perturbing kinetochore–microtubule interactions. This suggests that Mps1-dependent phosphorylation of Ndc80 contributes to the checkpoint signaling, and that these phospho-modifications need to be removed for the checkpoint to be inactivated.

On centromeres lacking tension, Aurora B-dependent phosphorylation of kinetochore substrates contributes to checkpoint functioning and microtubule destabilization. Similarly to the previous example with Ndc80 phosphorylation, in order to rapidly disable the checkpoint and activate APC/C, kinetochore-localized protein phosphatase 1 (PP1) reverses Aurora B-dependent and Mps1-dependent phosphorylation events at kinetochores (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009a). The substrates of Aurora kinase and PP1 phosphatase in this process remain unknown, but likely candidates are checkpoint components, APC/C^{Cdc20} or kinetochore components.

It remains to be proven, but it is possible that kinetochore-localized PP1 also counteracts the microtubule-destabilizing activity of Aurora B on the kinetochores, likely through reversal of the Aurora B-dependent modification of MCAK or Ndc80. PP1 phosphatase activity also potentiates kinetochore stripping of checkpoint components upon chromosome bi-orientation (Whyte et al., 2008). This means that PP1 contributes to the checkpoint silencing in several ways.

1.3.1.3 Balance between checkpoint signalling and APC/C activity

Once formed, the MCC tightly interacts with the APC/C, as the spontaneous dissociation rate of MCC-APC/C is small as observed *in vitro* and in mitotic extracts (Reddy et al., 2007). However, the presumed rate of dissociation, indirectly observed *in vivo* after all kinetochores have attached, is relatively rapid (Clute and Pines, 1999). The dissociation of the MCC from the APC/C, and the deactivation of Mad2, has been proposed by Reddy and colleagues to occur through Cdc20 ubiquitination in the context of the MCC-APC/C in complex with its E2 enzyme UbcH10 (Reddy et al., 2007). This process may itself be balanced by de-ubiquitination by the de-ubiquitinating enzyme USP44 (Stegmeier et al., 2007). The Cdc20 modification was shown to be a non-degradative ubiquitination, which is proposed to break the complex formed between Mad2 and Cdc20. Given that the binding of Cdc20 and Mad2 is expected to be a spontaneous process in living cells, this piece of data provides a potential source of energy needed to destabilize the inhibitory complex. These observations can be integrated into a model of the checkpoint whereby unattached kinetochores not only control the formation of the inhibitor but also its dissociation. Thus, the mitotic checkpoint would guarantee a more effective inhibition and faster release of Cdc20 as compared with the assumption that signalling only controls the formation of the inhibitor (Ciliberto and Shah, 2009).

1.3.1.4 Checkpoint adaptation

The "adaptation" (also known as "slippage" or "leakage") describes the escape from mitosis under the continued presence of conditions that normally activate mitotic checkpoint. For example, adaptation occurs

upon prolonged treatments with nocodazole (Rieder and Maiato, 2004). The causes of adaptation are generally unclear and might differ in different species. For instance, in rat kangaroo cells and human cells, adaptation correlates with degradation of cyclin B, but checkpoint proteins are retained at kinetochores, which indicates that the escape is not caused by a depletion of the checkpoint signal (Brito and Rieder, 2006).

1.3.2 Keeping the checkpoint silent during anaphase

At anaphase, tension is lost at kinetochores because of the loss of cohesion between sister chromatids. This loss of tension does not normally engage the checkpoint in anaphase, which indicates that mitotic checkpoint is inhibited during anaphase.

1.3.2.1 Dissociation of the checkpoint complexes

As was indicated in 1.3.1.3, UbcH10-dependent ubiquitynation of Cdc20 leads to its release from checkpoint complexes, including Mad2 and BubR1. This mechanism of checkpoint alleviation acts already in prometaphase, when it is effectively balanced by opposing de-ubiquitynation by USP44 to sustain the checkpoint. It is possible that after anaphase initiation this Cdc20 modification becomes stable as result of reduced de-ubiquitynation activity of USP44. This leads to dissociation of existing inhibitory complexes together with ceasing its further production at kinetochore scaffold.

1.3.2.2 APC/C-induced proteolysis

Anaphase proteolysis of cyclin B and the inactivation of Cdk1 – cyclin B kinase activity has an important role in the checkpoint

inactivation (Potapova et al., 2006). It was shown that mitotic checkpoint requires Cdk1 activity and inhibiting Cdk1 activity overrides the checkpoint-dependent arrest in Xenopus egg extracts and human cells. Moreover, Cdk1 inhibition also overcomes Mad2-dependent mitotic arrest (D'Angiolella et al., 2003). As soon as cell proceeds to anaphase, Cdk1 activity begin to decline, which might also affect the checkpoint.

In budding yeast, the checkpoint protein Mps1 is also degraded at anaphase by the APC/C (Palframan et al., 2006). Overexpression of Mps1 during anaphase is sufficient to re-engage the checkpoint. This indicates that removing Mps1 at anaphase might be essential to keep mitotic checkpoint inactive after chromosome segregation.

In vertebrates, anaphase is only triggered when securin and cyclin B are almost entirely degraded (Clute and Pines, 1999; Hagting et al., 2002). This indicates that the checkpoint re-activation in anaphase would probably have no consequences for cell cycle progression.

1.4 Metaphase-to-anaphase transition

The initiation of sister chromatids separation defines the transition from metaphase into anaphase. When chromosome bi-orientation is achieved, checkpoint-mediated blockage is removed by the ubiquitin ligase APC/C[Cdc20], which targets its anaphase substrates for destruction and enables irreversible progression into anaphase (Morgan, 1999). The most important targets of APC/C[Cdc20] are securin, whose destruction leads to the loss of sister chromatid cohesion, and cyclin B, whose destruction causes Cdk1 inactivation. This inactivation allows phosphatases to dephosphorylate Cdk1 targets and stimulates the completion of late mitotic events.

1.4.1 Cohesin cleavage by separase

Prior to anaphase, securin binds and inhibits a protease separase, which is in turn responsible for cleaving the cohesin subunit Scc1. Cohesin complexes hold sister chromatids together since their appearance in S phase. When mitotic checkpoint becomes satisfied, APC/C^{Cdc20}-dependent ubiquitynation and destruction of securin liberates separase, which then cleaves cohesin and allows sisters to separate (Nasmyth, 2002; Uhlmann et al., 2000).

1.4.2 Cdc14 activation and its function in anaphase

After sister chromatids have separated, dephosphorylation of Cdk1-substrates is the major regulatory mechanism driving the completion of mitosis. Cdk1 activity drives cell cycle progression to metaphase, thus some events in late mitosis may result from the direct reversal of Cdk-driven phosphorylation. Dephosphorylation of Cdk1 substrates is required for normal chromosome and spindle movements in anaphase, as well as for subsequent events of telophase: spindle disassembly, reformation of nuclei and decondensation of chromatin.

The protein phosphatases that dephosphorylate Cdk targets in late mitosis are not well understood, particularly in multicellular organisms. Some Cdk substrate dephosphorylation may be catalyzed by general phosphatases whose activities do not vary in the cell cycle. In budding yeast the late mitotic events largely depend on the Cdc14 phosphatase, which is the main antagonist of Cdk1. It remains unclear, however, if Cdc14-related phosphatases are important for the regulation of late mitosis in other organisms.

Before anaphase Cdc14 is found in the nucleolus, where it is held in an inactive state by its association with Net1. With anaphase onset, Cdc14 is activated, dissociated from Net1 and released from the nucleolus (Shou et al., 1999; Visintin et al., 1999). Cdc14 then diffuses throughout the nucleus and out into the cytoplasm to dephosphorylate targets in both locations. Cdc14 is activated by two regulatory mechanisms – first, the FEAR pathway, acting in early anaphase and then the MEN pathway, slightly later (D'Amours and Amon, 2004; Jaspersen et al., 1998; Stegmeier et al., 2002). With anaphase onset, Cdc14 is initially activated by separase. Thus, by causing separase activation, APC/C^{Cdc20} promotes both sister chromatids separation and a partial activation of the phosphatase that dephosphorylates Cdk1 targets (Morgan, 1999). Partial activation of Cdc14 is thought to be particularly important for the dephosphorylation of Cdk substrates involved in early-anaphase changes in spindle and chromosome behaviour. The later and full Cdc14 activation by MEN pathway drives complete Cdk1 inactivation and exit from mitosis.

1.4.3 Downregulation of Cdk1 promotes anaphase spindle stability

Although experimental disruption of sister chromatid cohesion alone can initiate chromosome separation, normal chromosome movements in anaphase also depend on regulated changes in the microtubule behaviour and chromosome attachment to the spindle. These changes are governed by downregulation of Cdk1 in anaphase. In animal cell, for example, expression of non-degradable cyclin B, which is no longer recognised and targeted for degradation by APC/C^{Cdc20}, does not prevent sister chromatid splitting but results in abnormal

chromosome movements and defects in anaphase spindle elongation (Parry et al., 2003; Parry and O'Farrell, 2001). This could be of course, due to expression of high levels of cyclin B, but also can indicate that Cdk1 activity affects microtubule dynamics. Similarly, artificial induction of cohesin cleavage in metaphase-arrested yeast cells results in abnormal chromosome movements and often broken spindles. This is due to the high dynamic instability of microtubules, which is unusual for anaphase. Artificial activation of the phosphatase Cdc14 in these cells stabilizes microtubules and restores normal anaphase spindle behaviour (Higuchi and Uhlmann, 2005). Inhibition of Cdk1 in analogous experiment in Drosophila embryos is also sufficient to drive proper segregation of chromosomes in artificially induced anaphase (Oliveira et al., 2010).

Anaphase spindle stability seems to be the prerequisite for normal chromosome segregation. In yeast, there are a few Cdc14 targets, which facilitate chromosome movement and spindle stability in early anaphase. Among them, the kinetochore component Ask1 regulate microtubule turnover at kinetochores and was shown to contribute to anaphase spindle stabilization. The microtubule binding proteins Stu1, Ase1 and Fin1 also promote reduction of microtubule dynamics at spindle midzone.

Another intriguing candidate responsible for anaphase spindle stability is the Aurora B kinase complex. As mentioned above, Ipl1/Aurora B and its binding partner Sli15/INCENP localize to kinetochores in metaphase, but relocate to the spindle midzone in anaphase. Here Aurora B kinase complex helps to stabilize the overlapping plus ends of interpolar microtubules. In metazoans, transfer

of Aurora B to the spindle midzone is blocked when Cdk1 activity is maintained at anaphase. Loss of Cdk1 phosphorylation on Sli15/INCENP triggers the passenger complex association with spindle microtubules (Hummer and Mayer, 2009). In budding yeast, Sli15/INCENP dephosphorylation by Cdc14 is necessary to locate Ipl1/Aurora B complex to spindle midzone and promote spindle stability (Pereira and Schiebel, 2003).

1.4.4 Cdc14 and mitotic exit

In most cell types the APC/C activator Cdc20 is replaced by the alternative activator Cdh1 in late mitosis. APC/C^{Cdh1} is required for the continued destruction of mitotic cyclins, and thus Cdk1 activity downregulation in G_1. Early released Cdc14 cannot fully activate Cdh1, and therefore cannot drive complete Cdk1 inactivation and completion of mitosis. Cdc14 is fully activated by an additional regulatory pathway called the mitotic exit network (MEN). It is possible that temporal regulation of Cdc14 activation controls stepwise progression of late mitotic events (Jin et al., 2008).

1.5   AIMS OF THIS STUDY

Tensionless kinetochores trigger checkpoint signaling and engage error-correction mechanism to destabilize incorrect attachments. This situation resembles the status of sister kinetochores in anaphase, when cohesin cleavage by separase also releases tension between sisters. However, re-engagement of the mitotic checkpoint due to tension loss at this stage would inhibit the APC/C, stabilize securin and cyclins again,

and thus impede further mitotic progression (Tinker-Kulberg and Morgan, 1999). Why the ubiquitous loss of tension at anaphase onset goes undetected by the checkpoint remains poorly understood (**Figure 1.4**).

We set out to investigate whether loss of cohesion at anaphase onset would in principle re-engage the mitotic checkpoint, and if so, how this is normally prevented.
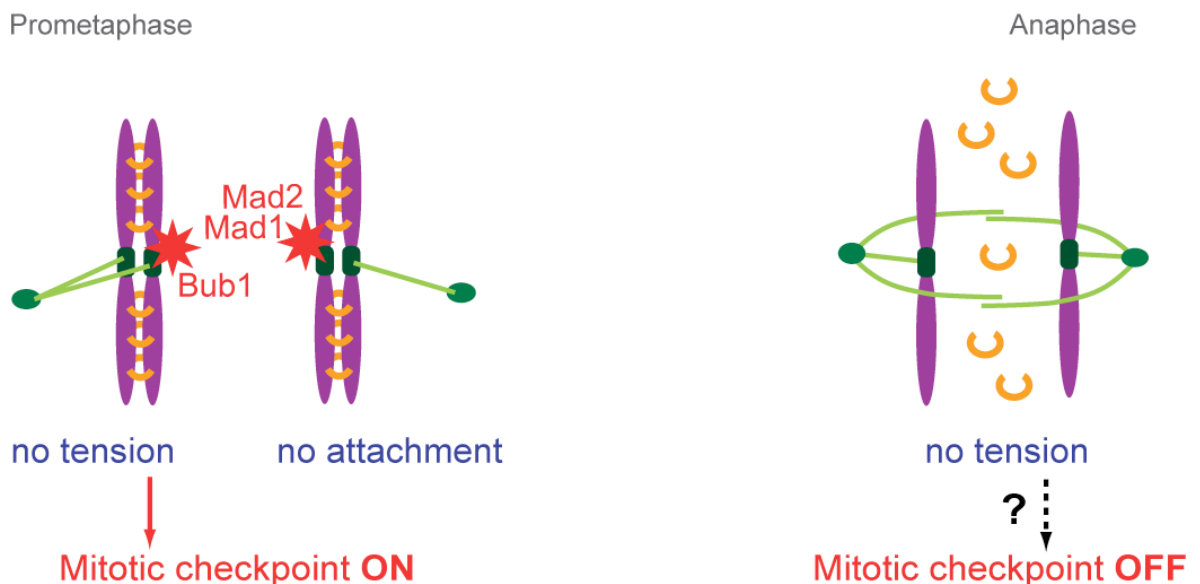
Figure 1.4   **Aim of this study:** *Why does mitotic checkpoint become insensitive to loss of tension at anaphase onset?*

2   MATERIALS AND METHODS

2.1   YEAST GROWTH AND MANIPULATION

2.1.1   Yeast strains and media

All budding yeast strains used in this study are listed in **Table 1**. Cells were grown in YP supplemented with 2% w/v glucose (YPD) or 2% w/v raffinose/galactose (YP-Raff/Gal). For growth of strains containing Cdc20 under the control of the methionine repressible promoter *MET3*, cells were grown in synthetic YNB media supplemented with either 2% w/v glucose or 2% w/v raffinose/galactose. For the selection of transformants, YNB agar plates were used lacking the auxotrophic amino acid used for selection. Diploid cells were sporulated on sporulation media (100 mM CH_3COONa, 20 mM NaCl, 25 mM KCl, 1.5 mM $MgSO_4$ and 1.5% w/v agar).

Strains were constructed by transformation with the appropriate DNA integration fragment designed for gene knockout or tagging. Affinity epitope tags were fused at the gene endogenous loci for Western blot detection, or a 3xGFP and mRFP cassette for detection by fluorescent microscopy, using polymerase chain reaction products (Bahler et al., 1998; Knop et al., 1999) Some strains were constructed by mating with another mutant followed by sporulation of diploids and selection on appropriate selective media (see 2.1.2). Genes knockout were created by one-step gene replacement of the entire ORF with the auxotrophic marker cassette.

Table 1.   Stains used in this study

Strain ID	Genotype
K699	*MAT*a (w303 "wild type" background)
Y360	*MAT*a *MET-HA3-CDC20::trp1 GAL-flag-ESP1-CBD::trp1(x6) MAD1-HA3::ura3 BUB1-3eGFP::his3*
Y657	*MAT*a *MET-HA3-CDC20::trp1 scc1Δ::his3 SCC1-TEV268-HA3::leu2 GAL-NLS-myc9-TEVprotease-NLS2::trp1(x10) GAL-CDC14-Pk3::his3 MAD1-HA3::ura3*
Y720	*MAT*a *MET-HA3-CDC20: trp1 SCC1-TEV268-Pk3::leu2 GAL-NLSmyc9-TEVprotease-NLS2:: trp1(x10) GAL-CDC14-Pk3::ura3 BUB1-3eGFP::his3*
Y721	*MAT*a *MET-HA3-CDC20::trp1 SCC1-TEV268-Pk3::leu2 GAL-NLSmyc9-TEVprotease-NLS2::trp1(x10) BUB1-3eGFP::his3*
Y850	*MAT*α *MET-HA3-CDC20::trp1 scc1Δ ::his3 SCC1-TEV268-HA3::leu2 GAL-NLS-myc9-TEVprotease-NLS2:: trp1(x10) MAD1-HA3::ura3*
Y851	*MAT*a *MET-HA3-CDC20::trp1 GAL-flag-ESP1-CBD:: trp1(x6) MAD1-HA3::ura3*
Y3025	*MAT*a *cdc14-1 MAD1-HA3::ura3 PDS1-myc18::trp1*
Y3026	*MAT*a *cdc15-2 MAD1-HA3::ura3 PDS1-myc18::trp1*
Y3147	*MAT*a *cdc14-1 MAD1-HA3::ura3 PDS1-myc18::trp1 mad2Δ::leu2*
Y4057	*MAT*a *cdc14-1 MAD1-HA3::ura3 PDS1-2A-myc18::trp1*
Y2669	*MAT*a *scc1-73 PDS1-HA6::ura3 sli15-6A-HA6::his3*
Y3774	*MAT*a *scc1-73 PDS1-HA6::his3 GAL-CDC14-Pk3::ura3*
Y3789	*MAT*a *PDS1-HA6::his3*
Y3595	*MAT*α *MET-HA3-CDC20::trp1 scc1Δ::his3 SCC1-TEV268-HA3::leu2 GAL-NLS-myc9-TEVprotease-NLS2::trp1(x10) MAD1-HA3::ura3 sli15-6A-HA6::his3*
Y2400	*MAT*a *PDS1-myc18::trp1 CDC14-HA6::his3*
Y4065	*MAT*a *PDS1-myc18:: trp1 GAL1-CDC14-Pk3::leu2*
Y1851	*MAT*a *MET3-HA-CDC20:: trp1 PDS1-myc18::ura3 CDC14-HA6::his3*

Y2761	*MATa MET3-HA-CDC20::trp1 PDS1-myc18::ura3 CDC14-HA6::his3 GAL1-CDC14-Pk3::leu2*
Y3594	*MATa sli15-6A-HA6::his3*
Y3967	*MATα GAL-CDC20::ura3 cdc14-1 PDS1-HA6::his3*
Y3968	*MATα GAL-CDC20::ura3 cdc15-2 PDS1-HA6::his3*
Y3969	*MATa GAL-CDC20::ura3 cdc14-1 mad2Δ:: leu2 PDS1-HA6::his3*
Y2671	*MATa PDS1-HA6::his3 MPS1-myc18::trp1*
Y3760	*MATa MET-HA3-CDC20::trp1 GAL-flag-ESP1-CBD::trp1(x6) MAD1-HA3::ura3 MPS1-myc18::leu2*
K6745	*MATa TetOs::ura3 TetR::leu2*
Y3947	*MATa sli15-6A-HA6::his3 TetOs::ura3 TetR::leu2*
LM46*	*MATa cdc14-1 Mad1-HA3::ura3 Sli15-6A-HA6::his3*
LM48*	*MATa GAL-Sli15-HA6::ura3*
LM49*	*MATa GAL-Sli15-6E-HA6::ura3*
Y3727	*MATa SLI15-6E-HA6::his3*
LM37*	*MATa GAL-Ndc10-(TEV)3-Sli15-HA3::ura3*
LM42*	*MATa SLI15-6A-HA6::his3*

Numbers listed in the table refer to strain number entries in the Uhlmann Lab database. Strain numbers marked with asterisk (*) refer to the numbers in Lesia's strain database.

2.1.2 Yeast mating and tetrad dissection

Mating was induced by incubation of opposite mating type yeast strains on YPD plates at 25°C for 24 hours. Diploids were selected then on appropriate selective media and grown again on YPD for 12 hours. The diploids were placed on a sporulation plate until tetrads appear. Spores were treated with Zymolase T-20 (MP Biomedicals) for 10 minutes to break asci and four released spores from each ascus were dissected using a Singer-MSM micromanipulator. The spores were incubated at 25°C until colonies formed.

2.1.3 Cell culture synchronizations

Yeast cells were arrested in G1 with the mating pheromone α-factor. To arrest cells, an early log phase culture (OD600 = 0.1) was treated with α-factor (provided by peptide services, Cancer Research UK) at a concentration of 1μg/ml. One and two hours later the same concentration of α-factor was added. Arrests were generally complete after two-generation times. Cell cycle arrest was determined both cytologically by the appearance of a pear-shaped "schmoo" and by FACS analysis of DNA content. G1 arrested cells were collected on a membrane filter (Schleicher & Schuell, ME28, 1.2mm) using a filtration apparatus from Millipore. Cells were extensively washed with YP before release into YP media supplemented with sugar.

For arrest in metaphase, nocodazole (Sigma) was added at 5 μg/ml. For arrest using *GAL*-Cdc20, cells were cultured in media containing 2% raffinose and 2% galactose before being filtered, washed and transferred to media containing raffinose as the sole sugar source. Protein expression was confirmed by western blotting.

For arrest using the repression of *MET3*-Cdc20, cells were grown in YNB supplemented with 2% glucose. To arrest cells, 2mM methionine was added. Cell cycle arrests were checked cytologically and by FACS analysis of DNA content.

2.1.4 Yeast transformation

Transformation of yeast was performed using PCR products as described in section 2.1.1. Mid-log phase culture (50 ml) was pelleted at 3,000 rpm for 5 minutes. The cell pellet was washed with 1 ml of deionized water, then with 1ml TEL (10 mM Tris/HCL pH 7.5, 100

mM EDTA and 100 mM Lithium acetate), before re-suspension in a final volume of 100ml TEL. 1 mg of either linearised DNA vector or PCR product was mixed with 2ml of a 10 mg/ml single stranded carrier DNA from salmon sperm and 600 µl TELP (TEL plus 40% PEG 3350 or 4000). Cell suspension was then added to this mix and followed by a short vortex. After incubation at 25°C for 2- 3 hours, cells were heat shocked at 42°C for 15 minutes. The cells were then pelleted at 6,000 rpm for 2 minutes, washed in 1 ml sorbitol and plated on selective media. Transformants were checked for the correct integration of the PCR cassette by western blot analysis or death on methionine (in the case of *MET3*-Cdc20) or glucose (*GAL*-Cdc20) containing media.

2.2   GENERAL MOLECULAR BIOLOGY TECHNIQUES

All standard molecular biology techniques, like DNA purification, PCR, restriction endonuclease digestion, bacterial plasmid purification were carried out as described in (Sambrook, 1989) or in corresponding kit manufacturer protocol.

2.3   PROTEIN ANALYSIS TECHNIQUES

2.3.1 Protein extract preparation

Whole cell extracts for the analysis of Mad1 phosphorylation was prepared using an alkaline extraction method (Kushnirov, 2000) In all other cases protein extracts were prepared using TCA method. 10ml of yeast cells culture was collected by centrifugation, resuspended in 1 ml of 20% trichloroacetic acid (TCA) and kept on ice for 15 minutes or till the end of the time-course experiment. Then cells were washed with 1

ml of 1M Tris-Base and finally cell pellets were re-suspended in 100ml of 2X SDS-PAGE loading buffer with DTT. 100ml of 0.5mm glass beads (BioSpec Products, Inc) were added and the cells were lysed using a FastPrep FP120 cell breaker (Bio101). Extracts were then spun down to separate them from the glass beads. Collected supernatant was transferred to a new tube, boiled for 5 minutes and cleared by centrifugation at 13,000 g for 5 minutes.

2.3.2 SDS-PAGE electrophoresis and western blotting

Protein samples were resolved on acrylamide/bis-acrylamide (37.5:1, amresco) 375 mM Tris-HCL pH 8.8 and 0.1% SDS. Small proteins of less than 30 kDa were typically resolved on 10% -12% and larger proteins over 100 kDa on 8% gels. A stacking gel was used on top of the separating gel and was composed of 125 mM Tris-HCL pH 6.8, 5% acrylamide/bis-acrylamide and 0.1% SDS.

Proteins were allowed to migrate at 50mA using SDS-PAGE running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS) in electrophoresis tanks from CBS scientific, CA. To monitor the position of the proteins in the gel and subsequently on the membrane, a broad range pre-stained protein marker (New England Biolabs) was used.

Separated proteins were transferred onto pre-equilibrated nitrocellulose membranes (Schleicher & Schuell) using a wet-transfer tank (Biorad). Transfer buffer contained 3.03 g/L Tris base, 14.1 g/L glycine, 0.05% SDS and 20% v/v methanol. The efficiency of transfer was then checked with Ponceau S solution (Sigma). The membrane was blocked with a 5% milk solution (Marvel) in PBST (170 mM NaCl, 3 mM KCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.01% tween 20) for 1 hour at

room temperature. Membranes were then incubated with primary antibodies diluted in milk solution for one hour at room temperature. The antibodies concentrations were as follows: anti-HA (12CA5, ICRF 1:5000), anti-myc (9E10, ICRF, 1:2000), anti-Clb2 serum (Santa Cruz, sc-9071, 1:2000), anti-PSTAIR recognizing Cdc28 (Santa Cruz, sc-53, 1:1000), anti-tubulin (AbD Serotec, YOL1/34, 1:1000) and anti-β-actin serum (Abcam, ab8227, 1:5000). Membranes were then washed in an excess of PBST three times for ten minutes. Horseradish peroxidase (HRP) coupled secondary antibodies (anti-mouse or anti-rabbit, Amersham, 1:5000) were then incubated with the membrane in PBST containing 5% milk for a further hour. Membranes were washed three times before developing with ECL (Amersham) according to the manufacturer's instructions.

2.3.3 Detection of Mad1 phosphorylation forms

Analysis of Mad1 phosphorylation was performed by electrophoresis of whole cell extracts on low crosslinking SDS-polyacrylamide gels, followed by Western blotting.

For the preparation of low crosslinking gel (acrylamide to bis-acrylamide ratio 33.5:0.3), 25% acryl solution was used to pour 8% separating gel and normal stacking gel. Protein extract samples were separated on a 14cm x 14cm gel. Proteins were allowed to migrate through the stacking gel at 100V and through the separating gel at 120V using SDS-PAGE running buffer. Transfer to the nitrocellulose membrane and western blotting was performed as usual.

2.4 DNA ANALYSIS AND MANIPULATION TECHNIQUES

All cloning and standard DNA manipulation procedures were carried out as described in Sambrook and Russell, Molecular Cloning, third edition, CSHL press, 2001 (Sambrook, 1989).

2.4.1 Plasmids

All plasmids created and used in this study are listed in **Table 2**.

Table 2.   List of DNA vectors

Number	Name	Description
Basic vectors for integration in yeast	
3	YIplac211	*URA3* based integrative vector
15	YIp211-GAL	*GAL1-10* promoter cloned between EcoRI and BamHI in YIp211
57	pJK171 Ndc10-GFP	*NDC10*-Superglow GFP under its own promoter in an integrating plasmid (*TRP1* marker)
272	YIplac211-Sli15prom	750bp PCR fragment of the *SLI15* promoter was cloned between Kpn1 and Xba1 sites of YIplac211
277	pYl211GAL-Sli15-HA6	2kb PCR fragment corresponding to the *SLI15* ORF was cloned in YIp211-GAL. HA3 cassete was then inserted into Not1 restriction site
281	YIp211-Sli15promSli15-HA6	2.2kb of *SLI15* ORF was sub-cloned from pYl211GAL-Sli15-HA6 into YIplac211-Sli15prom between Xba1 and Sph1 sites
452	pRS306	*URA3* based integrative vector
48	pBS- KlTRP1-MET	Construct for one step PCR promoter exchange. *MET3* promoter cloned between EcoRI and BamHI sites in pBS-KlTRP1

778	YIp(HIS3) GAL-CDC14-Pk3	pRS303 backbone with YIplac MCS, carrying GAL-CDC14-Pk3
815	YIp22 MET3-CDC20	*URA3*-based construct for MET3-CDC20 promoter exchange
LP 30*	pRS303-SLI15 term-Sli15-6E-HA6	*HIS3*-based plasmid for integration of *SLI15-6E* mutant version into the locus of endogenous *SLI15* gene
LP 33*	pYl211 GAL-Ndc10-TEV(3)-Sli15-HA6	Construct for expression of Ndc10-Sli15 fusion protein from *GAL* promoter. Based on pYl211GAL-Sli15-HA6
Vectors for epitope tagging in yeast		
30	pKlURA3	*K. lactis URA3* complementing *S. cerevisiae URA3* in unknown AmpR cloning vector
32	pBS-KlTRP1	*K. lactis TRP1* complementing *S. cerevisiae TRP1* in pBluescript
34	pUC19 myc18-KlTRP1	One step C-terminal myc18 tagging vector (*K. lactis TRP1* marker)
35	pUC19 HA3-KlTRP1	One step C-terminal HA3 tagging vector (*K. lactis TRP1* marker)
36	pUC19 HA6-SpHIS5	One step C-terminal HA6 tagging vector (*S. pombe HIS5* marker complements *S. cerevisiae HIS3*)
37	pUC19 HA3-SpHIS5	One step C-terminal HA3 tagging vector (*S. pombe HIS5* marker)
39	pUC19 myc18-KlURA3	One step C-terminal myc18 tagging vector (*K. lactis URA3* marker)
40	pUC19 HA3-KlURA3	One step C-terminal HA3 tagging vector (*K. lactis URA3* marker)
554	pUC19 Pk3-KlTRP1	One step C-terminal Pk3 tagging vector (*K. lactis TRP1* marker)
555	pUC19 Pk3-KlHIS3	One step C-terminal Pk3 tagging vector (*K. lactis HIS3* marker)
562	pUC19 HA3-Kl LEU2	One step C-terminal HA3 tagging vector (*K. lactis LEU2* marker)
628	pUC19 PK6-KlTRP1	One step C-terminal Pk6 tagging vector (*K. lactis TRP1* marker)

688	pRS303-eGFP3	3eGFP cassette in BamH1/Not1 site of pRS303
772	pUC19 HA6-kanMX6	One step C-terminal HA6 tagging vector (*kan^R* marker)
773	pUC19 HA6-KlURA3	One step C-terminal HA6 tagging vector (*K lactis URA3* marker)
774	pUC19 myc9-KlURA3	One step C-terminal myc9 tagging vector (*K. lactis URA3* marker)
776	pUC19 myc18-KlLEU2	One step C-terminal myc18 tagging vector (*K. lactis LEU2* marker)
653	pRS303-SLI15 term-Sli15-6A-HA6	*HIS3*-based plasmid for integration of *SLI15-6A* mutant version into the locus of endogenous *SLI15* gene
912	3eGFP-kan^R	Template (unknown) for one-step PCR tagging
211	pBS-KILEU2	*K. lactis LEU* gene in pBluescript between EcoR1 and Hind3 sites
295	pBKl-TRP1 mRFP	3eGFP cassette in Not1/Sal1 site of pBKl-TRP1

Numbers listed in the table refer to DNA number entries in the Uhlmann Lab database. Plasmid numbers marked with asterisk (*) refer to the numbers in Lesia's plasmid database.

2.4.2 Site-directed DNA mutagenesis

Site-specific mutagenesis was used to create a phospho-mimetic *sli15-6E* mutant. The integrative plasmid pRS303-SLI15 term-Sli15-6A-HA6, bearing Sli15 with 6 Cdk1 phosphorylation sites mutated to alanine (*SLI15-6A*) was used as template to replace the alanine coding nucleotide codons to those coding glutamic acid. Site-specific mutagenesis was carried out using QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer protocol. All oligonucleotides were synthesized by Sigma and were purified by HPLC.

2.4.3 Cloning of the Sli15-(TEV)3-Ndc10 fusion construct

The *NDC10* coding sequence was cloned into the vector pYl211GAL-Sli15-HA6 after the *GAL1* promoter. The C-terminus of *NDC10* was fused by overlap extension PCR to the *SLI15* open reading frame including 400bp of its downstream region using a linker sequence containing three TEV sites:

ggtggtggtggtccaagagaaaatttgtattttcaaggtccaagagaaaatttgtattttcaaggtgcttctga aaatttgtattttcaaggtggtggtggtggt. The plasmid pYl211GAL-Ndc10-(TEV)3-Sli15-HA6 was linearized in the *URA3* coding sequence and transformed into yeast for integration at the *URA3* locus.

2.4.4 DNA sequencing

For DNA sequencing 200 ng of plasmid DNA were added to 20 µl reaction mixed containing 3.2 pmol of the appropriate sequencing primer (and 8 µl of BigDye Terminator 3.1 (Applied Biosystems). We used the following thermal cycling conditions: 96˚C for 1 minute; 24 cycles of 96˚C for 30 seconds, 52˚C for 15 seconds and 60 ˚C for 4 minutes.

2.4.5 DNA sequence alignment

Multiple DNA sequences were aligned using Strider and ApE-A Plasmid Editor software. Strider software also was used for the alignment of protein sequences.

2.5 CELL BIOLOGY AND MICROSCOPY

2.5.1 Cell cycle analysis using flow cytometry

To determine cell cycle progression by DNA content, 1 ml of a mid log phase culture (OD_{600} = 0.4) were pelleted and fixed in 70% ethanol overnight at +4˚C. Cells were then RNAse treated in 1ml 200 mM Tris-HCL pH 7.5 containing 0.1 mg/ml RNAse A for at least 2 hours at 37°C. After pelleting, DNA was now stained using 400ml of a propidium iodide containing solution (200 mM Tris-HCL pH 7.5, 211 mM NaCl, 78 mM $MgCL_2$ 50mg/ml propidium iodide). Cells were sonicated (Sanyo, Soniprep 150) before being analysed on a FACScan (Becton Dickinson). Subsequent image preparation was performed using CellQuest software.

2.5.2 In situ immunofluorescence (IF)

Spindle elongation was analyzed in formaldehyde-fixed cells by indirect immunofluorescence. 2 ml of log-phase culture were re-suspended in 1 ml of ice cold 1% in IF-I buffer (100 mM KH_2PO_4/K_2HPO_4, 0.5 mM $MgCl_2$ pH6.4) and fixed overnight at 4˚C. Then cells were first washed in the same IF-I buffer lacking formaldehyde once and then re-suspended in a sorbitol based buffer IF-II (as IF-I but with sorbitol, 1.2 M, pH 7.4). Cells were re-suspended in 200µl of spheroplasting solution (as above IF-II, plus 2µl/ml of ß-mercaptoethanol and 2 µl/ml of 20 mg/ml Zymolase T-100) and incubated at 30˚C for 20 – 40 minutes. After this, spheroplasts were delicately washed once and re-suspended in IF-II buffer. 5µl of cells were loaded on polylysine-coated wells on 15 multi-well slides (MP Biomedical). Slides were blocked with

a blocking buffer (0.5% Bovine Serum Albumin in PBS) after the fixation in methanol for 3 minutes and in acetone for 10 seconds.

Incubation with primary and secondary antibodies was carried out in the dark in a humid chamber for 1 hour each. Wells were washed with blocking buffer 3 times between antibody staining and 4 times before addition of mounting media Fluoroguard with 0.1 µg/ml of DAPI in antifade medium. Slides were then covered with a cover slip and sealed. Antibodies used were: anti-tubulin clone YOL1/34 (Serotec), anti-GFP clone TP401 (Torrey Pines Biolabs, Houston, TX), anti–HA 16B12 (Babco).

2.5.3 Sister chromatid separation assay

Sister chromatid separation assay was performed using the tetracycline Operator/Repressor GFP system as described in (Michaelis et al., 1997). Under conditions when sister chromatids are tightly cohered, the GFP coated tetracycline arrays appear as one dot. Upon separation of sister chromatids, two GFP dots can be seen in anaphase cells. 2 ml culture was pelleted (13,000 rpm for 1 min) and resuspended in 1ml of ice-cold absolute ethanol. Cells were fixed on ice for 2 hours. An aliquot of the cell suspension was placed onto a thin 2% agar pad on a glass slide and covered with a coverslip. GFP dots were imaged on an Axioplan 2 microscope (Zeiss).

2.5.4 GFP-tagged protein visualization

Cells expressing Bub1-3xGFP were fixed in 100% ethanol for 2 hours on ice and mounted on 2% agarose pads for examination. Recruitment to kinetochores was confirmed by its colocalization with

Ndc80-mRFP. Fluorescent images were acquired using an Axioplan 2 imaging microscope (Zeiss) equipped with a 100x (NA = 1.45) Plan-Neofluar objective and an ORCA-ER camera (Hamamatsu).

2.6   ASSAYS FOR THE ANALYSIS OF THE MITOTIC CHECKPOINT IN BUDDING YEAST

We used a number of protocols to assay the mitotic checkpoint in budding yeast cells. We could assess the ability of cells to (1) establish and maintain the mitotic checkpoint arrest and (2) segregate chromosomes efficiently upon recovery from mitotic arrest.

2.6.1 Markers the mitotic checkpoint signalling

To assess the mitotic checkpoint state during cell cycle of synchronised yeast culture we monitored phosphorylation status of checkpoint protein Mad1. MAD1 was fused at its endogenous gene loci to an HA affinity epitope tag for western blot detection. Mad1 phosphorylation was assessed by retarded electrophoretic mobility, a sign for checkpoint engagement (Hardwick and Murray, 1995). Localization of the checkpoint protein Bub1 to kinetochores is also recognized as marker for checkpoint signaling (Gillett et al., 2004). Strains expressing Bub1-3xGFP alone or together with kinetochore protein Ndc80-mRFP were used for microscopic imaging of Bub1 localization.

The level of securin (Pds1) reflects the cell cycle stage: high (and maintained) levels of Pds1 indicate that the mitotic checkpoint is active and APC/C is being inhibited. Conversely, degradation of securin reflects

activation of APC/C as the consequence of checkpoint silencing (Fernius and Hardwick, 2009).

2.6.2 Induced checkpoint arrest by using microtubule-destabilizing drugs

Genetic screens in budding yeast identified components of the spindle checkpoint using drugs that inhibit microtubule polymerisation (Hoyt et al., 1991; Li and Murray, 1991). High levels of these drugs (e.g., benomyl, nocodazole) result in depolymerised spindle microtubules and therefore in lack of kinetochore-microtubule attachments. Wild-type cells respond to these unattached kinetochores and halt the cell cycle in metaphase. However, spindle checkpoint mutants, such as *mad2Δ*, used in this study, ignore the unattached kinetochores induced by the microtubule poison, and undergo anaphase prematurely.

To assess the checkpoint response of different cells to microtubule depolymerisation we used the drug nocodazole (Sigma, stock solution of 2mg/ml, dissolved in DMSO). 5μg of nocodazole was added per 1ml of G_1-synchronized culture. After 3 hours cells were collected and mitotic arrest was assessed cytologicaly, by FACS and western blot of checkpoint markers.

2.6.3 Mitotic checkpoint arrest induced by lack of tension at kinetochores

The assay to monitor efficient mitotic checkpoint arrest described above in 2.6.2, uses microtubule-depolymerising drugs to produce unattached kinetochores. Spindle checkpoint also causes mitotic delay in response to kinetochore attachments that lack tension. For example,

Aurora B, Sgo1 and the Bub1 kinase domain are all required for cells to respond to reduced cohesion at centromeres, but not the defects induced by high levels of nocodazole (Biggins and Murray, 2001; Fernius and Hardwick, 2007; Indjeian et al., 2005). We used a temperature-sensitive strain *scc1-73* carrying a mutation in the cohesin subunit Scc1, and also Pds1 protein tagged with HA or myc epitopes. Cell cycle progression in these cells that have attached, but not cohered (tensionless) sister chromatids can be monitored at the restrictive temperature (37°C).

2.6.4 Chromosome bi-orientation/mis-segregation assay

The fidelity of chromosome segregation upon mitotic checkpoint recovery can be monitored using the single chromosome tag method. Because only a pair of sister chromatids are tagged with GFP in mitosis (see 2.5.3), the chromosome mis-segregation can be easily detected and quantified by the observation of two chromosome dots in one daughter nucleus and no dots in the other.

Cells were synchronised in G_1 using α-factor, released into the corresponding media containing 10 μg/ml nocodazole and incubated at 25°C for three hours to allow for the spindle to disassemble and the cells to arrest. The microtubule drug was then carefully washed out from the media by culture filtration, as described in 2.1.3. The cells were then released into fresh media without the drug. The culture was incubated, shaking at 25°C for further 30-60 minutes to allow the spindle assembly and anaphase to occur. The cells were then fixed in 100% cold ethanol for 2 hours and analysed using an Axioplan 2 imaging microscope (Zeiss). Cell images were taken on DeltaVision RT system (Applied Precision) based on an Olympus IX71 microscope with a 100× oil

immersion lens, NA=1.4. Images were captured with a CoolSNAP HQ digital CCD camera (Roper scientific).

3 RESULTS

3.1 BUDDING YEAST AS A MODEL ORGANISM FOR CELL CYCLE ANALYSIS

We used the budding yeast *Saccharomyces cerevisiae* as a model organism for this study. Taking into account a number of experimental advantages and ease of genetic analysis, yeast have proved to be a valuable model system for the study of cell-cycle control. They can proliferate in a haploid state, in which only a single copy of each chromosome is present in the cell. This makes it easy to generate mutations in particular genes and to analyze them without the complications of a second (wild type) gene copy. It is also relatively easy to replace specific genes with mutant versions, or express them under the control of promoters that are responsive to the nutrient composition of growth medium.

3.2 TENSION LOSS AT ANAPHASE ONSET IS DETECTED BY THE MITOTIC CHECKPOINT

Our first goal was to find out whether loss of tension (as a result of cohesin cleavage) at anaphase onset is sensed by the mitotic checkpoint. Cells were arrested in metaphase by depletion of the APC activator Cdc20. In these cells, we artificially initiated anaphase onset by ectopic expression of either separase, or the foreign TEV protease that also triggers loss of cohesion by cleaving the accordingly engineered cohesin (Uhlmann et al., 2000). Mitotic checkpoint signalling was monitored by the phosphorylation status and kinetochore recruitment of the checkpoint components Mad1 and Bub1, respectively (Gillett et al., 2004; Hardwick and Murray, 1995).

Mad1 phosphorylation, accompanied by retarded electrophoretic mobility, a sign for checkpoint engagement, was not detectable during separase-triggered anaphase (**Figure 3.1,A**), consistent with the notion that the mitotic checkpoint remains silent. Only at later timepoints, some Mad1 phosphorylation became apparent, which was probably the consequence of progression into the next cell cycle after separase expression (Sullivan and Uhlmann, 2003). In contrast, when anaphase onset was triggered by TEV protease expression, Mad1 became phosphorylated concomitant with anaphase onset. Similarly, recruitment of Bub1 into distinct nuclear foci, a marker for recognition of tensionless kinetochores by the checkpoint, was observed at the time of anaphase onset in response to TEV protease expression, but not after separase expression (**Figure 3.2,B**).

This suggests that loss of cohesion at anaphase onset results in loss of tension, which is detected by the mitotic checkpoint, and triggers checkpoint signalling. This is prevented by separase activation at anaphase onset. Apart from cohesin cleavage, separase activity is required to keep the spindle checkpoint silent after chromosomes split apart. These observations are consistent with a recent report showing that the checkpoint protein BubR1 associates with anaphase chromosomes after TEV protease-induced cohesin cleavage in mitotically arrested Drosophila embryos (Oliveira et al., 2010).
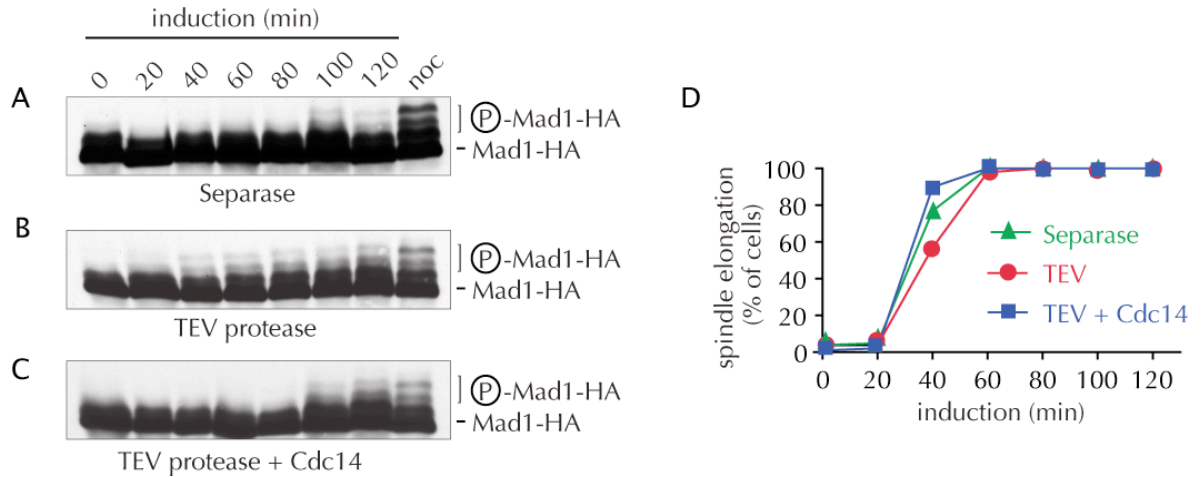
Figure 3.1   Tension loss, triggered by sister chromatid disjunction, is detected by the mitotic checkpoint.
*Cells were arrested in metaphase by Cdc20 depletion and expression of separase (**A**), TEV protease (**B**) or TEV protease together with Cdc14 (**C**), was induced. Signalling of the mitotic checkpoint was monitored by the phosphorylation-induced electrophoretic mobility shift of Mad1, fused to an HA epitope tag to facilitate Western detection. The same cells treated with the spindle poison nocodazole (5µg/ml; noc), but uninduced, served as a positive control for mitotic checkpoint engagement. (**D**) Anaphase spindles of 4 µm or longer were scored as elongated by indirect immunofluorescence staining of tubulin.*
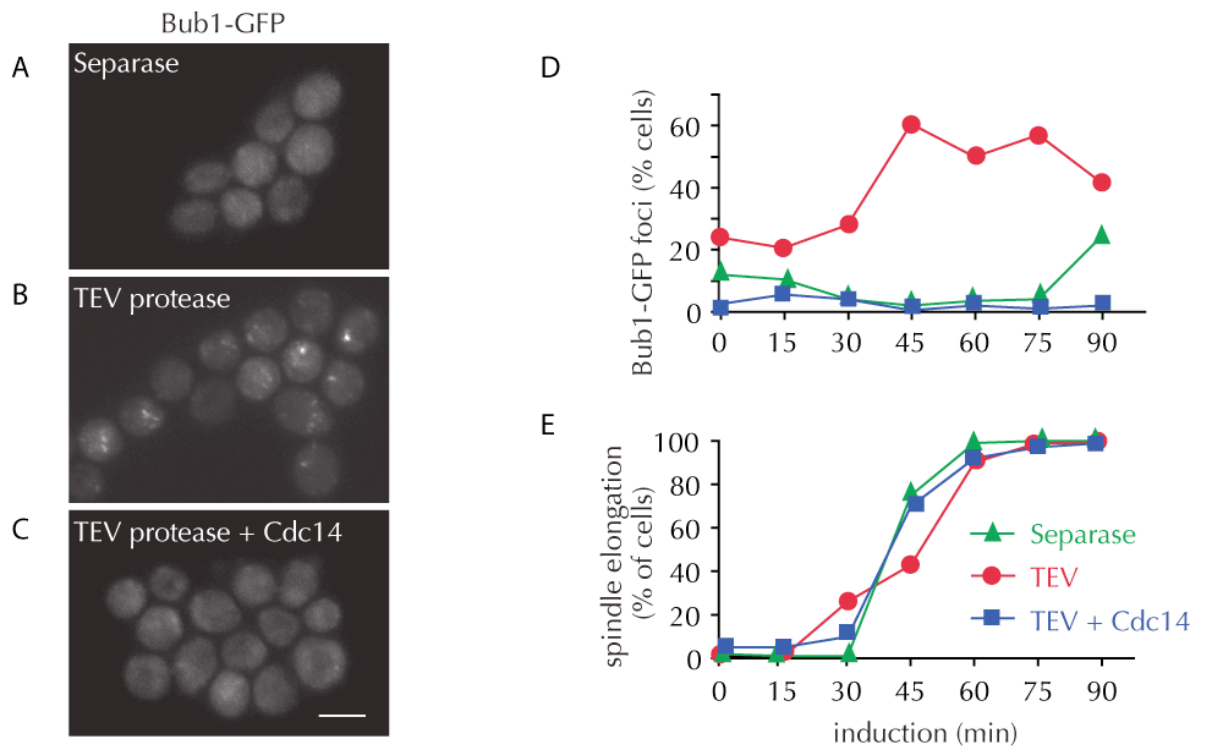
Figure 3.2   Bub1 kinetochore foci formation after sister chromatid disjunction indicates the checkpoint engagement.
*Cells were arrested in metaphase by Cdc20 depletion and expression of separase (**A**), TEV protease (**B**) or TEV protease together with Cdc14 (**C**), was induced. Checkpoint engagement was visualized by the appearance of Bub1-GFP nuclear foci. Images are of cells 45 min after induction, scale bar 5 μm. (**D**) Anaphase spindles of 4 μm or longer were scored as elongated by indirect immunofluorescence staining of tubulin.*

3.3 CDC14 INACTIVATES THE SPINDLE CHECKPOINT IN ANAPHASE

In addition to splitting sister chromatids, separase also has a non-proteolytic function in the cell (Sullivan and Uhlmann, 2003). It promotes activation of the Cdc14 phosphatase, a key Cdk1 opponent during budding yeast mitotic exit. From previous experiments we established that cohesin cleavage itself is not responsible for keeping the checkpoint silent as cells move into anaphase (**Figure 3.1,A,B** and **Figure 3.2,A,B**). The ability of separase to restrain mitotic checkpoint re-engagement in anaphase is independent of its protease function. Thus, next we wanted to address whether Cdc14 acts downstream of separase to make cells insensitive to loss of tension at anaphase onset.

We used the same experimental conditions as described in Figure 3.1, but ectopically co-expressed Cdc14 together with TEV protease in metaphase-arrested cells. This prevented both Mad1 phosphorylation and Bub1 foci formation in response to sister chromatid splitting (**Figure 3.1,C** and **Figure 3.2,C**).

This result indicates that Cdc14 can inactivate the response of the mitotic checkpoint to loss of tension.

3.3.1 Checkpoint engagement in anaphase lacking Cdc14

To confirm that Cdc14 is responsible for restraining the checkpoint in anaphase, we examined the checkpoint state in anaphase without Cdc14.

For this purpose we used a *cdc14-1* temperature sensitive strain. *cdc15-2* mutant cells served as a control that, like *cdc14-1* cells, arrest in telophase at the restrictive temperature but activate Cdc14 in early
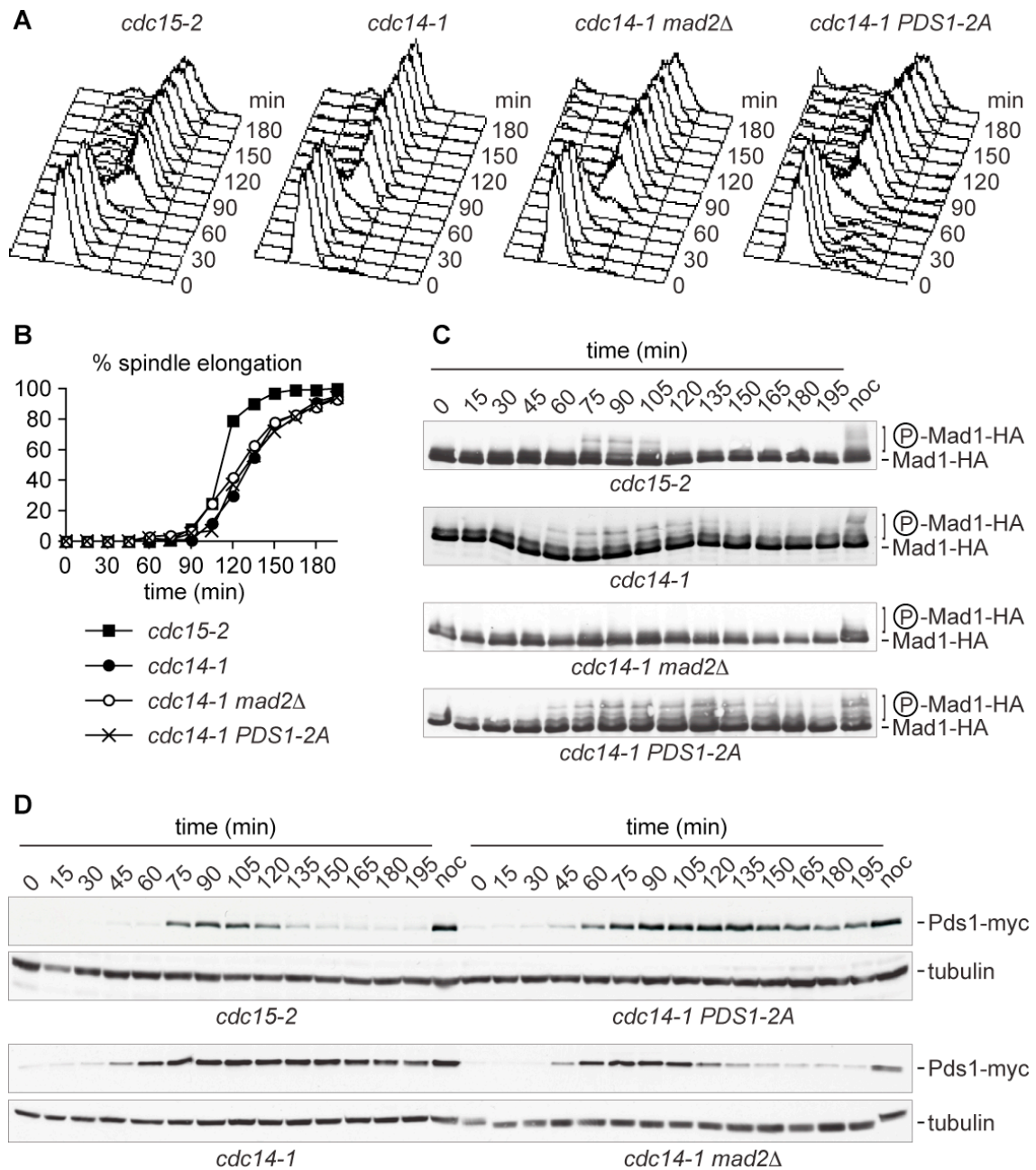
Figure 3.3 **Persistent mitotic checkpoint activity during anaphase in *cdc14-1* mutant.** *(A) Cells of the indicated genotypes were released from α-factor block in G1 into synchronous cell cycle progression at the non-permissive temperature (37°C) for the cdc14-1 and cdc15-2 alleles. Cell cycle progression was monitored by FACS analysis of DNA content. (B) Anaphase spindle elongation was analyzed by indirect immunofluorescence staining of tubulin. Spindles of 4 μm or longer were scored as elongated. (C) The Mad1 phosphorylation status in cells from the above experiment was analyzed by Western blotting. (D) Levels of securin (Pds1), fused to a myc epitope tag to facilitate detection, were analyzed by Western blotting. Tubulin served as a loading control.*

anaphase (Stegmeier et al., 2002). After synchronization in G_1 using yeast pheromone α-factor, both strains progressed through the early stages of cell cycle with similar kinetics (**Figure 3.3,A**). Anaphase spindle elongation started at the same time, but took longer to complete in the case of *cdc14-1* cells, most likely due to the Cdc14 requirement for stable spindle midzone formation, as described above (**Figure 3.3,B**). In *cdc15-2* control cells, Mad1 phosphorylation became detectable at the time of S-phase and disappeared again at the metaphase to anaphase transition (**Figure 3.3,C**). In contrast, Mad1 phosphorylation persisted long into anaphase in *cdc14-1* cells, indicating a failure to inactivate the mitotic checkpoint.

The above result suggests that the mitotic checkpoint is engaged in *cdc14-1* anaphase cells.

Checkpoint engagement during anaphase is expected to inhibit the APC and consequently stabilize securin. Consistently, we observed high levels of securin in *cdc14-1*, but not *cdc15-2*, anaphase cells (**Figure 3.3,D**). The persistence of securin was due to the mitotic checkpoint in *cdc14-1* cells, as it was no longer observed after deletion of the gene encoding the checkpoint component Mad2. Anaphase spindle elongation was not advanced in *cdc14-1* cells lacking Mad2, confirming that Cdc14 affected the rate of spindle elongation independently of mitotic checkpoint regulation.

This means that checkpoint signalling in *cdc14-1* cells results in securin stabilization due to the ability of the engaged checkpoint to inhibit APC/C.

3.3.2 Securin re-accumulation in anaphase is due to checkpoint signalling

The above results suggest that the mitotic checkpoint is engaged in *cdc14-1* anaphase cells. However, checkpoint silencing and securin destruction are thought to be a prerequisite for anaphase onset. Persistent Mad1 phosphorylation and securin in *cdc14-1* cells are therefore probably the consequence of checkpoint re-engagement after it had initially been satisfied prior to anaphase. An alternative explanation might be that securin degradation is delayed as a result of deficient checkpoint silencing already in metaphase.

3.3.2.1 *Retarded securin degradation or re-accumulation*

To discriminate between these two possibilities we decided to analyse more carefully the metaphase to anaphase transition. A transient decrease in Mad1 phosphorylation and securin levels would have been obstructed by the limited mitotic synchrony of the cell population after release from α-factor arrest. Thus, we performed a similar experiment with cells synchronised in metaphase by depletion and re-induction of Cdc20 activator of APC/C (**Figure 3.4**).

In *cdc15-2* cells, securin was largely degraded and Clb2 levels decreased to a lower steady state level in response to Cdc20 re-induction. Importantly, Cdc20 is thought to be insufficient for complete Clb2 destruction, which requires activation of APC^{Cdh1} (Yeong et al., 2000). Cdh1 remains inactive in *cdc15-2* mutants cells due to disruption of the mitotic exit network.
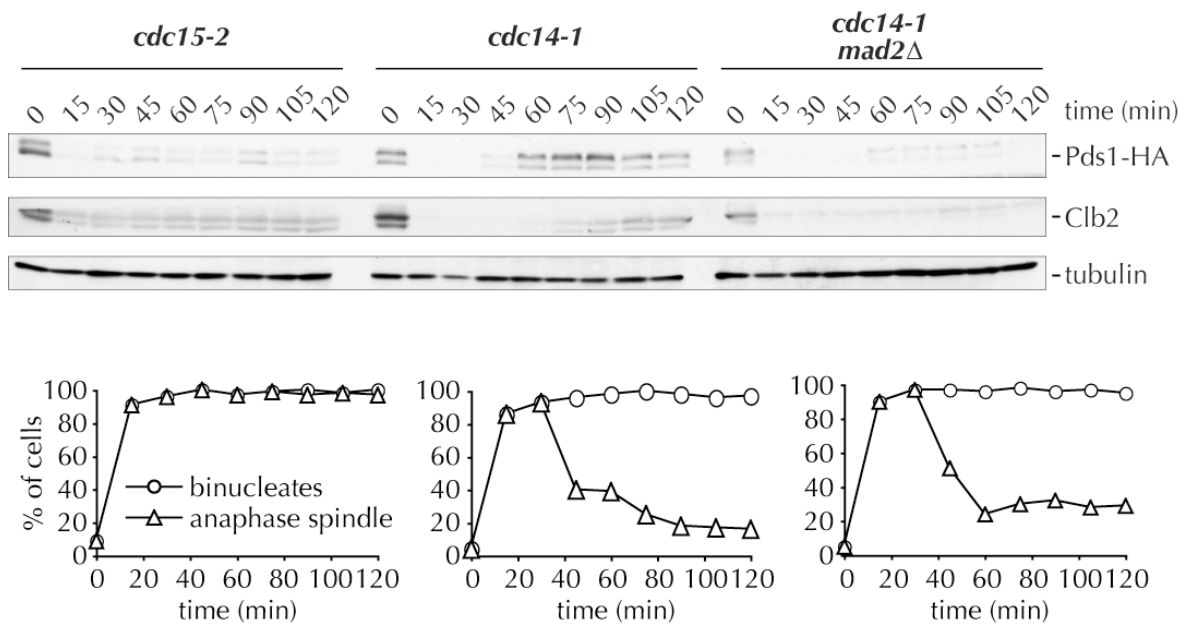
Figure 3.4   Checkpoint-dependent securin and Clb2 re-accumulation in a *cdc14-1* mutant.

Cells of the indicated genotypes were arrested in metaphase by depletion of Cdc20 under control of the GAL1 promoter. The temperature was raised to 37°C to inactivate the cdc14-1 and cdc15-2 alleles, and cells were released into synchronous anaphase progression by Cdc20 re-induction. Samples at the indicated times after release were processed for Western blotting against securin (Pds1) and Clb2. Tubulin served as a loading control. Anaphase progression was monitored by indirect immunofluorescence staining of tubulin, and by scoring nuclear division. Anaphase spindles break down soon after elongation in cdc14-1 cells due to defective spindle midzone assembly (Higuchi and Uhlmann, 2005; Uhlmann et al., 2000).

In *cdc14-1* cells we observed efficient securin and Clb2 destruction in response to Cdc20 re-induction, initially to levels lower than those observed in *cdc15-2* cells (**Figure 3.4**). This might be because Cdc14 is in part responsible for limiting the activity of Cdc20 during anaphase progression. However, after 45 min, securin, and later also Clb2 started to re-accumulate. Securin and Clb2 re-accumulation depended on a functional mitotic checkpoint and was strongly reduced in the absence of Mad2. The relatively long time (45 min) until checkpoint-dependent securin re-accumulation in *cdc14-1* mutant anaphase cells is probably due to the high levels of Cdc20 after its *GAL1* promoter-driven re-induction that must be overcome by the mitotic checkpoint before securin can re-accumulate.

This observation indicates that the mitotic checkpoint is satisfied (securin levels decline) and then becomes re-engaged in anaphase (securin re-accumulates), if Cdc14 is inactive.

3.3.2.2 Securin phosphorylation status does not affect its stabilization in response to the spindle checkpoint

It has been suggested that Cdc14 promotes securin destruction during anaphase by its direct dephosphorylation (Holt et al., 2008). In this case securin re-accumulation could be due to its persistent phosphorylation, which cannot be removed in a *cdc14-1* temperature-sensitive mutant.

To test this suggestion we introduced a non-phosphorylatable securin allele, *PDS1-2A*, that is no longer protected from degradation by Cdk1 phosphorylation (Holt et al., 2008). This, however, did not avert securin stabilization in *cdc14-1* anaphase cells (**Figure 3.3,D**).

In contrast to the dependence of securin stabilization on Mad2, this result suggests that securin accumulation in anaphase is mainly the consequence of mitotic checkpoint re-engagement.

3.3.3 Cdc14 overcomes checkpoint-dependent arrest

The above experiments have analyzed markers of the checkpoint and have suggested that Cdc14 is required to prevent its re-engagement due to loss of tension at anaphase onset. The important physiological consequence of checkpoint signalling is a mitotic delay.

Due to the essential requirement of Cdc14 for mitotic exit independently of checkpoint inactivation, we were unable to analyze a checkpoint-mediated delay in anaphase cells of temperature-sensitive mutant *cdc14-1*. To explore the potential of Cdc14 as a checkpoint regulator, we therefore analyzed its impact in a setting where mitosis is delayed in cells that fail to establish tension between sister chromatids due to defective sister chromatid cohesion.

3.3.3.1 Cdc14 overrides the tension checkpoint

As described earlier (Biggins and Murray, 2001), securin destruction and progression through mitosis was delayed in cells carrying the temperature-sensitive cohesin subunit *scc1-73* (**Figure 3.5,A,B**). We set out to analyze whether Cdc14 expression could inhibit the checkpoint in this situation and overcome the mitotic arrest.

We synchronized cells in G_1 and released them into mitosis at the restrictive temperature. *scc1-73* ts cells exhibited securin stabilization as a result of checkpoint-dependent mitotic arrest. In contrast, Cdc14
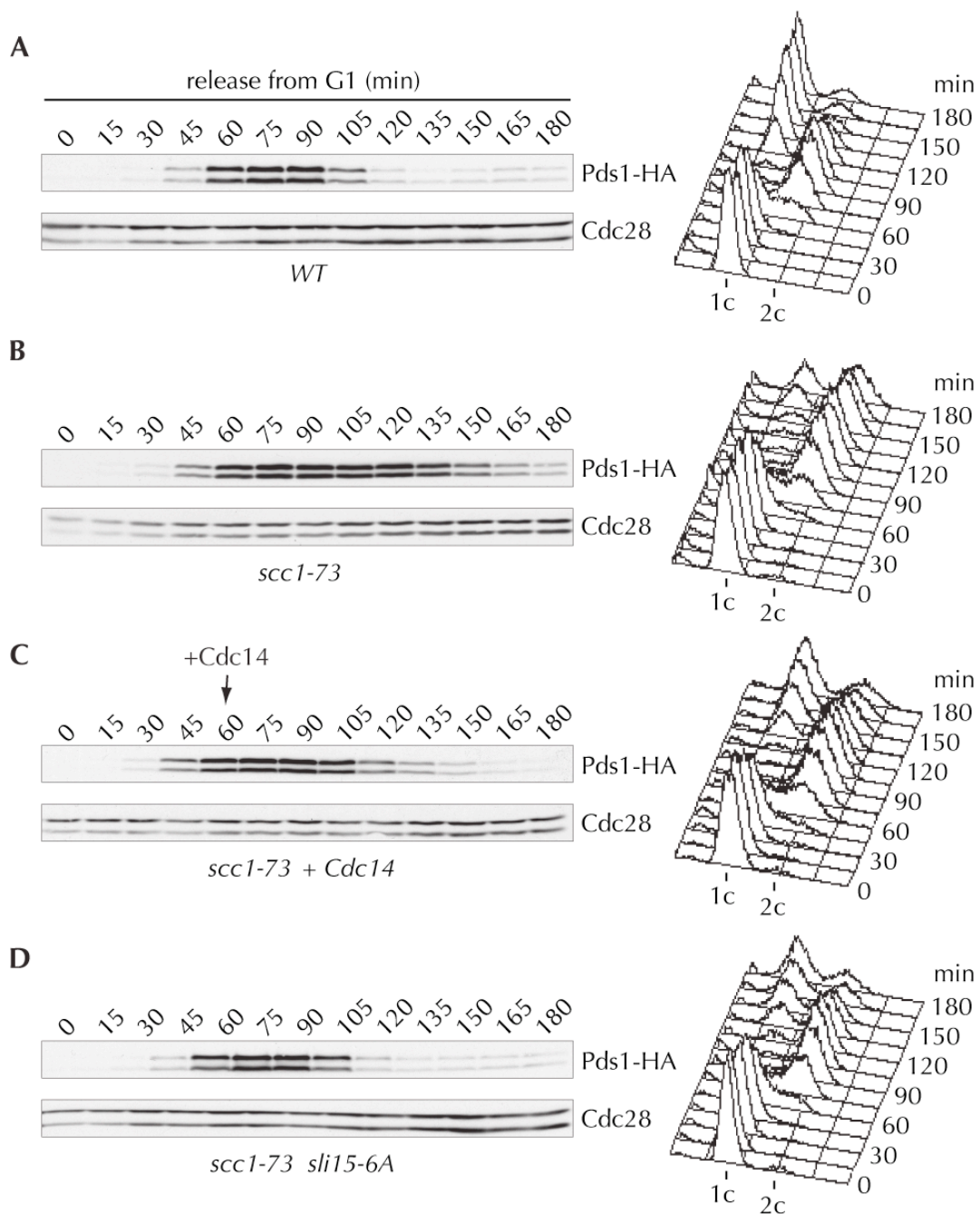
Figure 3.5   Cdc14 relieves the mitotic checkpoint delay due to absence of tension.
(A) wild type and (B) scc1-73 cells were grown in YP medium containing raffinose as carbon source, arrested in G1 using α-factor and released into synchronous cell cycle progression at the restrictive temperature (35°C). α-factor was added back at 75 min for re-arrest in the following G1. Cell cycle progression was monitored by Western blotting against securin (Pds1) fused to an HA-epitope tag, Cdc28 served as a loading control, and FACS analysis of DNA content. (C) In a second scc1-73 culture, Cdc14 expression from the GAL1 promoter was induced by galactose addition at 60 min. (D) A third scc1-73 culture carried the sli15-6A allele.

expressed in *scc1-73* cells largely overcame the delay to both securin destruction and mitotic progression (**Figure 3.5,C**).

This demonstrates that Cdc14 is able to override the checkpoint-dependent mitotic delay, caused by compromised cohesion and hence, a tension defect between sister chromatids. While in this experiment Cdc14 overcame the checkpoint response to lack of tension in prometaphase, Cdc14 would normally disable the response to loss of tension in early anaphase, its normal time of activation.

3.3.3.2   *Cdc14 overrides the attachment checkpoint*

In the experiments described above, we showed that Cdc14 is capable of downregulating the checkpoint, when it was engaged in response to tension loss between sister chromatidss. It was interesting to address whether Cdc14 can overcome the mitotic checkpoint also in response to an attachment problem.

G_1-syncronized cells were released into media containing the microtubule-destabilizing drug nocodazole. Wild type cells engaged the checkpoint in response to massive microtubule depolymerization and spindle disruption, and arrested in mitosis for the duration of the experiment (5 hours). Surprisingly, ectopic expression of Cdc14 overcame nocodazole-induced mitotic arrest and cells escaped into the next cell cycle (**Figure 3.6,A**).

A

B
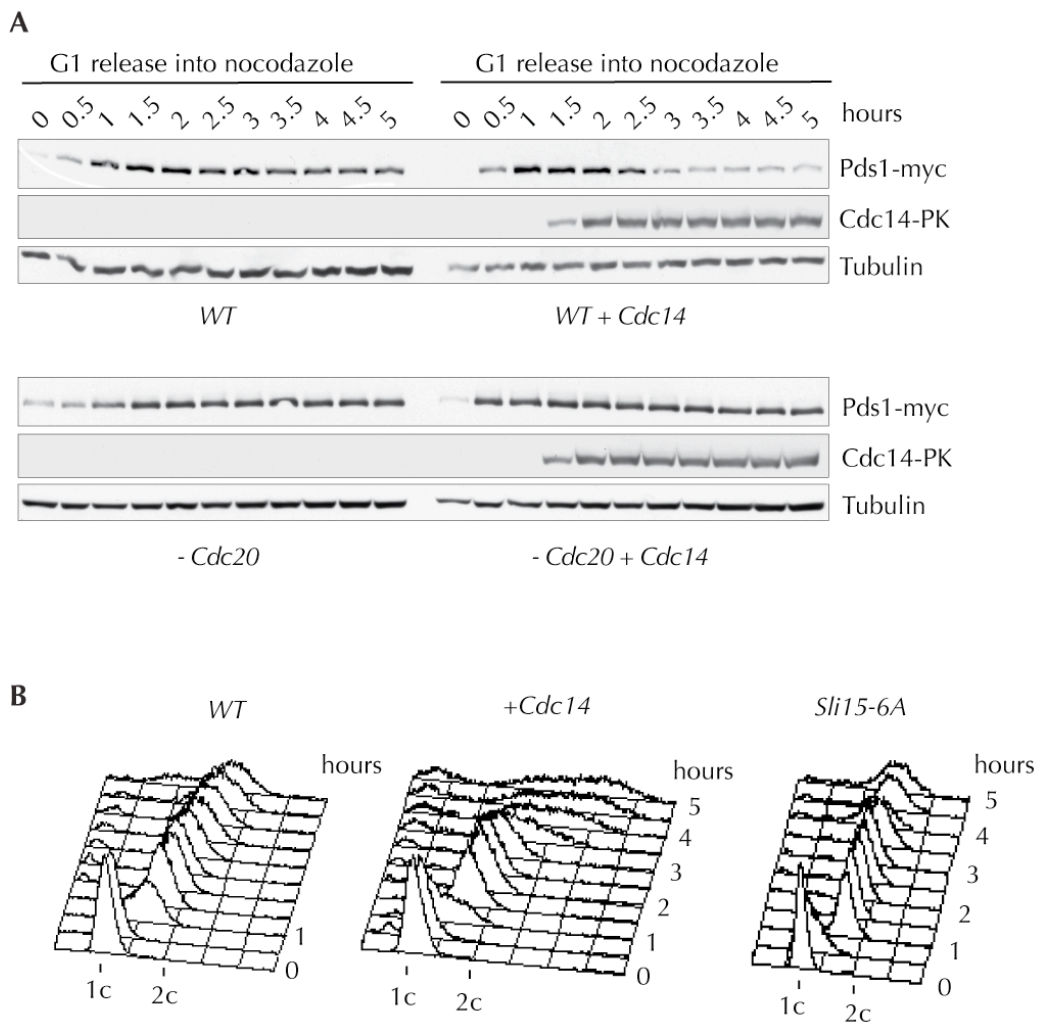
Figure 3.6   Ectopic Cdc14 expression overcomes a nocodazole-imposed mitotic checkpoint arrest.

(A) Cells were synchronized in G1 by α-factor treatment and released into nocodazole-containing medium. Cells harboring the MET3-CDC20 allele were grown in synthetic medium lacking methionine and were shifted to YP medium supplemented with 2mM methionine to repress Cdc20 expression at the time of release. One hour after release, Cdc14 expression was induced under control of the GAL1 promoter in half of the culture. After $1^{1}/_{2}$ hours, α-factor was re-added to prevent possible securin re-accumulation in the next cell cycle. (B) As (A), but α-factor was not re-added. Re-budding and re-replication in the presence of nocodazole was observed in response to ectopic Cdc14 expression, but not in sli15-6A cells.

We compared checkpoint-dependent mitotic block conditions with the arrest caused by the depletion of the APC/C regulator Cdc20. As in the previous experiment, cells were synchronized in G_1 and released into media containing nocodazole. In half of the cultures, Cdc14 was expressed one hour after G_1 release and securin levels were analyzed during one cell cycle. As expected, securin remained stable throughout the arrest in cells that did not express Cdc14, but was degraded in response to Cdc14 induction. In contrast, Cdc14-imposed securin destruction did not occur in cells depleted of Cdc20 (**Figure 3.6,A**). This result is consistent with the possibility that Cdc14 overcomes the mitotic arrest, caused by a checkpoint response to nocodazole treatment, but cannot overcome a mitotic arrest caused by other means. Moreover, release of this mitotic block requires functional Cdc20 excludes that Cdc14-activated Cdh1 is responsible for the mitotic exit.

3.4 A CONSERVED MECHANISM OF MITOTIC CHECKPOINT INACTIVATION AT ANAPHASE ONSET

The next question we faced was: how does Cdc14 inactivate the mitotic checkpoint? It has been suggested that APC/C-dependent degradation of the checkpoint kinase Mps1 disables the checkpoint in anaphase (Palframan et al., 2006). Mps1 degradation is partially mediated by the APC/C activator Cdh1. However, Cdh1 activation is a late event during mitotic exit and Cdc14-mediated dephosphorylation of Cdh1 is required for its binding to the APC. Consistently, we found that Mps1 levels declined only late and gradually in anaphase (**Figure 3.7,A**).
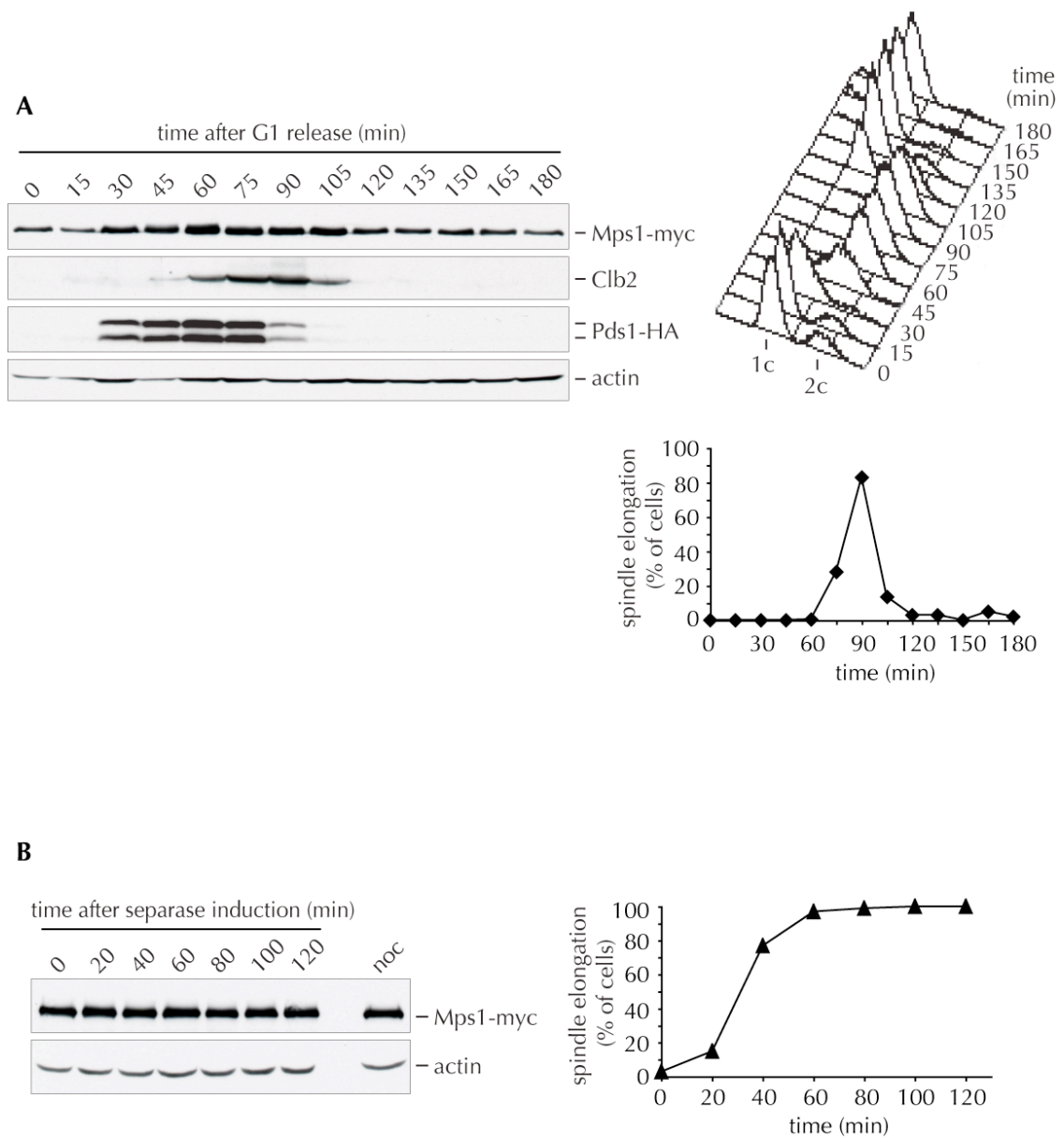
Figure 3.7   Mitotic checkpoint inactivation at anaphase onset in the presence of high Mps1 levels.
(A) Cells were arrested in G1 by α-factor treatment and released into synchronous cell cycle progression. At 60 min, α-factor was added back to halt cell cycle progression in the next G1 phase. Cell extracts were prepared at the indicated time points and protein levels of Mps1, fused to a myc epitope tag for detection, Clb2 and securin (Pds1), fused to an HA epitope tag, were analyzed by Western blotting. Actin served as a loading control. Cell cycle progression was monitored by FACS analysis of DNA content and indirect immunofluorescence staining of tubulin. (B) Separase expression was induced in cells arrested in metaphase by Cdc20 depletion, as in Figure 3.1. Spindle elongation was monitored by tubulin staining. Mps1 levels, as detected by Western blotting, did not change during the course of the experiment.

Mps1 degradation may therefore not act fast enough to render the mitotic checkpoint insensitive to loss of tension at anaphase onset. Furthermore, Mps1 remained stable, while the mitotic checkpoint was efficiently inactivated, in response to separase expression in mitotically arrested cells (**Figure 3.1** and **Figure 3.7,B**). These observations suggest that Cdc14 inactivates the mitotic checkpoint by an additional or different mechanism.

3.4.1 Sli15 – a candidate for Cdc14-dependent checkpoint inhibition

A candidate Cdc14 substrate for checkpoint inactivation is Sli15/INCENP. It forms part of the conserved Aurora B kinase complex at centromeres, required for conveying lack of tension to the mitotic checkpoint (Biggins and Murray, 2001). Its Cdc14-dependent dephosphorylation in anaphase mediates Sli15/INCENP relocation from centromeres to the spindle midzone. This prompt relocation of chromosome passengers at the beginning of anaphase makes Sli15 an excellent candidate for being responsible for disabling the mitotic checkpoint. Since Ipl1/Aurora B acts as a tension sensor if not removed from centromeres, Aurora B could also respond to the loss of tension when sister chromatids are split at anaphase onset.

3.4.2 Dephosphorylation of Sli15/INCENP inactivates the checkpoint

To test the contribution of Sli15/INCENP dephosphorylation to inactivation of the mitotic checkpoint, we employed cells carrying the *sli15-6A* allele in which 6 Cdk1 phosphorylation sites have been mutated, mimicking a dephosphorylated state independently of Cdc14 (Higuchi and Uhlmann, 2005; Pereira and Schiebel, 2003). The *sli15-6A*

mutant is proficient in its essential function in chromosome bi-orientation on the mitotic spindle (**Figure 3.8**) and arrests in response to nocodazole treatment (**Figure 3.6**). However, *sli15-6A* cells overcame the mitotic delay in response to defective sister chromatid cohesion in *scc1-73* cells (**Figure 3.5,D**). The ability of *sli15-6A* mutant to mount the checkpoint response to tension, but not attachment defects indicates that Sli15 has a specific function in tension-dependent checkpoint response. Sli15 dephosphorylation, which normally occurs in anaphase, and consequent departure of Aurora B complex from the kinetochores, makes the checkpoint unresponsive to loss of tension at anaphase onset.
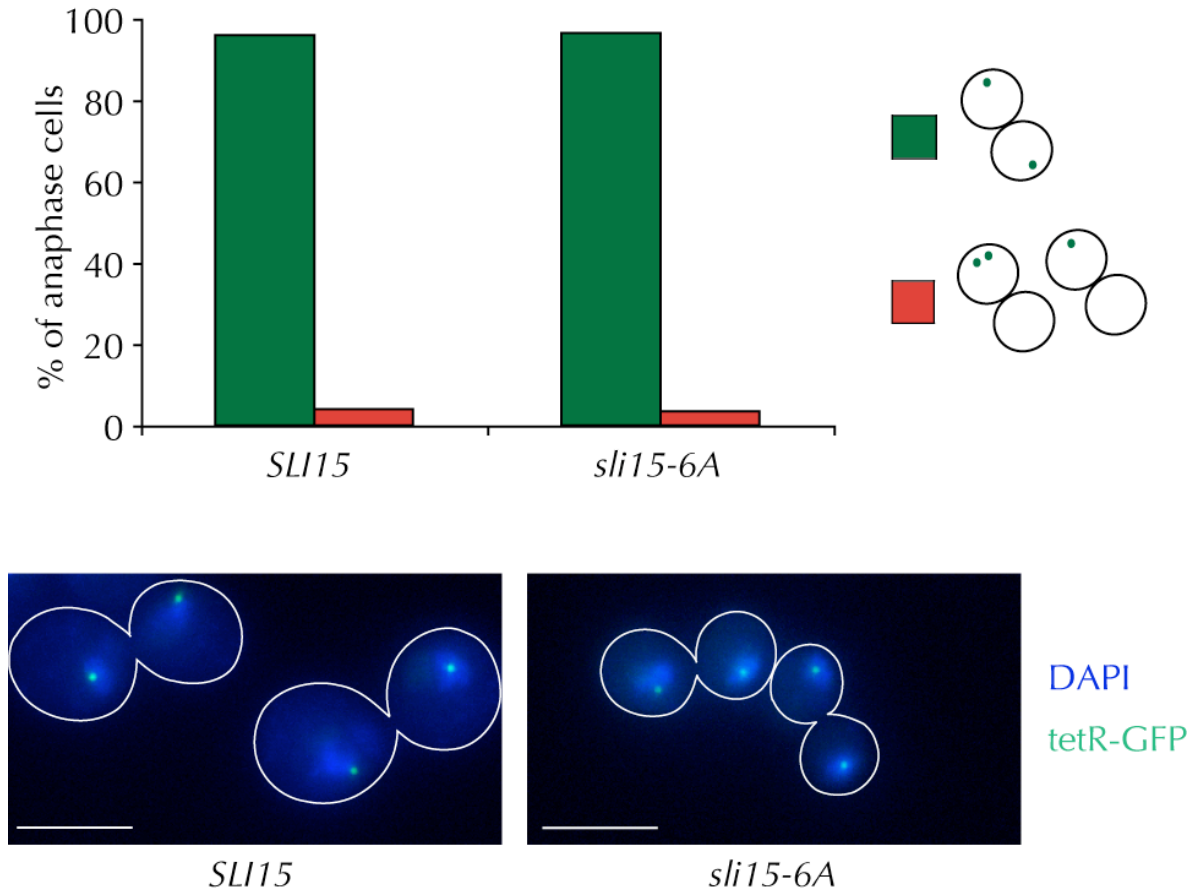
Figure 3.8   Sli15-6A is proficient in supporting chromosome bi-orientation.
*Wild type and sli15-6A cells were arrested in mitosis using the spindle poison
nocodazole that disrupts kinetochore microtubule interactions. After arrest for 2
hours, cells were released into fresh medium without nocodazole to resume mitotic
spindle formation, chromosome bi-orientation and sister chromatid segregation. One
hour after release, 52.5% and 61% of cells in the two cultures had entered
anaphase, respectively, seen as binucleated cells by staining with 4',6-diamidino-2-
phenylindole (DAPI). Correct segregation of sister chromatids of chromosome 5,
marked at the URA3 locus using the tetOs/tetR-GFP system, to opposite cell poles in
both cultures demonstrates the efficiency of Aurora B kinase-dependent chromosome
bi-orientation in wild type and sli15-6A cells. Scale bar 5µm.*

We next tested whether Sli15/INCENP dephosphorylation is indeed sufficient to prevent mitotic checkpoint re-engagement when tension between sister chromatids is lost at anaphase onset. We induced sister chromatid separation in metaphase-arrested *sli15-6A* cells by TEV protease expression. Unlike in *SLI15* control cells, in which Mad1 became phosphorylated at the time of anaphase onset, this response was no longer observed in *sli15-6A* cells (**Figure 3.9**)

These results suggest that Sli15 dephosphorylation turns off the ability of the mitotic checkpoint to respond to loss of tension between sister chromatids at anaphase onset.

Our study triggered the Petronczki group to test the existence of this mechanism in human cells. Similarly, Vázquez-Novelle and Petronczki showed that relocation of the Aurora B kinase complex is required to prevent untimely checkpoint protein recruitment to human centromeres in anaphase. Our study together with evidence provided from research in human cells (Vazquez-Novelle and Petronczki, 2010), suggest that a conserved mechanism prevents the mitotic checkpoint from re-engaging in anaphase.
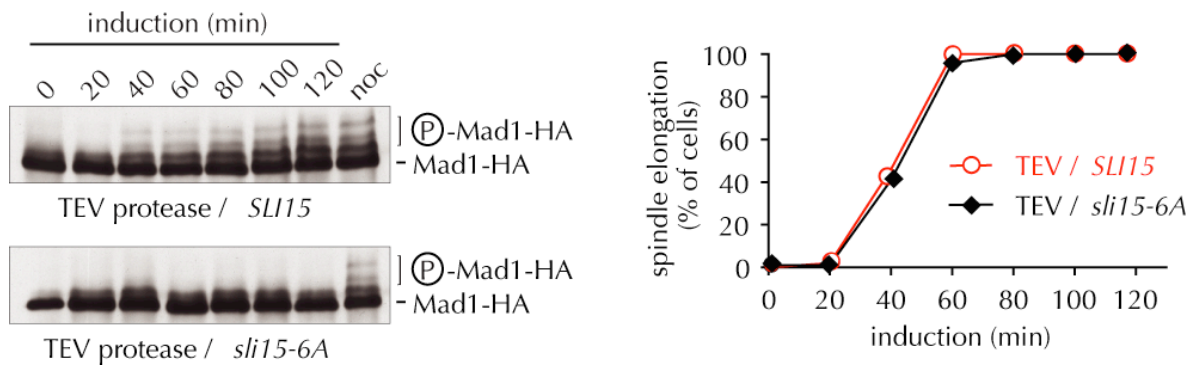
Figure 3.9   Non-phosphorylatable Sli15-6A prevents mitotic checkpoint re-engagement in anaphase.
Budding yeast cells harbouring wild type SLI15, or the sli15-6A allele, were arrested in metaphase by Cdc20 depletion. Loss of sister chromatid cohesion was triggered by TEV protease expression. Mad1 phosphorylation and anaphase spindle elongation were monitored as in Figure 3.1.

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3.5   Consequences of an engaged mitotic checkpoint in anaphase

Aurora B activity is required for the mitotic checkpoint and error correction of kinetochore-microtubule attachments. Its spatial proximity to protein targets in the outer kinetochore is thought to destabilize spindle attachments and generate a checkpoint signal until bi-polar orientation pulls sister kinetochores away from the Aurora B that resides at the inner centromere. Dephosphorylation-dependent CPC dissociation from centromeres apparently prevents the Aurora B kinase complex from gaining contact to its kinetochore targets when tension is lost in anaphase. Hence, it was interesting to explore, what consequences would cells face when Aurora B relocation from centromeres is prevented in anaphase. Will the checkpoint fire again in anaphase and delay mitotic exit?

3.5.1 Attempts to retain Ipl1/Aurora B at centromere in budding yeast

To prevent Ipl1/Aurora B relocation from centromeres in anaphase, we generated a *sli15-6E* phospho-mimetic mutant, in which six Cdk1 sites were mutated to mimic constant phosphorylation. A similar mutant was also generated in Schiebel's group (Pereira and Schiebel, 2003), using aspartic acid instead of glutamate (*sli15-6D*), to mimic phosphorylation. *sli15-6D* behaved similar to the wild type version of Sli15 and was not further analysed in detail.

A

DNA   Tubulin   Sli15-HA   Merged

B
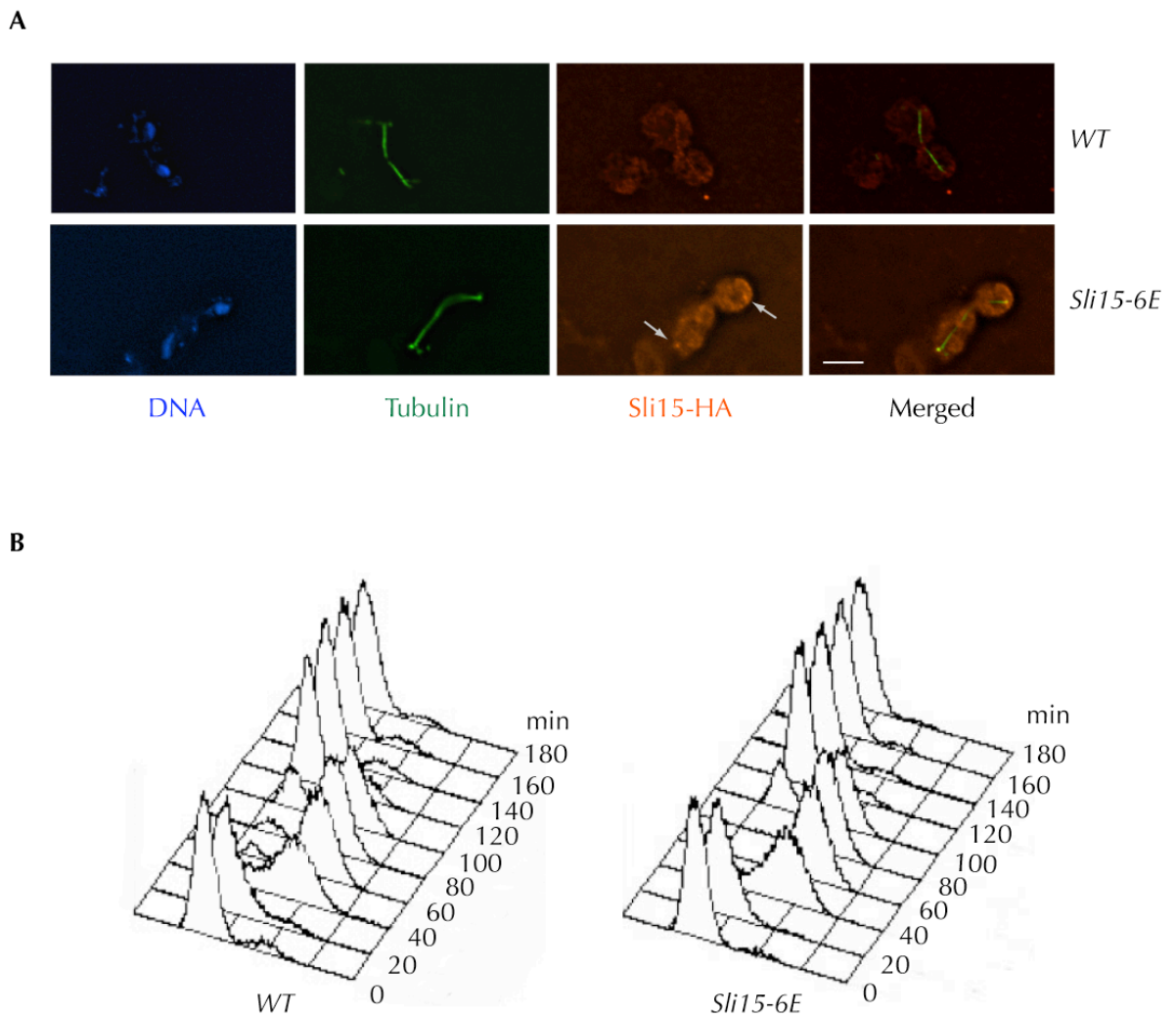
Figure 3.10   Expression of phospho-mimetic mutant Sli15-6E does not change progression through mitosis.
(A) Localization of Sli15 in anaphase cells expressing either wild type Sli15-HA or Sli15-6E-HA; scale bar 5 μm. (B) Budding yeast cells harbouring either wild type Sli15, or phospho-mimetic Sli15-6E, were synchronized in G1 using α-factor. Cells then were released into mitosis in media containing galactose to induce expression of the corresponding version of Sli15 protein. To prevent cells from escaping into the next cell cycle, α- factor was added again at 60min, when new cell buds appeared. Cell cycle progression of indicated strains was monitored by FACS analysis of DNA content.

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In a wild type strain, a tagged version of Sli15 was localized along the spindle in anaphase. This was confirmed by visualisation of the spindle with tubulin antibodies. In contrast, the localization pattern of *sli15-6E* was different from that seen for the wild type version of Sli15. The staining was rather weak in both cases, but *sli15-6E* was clearly absent from the anaphase spindle (**Figure 3.10**). Instead, we observed a weak staining of two dots at the end of spindle, most likely reflecting kinetochores of separated chromosomes in anaphase (showed by arrows). From this, we conclude that non-phosphorylated *sli15-6E* is retained at kinetochores and is not translocated to the spindle in anaphase. Surprisingly, progression through mitosis in this mutant was not changed in comparison to the wild type strain (**Figure 3.10**).

We also generated a fusion of Sli15 to the C-terminus of kinetochore protein Ndc10 under the inducible *GAL1* promoter, in order to tether Aurora B/Ipl1 complex to the kinetochore independently of the cell cycle stage. This Ndc10-Sli15 fusion version was successfully expressed, but failed to localize only to kinetochores in anaphase. Instead, staining of the mitotic spindle as well as spindle poles was observed. Accordingly, no effect on mitotic progression was seen, when expression of the fusion protein was induced from the *GAL1* promoter (**Figure 3.11**).

A

DNA   Tubulin   Sli15-HA   Merged

WT

GAL-Ndc10-Sli15

B

- Galactose   + Galactose
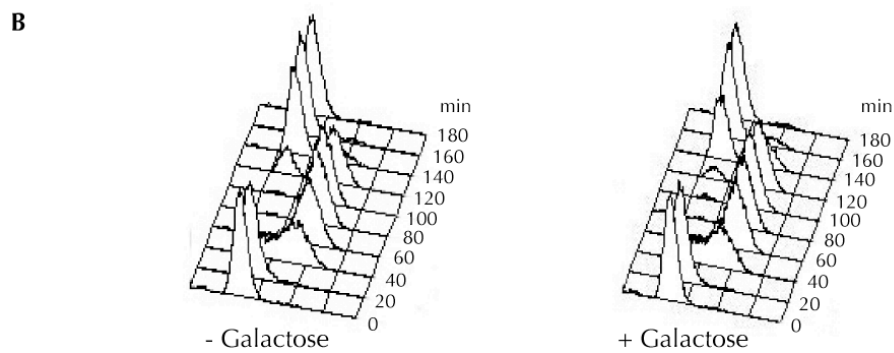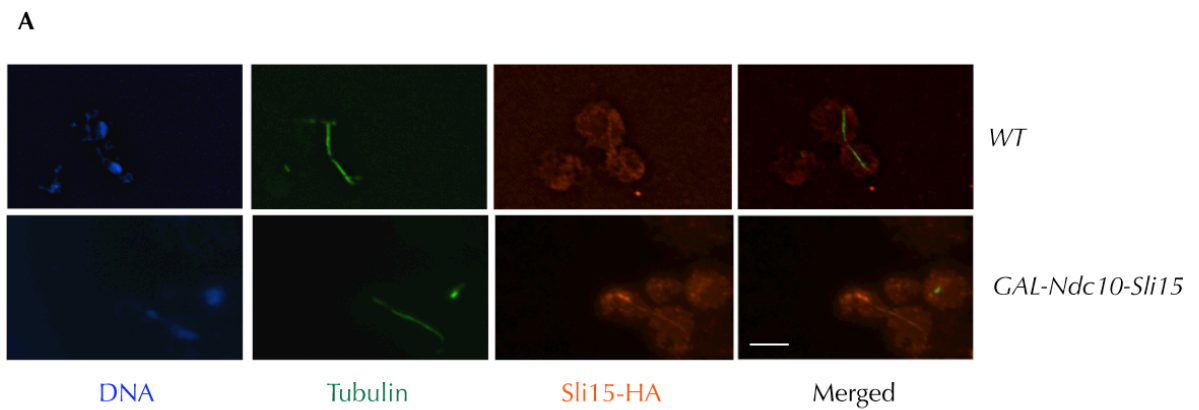
Figure 3.11   **Tethering Sli15 to kinetochore does not affect cell cycle progression.**
(A) Localization of Sli15 in anaphase cells expressing either wild type Sli15-HA or Ndc10-Sli15-HA fusion protein; scale bar 5 μm. (B) Budding yeast cells harboring Sli15 version fused to Ndc10, were synchronized in G1 using α-factor. Cells then were released into mitosis in media containing galactose to induce expression of Ndc10-Sli15 fusion, or lacking galactose to prevent its expression. To prevent cells from escaping into the next cell cycle, α- factor was added again at 60min, when new cell buds appeared. Cell cycle progression of corresponding cultures (with or without galactose induction) in the described experiment was monitored by FACS analysis of DNA content.

3.5.2 Re-engagement of the mitotic checkpoint in anaphase in higher eukaryotes

To test our proposed mechanism of checkpoint inactivation at anaphase, the Petronczki group looked for markers of the checkpoint in human anaphase cells (Vazquez-Novelle and Petronczki, 2010). They experimentally retained Aurora B and the CPC at the centromere throughout anaphase, using Mklp2 depletion. It had been previously reported that relocation of the Aurora B complex in mammalian cells requires the kinesin-6 family protein Mklp2 (Gruneberg et al., 2004). Preventing CPC translocation in this manner caused the untimely recruitment of mitotic checkpoint proteins to kinetochores at anaphase in an Aurora B-dependent manner.

They confirmed this result by using a version of INCENP carrying a T59E substitution that mimics constitutive Cdk1phosphorylation (Hummer and Mayer, 2009). In contrast to the wild type version of INCENP, which translocated to the spindle midzone at anaphase onset, INCENP$_{T59E}$ persisted at centromeres. This retention of INCENP$_{T59E}$ at centromeres was also accompanied by the recruitment of BubR1, Bub1 and Mps1 (but not Mad1 or Mad2) to kinetochores in anaphase. However, the retention of Aurora B at centromeres and recruitment of some checkpoint proteins to anaphase kinetochores was not sufficient to inhibit APC/C and block mitotic exit. They also showed that despite the presence of Aurora B at kinetochores, it did not cause kinetochore detachments from the spindle microtubules.

These experiments imply that CPC relocation is an efficient mechanism to switch the checkpoint off in anaphase. However, artificial

retention of the Aurora B kinase complex at kinetochores alone cannot lead to full checkpoint re-engagement after its initial satisfaction in metaphase. This raises the possibility of additional mechanisms involved in the checkpoint inactivation, acting in parallel to the Aurora B complex dissociation (see chapter 4. Discussion).

4 DISCUSSIONS

The mitotic checkpoint is a very sensitive mechanism that detects a single mis-attached kinetochore and efficiently blocks cell cycle progression in response to such a mistake. This also requires the checkpoint to be quickly inactivated to ensure irreversible progression and completion of the mitotic division when chromosomes begin to separate.

4.1 CHECKPOINT SILENCING VS. INACTIVATION

Aurora B actively contributes to the checkpoint signalling prior to anaphase: 1) as part of an error-correction mechanism, Aurora B produces detached kinetochores of misaligned chromosomes; 2) as a component of the spindle checkpoint, Aurora B directly recruits checkpoint proteins to kinetochores. The spatial proximity between inner centromeric Aurora B kinase and yet to be identified phosphorylation targets at the outer kinetochore, is thought to initiate the checkpoint signalling. Once bi-orientation is achieved, the kinetochore undergoes a conformational change in response to the exerted physical tension. This increases the distance between Aurora B kinase and the outer kinetochore, moving its phosphorylation targets out of reach. Protein phosphatase 1, resident at the outer kinetochore, now efficiently removes the phosphoepitopes and thereby contributes to the checkpoint silencing. Thus, checkpoint signalling diminishes upon bi-orientation and no longer restrains anaphase (Howell et al., 2004; Vanoosthuyse and Hardwick, 2009b).

However, this initial checkpoint silencing in metaphase is not irreversible. At anaphase onset kinetochores revert to their tensionless conformation. This would bring the outer kinetochore back into the proximity of Aurora B kinase and would lead to re-engagement of the mitotic checkpoint. The anaphase switch demands rapid checkpoint inactivation to ensure irreversible progression of mitosis after chromosome separation. We showed here that this is accomplished, at least partially, by moving Aurora B out from centromeres.

4.2 Mechanism of mitotic checkpoint inactivation at anaphase onset

The resulting model of Aurora B kinase regulation as part of the mitotic checkpoint is illustrated in **Figure 4.1** and **Figure 4.2**.

Localized at the centromere, Aurora B serves as a sensor for tensionless kinetochores. The spatial proximity of Aurora B to its kinetochore substrates facilitates mitotic checkpoint signalling. Upon bi-orientation of chromosomes on the metaphase plate, sister kinetochores become stretched in opposite directions and kinetochore targets of Aurora B are moved out of reach from the kinase. This allows checkpoint silencing and subsequent activation of the APC/C by its activator Cdc20.

Cohesin cleavage at this stage releases tension between sister kinetochores. This potentially can lead to checkpoint re-engagement at tensionless kinetochores, which we observed by inducing sister chromatid disjuncion with TEV protease expression. Untimely kinetochore localization of checkpoint proteins and re-inhibition of APC/C was monitored in anaphase lacking the Cdc14 phosphatase.
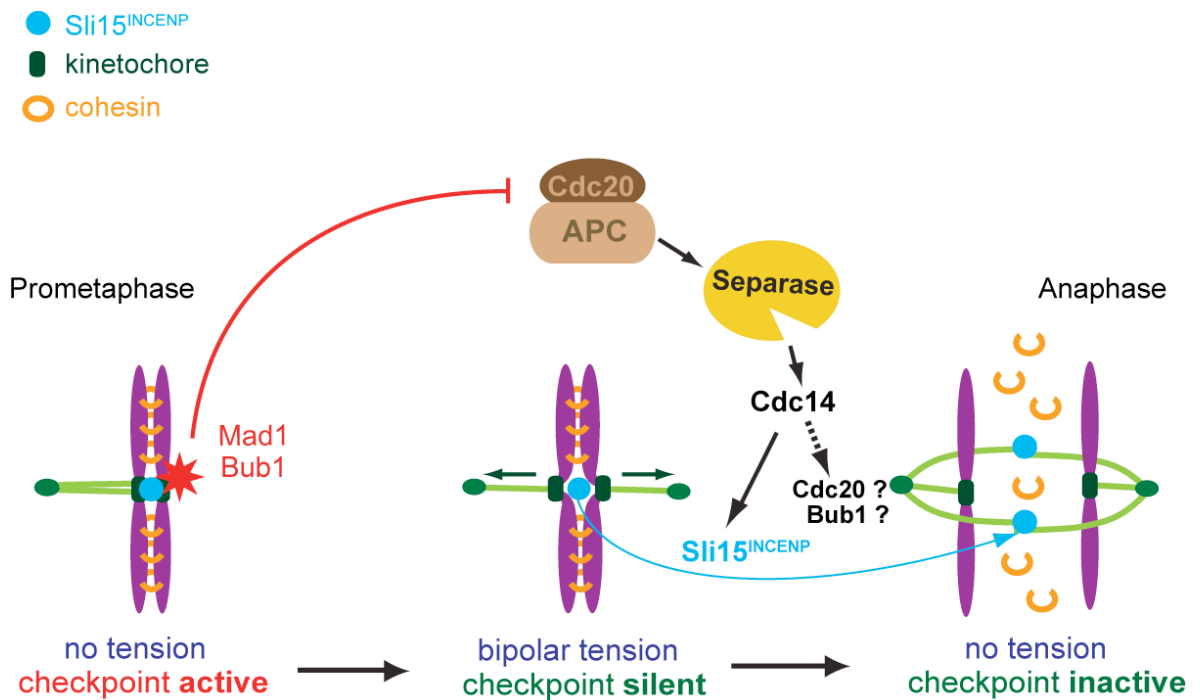
Figure 4.1 Model for mitotic checkpoint inactivation in anaphase.
During prometaphase, a mitotic checkpoint signal emanates from kinetochores that have not yet come under tension. This prevents APC activation by Cdc20. Once bipolar tension is established in metaphase, the checkpoint is silenced and the APC degrades securin to activate separase. Cohesin cleavage now triggers anaphase and tension is lost again from kinetochores. This would re-activate the checkpoint, but this is prevented by Sli15/INCENP dephosphorylation and consequent relocation of the Aurora B kinase complex to the spindle midzone. Dephosphorylation of additional Cdk1 targets might contribute to maintain an inactive checkpoint.
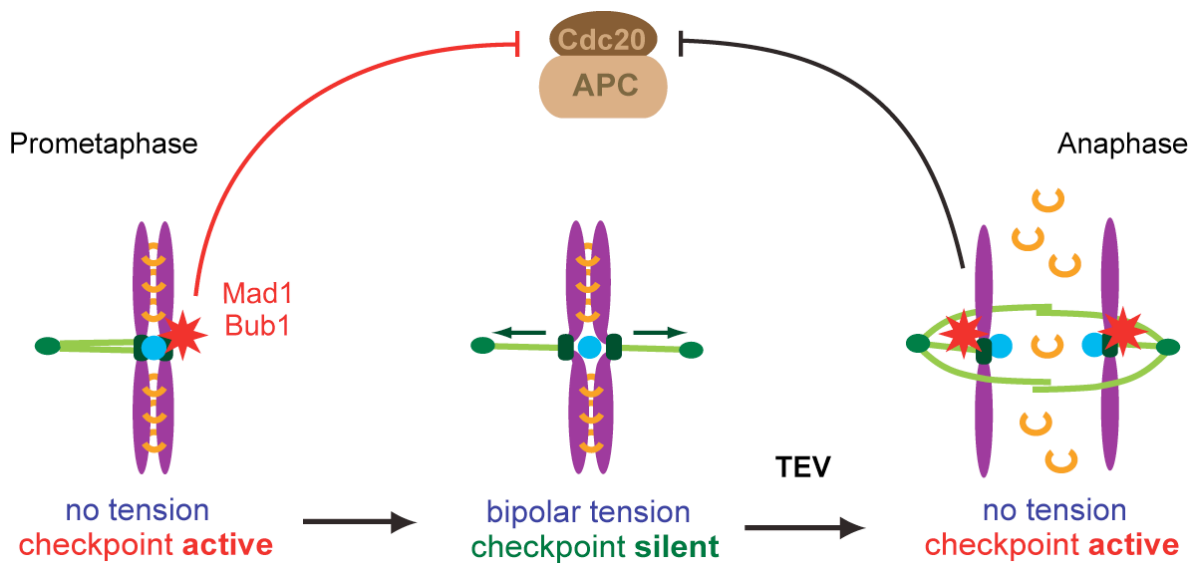
Figure 4.2   Tension loss after chromosome separation can trigger mitotic checkpoint re-activation.

During prometaphase, a mitotic checkpoint signal emanates from kinetochores that have not yet come under tension. This prevents APC activation by Cdc20. The checkpoint silencing upon chromosome bi-orientation on metaphase plate is not irreversible. Artificial cohesin cleavage by TEV protease at this stage triggers checkpoint signaling (marked by the red star), as tension is lost again between sister kinetochores. Although the chromosomes split apart, separase activation and following late mitotic events, including checkpoint inhibition, are prevented. Thus, mitotic checkpoint is still functional and inhibits APC/C even in anaphase.

This sequence of events is prevented during normal anaphase and the checkpoint remains silent. In budding yeast, concomitant with anaphase onset, Cdc14 phosphatase becomes active. Apart from Cdk1 downregulation, we showed that Cdc14 inactivates the mitotic checkpoint by removing the Aurora B complex from centromeres. Cdc14 dephosphorylates Sli15, a component of the chromosome passenger complex (CPC), and this event directs the whole complex to the spindle midzone. Thus, tensionless kinetochores in anaphase lack their tension sensor, which would otherwise trigger checkpoint re-engagement. Rapid removal of Aurora B from centromeres irreversibly inactivates the checkpoint and secures kinetochores attachments of separating chromosomes. Spatially separated from its kinetochore substrates, Aurora B is unable to affect stability of kinetochore-microtubule attachments and initiate checkpoint response. On the other side, localized to the spindle, Aurora B helps to stabilize it and reduce microtubule turnover.

4.2.1 Three states of mitotic checkpoint during cell division

The mitotic checkpoint can be characterised by three different states, which substitute each other as the cell goes through mitosis (**Figure 4.1** and **Figure 4.2**).

1) **Mitotic checkpoint is active and engaged.** During prometaphase, chromosome kinetochores establish their attachments to spindle microtubules. During this stochastic process unattached or erroneously attached kinetochores are produced. Absence of tension in sister kinetochores pair engages the mitotic checkpoint and triggers an error correction mechanism. This is the time when an active (from the beginning of mitosis) spindle checkpoint can be engaged due to the

possibility of mistakes occurring in chromosome orientation. The checkpoint engagement is relatively easy to observe at this stage, as it normally results in a delay of cell cycle progression.

2) **Checkpoint is silent (or satisfied), but still responsive.** Once stable bi-polar attachment of all chromosomes is achieved in metaphase, the mitotic checkpoint is satisfied. At this time checkpoint protein "stripping" from kinetochores is initiated and as a result, MCC (Mitotic Checkpoint Complex) formation is halted. Nevertheless, the checkpoint is still active and remains functional; it continues to monitor the status of kinetochore-microtubule attachments. If a detachment occurs at this stage, a checkpoint response will be renewed and further mitotic progression will be blocked. The Aurora B complex still resides at centromeres, where it can resume its error correction function as soon as tension between kinetochores is lost. In this study we experimentally showed that tension loss as a result of cohesin cleavage at this stage leads to checkpoint re-engagement. In spite of established bi-polar chromosome orientation and checkpoint satisfaction, the mitotic checkpoint is not inactivated. As soon as tension released due to cohesin cleavage, checkpoint can fire again. The checkpoint signalling, we monitored after sister chromatid disjunction, resulted in recurring inhibition of the APC/C and stabilisation of its anaphase targets.

3) **Checkpoint silent and inactive.** After the metaphase-to anaphase transition, the mitotic checkpoint, as well as error-correction pathways are disabled. Thus, loss of tension, which would have led to checkpoint engagement just moments earlier, no longer generates a response when sister centromeres are split in anaphase. The inactivation of these

mechanisms is mainly due to the downregulation of Cdk1 activity and Aurora B removal from centromeres. Budding yeast has a sole regulator of these events, the phosphatase Cdc14. Cells depleted of Cdc14 cannot reach this stage of complete checkpoint inactivation and hence, engage the mitotic checkpoint during anaphase. The point, at which the checkpoint becomes truly inactivated in a normal anaphase, marks a stage of "no return", after which progression of the cell cycle can no longer be prohibited.

To ensure successful chromosome segregation, eukaryotic cells tightly couple mitotic checkpoint inactivation to the disjunction of sister chromatids. This is achieved through the action of APC/C^{Cdc20}, which induces the simultaneous degradation of cyclin B and securin to downregulate Cdk1 and enable separase to cleave cohesin.

4.2.2 The mechanism of checkpoint inactivation is conserved

The Aurora B kinase complex is an integral part of the mitotic checkpoint also in human cells. Its sudden relocation from the inner centromere to the spindle midzone is a hallmark feature of this "chromosomal passenger" complex, promoted by INCENP dephosphorylation. In a collaborative study, Vázquez-Novelle and Petronczki confirmed that relocation of the Aurora B kinase complex is required to prevent untimely checkpoint protein recruitment to human centromeres in anaphase (Vazquez-Novelle and Petronczki, 2010). The Aurora B complex was also monitored at centromeres in Drosophila when checkpoint components were recruited during anaphase triggered by TEV protease cohesin cleavage or in the presence of non-degradable cyclin B (Oliveira et al., 2010; Parry et al., 2003). This suggests that a

conserved mechanism prevents the mitotic checkpoint from re-engaging in anaphase.

However, Petronczki's work also showed that centromere retention of Aurora B during anaphase was insufficient to generate other hallmarks of error correction and mitotic checkpoint engagement (such as destabilisation of kinetochore-microtubule attachments, Mad1 and Mad2 recruitment to kinetochores as part of a checkpoint signal generation, or inhibition of the APC/C). Thus, although Aurora B relocation appears to be an efficient mechanism to inactivate the mitotic checkpoint, additional pathways also contribute to complete inactivation of the checkpoint at anaphase.

4.2.3 Other pathways that keep the checkpoint inactive in anaphase

The departure of Aurora B from centromeres, caused by Sli15/INCENP dephosphorylation, is a fundamental and likely a universally conserved mechanism that inactivates the mitotic checkpoint in anaphase. However, it is not the only one. As mentioned above, retention of Aurora B at centromeres in anaphase does not lead to full checkpoint engagement and does not result in a mitotic exit delay. Elucidating the nature of the additional mechanisms will be an important task for future research.

Several observations suggest that many processes that inactivate the checkpoint at anaphase onset are linked to the downregulation of Cdk1 activity. For instance, Cdk1-dependent phosphorylation of fission yeast Bub1 and vertebrate Cdc20 are required for the functional spindle checkpoint (Chung and Chen, 2003; D'Angiolella et al., 2003; Yamaguchi et al., 2003). However, the dephosphorylation timing of

these proteins during mitotic exit and the phosphatases responsible remain to be identified. In budding yeast, the Cdc14 phosphatase is a good candidate for checkpoint inhibition in multiple ways.

An additional mechanism that contributes to checkpoint inactivation has been suggested by the Murray group. They suggested that Mps1 degradation during anaphase disables the checkpoint response (Palframan et al., 2006). The switch of APC/C activators (Cdc20 to Cdh1) is also likely to abolish the ability of the mitotic checkpoint to inhibit APC/C activity later in anaphase, as APC/C^{Cdh1} is not sensitive to checkpoint action (Vazquez-Novelle M.D., 2010).

Although Sli15/INCENP dephosphorylation inactivates the mitotic checkpoint at the very source of the checkpoint signal, we speculate that additional pathways contribute to the mitotic checkpoint inactivation at anaphase in order to keep it disabled until well into the next cell cycle. This ensures that loss of tension, which causes a robust block to mitotic progression in prometaphase, will not impede mitotic exit and return to G_1 once the signal to the separation of sister chromatids has been given.

4.3 FUTURE PERSPECTIVES

In budding yeast, Cdc14 phosphatase is probably responsible for multiple downstream pathways of checkpoint inhibition. As we have shown here, Cdc14 inactivates checkpoint upon Aurora B removal from centromeres in anaphase. In addition Cdc14 activation, being able to override the checkpoint response to nocodazole treatment, most likely affects other branches of the mitotic checkpoint.

Some checkpoint proteins are modified by phosphorylation during checkpoint engagement. It is very possible that Cdc14-mediated

dephosphorylation of these proteins at anaphase averts the checkpoint function. As a possible example, Mad1 is hyperphosphorylated when the mitotic checkpoint is engaged and serves as a marker of checkpoint signalling. During the cell cycle, Mad1 loses this modification at the metaphase-to-anaphase transition. The function and effect of this phospho-modification has not been elucidated, but it is very likely connected to checkpoint signalling. It would be very interesting to analyze which phosphatase contributes to checkpoint inhibition by removing this modification in metaphase and whether it also functions in anaphase to maintain dephosphorylated state of Mad1. Similarly, many other checkpoint components harbour phospho-modifications at the time of checkpoint signalling and can be potential targets for anaphase phosphatases.

Disassembly of checkpoint inhibitors is another factor, which can be influenced in order to quickly inhibit the checkpoint. This process likely begins already in metaphase, but could be facilitated with the onset of anaphase. It would be interesting to assess if Cdc14 contributes to disassembly of Mad2-Cdc20 and APC/C-MCC complexes.

Future studies will expand our understanding of mechanisms that regulate the checkpoint functioning and guard chromosome separation in every cell division. This will get us insight into the long-standing important biomedical question – the cause of aneuploidy and tumour formation.

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