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How to cite

DE MEDINA-REDONDO, María, MERALDI, Patrick. The spindle assembly checkpoint: clock or domino? In: Results and problems in cell differentiation, 2011, vol. 53, p. 75–91. doi: 10.1007/978-3-642-19065-0_4

This publication URL: https://archive-ouverte.unige.ch/unige:25827
Publication DOI: 10.1007/978-3-642-19065-0_4

Chapter 4
The Spindle Assembly Checkpoint: Clock or Domino?

María de Medina-Redondo and Patrick Meraldi

Abstract In each cell division, the newly duplicated chromosomes must be evenly distributed between the sister cells. Errors in this process during meiosis or mitosis are equally fatal: improper segregation of the chromosome 21 during human meiosis leads to Down syndrome (Conley, Aneuploidy: etiology and mechanisms, pp 35–89, 1985), whereas in somatic cells, aneuploidy has been linked to carcinogenesis, by unbalancing the ratio of oncogenes and tumor suppressors (Holland and Cleveland, Nat Rev Mol Cell Biol 10(7):478–487, 2009; Yuen et al., Curr Opin Cell Biol 17(6):576–582, 2005). Eukaryotic cells have developed a mechanism, known as the spindle assembly checkpoint, to detect erroneous attachment of chromosomes to the mitotic/meiotic spindle and delay the cell cycle to give enough time to resolve these defects. Research in the last 20 years, has demonstrated that the spindle assembly checkpoint is not only a pure checkpoint pathway, but plays a constitutive role in every cell cycle. Here, we review our current knowledge of how the spindle assembly checkpoint is integrated into the cell cycle machinery, and discuss some of the questions that have to be addressed in the future.

4.1 Introduction

Cell cycle progression is organized in such a way, that the initiation of a particular event is dependent on the completion of an earlier event. The cell cycle has been therefore described as being regulated by clocks and dominoes, which constitute the cell-cycle control system (Hartwell and Weinert 1989; Murray and Kirschner 1989). The former ones act as timers, which provide a fixed amount of time for the completion of each event. The latter ones constitute checkpoints (also known as surveillance mechanisms) that sense whether some malfunction prevents the successful completion of the processes, and delay the progression to the next phase.

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J.Z. Kubiak (ed.), *Cell Cycle in Development*, Results and Problems in Cell Differentiation 53, DOI 10.1007/978-3-642-19065-0_4,

Checkpoint systems consist of three types of units: a sensor detecting possible defects and triggering a signal, a signal-transduction cascade, and an effector that delays cell-cycle progression (Hartwell and Weinert 1989). Checkpoint mechanisms tend to sense negative intracellular signals that arrest the cell cycle, rather than counting all the positive signals, since the last arising positive signal in a sea of positive signals is harder to detect than the loss of the last negative signal. Under ideal conditions, prototypical checkpoints are not essential for normal cell-cycle progression.

An interesting example is the spindle assembly checkpoint (also known as spindle checkpoint or kinetochore attachment checkpoint), which ensures the fidelity of chromosome segregation during cell division in eukaryotes. To produce two genetically identical daughter cells, the replicated chromosomes have to be evenly segregated to each daughter cell. Accurate chromosome segregation depends on the proper attachment of chromosomes to spindle microtubules emanating from opposite spindle poles and their positioning in the middle of the spindle. Microtubule attachment is carried out by kinetochores, multiprotein complexes assembled on centromeric DNA that contain up to hundred different proteins (Cheeseman and Desai 2008; Musacchio and Salmon 2007; Santaguida and Musacchio 2009). The spindle assembly checkpoint senses the interactions between chromosomes and microtubules and delays anaphase onset until all chromosomes are attached in a bipolar manner. It is mediated by a set of evolutionarily conserved proteins that functions as a signal transduction system and includes Mad1, Mad2, Mad3/BubR1, Bub1, Bub3, and Mps1 (Hoyt et al. 1991; Li and Murray 1991; Weiss and Winey 1996).

The Bub and Mad proteins were first identified in the budding yeast *Saccharomyces cerevisiae* (Hoyt et al. 1991; Li and Murray 1991). The groups of Andrew Murray and Andrew Hoyt aimed to investigate the feedback control that prevents cells with incompletely assembled spindles from exiting mitosis. As by definition checkpoints are not necessary for cell cycle progression under normal conditions, screens were designed to find nonessential mutations, which override a mitotic arrest resulting from a perturbed spindle. Eight different mutants were described and named *bub* (for "budding uninhibited by benzimidazole") or *mad* (after "mitotic arrest defective"). Genetic analysis showed that they belonged to five different complementation groups that were termed *BUB1*, *BUB3*, *MAD1*, *MAD2*, and *MAD3*. In agreement with the checkpoint definition, budding yeast Bub and Mad genes are not essential (Hoyt et al. 1991; Li and Murray 1991). Here, we review the mechanisms governing the spindle assembly checkpoint, and explore to which extend it is a real "checkpoint". While we briefly discuss the molecular mechanisms controlling the spindle checkpoint (for more extensive reviews on this point we recommend Ciliberto and Shah 2009; Musacchio and Salmon 2007), our emphasis will lie on the general significance of this checkpoint, and the way it is integrated into the cell cycle machinery of embryonic and somatic cells of different model systems.

4.2 Brief Description of the Spindle Assembly Checkpoint

In a classical study of 1995, the group of Conly Rieder showed that the spindle assembly checkpoint is a kinetochore-microtubule attachment checkpoint, as it demonstrated that the presence of a single unattached kinetochore is sufficient to generate a "wait-anaphase" signal (Rieder et al. 1995). To segregate chromosomes sister kinetochores bind spindle microtubules (K-fibers) emanating from the two poles to ensure the proper distribution between the two daughter cells. Since kinetochore-microtubule attachment is a stochastic process, defective attachments can arise. These include attachments of both sister kinetochores to microtubules coming from the same pole (syntelic attachment), attachment of one sister kinetochore to microtubules from both spindles poles (merotelic attachment), lack of attachment to one sister kinetochore (monotelic attachment), and absence of kinetochore-microtubule attachments. With the exception of merotelic attachments, all these erroneous configurations activate the spindle checkpoint, either due to lack of microtubule occupancy at one or both sister kinetochores, or due to lack of tension between improperly attached sister kinetochores. It is still controversial whether lack of tension directly activates the checkpoint, or whether it leads to unattached kinetochores as a consequence of Aurora B activity, which detects and disrupts tension-free kinetochore-microtubule attachments (Nezi and Musacchio 2009). Merotelic attachments, in contrast, are corrected as cells progress through mitosis, including during chromosome segregation in anaphase (Cimini et al. 2001).

At the molecular level unattached kinetochores accumulate the conserved spindle assembly checkpoint proteins Mad1, Mad2, Mad3 (BubR1 in metazoans), Bub1, Bub3, and Mps1 (Chen et al. 1996; Hoffman et al. 2001). Although the proteins that act as landing pads for these proteins at kinetochores have been partially identified, the molecular mechanisms governing the specific accumulation of these proteins at kinetochores are still unclear. The Bub and Mad proteins and Mps1 function as a signaling cascade at kinetochores, with the protein kinases Mps1, Bub1, and BubR1 at the top of the cascade (Kops 2009; Musacchio and Salmon 2007). These proteins recruit through an unknown mechanism Mad1 to kinetochores. Mad1 then itself recruits Mad2, leading to the "activation" of the checkpoint cascade (Chen et al. 1998). Specifically, Mad1 forms a dimer with two Mad2 binding sites that can each bind one Mad2 dimer (De Antoni et al. 2005; Sironi et al. 2002). Interestingly, the two subunits within the Mad2 dimer are not equivalent, as each protein adopts a different conformation. One subunit binds tightly Mad1 in the so-called "closed" conformation, while the other subunit, in the so-called "open" conformation can bind to Cdc20, the target of the spindle checkpoint (De Antoni et al. 2005; Luo et al. 2000). This binding process releases open Mad2 from the Mad1:Mad2 complex and results in the closing of its conformation on Cdc20.

An active spindle assembly checkpoint delays the onset of anaphase by inhibiting the E3-ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C; Clute and Pines 1999). The APC/C controls anaphase onset by targeting Cyclin B

and securin for degradation by the 26S proteasome, resulting in the activation of separase, a protease that cleaves the cohesin complex between sister kinetochores (Peters 2006). The final consequence is the separation of sister chromatids and progression into anaphase. The spindle assembly checkpoint inhibits APC/C by targeting its activator Cdc20; however, the molecular mechanisms by which it inhibits APC/C^{Cdc20} are not fully elucidated. Originally it had been proposed that Mad2 alone acts as the effector of the spindle assembly checkpoint; however, its binding to Cdc20 is not sufficient to block APC/C activity at physiological concentrations (Fang 2002; Fang et al. 1998; Tang et al. 2001). A second model proposes that APC/C is inhibited by the Mitotic Checkpoint Complex (MCC), a complex that consists of Bub3, BubR1, Cdc20, and Mad2 (Fraschini et al. 2001; Sudakin et al. 2001). Biochemical experiments indicate that the inhibitory activity of the MCC is at least 2,000-fold greater that single recombinant Mad2. The model is further based on the idea that the signaling cascade at kinetochores catalyzes the formation of the MCC to inhibit APC/C. Interestingly the MCC is already present in the cytoplasm of G2 cells, before the onset of mitosis, in a state that is compatible with inhibition of the mitotic APC/C (Sudakin et al. 2001). This implies that cells already contain an APC/C-inhibitory activity at the onset of mitosis and that the activity of the spindle assembly checkpoint at kinetochores might be crucial to sustain the formation of MCC. Finally, a third attractive model has emerged recently, which proposes that the MCC is only a transient step in the activation of the spindle assembly checkpoint (Nilsson et al. 2008). In this model Mad2 bound to Cdc20 acts as a catalyst for the binding of Mad3/BubR1 to Cdc20 (Kulukian et al. 2009; Nilsson et al. 2008). Mad3/BubR1 not only acts a pseudosubstrate for Cdc20–APC/C, it can also target Cdc20 itself for ubiquitination by the APC/C, leading to the degradation of Cdc20 (Burton and Solomon 2007; Nilsson et al. 2008). In such a model APC/C activity is restrained by the degradation of its Cdc20 activator. Consistent with such a model, it was found that the mitotic progression of cells expressing a nondegradable Cdc20 could not be blocked by the spindle assembly checkpoint (Nilsson et al. 2008).

The increasing molecular complexity of the checkpoint signaling is also reflected at the level of its integration into cell cycle control. Indeed, experimental evidence in metazoans indicates that the checkpoint is much more tightly integrated into cell cycle progression than originally assumed. During the past 20 years we have learned many new aspects of this integration, but many questions remain unresolved.

4.3 A Checkpoint or Timing Mechanism?

The spindle assembly checkpoint was first described in budding yeast as a pure checkpoint that is nonessential for proper chromosome segregation or cell viability (Hoyt et al. 1991; Li and Murray 1991). However, when studied in other organisms it became rapidly clear that the spindle assembly checkpoint is essential in

mammalian systems. Knock-out of Mad2 in mice is embryonic lethal, and although it is possible to obtain Mad2$^{-/-}$ mouse embryo fibroblasts, this is only possible in cells that have at the same time lost the ability to induce p53-dependent apoptosis (Burds et al. 2005; Dobles et al. 2000). The decisive difference between murine and budding yeast cell division is that *S. cerevisiae* cells establish kinetochore-microtubule attachments immediately after centromere replication and undergo a closed mitosis with intranuclear microtubules (Westermann et al. 2007). In contrast, metazoans undergo an open mitosis, in which microtubules only reach kinetochores after nuclear envelope breakdown in prometaphase. The spindle checkpoint is therefore active in every single metazoan cell division, since at the onset of prometaphase cells will have only unattached kinetochores. In contrast, in budding yeast, it is estimated that the spindle assembly checkpoint is only active in about one division out of thousand, a rare event in case kinetochore attachments go wrong (Warren et al. 2002). This indicates that the spindle assembly checkpoint in metazoan cells is not a pure checkpoint mechanism, but rather an intrinsic part of the cell cycle machinery.

The view that the spindle assembly checkpoint is not just a checkpoint mechanism was further strengthened by the findings that microinjection of anti-Mad2 or anti-BubR1 antibodies in human cells, not only prevented a mitotic arrest in the presence of microtubule poison, such as nocodazole, but also led to a very premature anaphase onset when compared to mock-injected cells (Gorbsky et al. 1998). This indicated that the checkpoint proteins control the timing of cell division (Canman et al. 2002; Gorbsky et al. 1998; Shannon et al. 2002; Taylor and McKeon 1997). A more detailed study based on RNAi depletions of all the human Mad and Bub proteins revealed the existence of two different, separable phenotypes: while all the Mad and Bub proteins are necessary for delaying anaphase in the presence of unattached kinetochores, only Mad2 and BubR1 depletion also resulted in a significant acceleration of mitosis when compared to control-depleted cells (Meraldi et al. 2004). This suggested that Mad2 and BubR1, in addition to their checkpoint function, also act as mitotic "timers", while the other proteins, Bub1, Bub3, and Mad1, have a pure checkpoint role with regard to mitotic timing. Later studies further indicated that the remaining checkpoint kinase Mps1, participated as Mad2 and BubR1 in the timing mechanism (Tighe et al. 2008). Interestingly, the dual functions of Mad2 and BubR1 correlated with different protein pools, localized at kinetochores (checkpoint) or in the cytoplasm (timer), respectively (Meraldi et al. 2004).

Based on these results, a model was proposed in which APC/C activity is hold in check by a series of inhibitors as cells approach anaphase. First, APC/C is inhibited by Emi1 during G2 and prophase (Reimann et al. 2001a, b). In late prophase, Emi1 is phosphorylated by Plk1, leading to its recognition by the ubiquitin ligase SCF and its subsequent proteasome-dependent degradation (Guardavaccaro et al. 2003; Margottin-Goguet et al. 2003). At this stage, the spindle assembly checkpoint is not yet fully active, as kinetochores are still recruiting many of their components, which could lead to a premature activation of APC/C (Sudakin et al. 2001). However, to prevent such a premature activation of APC/C towards Cyclin B and securin a cytosolic pool of Mad2 and Mad3/BubR1 is present that inhibits

APC/C^{Cdc20} and thus functions as a mitotic timer, by inhibiting anaphase onset. As kinetochores rapidly recruit all their components, they will activate the spindle assembly checkpoint machinery in the absence of microtubule attachment, resulting in a sustained APC/C^{Cdc20} inhibition until all kinetochores are attached and the cell is ready to segregate their sister chromatids accurately (Meraldi et al. 2004). An alternative hypothesis is that such a timing mechanisms represent a very basic form to prevent premature anaphase onset, without direct necessary input from the kinetochores. Such a basic timing mechanism might be useful in the very early embryonic cell cycles, which often do not have a spindle assembly checkpoint, or only a very weak checkpoint. Indeed, *Caenorhabditis elegans* embryos have only a very weak spindle checkpoint, that can only delay anaphase onset by a few minutes (Essex et al. 2009; Kitagawa 2009; Kitagawa and Rose 1999), while *Xenopus* egg extracts do not have a functional spindle checkpoint at all, unless one increases massively the nucleus to cytoplasm ratio (Chen et al. 1996; Minshull et al. 1994). Nevertheless, these extracts contain all the spindle assembly checkpoint proteins, suggesting that they fulfill a function at this stage of embryonic development.

It is important to note that another nonspindle assembly checkpoint-related protein has been suggested as a putative mitotic timer: Cyclin A (Geley et al. 2001). Indeed, Cyclin A degradation is independent of the spindle checkpoint and required for anaphase onset (Geley et al. 2001). Cyclin A degradation by the APC/C already starts in prometaphase and is a relative long process, providing cells with enough time to attach all their chromosomes and form proper metaphase plates (den Elzen and Pines 2001; Geley et al. 2001). Thus it seems clear that time is such a crucial parameter for mitosis in many organisms, that cells might have developed different, independent pathways that cooperate to ensure each cell has enough time to attach and align chromosomes before proceeding to anaphase.

The existence of the timing function of Mad2 and BubR1 has been confirmed in other organisms, in particular to the fly *Drosophila melanogaster*. *Mad2*- or *BubR1*-null mutants have an accelerated mitosis, whereas the disruption of other spindle checkpoint genes does not lead to such short cell divisions (Buffin et al. 2007; Rahmani et al. 2009). However, despite a shortened mitosis and a lack of a spindle assembly checkpoint activity, mad2-null mutants in *Drosophila* are viable and fertile. This suggests that in the vast majority of the cells, kinetochores become bipolarly attached to microtubules and aligned on the metaphase plate before Cyclin B and securin are degraded to an extent that allows chromosome segregation and anaphase onset. Therefore, these cells do not need any mitotic timer or checkpoint to prolong metaphase to fulfill all these requirements before exiting mitosis (Buffin et al. 2007). Indeed, the duration of prometaphase (time to bind and align all kinetochores) is only 5 min in wild-type *Drosophila* cells, compared to the average 20 min or more in vertebrate cells, most likely due to the very low numbers of chromosomes ($n = 4$). However, in conditions in which spindle formation is perturbed, such as loss of centrosomes, the spindle assembly checkpoint becomes also essential in *D. melanogaster* (Buffin et al. 2007). This demonstrates that, despite the fact that the molecular mechanisms of the spindle assembly checkpoint are highly conserved, the biological and cell cycle context determines whether the

checkpoint plays an essential role, as in mammals, or a more subsidiary role, as in fly and fungi. Another important question is whether the timing mechanisms associated with Mad2 and BubR1 is specific for metazoans or whether it is also present in fungal systems. This is difficult to evaluate at the current stage, since this timing mechanism becomes only visible in mammalian cells when using nuclear envelope breakdown as a clear morphological starting point $t = 0$ at the onset of mitosis. In the absence of a dramatic cytoskeletal and nuclear reorganization in fungal systems it might be difficult to test for the importance of a timing mechanisms. Finally, it is presently unclear how this timing mechanism is shut off, and whether this follows similar molecular mechanisms as for the inactivation of the spindle assembly checkpoint itself (see Sect. 4.7).

4.4 Is It Only Checking Kinetochore-Microtubule Attachments?

According to their original definition, checkpoints arrest the cell cycle for the cell to repair errors before resuming cell cycle progression. Although the spindle assembly checkpoint clearly fulfills this requirement, it has also become evident that the spindle checkpoint machinery goes beyond that, as its components are also involved in regulation and repair of kinetochore-microtubule attachments. Indeed, all three spindle checkpoint kinases, Mps1, BubR1, and Bub1, regulate and sometimes correct defective kinetochore-microtubule attachments (Kops 2009; Kops et al. 2010; Musacchio and Salmon 2007). Importantly, Bub1 and BubR1 separation of function mutants have demonstrated that this ability to regulate kinetochore-microtubule attachments is independent of their capacity to control spindle checkpoint signaling (Elowe et al. 2010; Klebig et al. 2009; Malureanu et al. 2009; Warren et al. 2002). BubR1 controls the dynamics of kinetochore-bound microtubules, while Bub1 has been proposed to contribute to the transition of lateral kinetochore-microtubule attachments to end-on attachments during chromosome alignment (Gillett et al. 2004; Lampson and Kapoor 2005; Meraldi and Sorger 2005). Finally, Mps1 has been involved in the correction of defective kinetochore-microtubule attachments, possibly by regulating Aurora B at kinetochores (Jelluma et al. 2008; Maure et al. 2007). Such a role is very reminiscent of the function ascribed to the so-called DNA-damage checkpoint (Rouse and Jackson 2002; Zhou and Elledge 2000). DNA-damage checkpoint proteins not only arrest the cell cycle in the presence of DNA damage, such as double-strand breaks, but also act as landing pads for DNA repair proteins at the site of the DNA damage, thus acting in a dual role of checkpoint signaling and repair mechanisms (Martin et al. 1999; Mills et al. 1999). The situation is even more extreme for spindle checkpoint kinases, as the functional data suggest that they not only contribute to the repair of defective attachments but also play a constitutive role in every cell division. This is not only the case in systems with an open mitosis, which always go through a stage with 100% of unattached kinetochores, but also in fungi with closed mitosis that have

microtubules bound to kinetochores during the whole cell cycle: budding yeasts lacking *BUB1* show severe chromosome segregation defects, that occur at a much higher rate than in yeasts lacking *MAD2* (Warren et al. 2002). This indicates that Bub1 is not just repairing defective attachments, but really systematically contributes to the establishment of correct attachments to kinetochores.

The dual role in checkpoint signaling and attachment regulation also raises the question as to where to draw the boundary between "checkpoint" proteins and other components of the kinetochore machinery. Several other components of the kinetochores are essential for the spindle assembly checkpoint, such as the so-called KMN (*K*nl-1-*M*ind-*N*dc80) network, which is required for kinetochore-microtubule attachment and acts as landing pad for most classical spindle checkpoint proteins on kinetochores (Cheeseman et al. 2004, 2006; Kiyomitsu et al. 2007; Martin-Lluesma et al. 2002). Should such proteins be considered as a part of the checkpoint or not? Given their essential role for kinetochore-microtubule attachment in every organism, probably not, but this shows that when analyzing every aspect at the molecular level, it becomes more difficult to define "checkpoint" proteins. The most ambivalent example is the protein kinase Aurora B (Ipl1 in budding yeast or Ark1 in *Schizosaccharomyces pombe*). This protein kinase, which is located at the centromeres between the two sister-kinetochores, plays an essential role in the correction of erroneous kinetochore-microtubule attachment (Liu and Lampson 2009; Nezi and Musacchio 2009). Specifically, it phosphorylates the microtubule-binding KMN network at kinetochores in a proximity-dependent manner if kinetochores are not stretched apart through a bipolar attachment (Liu et al. 2009; Welburn et al. 2010). But it has also been implicated directly in the spindle checkpoint signaling, as it contributes to the loading of Mad2 to kinetochores in human cells and is essential for the spindle checkpoint in *S. pombe* (Johnson et al. 2004; Vanoosthuyse and Hardwick 2009). Given that Ipl1/Ark1 is essential in fungi, it is not considered a checkpoint protein. However, its molecular functions are so close to the other checkpoint kinases, that it becomes very difficult to rationalize this strict separation.

One critical tool to understand these dual functions in the future will be to use separation of function mutants to elucidate the relative contributions to each pathway of these proteins. Moreover, there is a strong need for the identification of substrates of the checkpoint kinases to obtain more precise molecular investigative tools such as phospho-deficient or phospho-mimetic mutants. While multiple Aurora B substrates at kinetochores have been identified, we only know very few targets of Bub1, BubR1, or Mps1 involved in checkpoint signaling or kinetochore-microtubule attachments.

4.5 The Meiotic Spindle Assembly Checkpoint

Errors in chromosome-microtubule attachments are not exclusive to mitosis, but occur also during meiosis. Therefore, it is not surprising that the spindle checkpoint also functions during meiotic divisions (Shonn et al. 2000). Absence of tension at

kinetochores can activate the spindle checkpoint in meiosis (Li and Nicklas 1995) and, similar to mitosis, the checkpoint is required to induce metaphase arrest in the presence of perturbed kinetochore-microtubule attachments. In addition, there is considerable evidence that, as in mitosis, the spindle checkpoint machinery is not only acting as a pure checkpoint, but rather plays a constitutive role that is important for the distinct features of equational division (mitosis, meiosis II) and reductional division (meiosis I). This is particularly evident in *S. cerevisiae*, as the spindle checkpoint machinery is nonessential for mitotic division in this organism, but essential for meiotic divisions (Shonn et al. 2000).

S. cerevisiae Mad2 and Bub1 are required for proper chromosome segregation in meiosis I (coincident with APC^{Cdc20} activity), although their absence does not compromise chromosome distribution in meiosis II (Bernard et al. 2001; Shonn et al. 2000). Bub1 in particular, has multiple roles, as it monitors kinetochore-microtubule attachments, contributes to the unification of sister chromatids, and prevents the removal of the cohesin Rec8 from centromeric regions (Bernard et al. 2001). At the molecular level, Bub1 regulates this latter function by controlling the centromeric localization of Sgo1 through the phosphorylation of Histone H2A (Kawashima et al. 2010). The recruitment of Sgo1 in turn protects Rec8 from degradation during meiosis by safeguarding it from the separase (Kitajima et al. 2004). This Bub1 function relies on its Cdc2-dependent phosphorylation during meiosis I (Yamaguchi et al. 2003). Budding yeast cells lacking Mad2 showed a nondisjunction phenotype of chromosomes in meiosis I, which can be reverted by delaying anaphase I. More generally, the spindle checkpoint also detects lack of tension in meiosis I and delays the degradation of the securin Pds1 and thus prevents meiotic progression (Shonn et al. 2000).

Similar results were also obtained in vertebrate systems, in particular in murine oocytes. Loss of Bub1 leads to a premature resolution of chiasmata, massive chromosome segregation errors due to a failure to biorient bivalent chromosomes and a premature anaphase onset (McGuinness et al. 2009). A similar role is also known for BubR1, since its loss generally disrupts the ability of the spindle assembly checkpoint to inhibit APC/C^{Cdc20} (Homer et al. 2009), but also leads to chromosome alignment defects in meiosis II (Baker 2004). The most surprising finding is that BubR1 depletion in mouse oocytes results in the override of the physiological prophase I arrest, leading to germinal vesicle breakdown, and a subsequent arrest before anaphase I onset. At first sight this is inconsistent with its spindle checkpoint function, according to which its depletion should accelerate anaphase I entry, indicating that BubR1 must have other functions apart from its spindle assembly checkpoint role. Consistently, the presence of BubR1 is necessary to stabilize the APC/C activator Cdh1, which is crucial to prevent premature entry into meiosis I (Homer et al. 2009). BubR1/Mad3 is also essential for prophase I in both yeast and fly meioses, suggesting that this additional meiotic role is conserved (Cheslock et al. 2005; Malmanche et al. 2007). Again, as for the mitotic cycle, it will be important to identify the meiotic targets of Bub1 and BubR1 and to compare them to their mitotic counterparts, to better understand how these kinases adapt to the specific context of meiosis.

4.6 Checkpoint Function at Kinetochores Versus Cytoplasm

The spindle assembly checkpoint is defined as a kinetochore-attachment checkpoint. This is based to the fact that the checkpoint remains active as long as cells have erroneous kinetochore-microtubule attachments, and the fact that disruption of the kinetochore structure, either through laser ablations, genetic means or RNAi depletion, abrogates checkpoint signaling (Meraldi et al. 2004; Rieder et al. 1995; Tavormina and Burke 1998). This had led, for good reasons, to a very kinetochore-centric view of the spindle assembly checkpoint. However, at the molecular level the checkpoint has to also function outside of kinetochores. Indeed, if one single unattached kinetochore results in APC/C^{Cdc20} inhibition, this kinetochore must emit a diffusible signal that can inhibit cellular APC/C^{Cdc20} activity. Elegant experiments with fused cells containing two neighbouring bipolar spindles indicate that the diffusion rate of this signal is limited, as an unattached kinetochore in one spindle could not inhibit anaphase progression in the neighbouring spindle, despite the presence of a common cytoplasm (Rieder et al. 1997). Overall, this leads to the question which part of the checkpoint requires the kinetochore as a catalyst and which part occurs or can occur in other parts of the cells.

The strongest evidence for a nonkinetochore function comes from the set of proteins that act as inhibitors of APC/C^{Cdc20}. These include the MCC, comprising Mad2, BubR1, Bub3, and Cdc20. This complex can already be detected during interphase in human and yeast cells (Sudakin et al. 2001), at a time where functional kinetochores are not yet assembled, indicating that it acts outside of kinetochores. The same is true if BubR1 is the effector that ultimately inhibits APC/C^{Cdc20}, as both in a reconstituted functional *Xenopus laevis* assay, human cell extracts or mouse embryo fibroblasts, BubR1 appears to act as a cytoplasmic APC/C inhibitor (Kulukian et al. 2009; Malureanu et al. 2009; Nilsson et al. 2008). These data are also consistent with functional data in human cells, which are based on the RNAi-mediated depletion of Mad2 and BubR1 or on the depletion of the landing pads of these proteins on kinetochores (Meraldi et al. 2004). While complete depletion of Mad2 or BubR1 abrogates the mitotic timing and checkpoint function of these proteins, disruption of their kinetochore localization only disrupts the checkpoint function, indicating that their timing function must reside outside of kinetochores.

Recent experiments both in fission yeast and human cells suggest that such a behavior is not only limited to the effector proteins, but can also be found for the more upstream kinase Bub1 (Klebig et al. 2009; Windecker et al. 2009). A first study based on a structure-function study of human Bub1 mutants in an Bub1 RNAi background, indicates that Bub1 does not need to be located at the kinetochores to control chromosome congression and spindle assembly checkpoint even though there is a minor impairment of each function (Klebig et al. 2009). The same is true in *S. pombe*, where *bub3* mutants, which fail to localize Bub1 to kinetochores, are still able to mount a Bub1-dependent checkpoint response and to control Shugoshin localization at centrosomes through Bub1 (Windecker et al. 2009). This behavior is not restricted to Bub1, since BubR1 does not need to localize to kinetochores to

control chromosome alignment (Klebig et al. 2009). These data suggest that Bub1 and BubR1 are not mere scaffold proteins at the kinetochores and that their binding to kinetochores only increases the efficiency by which they control kinetochore-microtubule attachment and spindle checkpoint signaling. Although these two kinases are likely to act mostly at kinetochores in wild-type conditions, these results suggest that a more thorough investigation of the nonkinetochore bound pathways of the spindle assembly checkpoint machinery might reveal more surprising results.

4.7 How Is the Spindle Assembly Checkpoint Turned Off?

Once all chromosomes are bipolarly attached to microtubules emanating from both spindle poles through their sister kinetochores, the spindle assembly checkpoint is satisfied and must be inactivated to allow anaphase onset. However, spindle assembly checkpoint inactivation is clearly the least understood aspect of this pathway.

A first question is whether checkpoint inactivation is an active, dominant process, in which a molecular entity is turned on, once the checkpoint is satisfied, or whether it has a more passive nature, i.e. if the checkpoint signal is not maintained, it will fade away. Experiments with fused cells containing two mitotic spindle in a shared cytoplasm, found that spindle assembly checkpoint satisfaction in one spindle could induce anaphase onset in the other spindle, even if it still had unattached kinetochores (Rieder et al. 1997). This suggested that once the spindle checkpoint is satisfied, cells emit a dominant diffusible signal that will suppress the inhibitory signal of the spindle assembly checkpoint. However, the molecular nature of this suppression is not clearly defined.

A first system that was found to stop the checkpoint signal at kinetochores is the dynein/dynactin system. The spindle assembly checkpoint proteins Mad1, Mad2, and BubR1, are removed from kinetochores upon microtubule attachment and transported along microtubules to the spindle pole (Howell et al. 2000, 2001), a transition that is supposed to silence the signal emanating from kinetochores. This spindle checkpoint protein depletion system uses the dynein/dynactin motor protein and specialized dynein binding partners on kinetochores, composed of the RZZ complex (*R*od, *Z*W10 and *Z*wilch) and Spindly (Barisic et al. 2010; Chan et al. 2009; Clute and Pines 1999; Gassmann et al. 2008, 2010; Griffis et al. 2007; Howell et al. 2001; Karess 2005; Yamamoto et al. 2008). However, experimental evidence suggest that these proteins cannot be the only silencing system, as cells lacking Spindly still silence the checkpoint upon chromosome alignment (Gassmann et al. 2010). Studies both in *S. cerevisiae* and *S. pombe* demonstrate that checkpoint inactivation requires the protein phosphatase 1, indicating the need for a counterbalance to the checkpoint kinases, in particular to the Aurora B type of protein kinases (Pinsky et al. 2009; Vanoosthuyse and Hardwick 2009). Another study in *S. cerevisiae* proposed that, while the spindle assembly checkpoint inhibits APC/C^{Cdc20} activity, the checkpoint protein Mps1 is in turn a target of the APC/C, indicating that these activities counteract one each other in a double negative

feedback loop (Palframan et al. 2006). This led to a model in which the checkpoint efficiently inhibits APC/C^{Cdc20} due to the combination of preformed cytoplasmic MCC and unattached kinetochores emitting the "wait anaphase" signal. As kinetochores are bound by microtubules, the negative signal is reduced and APC/C activity increases, leading to the destruction of Mps1 and possibly other checkpoint proteins. Such a mechanism might be particularly important to permanently inactivate the spindle checkpoint once cells have initiated anaphase. A conceptually similar model has been proposed in human cells, with this time Cdc20 as the critical element of checkpoint maintenance and silencing. Jonathan Pines and coworkers found that the Cdc20 protein is constantly synthesized during mitosis, but that at the same it is rapidly degraded in a spindle-checkpoint dependent manner. Importantly, expression of a nondegradable Cdc20 led to an override of the spindle checkpoint machinery, strongly suggesting that Cdc20 concentration is the critical element that controls checkpoint silencing (Nilsson et al. 2008). Finally, the $p31^{comet}$ protein was proposed as a physiological negative regulator of the spindle assembly checkpoint. At the biochemical level, $p31^{comet}$ binds to the closed Mad2 conformation, as it mimics the structure of the open Mad2 conformation, and thus acts as a competitive inhibitor of open-closed Mad2-binding (Mapelli et al. 2006; Xia et al. 2004). However, $p31^{comet}$ depletion led to a weak defect in checkpoint inactivation, raising the question of the importance of this mechanism for spindle checkpoint silencing (Habu et al. 2002; Xia et al. 2004).

As several, confluent pathways have been suggested to cooperate in the spindle checkpoint activation, it is tempting to speculate that several mechanisms are also required for its silencing, providing a tight control over this essential transition. One puzzling fact though is that in contrast to the highly conserved checkpoint activation mechanisms, the silencing pathways are less well conserved: dynein is not present at kinetochores in fungi, no $p31^{comet}$ orthologue could be identified in yeast, and protein phosphatase 1 has not been involved in checkpoint silencing in vertebrates so far. Moreover, even though we have now identified several molecular players in this system, the nature of the diffusible checkpoint inhibitor proposed is unclear at this stage (Rieder et al. 1997).

4.8 Conclusion

The spindle assembly checkpoint has been defined as the crucial mitotic checkpoint mechanism 20 years ago. This has led to a very intense field of research and to the discovery of very elegant regulatory mechanisms controlling this signaling cascade. However, after all this time we still have only a very partial molecular view of the signaling system itself and the ways it is integrated more generally into the cell cycle machinery. From our subjective viewpoint we believe that for a better understanding of the checkpoint we will need to unravel in the future two critical crossroads between the checkpoint and the cell cycle: First we need to understand how the checkpoint kinases Mps1, Aurora B, Bub1, and BubR1 translate a mechanical

signal of unattached kinetochores into an active Mad2/BubR1 inhibition signal, a question that is very diffuse at this stage. Second, we will need to better define the inhibitory mechanisms that control checkpoint silencing, including the putative feedback loops emanating from the cell cycle that guarantee that such a step is irreversible once cells have initiated anaphase.

Acknowledgments We would like to thank all the Meraldi laboratory members for the helpful discussions. MDMR is supported by an Oncosuisse grant, PM is supported by a Förderungsprofessorship or the Swiss National Research Fund, a EURYI-award by the European Research Council and the ETH Zurich.

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