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Cdk1 kinase counteracts PP2A^{Cdc55} phosphatase to induce APC/C phosphorylation and adaptation to the Spindle Assembly Checkpoint (SAC) in budding yeast

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List of abbreviations: SAC, Spindle Assembly Checkpoint; PFL, positive feedback loop; Cdk1, Cyclin dependent kinase 1; APC/C, Anaphase Promoting Complex or Cyclosome; MEN, mitotic exit network; FEAR, Cdc14 early release.

Abstract

The spindle assembly checkpoint (SAC) monitors that all sister chromatids are correctly attached to microtubules of the mitotic spindle during prometaphase. The correct attachment is known as biorientation, and it is the prerequisite for proper partitioning of the duplicated DNA from the mother to the two daughter cells. Until the last kinetochores are bioriented, the SAC arrests progression into anaphase by inhibiting the Anaphase Promoting Complex or Cyclosome (APC/C) bound to its coactivator Cdc20, therefore stabilizing the cohesin rings that hold pairs of sister chromatids together.

When the SAC is continuously activated, cells remain arrested in prometaphase for some hours, but not indefinitely; even if the checkpoint is not satisfied, eventually cells separate the duplicated DNA material and progress into anaphase. This phenomenon is known as adaptation to the SAC and is poorly understood from the molecular viewpoint.

By using budding yeast (*S. cerevisiae)* as a model organism, in this work I show that adaptation to the SAC requires phosphorylation of the APC/C, which is stimulated by the Cyclin dependent kinase 1 (Cdk1) bound to its mitotic regulatory subunit, Clb2, and is opposed by the phosphatase $PP2A^{Cdc55}$.

I propose that $PP2A^{Cdc55}$ and the APC/C are implicated in a double negative feedback loop of reciprocal inhibition, which regulates transition into anaphase in adapting cells: when accumulating Clb2 provides sufficient Cdk1:Clb2 activity to allow initial activation of APC/C^{Cdc20}, the antagonist of the APC/C, $PP2A^{Cdc55}$, starts to be inhibited. This strongly reinforces APC/C^{Cdc20}, and leads to a rapid and irreversible transition into anaphase.

Introduction

The cell cycle is the ordered sequence of events by which a cell grows and then divides into two daughter cells containing the same genetic information as the mother cell.

Basics of the cell cycle

In eukaryotic cells, the cell cycle is divided into two phases: interphase and mitosis. During interphase cells grow, duplicate their organelles (e.g., ribosomes, mitochondria, endoplasmic reticulum) and, more importantly, their DNA material. In mitosis the duplicated DNA is segregated into two equal parts, which are then inherited by the daughter cells.

Interphase can be further divided into three phases:

1) Gap 1 (or G1) phase: in this phase cells monitor their size and the external environment (e.g., nutrients, temperature, presence of other similar or different cells) and decide if conditions are appropriate to initiate DNA replication. If not, they can enter a resting state, called G0, characterized by low metabolic activity. If instead conditions are favorable, G1 or G0 cells transit into S phase. Entry into S-phase is an important decision that cells have to face: once they bypass this point, also known as Start (in yeast) or restriction point (in animals), they cannot revert to the previous state and are obliged to complete the cell cycle;
2) S phase: in this phase cells duplicate their DNA, with each chromosome producing an identical copy of itself; at the end of S-phase, these identical chromosomes remain physically associated to one another to form couples of sister chromatids. During DNA replication a surveillance mechanism, known

as the S-phase checkpoint, monitors that the genetic material is duplicated properly. If errors occur during this process, the checkpoint arrests cell cycle progression and activates mechanisms that allow correction of these errors;

3) Gap 2 (or G2) phase: in this phase cells monitor that duplication of organelles and DNA occurred without mistakes and that they are ready to divide their DNA material and other subcellular components into the two daughter cells.

In the next section, I will address mitosis, the phase of the cell cycle most relevant for my studies.

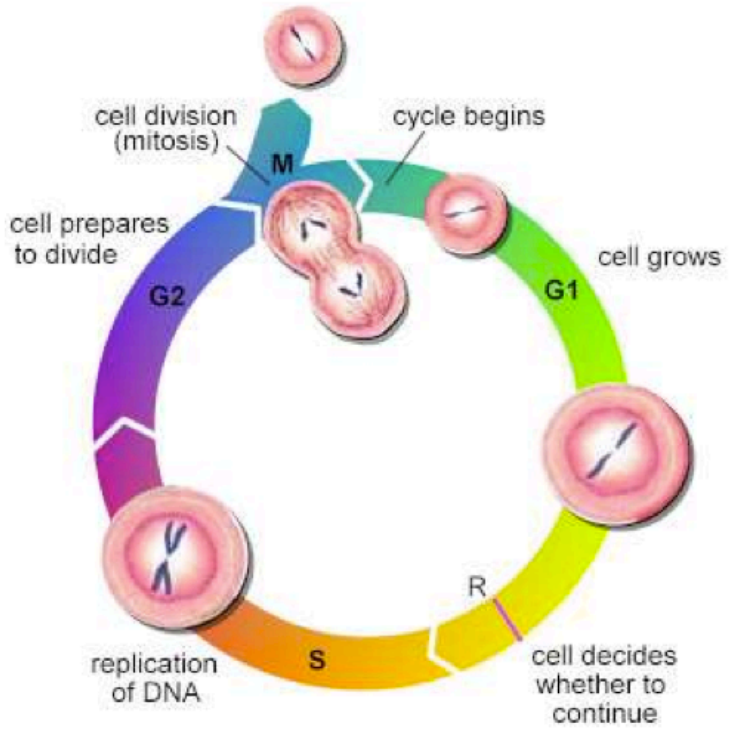

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Figure 1. Cell cycle phases. The eukaryotic cell cycle is divided in four phases: G1 phase, when cells check that internal/external conditions are appropriate for DNA replication; S phase, in which replication of the genetic material occurs; G2 phase, when cells control that DNA and other subcellular components have been properly duplicated; M phase or mitosis, in which duplicated DNA material and cytoplasmic components are finally partitioned between the two newborn daughter cells.

1. Mitosis

Mitosis is the phase of the eukaryotic cell cycle where the DNA material, which has been duplicated during S phase, is equally segregated into the two daughter cells. After DNA replication, chromosomes are organized as pairs of sister chromatids, each of which consisting of two identical chromosomes originated from a single one. The two sister chromatids are held together by cohesin, a hetero-tetrameric complex composed of the four subunits Scc1, Scc3, Smc1 and Smc3 (Nasmyth and Haering, 2009). Interaction of these four subunits results in the formation of a closed ring structure that entangles the two sister chromatids in a single macromolecule. Such a ring needs to be opened in mitosis to allow the separation of sister chromatids.

Mitosis can be schematically divided into different phases, each of which attains a specific goal and is characterized by a specific balance of key molecular regulators (see paragraphs 1.1, 1.2, 2). During *prophase*, chromosomes, which in interphase are present in the form of dispersed DNA, condense in compact, topologically supercoiled structures, which have the advantage of being more manageable for the physical division of the two chromatids. At this point, structural changes occurring in the organization of microtubules, long fibers that originate from two microtubule-organizing centers (MTOCs), lead to the formation of the mitotic spindle: a dynamical, symmetric structure apt to capture each pair of sister chromatids (*prometaphase*). Once all sister chromatids pairs have been bound by microtubules emanating from the opposite poles of the cell, they align on the same plane (the so-called *metaphase* plate). The stable positioning of chromosomes on the metaphase plate is due to a balance of forces that pull them towards the opposite poles of the cell. This situation is known as biorientation, and characterizes a physiological metaphase. When all chromosomes are bioriented, cleavage of the cohesin subunit Scc1 by the protease separase allows physical separation of the sister chromatids of each chromosome.

Separation of sister chromatids is completed during *anaphase*, when the mitotic spindle elongates and pulls separated sister chromatids apart. After each daughter cell has inherited the same chromosome set, mitosis terminates with the decondensation of divided chromosomes and the physical separation of the cytoplasmic components in the daughter cells (*telophase* and *cytokinesis*, respectively).

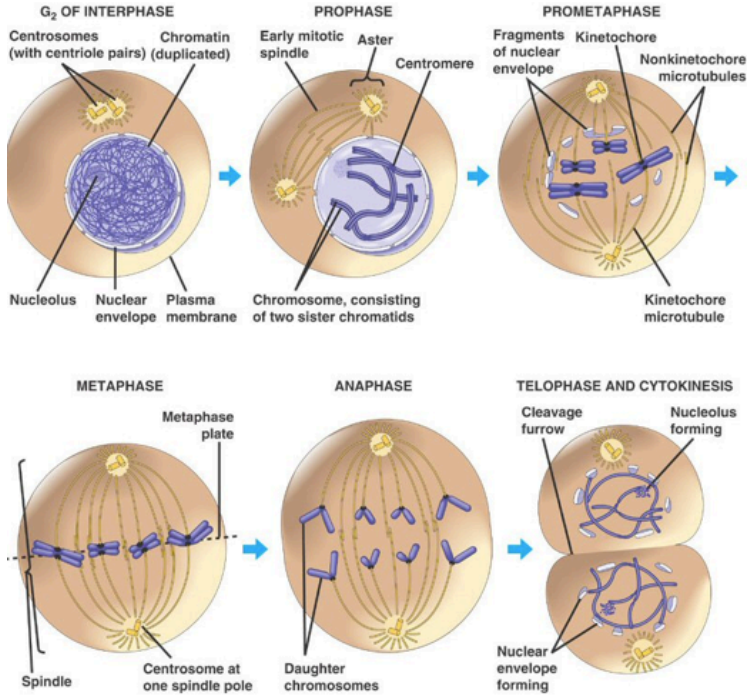

Figure 2. The different phases of mitosis. After G2 phase, *prophase* initiates mitosis with condensation of chromosomes and separation of microtubules organizing centers or MTOCs (centrosomes in vertebrates and spindle pole bodies in budding yeast). In *prometaphase*, the highly dynamic mitotic spindle tries to capture each condensed pair of sister chromatids. In *metaphase* all sister chromatids are finally bioriented on the metaphase plate, and equidistant from the two centrosomes. *Anaphase* consists in the physical separation of metaphase plate-aligned sister chromatids, which are pulled apart to the opposite poles of the cells. In *telophase* also cytoplasmic components are partitioned between the two nascent daughter cells.

1.1 The role of Cdk1 in mitosis establishment and progression

The master regulator of the whole mitotic process is the Cyclin-dependent kinase 1 (Cdk1) bound to its regulatory subunits, called mitotic cyclins (Clbs in budding yeast) (Sullivan and Morgan, 2007). By phosphorylating key substrates (e.g., condensins and microtubules-associated proteins), Cdk1:Clbs initiate mitosis by driving its major events: chromosomes condensation, nuclear envelope breakdown (only in vertebrates), mitotic spindle formation, spindle elongation and separation of DNA masses. The same substrates need to be then dephosphorylated to allow exit from mitosis.

Proteins known as Cyclins are essential for Cdk1 kinase activity and they also determine its substrate specificity. The four mitotic cyclins in budding yeast, Clb1, Clb2, Clb3 and Clb4, have partially overlapping substrate specificity, with a certain level of redundancy among them (Rahal and Amon, 2008). Clb2 is responsible for most (about 70%) of total mitotic activity of Cdk1 and for this reason it is considered the main mitotic cyclin (Rahal and Amon, 2008). In this work, I will refer to Cdk1:Clb2 to collectively indicate all the mitotic Cdk1:Clbs complexes.

Given that Cdk1 activity is mainly regulated through its binding to cyclins, regulation of cyclins levels is crucial for Cdk1 activity. Mitotic cyclins are synthetized throughout G2 phase and mitosis, whereas their degradation is inhibited from G2 to metaphase, when they reach their peak levels. The transition from metaphase to anaphase corresponds to the initiation of Clb2 degradation, which is then completed by the end of mitosis. Accordingly, Cdk1:Clbs enzymatic activity peaks at metaphase and progressively decreases during anaphase, until it becomes minimal just before mitotic exit (ME).

Inhibition of Cdk1 activity by cyclin degradation is not sufficient for ME, which also requires the main substrates of Cdk1 to be dephosphorylated. Specific phosphatases

are responsible for dephosphorylating Cdk1 substrates and for definitively inhibiting Cdk1 at the end of mitosis (see Introduction, Paragraph 2). In budding yeast, the main antagonist of Cdk1:Clb2 is the Cdc14 phosphatase (Stegmeier and Amon, 2004); Introduction, Paragraph 2) . Among the substrates that Cdc14 dephosphorylates are Cdh1 and Sic1. Dephosphorylation of Cdh1 induces its activation, which results in degradation of Clb2 and inactivation of Cdk1 in late anaphase (Stegmeier et al., 2004). Dephosphorylation of Sic1 instead increases its stability and leads to accumulation of the same protein, which acts as a competitive inhibitor of Cdk1: sequestration of Cdk1 by Sic1 is another mechanism that contributes to complete Cdk1 inactivation at the end of mitosis (Stegmeier et al., 2004).

The activation of Cdk1-counteracting phosphatases is insufficient *per se* to trigger exit from mitosis, unless it is associated with Clb2 degradation and Cdk1 inactivation; this is confirmed by the fact that ectopically inhibiting Clb2 degradation stabilizes Clb2 and strongly delays or even inhibits exit from mitosis (Drapkin et al., 2009).

1.2 The metaphase-to-anaphase transition

During G2 phase and early mitosis, centromeric cohesin cannot be cleaved because the protein securin (Pds1 in budding yeast) sequesters separase and inhibits its protease activity against cohesin (Cohen-Fix and Koshland, 1999). Securin/Pds1 is therefore the main inhibitor of anaphase in all eukaryotic cells. As a consequence, ectopic expression of a non-degradable form of Pds1 prolongs metaphase arrest in yeast cells (Cohen-Fix and Koshland, 1999; Stemmann et al., 2001).

At the metaphase-to-anaphase transition, the E3 ubiquitin ligase known as the Anaphase Promoting Complex/Cyclosome (APC/C), bound to its coactivator Cdc20, ubiquitinates securin/Pds1, leading to its degradation via the proteasome 26S. When securin/Pds1 is degraded, separase targets cohesin, whose closed ring is cleaved and separation of sister chromatids occurs. APC/C^{Cdc20} activation is therefore the crucial

event that starts anaphase and this explains why eukaryotic cells lacking Cdc20 are permanently arrested in metaphase, where they ultimately die (Huang et al., 2009; Lim et al., 1998; Malureanu et al., 2010; Shirayama et al., 1999). In turn, Cdk1:Clb2 directly promotes anaphase onset by activating APC/C^{Cdc20} against Pds1 (see Introduction, Paragraph 1.2).

Once activated, APC/C^{Cdc20} is also responsible for the initial phase of Clb2 degradation, which occurs in early anaphase, concomitant with securin/Pds1 degradation. In late anaphase and in telophase, Cdc20 is replaced by the other APC/C coactivator, Cdh1, in driving Clb2 ubiquitination by the APC/C. In this way, APC/C^{Cdh1} is responsible for the complete Clb2 degradation and Cdk1 inactivation that is essential for the exit from mitosis (Yeong et al., 2000) .

Contrarily to Cdc20, Cdh1 is not essential for cell viability and mitotic exit: the contribution to Clb2 degradation given by APC/C^{Cdc20} is strong enough to allow sufficient inhibition of Cdk1 activity that is compatible with mitotic exit. The main role of APC/C^{Cdh1}-induced degradation of Clb2 is to avoid that cells exit mitosis with residual levels of Cdk1:Clb2 activity, which could result in DNA replication defects in the next cell cycle (Wasch and Cross, 2002).

1.3 Structure and regulation of the Anaphase Promoting Complex or Cyclosome (APC/C)

Ubiquitination is a post-translational modification that targets proteins for degradation by the proteasome. Long chains of pre-activated ubiquitin monomers are attached by ubiquitin-binding enzymes (known as ubiquitin ligases) to lysines of substrates to be degraded by the proteasome.

1.3.1 Structure of the APC/C

The APC/C is the E3 ubiquitin ligase that plays an essential role in the metaphase-to-anaphase transition and in mitotic exit. It is composed by 13 subunits that assemble

to form three functionally distinct subdomains: a catalytic core, a structural platform and a sandwich of subunits containing multiple repeats of the 34 amino acid tetratricopeptide residue (TPR) motif (Primorac and Musacchio, 2013).

The catalytic core of the APC/C consists of Apc11 and Apc2, which catalyze ubiquitination of substrates, such as securin/Pds1 and mitotic cyclins. The structural platform is made of Apc1, Apc4 and Apc5.

The TPR subunits Cdc27/Apc3, Cdc16/Apc6 and Cdc23/Apc8, which are conserved among all eukaryotes, are assembled in the so-called "arc lamp" structure of the APC/C (Schreiber et al., 2011). They are responsible for the binding of Cdc20 and Cdh1 to the APC/C and, consequently, for APC/C activation against specific substrates (Matyskiela and Morgan, 2009; Vodermaier et al., 2003). Binding of Cdc20 and Cdh1 to the APC/C is mediated by specific aminoacidic sequences; in particular a motif called the C-box, and a conserved isoleucine-arginine (IR) dipeptide at the C-terminus are considered essential for the binding and for the ubiquitin-ligase activity of the APC/C (Thornton et al., 2006; Vodermaier et al., 2003).

Cdc20 and Cdh1 are essential to present substrates to the APC/C and they also contribute to enhance APC/C catalytic ubiquitin-ligase activity against the same substrates (Kimata et al., 2008; Schwab et al., 2001). Cdc20 and Cdh1 recognize specific aminoacidic consensus sequences in the substrates of APC/C, which are important for their interaction with the E3 ligase: among them, the destruction box (D-box) and the KEN-box are the most common and characterized. The former consists of the sequence RXXLXXXXN (where R is arginine, X is any aminoacid, L is leucine and N is asparagine), the latter of the sequence KENXXXN (where K is lysine and E is glutamic acid). APC/C^{Cdc20} mainly recognizes the D-box, while APC/C^{Cdh1} preferentially binds and ubiquitinates KEN-box-containing substrates (Pfleger and Kirschner, 2000).

1.3.2 APC/C^{Cdc20} regulation

In yeast cells, Cdk1:Clb2 takes part in APC/C^{Cdc20} activation during prometaphase and metaphase through at least two mechanisms. Cdk1:Clb2 induces *CDC20* transcription by inhibiting the transcriptional repressor of *CDC20*, Yox1 (Liang et al., 2012; Primorac and Musacchio, 2013). Moreover, Cdk1 phosphorylates Cdc16, Cdc27 and Cdc23 on multiple serines and threonines; this phosphorylation increases Cdc20 binding affinity for the APC/C and thus ultimately APC/C activity (Rudner et al., 2000; Rudner and Murray, 2000).

Cdk1-induced transcription of *CDC20* is thought to be essential for the metaphase-to-anaphase transition: indeed, when Cdk1 is inhibited, mRNA and protein levels of Cdc20 rapidly drop and cells remain arrested in metaphase with stable securin/Pds1 (Liang et al., 2012; Vernieri et al., 2013). On the contrary, phosphorylation of TRP subunits of the APC/C is not essential for cell viability: mutant cells carrying substitutions of serines and threonines into alanines in Cdk1 consensus sequences of Cdc16, Cdc27 and Cdc23 (*cdc16-6A, cdc27-5A cdc23-A*) only show mild delay (about 30 minutes) in cell cycle progression (Rudner and Murray, 2000).

As opposed to Cdc20, Cdh1 is negatively regulated by Cdk1 activity: Cdk1:Clb2 phosphorylates Cdh1 and inhibits its binding to the APC/C (Kramer et al., 2000; Zachariae et al., 1998). Only when APC/C^{Cdc20}-mediated degradation of Clb2 partially inactivates Cdk1 and the phosphatases involved in mitotic exit start to remove phosphate groups from Cdh1, APC/C^{Cdh1} is fully activated against Clb2; this event leads to the complete degradation of Clb2 and, consequently, to full inactivation of Cdk1.

In summary, after initiating mitosis, Cdk1:Clb2 contributes to progression from metaphase to anaphase by promoting securin/Pds1 degradation by APC/C^{Cdc20} (Rahal and Amon, 2008). Moreover, by activating APC/C^{Cdc20} against Clb2, Cdk1:Clb2 also

starts its own inactivation that is essential for mitotic exit; this process is further enhanced by APC/C^{Cdh1}-mediated degradation of Clb2 (Wasch and Cross, 2002; Yeong et al., 2000).

1.4 Cell cycle regulation of Cdc20 levels

Given the relevance of Cdc20 for the metaphase-to-anaphase transition, the main subject of the work presented here, it is worth discussing its regulation in some detail.

Cdc20 is continuously synthesized during S-phase and mitosis. As anticipated, its synthesis in yeast cells at least partially depends on Cdk1-dependent inhibition of Cdc20 transcriptional inhibitor, Yox1 (Liang et al., 2012). At the end of mitosis and during G1 phase, when Cdk1 activity drops to its minimum due to cyclin degradation, Cdc20 synthesis is practically absent, and also protein levels become negligible (Pan and Chen, 2004; Robbins and Cross, 2010a). In S-phase and mitosis, concomitantly with higher synthesis, Cdc20 is also highly turned-over: indeed, inhibition of protein synthesis through cycloheximide in these phases leads to very fast degradation of Cdc20, with an half-life of less than 10 minutes (Foe et al., 2011). These high rates of protein synthesis and degradation allow maintenance of constant steady state levels of Cdc20 in mitosis.

Foe et al. showed that Cdc20 degradation in cycling cells completely relies on APC/C activity, because APC/C inhibition stabilizes its protein levels when synthesis is blocked (Foe et al., 2011). They showed that Cdc20 ubiquitination by the APC/C occurs through an *in cis* mechanism: the same Cdc20 molecule that binds APC/C as a coactivator is ubiquitinated by APC/C and primed for degradation. Both the C-box and IR tail domains of Cdc20, which are important for binding to and activating the APC/C against securin/Pds1 and Clb2 (see Paragraph 1.2), are also important for APC/C-mediated ubiquitination of Cdc20.

At the end of mitosis, when APC/C^{Cdh1} activity increases**,** APC/C^{Cdh1} is mainly responsible for Cdc20 ubiquitination (and degradation) through an *in trans* mechanism: Cdh1 binds APC/C as a coactivator and recognizes Cdc20 as a substrate (Foe et al., 2011; Huang et al., 2001). In line with this, Cdc20 D-boxes, which are specifically recognized by APC/C^{Cdh1} as substrate sequences, are essential for Cdc20 ubiquitination by APC/C^{Cdh1}(Robbins and Cross, 2010a). Finally, during G1 phase, when Cdc20 synthesis is missing due to Cdk1 inactivation, APC/C^{Cdh1} completely degrades Cdc20 (Huang et al., 2001).

2. Cdc14 phosphatase controls exit from mitosis in budding yeast

As previously said, the essential phosphatase Cdc14 reverts the major mitotic events induced by Cdk1:Clb2 and induces mitotic exit. To do so, Cdc14 dephosphorylates key substrates of Cdk1 (e.g., Cdh1 and Sic1) both in the nucleus and in the cytoplasm.

Regulation of Cdc14 activity essentially occurs via localization. During interphase and early mitosis (until metaphase), Cdc14 is sequestered (and kept inactive) in the nucleolus by its inhibitor, the nucleolar protein Net1 (Shou et al., 1999; Visintin et al., 1999). During anaphase, the phosphorylation of Net1 catalyzed by several kinases (including Polo Kinase or Cdc5, Cdk1 and Dbf2-Mob1) induces progressive release of Cdc14 from the nucleolus and activation of its phosphatase activity against its substrates (Visintin et al., 1998).

2.1 The Cdc14 Early Anaphase Release (FEAR) network

Cdc14 activation occurs via two temporally and molecularly distinct mechanisms, corresponding to two waves of release from the nucleolus. The first wave of Cdc14 release occurs at the onset of anaphase, when several proteins (Cdk1:Clb2, Cdc5,

Slk19, separase), belonging to the so-called FEAR (CdcFourteen Early Anapahse Release) network, directly or indirectly promote partial phosphorylation of Net1 and release of the phosphatase in the nucleus, but not in the cytoplasm (Stegmeier et al., 2002; Visintin et al., 2003). In particular, Net1 phosphorylation status at the anaphase onset is regulated by a balance between kinase and phosphatase activities: FEAR kinases, such as Cdk1 and Cdc5, phosphorylate Net1 and stimulate Cdc14 release (Azzam et al., 2004; Visintin et al., 2003); phosphatases, such as $PP2A^{Cdc55}$ (see Introduction, Paragraph 3), remove these phosphate groups and inhibit Cdc14 release (Queralt et al., 2006) (see Figure 3). As long as $PP2A^{Cdc55}$ is active, mitotic kinases cannot phosphorylate Net1. Crucially, at the onset of anaphase, separase activation not only induces the separation of sister chromatids, but also inhibits $PP2A^{Cdc55}$ by binding and sequestrating the phosphatase; this leads to significant increase in Net1 phosphorylation and to Cdc14 release in the nucleus (Queralt et al., 2006). For these reasons, even if not directly involved in Net1 phosphorylation, separase is considered an important component of the FEAR network (see Figure 3).

The main function of FEAR-induced Cdc14 release is the proper condensation of ribosomal DNA (rDNA) and segregation of late-segregating chromosomal regions such as telomeres (Sullivan et al., 2004). Nevertheless, this first wave of Cdc14 release is not essential to complete the cell cycle and exit from mitosis.

2.2 The Mitotic Exit Network (MEN)

Complete release of Cdc14 in the nucleus and in the cytoplasm occurs in late anaphase, and is mediated by the Mitotic Exit Network (MEN). The MEN consists of a Ras-like signaling cascade, whose crucial, upstream component is the GTPase Tem1.

Active Tem1 induces activation of Cdc15 kinase, which in turn phosphorylates and activates the final effector of the cascade: Dbf2-Mob1 kinase. Dbf2-Mob1 promotes massive phosphorylation of Net1 in late anaphase, leading to the complete release of

Cdc14 from the nucleolus and to full activation of the phosphatase (Bardin et al., 2003; Lee et al., 2001; Visintin and Amon, 2001) (see Figure 3).

Regulation of the Tem1 GTPase activity is therefore crucial for Cdc14 release. Like other GTPases, Tem1 is negatively regulated by GAPs (GTPase activating proteins), and activated by GEFs (guanine-nucleotide exchange factors). In the MEN cascade the GAPs are Bub2 and Bfa1, the GEF is Lte1 (Bardin et al., 2000). To allow mitotic exit only if proper segregation of DNA masses into the daughter cells has occurred, Tem1 activation is linked to the entry of DNA into the bud. Tem1 and its inhibitors Bub2-Bfa1 are localized at the daughter-directed spindle pole body (dSPB). On the contrary, Tem1 activator, Lte1, localizes at the bud cortex (the inner plasma membrane of the daughter cell). Until Tem1 reaches Lte1 in the daughter cell, it is hostage of Bub2/Bfa1 at the dSPB. Only when the dSPB (and the DNA that it pulls into the bud) moves into the future daughter cell and comes in contact with Lte1 localized at the bud-cortex, Bub2 and Bfa1 are inhibited, and Tem1 activated by Lte1 (Bardin et al., 2000; Geymonat et al., 2009). At this point, the MEN cascade can induce massive release of Cdc14 from the nucleolus and dephosphorylation of Cdk1 substrates takes place. Once activated, the MEN cascade reinforces its own activation through a positive-feedback-loop between Cdc15 and Cdc14: Cdc15 induces Cdc14 release and activation via Dbf2-Mob1, and Cdc14-mediated dephosphorylation of Cdc15 increases its kinase activity towards the downstream effector of the MEN, Dbf2-Mob1 (Jaspersen and Morgan, 2000).

While FEAR-induced Cdc14 activation is neither essential nor sufficient for mitotic exit, MEN-induced massive release of the phosphatase is both necessary and sufficient. Accordingly, inactivation of MEN components such as Cdc15, induces permanent arrest of the cell cycle in anaphase just as Cdc14 inactivation does, due to

the inability of cells to disassemble the mitotic spindle and to inactivate Cdk1:Clb2 (Rock and Amon, 2009).

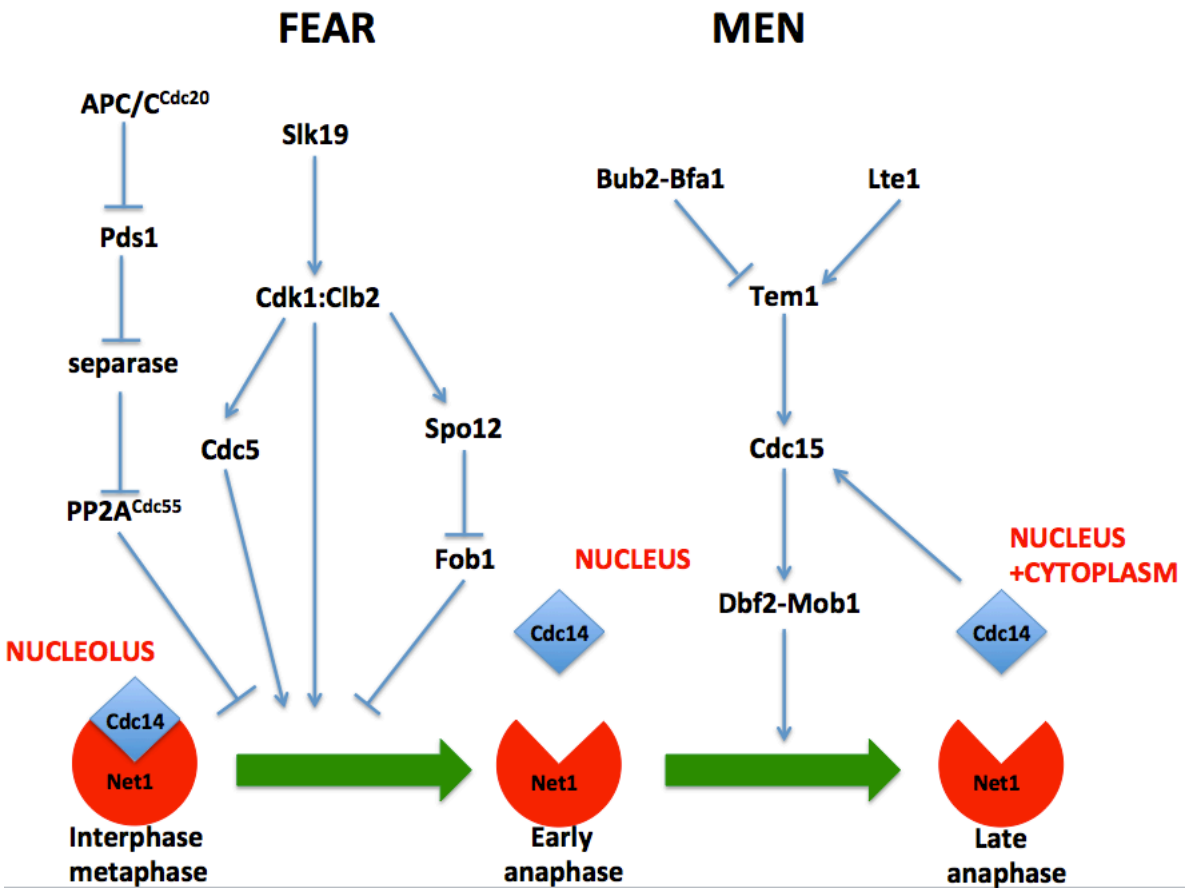

Figure 3. Cdc14 regulation in mitosis. During interphase and metaphase, unphosphorylated Net1 binds and sequesters Cdc14 in the nucleolus. At the metaphase-to-anaphase transition, peak-levels of Cdk1:Clb2 and Cdc5 activities, together with inhibition of $PP2A^{Cdc55}$ phosphatase by separase, result in partial phosphorylation of Net1 and, consequently, in partial, nuclear release of Cdc14. In late anaphase, entry of duplicated DNA into the daughter cell induces inactivation of Bub2-Bfa1 and activation of Lte1. This activates Tem1 and the MEN cascade whose final effector is Dbf2-Mob1 kinase. The consequent, massive phosphorylation of Net1 induces complete release of Cdc14 in the nucleus and in the cytoplasm, where the phosphatase promotes dephosphorylation of key substrates and initiates exit from mitosis. MEN activation is robustly enhanced by a positive feedback loop: released Cdc14 dephosphorylates and activates Cdc15, which reinforces Net1 phosphorylation and Cdc14 release.

3. PP2A phosphatase

PP2A phosphatase is composed of three subunits: a scaffold subunit A, a catalytic subunit C and two main regulatory subunits, Cdc55 and Rts1 (respectively B and B' subunits in mammalian cells). The scaffold subunit has an important structural role and associates with the catalytic subunit to form the core enzyme. The catalytic subunit in yeast consists of either Pph1 or Pph2, which are almost identical and have interchangeable functions: cells deleted for one both these subunits are viable and healthy, while double deleted are slow growing, thus sick (Jiang, 2006). The regulatory subunits of PP2A determine its substrate specificity. $PP2A^{Cdc55}$ plays many roles in mitosis, favoring mitotic entry (Minshull et al., 1996) and inhibiting both the metaphase-to-anaphase transition and mitotic exit (Clift et al., 2009; Rossio and Yoshida, 2011; Vernieri et al., 2013; Wang and Ng, 2006a; Wang and Ng, 2006b). $PP2A^{Rts1}$ is mainly active during meiosis, when it protects cohesin and avoids premature separation of sister chromatids (Bizzari and Marston, 2011; Yu and Koshland, 2007).

3.1 $PP2A^{Cdc55}$ inhibits the metaphase-to-anaphase transition

Several lines of evidence suggest that $PP2A^{Cdc55}$ is an inhibitor of the metaphase-to-anaphase transition in budding yeast. First of all, deletion of *CDC55* partially rescues the temperature sensitivity of a hypoactive Cdc20 mutant protein, cdc20-1 (Sethi et al., 1991; Wang and Burke, 1997). This suggests that $PP2A^{Cdc55}$ acts as an inhibitor of APC/C^{Cdc20} activity and, consequently, that *cdc55Δ* cells activate APC/C^{Cdc20} with enhanced ability (Jiang, 2006). Consistent with this observation, Cdc55 overexpression from the *GAL1* promoter arrests cells in metaphase (Chiroli et al., 2007). It has also been reported that overexpression of shugoshin (Sgo1 in yeast) induces a metaphase arrest that is dependent on $PP2A^{Cdc55}$ (Clift et al., 2009).

According to Clift and collaborators, Sgo1 activates PP2A^{Cdc55}, which inhibits separase and protects cohesion cleavage.

The substrates that PP2A^{Cdc55} dephosphorylates to delay transition into anaphase are not known. Also, it is as yet unclear if PP2A^{Cdc55} inhibits transition into anaphase in an unperturbed mitosis, or only in specific conditions, such as spindle checkpoint assembly activation (see paragraph 4.7).

3.2 PP2A^{Cdc55} inhibits exit from mitosis

PP2A^{Cdc55} restrains Cdc14 release from the nucleolus, therefore inhibiting mitotic exit. Until metaphase, PP2A^{Cdc55} dephosphorylates Net1 and inhibits Cdc14 release; only when activated at the metaphase-to-anaphase transition, the protease separase inhibits PP2A^{Cdc55} in a protease-independent manner (likely by simple binding) and allows phosphorylation of Net1 by FEAR and MEN kinases. Full phosphorylation of Net1 induces full release of Cdc14, which is preparatory for reverting the main phosphorylation events induced by Cdk1 and other mitotic kinases (Queralt et al., 2006).

PP2A^{Cdc55}'s role in mitotic exit is also regulated via localization: in interphase the phosphatase is localized both in the nucleus and in the cytoplasm, where it promotes entry into mitosis; at the metaphase-to-anaphase transition, the cytoplasmic proteins Zds1 and Zds2 are responsible for PP2A^{Cdc55} extrusion from the nucleus, which contributes to mitotic exit (Rossio and Yoshida, 2011).

Coherent with the negative regulation of Cdc14 by PP2A^{Cdc55} is the fact that *cdc55Δ* cells release Cdc14 precociously from the nucleolus due to hyper-phosphorylated Net1, and also that *CDC55* deletion suppresses the effects of mutations causing defects in the mitotic exit process, such as *spo12*, *slk19*, *lte1* and *tem1* (Wang and Ng, 2006a; Wang and Ng, 2006b; Yellman and Burke, 2006). Finally, mutant isoforms of Cdc55 that are not extruded from the nucleus in anaphase are also impaired in

mitotic exit (Rossio and Yoshida, 2011), which confirms that both inhibition by separase and shuttling to the cytoplasm are essential to inhibit $PP2A^{Cdc55}$.

4. The Spindle Assembly Checkpoint (SAC): a barrier against aneuploidy

The metaphase-to-anaphase transition is one of the most delicate phases of the cell cycle. Errors occurring during chromosomes segregation can lead to unequal partitioning of DNA material in the two daughter cells, with one cell inheriting more and the other less DNA. This excess/defect of inherited DNA can consist of one or more entire chromosomes, or pieces of chromosomes. In any case, the consequence of unequal DNA partitioning is aneuploidy, which can be defined as "the presence of an abnormal number of chromosomes, either more or less than the normal, euploid number" (Gordon et al., 2012). Aneuploidy is a very harmful condition for both unicellular and multicellular eukaryotic organisms: unbalanced gene numbers can result in unbalanced levels of key proteins. If this condition affects proteins that are involved in key processes, such as the cell cycle or metabolic processes, it can induce serious dysfunctions, which can result in cell death (Tang and Amon, 2013). In the case of multicellular organisms, aneuploidy has also been proposed to cause genomic instability and cancer (Gordon et al., 2012; Oromendia et al., 2012; Tang and Amon, 2013).

The mechanics of chromosome segregation

The main function of the mitotic spindle is to bind pairs of sister chromatids and to pull them apart to the opposite poles of the cell during anaphase. For this reason the spindle is a symmetric structure: microtubules emanate from microtubules organizing centers called centrosomes (spindle pole bodies or SPBs in budding yeast) that in mitosis localize in opposite positions within the dividing cell, perpendicular to

the metaphase plate. In this way, each pair of sister chromatids can be bound by microtubules coming from the two poles of the cell. During anaphase, the spindle elongates in opposite directions, leading to the physical separation of the sister chromatids and to equal partitioning of DNA masses in the two daughter cells. The bipolar nature of the mitotic spindle is therefore essential to ensure equal segregation of the DNA material in the two daughter cells (Vitre and Cleveland, 2012).

How do microtubules interact with chromosomes? Chromosomes contain multi-proteinaceous structures called kinetochores, that assemble in prometaphase at centromeres and are responsible for binding microtubules of the mitotic spindle. The process of microtubules binding to kinetochores is an intrinsically stochastic process, known as the ''search and capture'' process: due to high dynamicity of microtubule fibers assembly/disassembly, the mitotic spindle continuously attaches and detaches kinetochores (Huang and Huffaker, 2006).

Given the stochastic nature of microtubules attachment to kinetochores, some time is needed before both kinetochores of a sister chromatid pair contemporarily bind microtubules spindles emanating from opposite centrosomes. Only when this event occurs, a proper level of physical tension develops at the kinetochore as a result of the force that microtubules from opposite poles exert in the attempt to part the sisters. The kinetochore structure is such that some kinetochore components are able to sense proper attachment of microtubules and proper physical tension (see paragraph 4.1). These sensor proteins can verify that conditions to guarantee proper segregation of the DNA material in the two daughter cells are satisfied. If this is the case, cells can activate the chromatids segregating machinery, composed of the elongating spindle and associated motor proteins, which leads to separation of sister chromatids and entry into anaphase.

The onset of anaphase must instead be inhibited in situations that would cause unequal DNA segregation between daughter cells. In particular, in prometaphase, before all chromosomes are bioriented on the metaphase plate, some transient, wrong attachments between microtubules and kinetochores normally occur. Among them are monotelic, syntelic and merotelic attachments, which I briefly describe. Monotelic attachments occur when only one kinetochore of a pair of sister chromatids is bound to the mitotic spindle. Syntelic attachments instead occur when both kinetochores are bound by microtubules emanating from the same centrosome. Finally, merotelic attachments consist of one kinetochore binding microtubules coming from both centrosomes. Of course, a fourth and equally dangerous condition occurs before biorientation, when both kinetochores of a sister chromatids pair are unattached. If anaphase occurred in the presence of any of these situations, daughter cells could inherit different amounts of DNA, and consequently develop aneuploidy (see Figure 4).

Eukaryotic organisms have evolved a conserved surveillance mechanism, called the Spindle Assembly Checkpoint (SAC), which halts cell cycle progression in prometaphase in the case of lacking/incorrect microtubules-kinetochores attachments. By inhibiting progression into anaphase in such dangerous conditions, the SAC reduces the risk of aneuploidy. Only when incorrect attachments are corrected and all chromosomes are properly bioriented on the metaphase plate, the checkpoint is satisfied, and the anaphase-wait signal is switched off. At this point anaphase can start, with a very low risk of unequal partitioning of DNA in the two daughter cells.

The prometaphase arrest imposed by the SAC is obtained by inhibiting the main actor of the metaphase-to-anaphase transition: APC/C^{Cdc20}. Inhibition of APC/C^{Cdc20} activity

by the SAC leads to securin and cyclin B stabilization and separase inhibition (Figure 5).

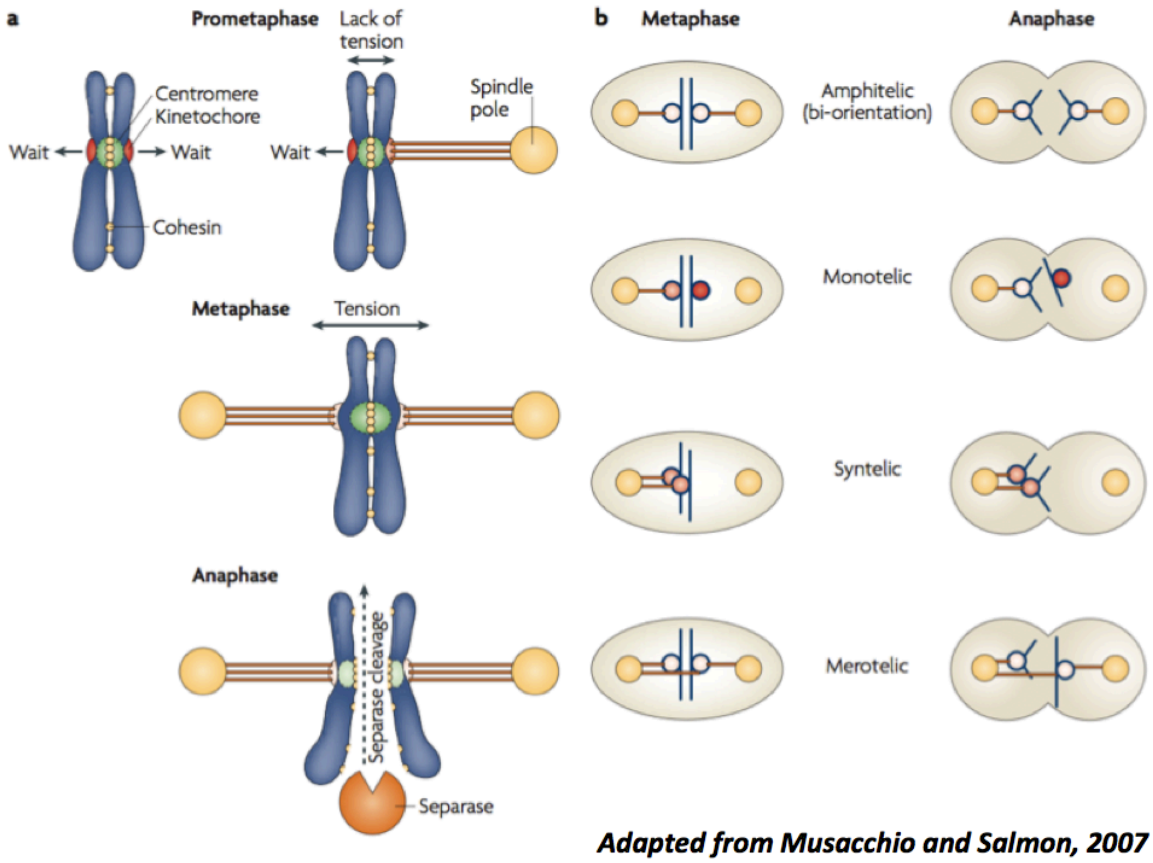

Figure 4. Kinetochore components sense the presence of unattached/incorrectly attached microtubules and activate the SAC. a) In prometaphase sister chromatids are held together by cohesins; when one (right) or both (left) of them are unattached to the mitotic spindles, the kinetochores produce an anaphase-wait signal that activates the SAC and arrests progression to anaphase. Only when both sister chromatids are attached to the spindle and are under tension on the metaphase plate, the anaphase-wait signal is switched off, separase is activated against cohesion, and anaphase can occur. **b)** Only amphitelic attachments (the two members of a sister chromatids pair bound to microtubules emanating from opposite poles of the spindle) are compatible with equal partitioning of the DNA material into the two daughter cells. If anaphase occurred in the presence of monotelic (only one member of the couple bound to the spindle), syntelic (both members bound to microtubules from the same pole) or merotelic (one kinetochores bound to both poles via the spindle) attachments, the DNA would be unequally segregated and aneuploidy would be generated.

4.1 The molecular network of the SAC

In 1991, two screens in Murray's and Roberts' labs identified budding yeast genes whose deletion caused lack of mitotic arrest induced by spindle depolymerizing drugs (Hoyt et al., 1991; Li and Murray, 1991). These genes were called *MAD* (mitotic-arrest deficient) and *BUB* (budding uninhibited by benzimidazole). The MAD family of SAC components contains Mad1, Mad2 and Mad3 proteins, while the BUB family contains Bub1 and Bub3 proteins. Bub2, which was also identified in one of those screenings, is part of another mitotic checkpoint that monitors the correct orientation of the anaphase spindle (the Spindle Positioning Checkpoint or SPoC) (Caydasi et al., 2010; Piatti et al., 2006).

In the following 20 years, it has become clear that MAD and BUB proteins are conserved components of the SAC in all eukaryotes; moreover, how these and other checkpoint proteins interact to induce the formation of the final APC^{Cdc20} inhibitor has been quite well elucidated. However, several gaps still need to be filled as I am going to explain below.

Even if the very upstream event that initiates checkpoint activation remains unclear, kinetochore proteins such as Aurora B and members of the KMN (Knl1/Mis12/Ndc80) kinetochore complex are essential to sense unattached/incorrectly attached kinetochores, and indeed the SAC signal originates at the kinetochores (Rieder et al., 1994). Nevertheless, it is as yet poorly understood how kinetochore proteins sense lack of biorientation. One of the current models (Santaguida and Musacchio, 2009) proposes that lack of biorientation causes lack of tension at the kinetochores which leads the Aurora B kinase, associated with the kinetochore, to come in contact with the mitotic spindle. This results in phosphorylation of Ndc80 and microtubules-associated proteins (such as MCAK and Kif2a) by Aurora B, with the consequence that incorrectly attached microtubules are

detached from the kinetochores and that essential SAC components are localized at unattached kinetochores (Musacchio and Salmon, 2007).

Among SAC components, the best understood are Mad1 and Mad2, whose localization at the unattached kinetochores (UK) depends on Mps1 and Aurora B, the two kinases acting synergistically (Ditchfield et al., 2003; Hardwick et al., 1996; Morin et al., 2012; Murata-Hori et al., 2002; Santaguida et al., 2011; Vigneron et al., 2004).

Mps1 in yeast cells phosphorylates the kinetochore protein Knl1 in threonines belonging to repeated motifs called MELT, which conform to the consensus M-[E/D]-[L/I/V/M]-T; phosphorylated MELT sequences are recognized by Bub3, which is consequently recruited to kinetochores in complex with either Bub1 or Mad3 (Primorac et al., 2013; Shepperd et al., 2012; Yamagishi et al., 2012).

Bub3-mediated localization of both Bub1 and Mad3 at UK is essential for SAC activation. Indeed, Bub3-mediated recruitment of Bub1 contributes to Mad1 and Mad2 localization at UK via as yet unknown mechanisms, while Mad3 recruitment is important in a more downstream event: the formation of the final effector of the checkpoint, the MCC (see below) (Johnson et al., 2004; Vigneron et al., 2004).

Mad1 in interphase exists in complex with Mad2, and is localized in the nucleus, with a specific enrichment at the level of the nuclear envelope (Brady and Hardwick, 2000; Hardwick and Murray, 1995). In prometaphase, in the absence of sister chromatids biorientation, Mad1 is recruited (in a way that depends on Mps1, Aurora B, Bub1 and Bub3, as previously explained) at UK, where it induces a conformational change in Mad2, which is converted from the so-called open conformation, $Mad2^{O}$, to the closed one, $Mad2^{C}$ (De Antoni et al., 2005a; De Antoni et al., 2005b; Mapelli et al., 2007). $Mad2^{C}$ is the active form of Mad2 that binds Cdc20 to form the $Mad2^{C}$:Cdc20 heterodimer, the seed on which the other SAC components Mad3 and Bub3 assemble

and form the final effector of the SAC: the Mitotic Checkpoint Complex (MCC) (De Antoni et al., 2005b).

The MCC, which is a complex of $Mad2^{C}$, Cdc20, Mad3 and Bub3, binds the APC/C and prevents it from ubiquitinating securin and cyclin B (Figure 5). In order to assemble a functional MCC and to fully inhibit APC/C^{Cdc20} upon SAC activation, Mad2, Mad3 and Bub3 are all required; although both Mad2 and Mad3 can bind Cdc20 independently from each other, the presence of all three components produces a synergistic effect that likely stabilizes the MCC and prevents its rapid disassembly (Chao et al., 2012; Mariani et al., 2012).

The conformational change from $Mad2^{O}$ to $Mad2^{C}$ that occurs upon Mad1 binding at UK is essential to activate the SAC cascade. $Mad2^{O}$ binding affinity for Cdc20 is too low to allow assembly of the MCC. That is why cells carrying Mad2 mutant forms that are locked in the open conformation are unable to arrest cell cycle progression upon disruption of microtubules-kinetochores interactions (De Antoni et al., 2005a; De Antoni et al., 2005b). For the same reason, cells that are unable to localize Mad1 at UK also fail to localize and activate Mad2, and are therefore SAC-deficient; viceversa, localization of Mad1 to kinetochores is *per se* sufficient to induce a metaphase arrest even in the presence of correctly aligned chromosomes; this arrest depends on the interaction between Mad1 and Mad2, which confirms the essential role of Mad1 in localizing Mad2 at kinetochores during SAC induction (Maldonado and Kapoor, 2011).

When measured *in vitro*, conversion from $Mad2^{0}$ to $Mad2^{C}$ is very slow, which cannot explain the very rapid MCC formation and APC/C^{Cdc20} inhibition that occurs upon disruption of microtubules-kinetochores interaction (Simonetta et al., 2009). It has been shown that Mad1: $Mad2^{C}$ dimers in vitro (Simonetta et al., 2009) and at the kinetochores (Kulukian et al., 2009) can act as catalysts that speed up $Mad2^{C}$

formation. These results confirmed the previously formulated ''Template model'' (De Antoni et al., 2005a; De Antoni et al., 2005b; Mapelli et al., 2007), which postulates that $Mad2^{O}$ binds to Mad1: $Mad2^{C}$ dimers at UK to form the heterotrimeric complex Mad1:Mad2C:$Mad2^{O}$, which strongly accelerates conversion of $Mad2^{O}$ to $Mad2^{C}$. $Mad2^{C}$ is then released from the kinetochore and easily binds Cdc20 to promote final MCC assembly together with Mad3 and Bub3.

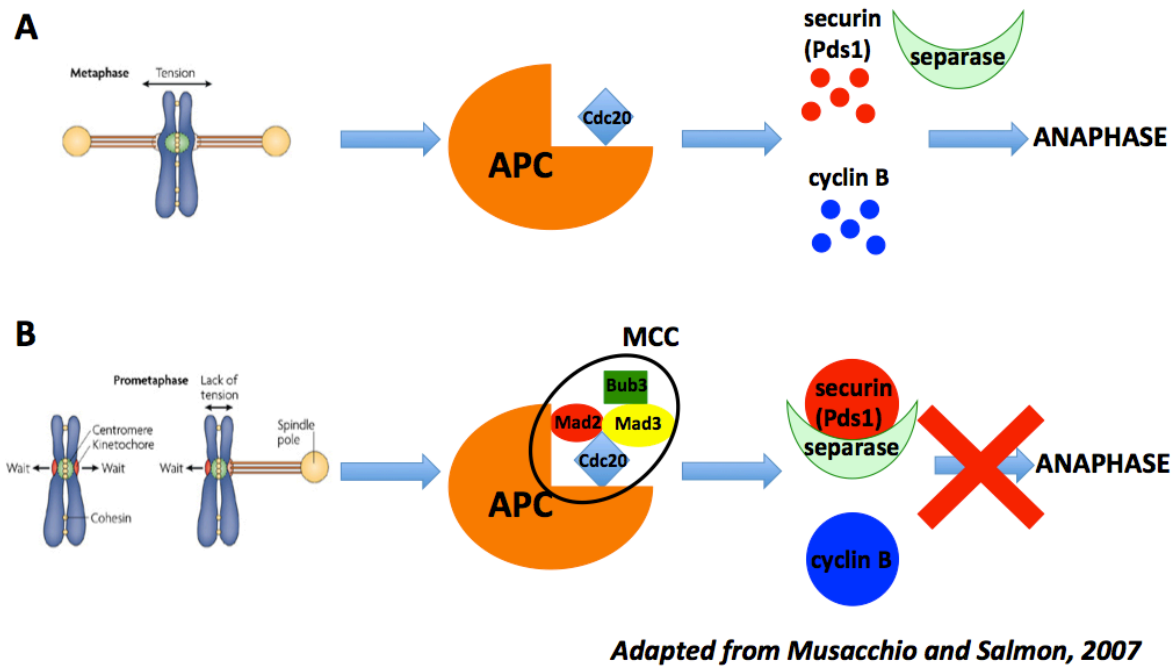

Figure 5. Assembly of the MCC by the SAC induces sequestration of the APC/C and inhibition of its ubiquitin ligase activity towards securin/Pds1 and cyclin B. A) When sister chromatids are bioriented and under tension on the metaphase plate, activation of APC/C^{Cdc20} induces degradation of securin/Pds1 and cyclin B; this leads to separase activation and to cohesion ring cut, whit the consequence that cells transit into anaphase; B) When sister chromatids are not bioriented, lack of attachment/tension activates the SAC, which induces the formation of the Mitotic Checkpoint Complex (MCC), composed of Mad2, Mad3, Bub3 and Cdc20. The MCC sequesters the APC/C and prevents ubuitination and degradation of serurin/Pds1 and cyclin B. As a consequence, separase is not activated, cohesin is not cleaved, and anaphase onset is prevented.

4.2 Role of the SAC during unperturbed mitoses

The evolutionary conservation of the SAC and its components in all eukaryotic organisms points to the importance of this checkpoint during a regular cell cycle.

As previously said, during each physiological mitosis in all eukaryotic cells there is a time when condensed chromosomes are not yet attached to the segregation machinery. At this time, the SAC is activated to delay anaphase onset until the last pair of sister chromatids is bioriented on the metaphase plate. Abrogation of the SAC response in this phase (by interfering with the production/kinetochore localization of essential SAC components such as Mad1, Mad2 or BubR1, or by inhibiting essential kinases of the SAC cascade, such as Aurora B or Mps1) increases the rate of chromosome missegregation and aneuploidy in human cells (Holland and Cleveland, 2009; Kwiatkowski et al., 2010; Lengauer et al., 1997; Michel et al., 2001; Rajagopalan et al., 2003).

In budding yeast cells the role of the SAC during an unperturbed cell cycle is less important than in vertebrates: in physiological mitoses of *S. cerevisiae* cells deleted for Mad1, Mad2 or Mad3, the rate of artificial chromosomes missegragation is quite low (about 1% *per cycle* for *mad1Δ* and *mad2Δ* mutants and 0.3% for *mad3Δ* ones), yet significantly higher than in wild-type cells (about 0.1% *per cycle*) (Warren et al., 2002).

The reason for this apparent discrepancy for the physiological importance of the SAC in lower (yeast) and higher eukaryotes (mammals) is not clear, but it is likely that chromosomes segregation mistakes are less common in less complex organisms. The SAC may have evolved in simpler organism, but probably it has become more and more important in evolved organisms, in parallel with the increasing complexity of the DNA segregation process.

4.3 Cdk1 and the SAC

The involvement of Cdk1:cyclin B in SAC activation seems reasonable on the basis of the following considerations:

1) Cdk1:cyclin B is regulates most mitotic processes, and the SAC is the main mitotic checkpoint;
2) Many essential components of the SAC cascade (Mps1, Aurora B, Bub1) are kinases whose most upstream activator is not known:

It is thus conceivable that Cdk1 might be one such initiating event of the checkpoint, in this way coordinating progression through mitosis with the control on the physiological attachments of chromosomes to the segregation machinery. Not surprisingly, several works explored the role of Cdk1 in the SAC.

Using HeLa cells, D'Angiolella et al. showed that inhibition of Cdk1:cyclin B activity in cells arrested by nocodazole induced MCC disassembly, APC/C^{Cdc20} activation against securin and cyclin B, and mitotic exit (D'Angiolella et al., 2003). This led the authors to conclude that at least partial mitotic Cdk1 activity is essential to maintain the SAC active and to inhibit APC/C^{Cdc20}. What are the substrates that Cdk1 phosphorylates to maintain the SAC arrest is a question that remained unanswered in this study.

In fission yeast, Cdk1 is implied in the maintenance of the SAC: Bub1 phosphorylation by Cdk1 was indeed shown to be essential for fully activating Bub1 as a SAC component and thus for mounting a full SAC response (Yamaguchi et al., 2003).

Works on budding yeast also suggested a role for Cdk1 in SAC activation/maintenance (Amon, 1997; Kitazono et al., 2003). In particular, Amon showed that overexpression of the physiological Cdk1:Clb2 inhibitor, Sic1, in SAC-arrested yeast cells leads to sister chromatids separation due to APC/C reactivation (Amon, 1997).

Put together, these studies suggest a model in which Cdk1:Clb2 is essential to keep APC/C^{Cdc20} inhibited, at least in the case of SAC activation. If this were true, and bearing in mind that APC/C^{Cdc20} contributes to Clb2 degradation and Cdk1 inactivation, APC/C^{Cdc20} and Cdk1:Clb2 would be involved in a double negative feedback loop (Figure 6A). Such a topological connection can give rise to a bistable system, which can alternate between two opposite steady states: one in which Cdk1 activity is high and APC/C^{Cdc20} is completely inhibited (which corresponds to metaphase), and the other one in which APC/C^{Cdc20} is fully active and inactivates Cdk1 by inducing Clb2 degradation (which corresponds to anaphase) (see Figure 6A). Transition between these two opposite states would be rapid and irreversible, therefore justifying the switch-like nature of the metaphase-to-anaphase, as proposed by some authors (He et al., 2011; Holt et al., 2008; Palframan et al., 2006).

Whether the double negative feedback loop between Cdk1 and APC/C^{Cdc20} is conserved among all eukaryotic cells and if it plays a role in making transition into anaphase switch-like is a reasonable hypothesis that has not been experimentally tested yet. Even the problem of the irreversibility of metaphase-to-anaphase transition remains essentially unsolved, because there are no clear experimental data showing that this transition is actually switch-like.

To generate even more uncertainty about the reciprocal regulation of Cdk1 and APC/C^{Cdc20} is the fact that Cdk1:Clb2 is also involved in APC/C^{Cdc20} activation by inducing APC phosphorylation and Cdc20 synthesis (see Introduction, Paragraph 1.3 and (Hegemann et al., 2011; Kramer et al., 2000; Liang et al., 2012; Rudner and Murray, 2000; Steen et al., 2008)). If we also take into account the aforementioned negative regulation of APC/C^{Cdc20} by Cdk1, we are in the presence of an incoherent regulation, where Cdk1 both inhibits and activates APC/C^{Cdc20} (see Figure 6B). The biological significance of such a putative incoherency is not clear. One hypothesis is

that the role of Cdk1 in APC/C^{Cdc20} activation/inactivation could be organism-specific or even condition-specific. In the latter case, for example, the main activity of Cdk1 on APC/C^{Cdc20} could depend on the activation status of the SAC: in conditions of SAC activation Cdk1 could potentiate MCC-mediated APC/C inhibition, while during a normal metaphase-to-anaphase transition or during release from the SAC the main role of Cdk1 could be to activate APC/C^{Cdc20}.

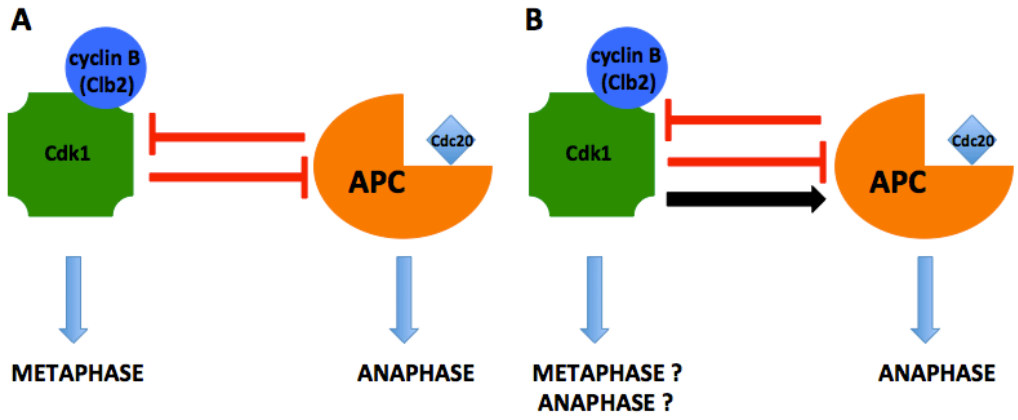

Figure 6. Different models of APC/C regulation by Cdk1:cyclin B: coherence (negative regulation) versus incoherence (negative plus positive regulation). A) Cdk1:cyclin B is implicated in a double-negative feedback loop with APC/C^{Cdc20}: Cdk1 contributes to SAC inactivation of APC/C^{Cdc20}, while APC/C^{Cdc20} induces Cdk1 inactivation via cyclin B ubiquitination and degradation: when Cdk1 prevails over APC/C^{Cdc20}, cells remain arrested in metaphase with inactive APC/C; when instead APC/C^{Cdc20} prevails, cells transit into anaphase and completely inactivate Cdk1; B) In this model, the regulation of APC/C^{Cdc20} by Cdk1 is incoherent, because Cdk1 both inhibits and activates APC/C^{Cdc20}: if both mechanisms are contemporarily active, it is very difficult to predict the main role of Cdk1 on APC/C activity and in the SAC (activation versus inactivation of the checkpoint).

4.4 Activation of the SAC downstream of the kinetochores

When the SAC is induced by disrupting attachment between microtubules and kinetochores, the full biochemical cascade, starting from kinetochores and propagating within the nucleus, is activated to produce MCC and to inhibit APC/C^{Cdc20}.

Alternatively, MCC production can be ectopically promoted by overexpressing some SAC components in the presence of correctly attached microtubules and bioriented chromosomes.

It is has been known from many years that Mps1 overexpression arrests budding yeast cells in metaphase: the arrest is mediated by the formation of the MCC and requires all essential SAC components: Mad1, Mad2, Mad3, Bub3 (Hardwick et al., 1996).

More recently, it has been shown that MCC formation can be induced downstream of the kinetochores, by forcing Mad2:Cdc20 binding ectopically (Lau and Murray, 2012b; Mariani et al., 2012). In particular, Mariani et al. showed that Mad2 overexpression from the *GAL1* promoter arrests yeast cells in metaphase by inducing MCC formation and independently from Mad1 and the kinetochores. Similar results were obtained in fission yeast (Millband and Hardwick, 2002). These data are coherent with the fact that, in conditions of unattachment between chromosomes and microtubules, kinetochores simply act as a catalytic platform that spatially concentrates and activates SAC proteins to induce MCC formation. According to this view, upon Mad2 overexpression, kinetochores are bypassed likely because highly concentrated Mad2 can bind enough Cdc20 to form the initial seed of Mad2:Cdc20 that leads to MCC assembly and APC/C sequestration. Indeed, coherently with the acknowledged essential role of Mad3 and Bub3 in MCC formation, Mad2 overexpression requires both these proteins to arrest cells in metaphase (Mariani et al., 2012). Mariani and collaborators also showed that progressively increasing levels of Mad2 are associated to increasing arrest in metaphase. These facts show that SAC activation is not a ''all or none'' event; on the contrary, it can be gradually modulated to obtain progressively increasing responses as a result of progressively increasing stimuli (Collin et al., 2013).

Lau and Murray used a very different experimental system but obtained quite similar results: if Mad2 is ectopically induced to bind Cdc20, budding yeast cells are arrested in metaphase with bioriented chromosomes and stabilized securin (Lau and Murray, 2012b). This result suggests that the main goal of the SAC is to induce Mad2 binding to Cdc20, and that the role of activated, unattached kinetochores is to facilitate this binding by recruiting Mad2 and Cdc20. Curiously, Mad3 and Bub3 are dispensable to arrest cell cycle progression in this system, suggesting that the main role of Mad3:Bub3 sub-complex is to stabilize and/or to reinforce Mad2:Cdc20 association: when this binding is strong enough, as occurs in the fusion protein, Mad3:Bub3 presence becomes unessential (Lau and Murray, 2012b).

4.5 The importance of Cdc20 destabilization by the SAC

SAC components Mad1, Mad2, Mad3 and Bub3 are very stable proteins. Indeed, the whole SAC cascade relies on post-translational events, be it protein binding or phosphorylation. Regulation of protein synthesis does not seem to play a major role in the SAC with the notable exception of Cdc20.

4.5.1 The SAC helps Cdc20 degradation

Cdc20 is continuously synthesized during mitosis and also during a SAC arrest (Liang et al., 2012). As a consequence, if there were no Cdc20 degradation when the checkpoint is activated, Cdc20 levels would increase and probably saturate SAC components quickly, until newly synthetized Cdc20 could not be sequestered by the MCC anymore. This could lead to activation of APC/C^{Cdc20} and escape from the SAC arrest. Such a possibility is confirmed by the fact that Cdc20 overexpression bypasses the prometaphase arrest induced by the SAC and produces premature APC/C^{Cdc20} activation (Hwang et al., 1998). To avoid precocious override of the checkpoint, eukaryotic cells degrade Cdc20 during a SAC arrest with the aim of matching protein synthesis and keeping Cdc20 levels low (Nilsson et al., 2008; Pan and Chen, 2004).

Indeed, Cdc20 turnover is so high during the SAC arrest, that Cdc20 half-life was estimated to be of about 5 minutes in budding yeast cells (Foster and Morgan, 2012; Pan and Chen, 2004).

The SAC has been proposed to have an active role in Cdc20 degradation. Pan and Chen showed that, compared to wild type cells, mutant yeasts lacking essential SAC components (Mad1, Mad2 or Mad3) accumulate mitotic Cdc20 to levels that are sufficient to bypass the checkpoint arrest. This accumulation is independent from Cdc20 synthesis, which is similar in wild-type and mutant cells; it only depends on degradation. Cdc20 degradation fully depends on active APC, because APC inactivation leads to complete inhibition of Cdc20 degradation (Pan and Chen, 2004). Nilsson et al. confirmed similar findings in mammalian cells, and they also showed that Cdc20 mutant protein lacking ubiquitination sites recognized by the APC/C is resistant to checkpoint arrest.

Curiously, Mad3-deleted (*mad3Δ*) cells accumulate more Cdc20 than both the other SAC mutants Mad1 and Mad2 (Pan and Chen, 2004). The origin and biological meaning of this difference is not known, but it suggests a special role of Mad3 in regulating Cdc20 levels during a SAC arrest.

4.5.2 Regulation of Cdc20 ubiquitination within the MCC

Cdc20 ubiquitination is probably mediated by the same APC/C that binds the MCC, by which it is inhibited. This *in cis* ubiquitination of Cdc20 leads to Cdc20 degradation via the proteasome and likely to the disassembly of MCC. Following MCC disassembly, the other components of the complex (Mad2, Mad3 and Bub3) can be recycled to bind newly synthetized Cdc20 (Mansfeld et al., 2011; Varetti et al., 2011a; Varetti et al., 2011b). Work by Foster and Morgan showed that the non-essential APC subunit, Mnd2 (APC15 in higher eukaryotes) plays an important role in Cdc20 ubiquitination during a SAC arrest. *MND2*-deleted (*mnd2Δ*) budding yeast cells ubiquitinate Cdc20

less than wild-type cells. This results in partial Cdc20 stabilization upon synthesis inhibition and in significant increase of its half-life (from 10 minutes in the wild-type to 25 minutes in the *mnd2Δ* mutant). Although the steady state levels of Cdc20 increase in *mnd2Δ* compared to wild-type cells, mutants lacking Mnd2 are perfectly arrested by the SAC.

If it is true that moderate overexpression of Cdc20 results in bypass of the checkpoint (Pan and Chen, 2004), the SAC proficiency of *mnd2Δ* cells could seem surprising. It can be explained in two different ways: either *mnd2Δ* mutants do not accumulate enough Cdc20 to bypass the arrest, or Mnd2 deletion impacts on the molar ratio of different sub-complexes (Mad2:Cdc20, MCC, APC/C:MCC, Mad3:Bub3:Cdc20, free Cdc20) in a way that implies full sequestration and inhibition of the APC/C even in the presence of increased Cdc20 levels.

The former hypothesis is plausible but it apparently contradicts the observation that transition into anaphase is actually delayed in *mnd2Δ* cells upon SAC inactivation. This latter result seems counter-intuitive because increased levels of Cdc20 in *mnd2Δ* cells would be expected to accelerate transition into anaphase when the SAC is switched off. The fact that exactly the opposite occurs in budding yeast suggests that the second hypothesis may also be true: reduction of Cdc20 degradation in *mnd2Δ* mutants could be associated to reduction of MCC recycling and to stabilization of APC/C:MCC, as suggested also by Foster and Morgan. This would in turn delay APC/C release and activation upon checkpoint switch off, even in the presence of increased total levels of Cdc20.

This last interpretation is supported by data obtained in studies on mammalian cells. Mammalian APC15 promotes Cdc20 turnover similarly to its yeast homolog, Mnd2 (Mansfeld et al., 2011). Moreover, APC15 depletion increases MCC stability (i.e., the binding of Mad2, Bub3 and of the homolog of Mad3, BubR1, to Cdc20 and APC/C) and

delays anaphase following chromosomes biorientation, but does not affect APC/C ubiquitination on mitotic substrates. Whether Cdc20 degradation is required for destabilizing the MCC also in yeast remains unproven. It is surely plausible, especially given the similar delay observed in mammalian and yeast when APC15 (i.e., Mnd2) is not expressed.

To summarize all these data, the role of Cdc20 destabilization by the SAC may be dual: on the one hand it avoids excessive accumulation of Cdc20 and the consequent precocious activation of the APC/C, which would result in checkpoint override. On the other hand, it allows timely disassemble of APC/C:MCC and reactivation of the APC/C upon SAC silencing (see paragraph 4.6). If this interpretation is correct, we can hypothesize that, while deletion of Mnd2/APC15 increases Cdc20 to a level that is insufficient to bypass the checkpoint, it can be detrimental for silencing of the checkpoint, because it stabilizes the APC/C:MCC complex and delays APC/C^{Cdc20} reactivation.

4.6 SAC silencing

With Cdc20 degradation, I have introduced the subject of checkpoint silencing. When all sister chromatids are properly attached to microtubules and bioriented on the metaphase plate, the SAC is satisfied: the spindle checkpoint cascade is switched off, the MCC disassembled, and cells can proceed into anaphase. Whether it is sufficient to switch off the signal to rapidly revert the biochemical events that led to MCC assembly or, on the contrary, some specific pathways must be activated to favor SAC silencing, is a debated topic (Kops and Shah, 2012). It is surely true that the top of the signaling pathway is switched off when microtubule properly attach to kinetochores. Simple 'stripping' of essential checkpoint components from the kinetochores upon biorientation and consequent reduction of their local concentration can indeed contribute to reduce the speed of MCC formation (Wojcik et al., 2001). In particular,

Mad1 and Mad2 removal from attached kinetochores is mediated via the minus-end directed microtubule motor dynein (Howell et al., 2001).

Anyway, the fact that securin degradation and progression into anaphase occur with very fast kinetics upon nocodazole removal (around 20 minutes in budding yeast (Foster and Morgan, 2012)) suggests that active enzymatic processes can accelerate reversion of crucial biochemical events that maintain the SAC active. This conclusion is further reinforced by the fact that in physiological conditions Cdc20/Mad2 binding is energetically favoured (Simonetta et al., 2009).

Given that the upstream events that lead to SAC activation are promoted by essential kinases (Mps1, Aurora B, Bub1), the most plausible candidates for actively reverting the SAC cascades are phosphatases.

4.6.1 Cdc14

A phosphatase that has been implicated in SAC silencing is Cdc14: if Cdc14 is inhibited at the metaphase-to-anaphase transition in a normal cell cycle, cells transit into anaphase but then they stabilize Pds1 in Mad2-dependent manner (Mirchenko and Uhlmann, 2010). This means that in the absence of Cdc14, SAC silencing is not permanent and the checkpoint can be re-engaged as soon as cohesin cleavage reduces tension at the kinetochores. Cdc14 inhibits SAC reactivation in anaphase by dephosphorylating and delocalizing Sli5^{INCENP}, a subunit of the Aurora B complex, from the centromeres.

4.6.2 Glc7

Glc7, the budding yeast homolog of the catalytic subunit of protein phosphatase 1 (PP1), is essential for timely switching off the checkpoint upon sister chromatids biorientation (Pinsky et al., 2009). Cells released from a nocodazole-induced arrest stabilize Pds1 and do not separate sister chromatids if Glc7 is partially inactivated. In the same way these cells cannot recover from Mps1 overexpression-induced SAC

arrest. Given the role of PP1 in reversing Aurora B-mediated phosphorylations, it is plausible to hypothesize that some of Aurora B substrates could be shared by Pp1.

4.6.3 Mps1 degradation

Mps1-dependent phosphorylation of Knl1 has been already mentioned as a key event in Bub1 and Bub3 localization at the kinetochores. Thus, Mps1 degradation is likely relevant for SAC inactivation. Even more, Mps1 degradation by APC/C^{Cdc20} has been implicated in irreversibly switching off the SAC at the metaphase-to-anaphase transition in yeast (Palframan et al., 2006). Mps1 and APC/C^{Cdc20} are involved in a double negative feedback loop: Mps1 is essential for SAC activation and APC/C^{Cdc20} inhibition, while APC/C^{Cdc20} contributes to Mps1 degradation and consequent inhibition. This circuitry is compatible with the existence of a bistable system, in which only two states are possible: one with active Mps1 and inhibited APC/C^{Cdc20}, which corresponds to an active SAC, and the other one with active APC/C^{Cdc20} and inactive Mps1, which corresponds to transition into anaphse. The metaphase-to-anaphase transition corresponds to the movement from the first to the second state: once Mps1 has been degraded by active APC/C^{Cdc20}, it is impossible for cells to reactivate the checkpoint, even in presence of lack of attachment/tension at the kinetochores. According to this model, Mps1 degradation makes SAC silencing irreversible.

The role of PP1 and Cdc14 phosphatases and of Mps1 degradation in silencing the SAC has not been tested in higher eukaryotes.

4.6.4 Cdc20 degradation

One mechanism that seems to contribute to timely SAC silencing in both yeast and mammalian cells is APC/C:MCC-mediated degradation of Cdc20: as described in Paragraph 4.5, Cdc20 is constantly destabilized during the SAC arrest and the Mnd2 yeast subunit (APC15 in mammals) of the APC/C is crucial for Cdc20 ubiquitination

within the APC/C:MCC complex. Cdc20 continuous degradation during the SAC arrest could be important to increase the rate of MCC destabilization and APC/C release from the APC/C:MCC complex. This would lead to continuous recycling of free APC/C, and to consequent, rapid APC/C^{Cdc20} reactivation and anaphase execution upon release from the SAC (Foster and Morgan, 2012; Mansfeld et al., 2011).

4.7 PP2A phosphatase and the SAC

As previously described, kinases play an important role in SAC activation/maintenance at many steps of the checkpoint cascade. On the contrary, phosphatases are usually considered to revert the major phosphorylation events that activate the checkpoint and to be implicated in silencing the SAC (see Introduction, Paragraph 4.6). PP2A is an exception to this paradigm: yeast cells deleted for the non-essential PP2A regulatory subunit Cdc55 are SAC defective, similarly to MAD mutants (Minshull et al., 1996). In other words, $PP2A^{Cdc55}$ helps the SAC to remain active .

The substrates that $PP2A^{Cdc55}$ dephosphorylates to keep the checkpoint arrest are unknown. The fact that $PP2A^{Cdc55}$ contributes to restrain Cdc14 release from the nucleolus in metaphase led some authors to hypothesize that *cdc55Δ* cells are resistant to the SAC because they prematurely release Cdc14 from the nucleolus (Yellman and Burke, 2006), in agreement with the role of Cdc14 in SAC silencing that I have just mentioned (Introduction, Paragraph 4.6, (Mirchenko and Uhlmann, 2010)). Taking into account the role of Cdc14 in activating Cdh1, the precocious activation of the phosphatase, observed in the *cdc55Δ* mutant, could ectopically activate APC/C^{Cdh1}, thus bypassing the requirement for APC/C^{Cdc20} in inducing the prometaphase-to-anaphase transition in nocodazole-treated cells.

In favor of this hypothesis is that *cdc55Δ* mutants do release Cdc14 prematurely from the nucleolus, but whether this precocious release of Cdc14 is the cause of SAC-deficiency remains unproven (Yellman and Burke, 2006).

4.8 Targeting the SAC as an effective anti-cancer therapy

In addition to being activated during unperturbed mitoses before the establishment of chomosomes biorientation, the SAC can also be forcibly and strongly induced through the use of drugs that perturb the correct attachment between microtubules and kinetochores at prometaphase. Among these drugs are the so-called spindle poisons, which interfere with mitotic spindle dynamics by interacting with Tubulin subunits. The result of this interaction is the alteration of the physiological dynamics of spindle assembly or disassembly, with some drugs mainly causing inhibition of spindle assembly (the so-called spindle depolymerizers) and others slowing down spindle disassembly (the so-called spindle stabilizers) (Gascoigne and Taylor, 2009).

Among drugs that inhibit polymerization of microtubules are nocodazole, colcemid and vinca alkaloids (vinblastine, vincristine and vinorelbine). The best known chemicals that induce microtubule stabilization are the taxanes (including paclitaxel and docetaxel). Although they produce opposite effects on microtubules dynamics, these two wide categories of drugs are able to powerfully activate the SAC in all eukaryotic cells. When used at high concentrations, microtubules depolymerizers completely destroy the mitotic spindle and produce lack of attachment at the level of each kinetochore, thus activating the checkpoint (Brito and Rieder, 2006a; Jordan et al., 1991; Weaver and Cleveland, 2005). On the contrary, microtubules stabilizers strongly reduce the inherently high rate of microtubules depolimerization. As a consequence, the correction of attachment mistakes that frequently occur during the random ‘’search and capture’’ process of microtubules binding to kinetochores is significantly impaired, and aberrant interactions are stabilized. These aberrant attachments are not compatible with the development of the proper level of tension across the kinetochores, and therefore microtubule stabilizers also activate the SAC (Kelling et al., 2003; Musacchio and Salmon, 2007).

All spindle poisons arrest cells in prometaphase for several hours by activating the SAC. Cells lacking essential elements of the SAC cascade bypass the arrest and, even in the absence of bioriented chromosomes, they separate their DNA, due to the inability to sequester and inhibit APC/C^{Cdc20} and to restrain separase activation. The fate of these cells is quite dramatic: in the absence of the mitotic spindle, segregated DNA masses cannot be pulled apart to the opposite poles of the cell and cytokinesis cannot occur. These cells can then start a new round of DNA duplication, thus becoming tetraploid and then dying (Fesquet et al., 1999).

Budding yeast cells, which do not seem to significantly benefit from SAC activation during unperturbed mitoses, are instead strongly arrested in prometaphase upon treatment with the microtubule depolymerizing agent nocodazole, similarly to mammalian cells.

Spindle poisons have been used not only to study the biology of the SAC but are among the most effective drugs used in the daily clinical practice to treat both liquid and solid tumors. Just to give some examples, the taxanes paclitaxel and docetaxel are used both in the adjuvant and metastatic setting of breast, ovarian, lung and prostate cancers (Chu et al., 2005; Ghersi et al., 2005; Jordan and Wilson, 2004). Vinorelbine is used to treat lung and breast cancers, while vincristine is included in many polichemotherapic schemes for the treatment of Non-Hodgkin's lymphomas (Fossella et al., 2000; Marcus et al., 2008; Niitani, 1999; Winton et al., 2005).

These drugs induce prolonged mitotic arrest of cancer cells (this is why they are also called anti-mitotics) and, eventually, their death in mitosis through activation of apoptotic pathways (Gascoigne and Taylor, 2009). It is clear that their mechanism of action relies on a functional checkpoint that can induce a prolonged mitotic arrest upon impairment of the connection between kinetochores and microtubules. For this reason, full insight into the molecular mechanisms of SAC activation and inactivation

is not only important to understand the biology of eukaryotic cells, but it can also suggest strategies to improve clinical effectiveness of this effective class of chemotherapeutical drugs.

5. Adaptation to the SAC

During unperturbed cell cycles, chromosomes are only transiently unattached/incorrectly attached to microtubules: biorientation is usually reached within a range of minutes. At this point the spindle checkpoint is rapidly silenced, APC/C^{Cdc20} is activated against securin, and cohesin is degraded to allow anaphase (see paragraph 4.6). Conditions that prolong activation of the SAC (in the range of hours) are not physiological; they are usually induced by drugs (e.g., spindle poisons) that interfere with microtubules dynamics as described above and profoundly disrupt most (if not all) microtubules interactions with kinetochores. Even in conditions of such a harsh induction of the checkpoint, the mitotic arrest due to SAC activation is not endless: after some hours of prometaphase arrest, some cells are able to exit mitosis even in the presence of the drug that activates the checkpoint. This phenomenon is known as *adaptation* to the SAC and it is conserved in eukaryotes from budding yeast to mammals, (in both normal and transformed cells) (Brito and Rieder, 2006b; Rossio et al., 2010a). It can be considered as a sort of defense mechanism that eukaryotes evolved to avoid indefinite mitotic arrest that would result in cell death: eventually cells accept the high risk of DNA missegragation, instead of remaining permanently arrested and therefore dying in mitosis (Rossio et al., 2010b). The time that cells remain arrested in mitosis before adapting is highly variable and depends on both the cell type and on the drug that induced the SAC (Gascoigne and Taylor, 2008; Gascoigne and Taylor, 2009; Rossio et al., 2010a).

It is important to stress the difference between satisfaction of the SAC, followed by mechanisms of SAC silencing discussed in the previous chapter, and adaptation. Silencing occurs as soon as the last kinetochore is properly attached to the mitotic spindle, and is associated with a rapid turning off of the full SAC cascade, from kinetochores to the MCC. At this point SAC components dissociate from kinetochores, the MCC is rapidly dissociated and APC/C^{Cdc20} can activate against securin and cyclin B, just as during an unperturbed metaphase-to-anaphase transition.

On the contrary, adaptation occurs in the presence of an activated SAC, while the kinetochores are still signaling due to the lack of chromosomes biorientation. Indeed, as long as vertebrate cells adapt to the SAC, the checkpoint components Mad1, Mad2 and BubR1 are still bound to kinetochores, and presumably they continue to emit the anaphase-wait signal (Brito and Rieder, 2006b).

5.1 Adaptation to different SAC-activating stimuli

Adaptation to the SAC is conserved among eukaryotes, and takes place regardless of the mechanism that induces the checkpoint. Budding yeast cells adapt to Mad2 over-expression with times that are comparable to those in nocodazole (Rossio et al., 2010a) and longer than in the presence of benomyl, a drug that interferes with microtubules polymerization similarly to nocodazole. Also the SAC arrest induced by Mps1 overexpression in yeast is eventually bypassed and cells proceed into anaphase (Hardwick et al., 1996; Rudner and Murray, 2000).

Studies on human cells lines uncovered that cells where the SAC is activated for a long time can either die during the SAC arrest, or adapt to the checkpoint and enter a new round of DNA replication. The percentage of cells that die/adapt depends on the cell type and on the drug used to activate the SAC. However, there is always a percentage of cells that adapt to the checkpoint, independently from the fact that nocodazole,

taxol or AZ138 (an inhibitor of the bipolar mitotic kinesin Eg5) is used to induce the checkpoint (Gascoigne and Taylor, 2008).

5.2 Molecular mechanisms of adaptation to the SAC

5.2.1 The slippage model

In human cells, adaptation to the SAC has been ascribed to the intrinsic inefficiency of the checkpoint to completely inhibit APC/C^{Cdc20} (Brito and Rieder, 2006b). Some residual activity of APC/C^{Cdc20} remains during the SAC arrest, which slowly and progressively induces cyclin B degradation, until some low level of cyclin B (and consequently of Cdk1 activity) is reached that is no more compatible with the maintenance of the mitotic state. At this point cells slip out of mitosis without undergoing cytokinesis and therefore without dividing their DNA; nevertheless they are ready to start a new round of DNA replication and, in case, of cell division (Figure 7A). This model, known as the ''slippage model'', is coherent with the positive role that mitotic Cdk1 plays in activating the SAC in human cells (D'Angiolella et al., 2003). Progressive cyclin B degradation before mitotic slippage could consequentially reduce the strength of APC/C^{Cdc20} inhibition due to the checkpoint: as a result, the initial inefficiency of the SAC in inhibiting APC/C^{Cdc20} would be progressively reinforced by the drop in cyclin B levels (and Cdk1 activity) and would speed up slippage in the last phases of cyclin B degradation.

5.2.2 Adaptation as specific case of SAC satisfaction

Apart from the slippage model, other mechanisms have been proposed to explain adaptation to the SAC. In principle, some of the events that are implicated in SAC silencing could also be active in promoting adaptation. Among them is the degradation of Mps1 or Bub1. As far as Mps1 is concerned, it is important to remember that this kinase, which essentially contributes to APC/C^{Cdc20} inhibition upon SAC activation, is itself degraded in APC/C^{Cdc20}–dependent manner when cells

transit into anaphase (Palframan et al., 2006). If a little APC/C^{Cdc20} were stochastically activated during a SAC arrest, it could drive Mps1 degradation under a certain threshold that is required for SAC maintenance. Due to the double negative feedback loop between these two molecules, initial degradation of Mps1 could further reinforce APC/C^{Cdc20} activation, driving cells in anaphase very quickly (see Introduction, Paragraph 4.6).

Bub1 degradation has also been proposed as a mechanism of adaptation to the SAC in budding yeast cells: inhibition of Cdk1 phosphorylation-mediated degradation of Bub1 prolongs the mitotic arrest in benomyl (Goto et al., 2011).

The involvement of both Mps1 and Bub1 inactivation in adaptation assumes that the SAC cascade is silenced at the level of kinetochores when cells adapt. This possibility implies that adaptation occurs as a consequence of the delocalization of SAC components (e.g., Mad1, Mad2, Bub1, Bub3) from kinetochores, similarly to silencing that follows SAC satisfaction (Figure 7B).

This hypothesis is in contrast with the fact that delocalization of SAC proteins from kinetochores does not occur in adaptation (Brito and Rieder, 2006b). Rather, the fact that SAC components remain associated to unattached kinetochores during adaptation suggests that the molecular event that triggers adaptation occurs rather downstream in the SAC cascade, and has probably to do with the dynamics of MCC disassembly.

In agreement with this hypothesis are published data of adaptation to Mad2 overexpression in budding yeast (Rossio et al., 2010a). As previously said, Mad2 overexpressing cells adapt with kinetics that are comparable to those in nocodazole (Rossio et al., 2010a). Given that the SAC arrest in Mad2 overexpressing cells does not depend on the kinetochores, the molecular event that triggers adaptation in this

system occurs downstream of the kinetochores, and likely has a direct impact on MCC stability.

5.2.3 Activation of pathways that bypass APC/C^{Cdc20}

Other mechanisms that have been proposed to explain SAC adaptation imply the activation of pathways that bypass the requirement of APC/C^{Cdc20} and activate the APC/C through parallel ways. The most obvious way to bypass APC/C^{Cdc20} would be to activate APC/C^{Cdh1} (Figure 7C).

Cdh1 has been shown to be necessary for adaptation to the checkpoint in budding yeast cells deleted of *BUB2* (Toda et al., 2012). *bub2Δ* cells bypass the SAC arrest because they ectopically activate APC/C^{Cdh1} through the MEN pathway (see paragraph 2). As expected, the deletion of Cdh1 restores a physiological, prolonged SAC arrest in these cells, which is interpreted by the authors as inability of *bub2Δ cdh1Δ* cells to adapt compared to *bub2Δ* cells. The role of Cdh1 in SAC adaptation in wild-type cells, however, has never been addressed.

In the same logic of a molecular mechanism that bypasses the requirement for APC/C^{Cdc20}, the phosphatase Cdc14 is potentially a good candidate for driving adaptation in budding yeast. In support of this possibility is that overexpression of Cdc14 bypasses the SAC and that Cdc14 is required to inhibit SAC re-activation in anaphase (Mirchenko and Uhlmann, 2010). Moreover, Cdc14 ectopic activation in *cdc55Δ* cells has been suggested as the reason why these mutants are SAC-defective (Yellman and Burke, 2006). However, whether SAC silencing driven by Cdc14 precocious activation can lead to adaptation to the checkpoint has never been proven. In conclusion, we still do not know whether adaptation to the SAC requires silencing of the checkpoint or bypassing the requirement for APC/CCdc20. Even less, we are sure about the molecular mechanism that drives adaptation to checkpoint.

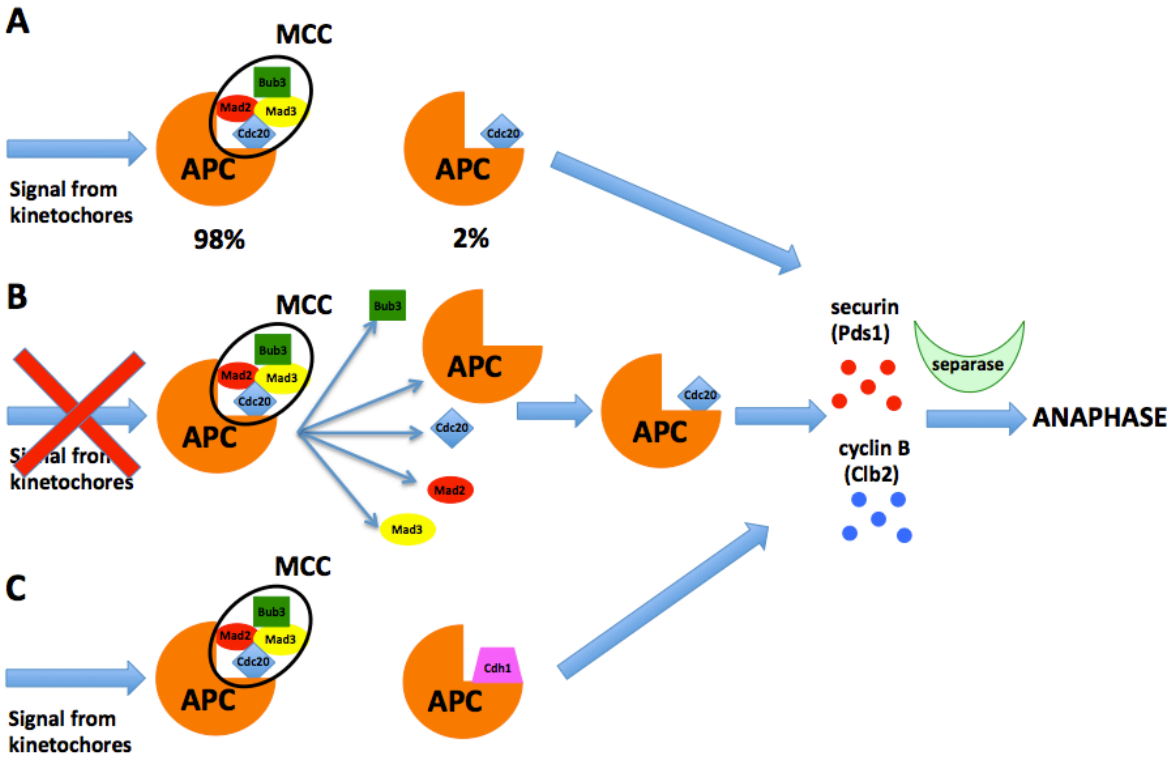

Figure 7. Multiple mechanisms of adaptation to the SAC. A) While most of Cdc20 (98% in the picture) is sequestered by the MCC within the APC/C:MCC complex, a small amount of it (2% in the picture) remains free and can bind and activate APC/C. The resulting, small amount of APC/C^{Cdc20} is sufficient to trigger slow, but progressive degradation securin and cyclin B, and transition into anaphase. B) Abrogation of the signal coming from unattached kinetochores (due to Mps1 or Bub1 degradation, for instance) inhibits MCC formation and causes Cdc20 release from the APC/C:MCC complex. This leads to APC/C^{Cdc20} activation, similarly to when the SAC is satisfied. C) In this case, Cdc20 is completely sequestered by the MCC, but ectopic activation of APC/C^{Cdh1} bypasses the requirement of Cdc20 and induces transition into anaphase by promoting degradation of securin and cyclin B.

Aim of the thesis

The main goal of this thesis is to clarify the role of Cdk1 in the Spindle Assembly Checkpoint (SAC) in budding yeast cells and to investigate the mechanisms that underlie adaptation to the SAC. Another major aim of the work is to understand how Cdc20 levels can impact on the fate of SAC-arrested yeast cells, influencing the time that yeasts remain arrested in prometaphase before adapting to the checkpoint and exiting mitosis.

The results contained in the first chapter of the work have been recently published. See "*Adaptation to the spindle checkpoint is regulated by the interplay by Cdc28/Clbs and PP2A^{Cdc55}*". Vernieri C, Chiroli E, Francia V, Gross F, Cilibero A. *J Cell Biol.* 2013 Sep 2;202(5):765-78.

Materials and methods

Yeast strains, growth media and reagents

All yeast strains used were derivatives of or were backcrossed at least three times with strains of W303 background (*ade2-1*, *trp1-1*, *leu2-3*, *112*, *15*, *ura3*, *ssd1*).

The population experiment in Figure 10 was performed using synthetic medium lacking methionine. In all other population experiments I used YEP medium (1% yeast extract, 2% Bacto Peptone and adenine 50mg/liter) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR) or 2% raffinose plus 2% galactose (YEPRG).

Single cell adaptation experiments with overexpressed Mad2 were performed using synthetic complete medium supplemented with ammonium sulfate.

α-factor was used at a concentration of 5 μg/ml for first G1 synchronization and at 20 μg/ml for re-synchronization after release from G1 phase. Nocodazole was used at 15 μg/ml and readded after 3 hours at 7.5 μg/ml. Methionine was added at a final concentration of 20 mM. All experiments were performed at 30°C, unless otherwise stated. In population experiments with Mad2 and Pds1Δdb over-expression, galactose was added to YEPR medium 1 hours before release from α-factor arrest. In population experiments of adaptation, α-factor was readded to the medium 2 hours after release from α-factor, unless otherwise described.

Western blot analysis

In all Western blot experiments, cells were collected at indicated times and centrifuged for 2 minutes at room temperature. The pellet was then added 100 μl trichloroacetic acid (TCA) to precipitate proteins. Cells were mechanically broken

through glass beads and the obtained material was then collected and centrifuged. Pellets were then added Laemmli buffer (composed of SDS 2%, Tris-HCl pH 6.8 60 mM, glycerol 10% and bromophenol blue 0.01%) also containing β-mercaptoethanol as a reducing agent and basic TRIS to neutralize the TCA; extracts were finally boiled to denature proteins (Fraschini et al., 1999). Proteins were then loaded and separated in 10% or 12.5% polyacrylamide gels containing an acrylamide to bisacrylamide ratio of 29/1. The voltage of the separation apparatuses was fixed at 120-140 V for the duration of the run. To visualize phosphorylation shifts of Cdc16 in Figure 27, 7.5% polyacrylamide gels (with an acrylamide to bisacrylamide ratio of 80/1) were used. To discriminate single phosphor-specific bands of Cdc16 and Cdc27 (Figures 25 and 27, respectively) the Phos-Tag system was used: P-tag reagent 50μM was added, together with MnCl2 50μM, to 7.5% polyacrylamide gels with an acrylamide-to-bisacrylamide ratio of 37/1; small gels were run for 4 hours by keeping constant (15 mA/gel) the amperage settings of the run apparatus (Kinoshita et al., 2008).

Proteins were transferred from gels to Protrane membranes for 1-1.5 hours, with the voltage of the transfer apparatuses set at 100V.

Membranes were kept overnight in TBS-Tween plus milk 5% at 4°C and the next day incubated with primary antibodies. In the case of anti-Myc and anti-Pgk1 antibodies (for detection of Pds1-Myc18, Cdc16-Myc6, Cdc27-Myc9 and Pgk1), membranes were exposed to the primary antibodies for 2 hours at room temperature. In the case of Mad2, Clb2 and Cdc20, membranes were exposed to the primary antibodies for 8 hours at 4°C. Then three washes from the primary antibody were done for 45 minutes, and the secondary antibody was added for 30 minutes at room temperature. After another cycle of three washes in TST-Tween, proteins were detected by an enhanced chemiluminescence system (Pierce ECL; Thermo Fischer Scientific). Blots

were acquired as digitalized images through a Chemidoc XRS+System (Bio-Rad Laboratories).

For the detection of Myc-tagged proteins (Pds1, Cdc16, Cdc27) and Mad2, I used, respectively, 9E10 anti-Myc and anti-ScMad2 antibodies, produced at the Monoclonal Antibodies Facility at the IFOM-IEO Campus and diluted 1:1000 in TBS-Tween plus 5% milk. Commercial antibodies were used for Cdc20 (yC-20 from Santa Cruz Biotechnology; diluted 1:1000 in TBS-Tween plus 5% milk), Clb2 (y180 from Santa Cruz Biotechnology; diluted 1:1000 in PBS-Tween plus 1% milk plus 1% bovine serum albumin) and Pgk1 (D660 from Invitrogen; diluted 1:5000 in TBS-Tween plus 5% milk). Secondary antibodies came from Bio-Rad Laboratories.

The software Imagelab was used to quantify the acquired, digitalized images. Quantification of protein signals was performed by enclosing each protein band within a rectangle of constant area and recording the respective signal; then a background signal form an empty zone of the same membrane was subtracted to each protein band signal. In the end such signals were normalized to background-subtracted Pgk1 signals corresponding to the same times. To quantify Cdc16 and Cdc27 phospho-specific bands from P-Tag gels shown in Figures 25 and 27, the signal corresponding to the phosphorylation shift (above the second, fast migrating band clearly visible during the G1 arrest) was normalized over the total amount of protein (first two bands plus entire shift) in the same membrane at the same time point.

Fluorescence-activated Cell Sorting (FACS) analysis of DNA content

FACS analysis was used to analyze the DNA content of populations of cells. For FACS analysis, 1 ml of cell culture was fixed in 70% ethanol overnight at 4^{o}C. The day after

ethanol was washed with Tris-HCl 50mM pH 6.8 and then samples were added RNAase and put at 37°C to induce RNA degradation. After at least 4 hours, samples were centrifuged, and washed with PBS. After another centrifugation to remove PBS, 200μl of Propidium Iodide (PI) were added to color DNA. 100 μl of PI-exposed cells were then diluited in 1 ml of Tris 50mM pH 6.8, and samples were read at a flow cytometer machine (FACScan or FACScalibur; BD). For each sample, 10,000 events were counted and acquired data were analyzed with FlowJO Software.

Quantification of DNA content was used in many experiments to confirm uniform G1 arrest (single DNA content) induced by α-factor treatment, or G2/M arrest (double DNA content) due to treatment with nocodazole or to Mad2 overexpression. In most control and in some adaptation experiments, FACS analysis was useful in comparing times of progression through unperturbed or SAC-delayed cell cycles between different mutants.

Phosphatase assay

I used Calf Intestine Phosphatase (CIP) to confirm that shift observed on the blot profiles of Cdc16-Myc6 observed in *cdc55Δ* mutants on 7.5% polyacrylamide gels are specific for phosphorylated isoforms of the protein (Figure 27). To allow activation of the CIP I needed to eliminate the SDS contained in Laemmli buffer-diluited TCA extracts. For this reason samples containing protein extracts were centrifuged 5-6 times in Amicon Ultra Centrifugal filters (Millipore) and washed with buffer composed of: MgCl2 10 mM, NaCl 100 mM, TRIS 50 mM, DTT 1 mM, pH = 7.9). Extracts were then incubated at 30°C for 30 minutes with buffer alone, CIP, or CIP plus 10 mM sodium orthovanadate (to inhibit the CIP). Laemmli was added again and the sampled boiled at 98°C for 3 minutes before being loaded on 7.5% polyacrylamide gels.

Immunofluorescence analysis

In this study, indirect immunofluoresce (IF) analysis was essentially used to label tubulin, so to visualize the mitotic spindle and to distinguish between interphasic cells (no mitotic spindles), metaphasic cells (thick, short spindle), anaphasic/telophasic spindle (thin, elongated structure), absence of polymerized microtubules (treatment with nocodazole). This analysis allowed us to follow progression through the cell cycle or to study arrest and adaptation kinetics in Mad2 over-expressing cells. Moreover, in nocodazole-treated cells, IF analysis of spindle confirmed absence of mitotic spindle if the drug was working properly.

For IF analysis of microtubules spindles, 1 ml of culture was taken and fixed with formaldehyde 4% overnight at 4^0C. The day after three washes were done with a buffer solution of phosphate salts (KH2PO4 plus K2HPO4) and one additional wash with a sorbitol solution 1.2M containing the same phosphate salts plus MgCl2. Then cells were exposed to zymolase 10 mg/ml for 30 minutes to digest the cell wall. After this treatment cells were washed again with sorbitol solution and then loaded on glass slides. Then they were incubated with the primary antibody (anti α-tubulin YOL34 monoclonal antibody from AbD Serotec) for 90 minutes at room temperature. After 5 washes with a solution of PBS plus bovine serum albumin (BSA), the secondary antibody (FITC-conjugated anti-rat antibody from Jackson ImmunoResearch Laboratories) was added for additional 90 minutes at room temperature. After washing the secondary antibody with PSB/BSA for 5 times, DAPI was added to also detect DNA masses, and the slide was closed.

Percentages of metaphase/anaphase spindles were counted at a DeltaVision machine using a UPlan-Apochromat 100x (1.4 NA) oil immersion objective lens (Olympus).

Colocalization of Mad2-3GFP and Nuf2-mCherry

For studies of colocalization of Mad203GFP and Nuf2-mCherry, 1 ml of culture were fixed in cold (4°C) 100% ethanol overnight. The day after images were acquired on the DeltaVision Elite imaging system using a UPlan-Apochromat 100x (1.4 NA) oil immersion objective lens (Olympus). Colocalization was defined as the spatial correspondence between the strongest of Mad2-3GFP observed dots and one among the three visible Cherry dots (Nuf2 also localized at the two Spindle Pole Bodies, so usually three dots are visualized in each cell).

Single cell quantification of Clb2-GFP levels

Single cell experiments were performed in microfluidic chambers (CELLASIC), and cells were grown at 30°C in synthetic medium containing raffinose, glucose, or raffinose plus galactose. For single cell experiments in nocodazole (Figure 23) cells were grown and arrested in YEPD medium. Time-lapse movies were recorded by a DeltaVision Elite imaging system (Applied Precision) based on an inverted microscope (IX71; Olympus) with a camera (CoolSNAP HQ2; Photometrics) and a UPlan-Apochromat 60x (1.4 NA) oil immersion objective lens (Olympus).

Images were acquired every ten minutes.

Segmentation of cells and quantification of whole-cell Clb2-GFP signal

Analysis of single cell movies was performed through a software written in MATLAB (MathWorks). Single yeast cells were segmented and tracked through the program phyloCell, written by Gilles Charvin (unpublished results). From the averaged fluorescence Clb2-GFP signal of each cell I subtracted an averaged background signal deriving from cells of the same genotype and exposed to the same experimental conditions, but lacking the fluorescent markers. In this way, I was sure to subtract not

only the background signal deriving from the flow chamber bottom, but also intracellular auto-fluorescence signals due to non fluorophore-tagged intracellular components. In adaptation experiments I observed a progressive, constant increase of background signal over time. If not subtracted to fluorescent cells, this increasing background level would lead to overestimation of Clb2-GFP fluorescent signals.

Nuclear Clb2-GFP quantification

To precisely measure nuclear Clb2-GFP signals, we would need a nuclear marker capable of determining the exact boundaries of the area to quantify. In the absence of such a nuclear membrane marker, and given that Clb2 localizes in the nucleus, I estimated nucleus boundaries based on the same Clb2-GFP signal. When this signal was significantly higher than fluorescence in other parts of the cells, the indirect estimation of nuclear area was quite good. In these conditions it was in fact possible to divide the entire acquired field into three progressively more intense clusters of GFP signals: the highest corresponding to Clb2, the intermediate corresponding to the rest of the cell, and the lowest being represented by the extracellular space. The mathematical method that allows such a partition into three clusters is called 3-means clustering method. When Clb2-GFP is not sufficiently intense, the method does not distinguish between Clb2-localized areas and other intracellular zones where Clb2 is absent. In such conditions, nuclear size estimation by the clustering is unrealistically high. The moment in which the clustering is reliable is characterized by a clear drop in the ratio between the estimated nuclear area and the whole-cell area (Figure 8, left panel). I fixed to 30% the ratio threshold below which the clustering was considered reliable. Within this reliability region, I considered the averaged Clb2-GFP signal as the genuine nuclear Clb2-GFP signal. This signal was then smoothed and plotted. Smoothing of resulting time series signals was performed by averaging each time point signal with the previous and the following ones. In this

way, the effects of signal fluctuations due to noisy components were reduced. The Clb2-GFP signal was not smoothed in cycling cells.

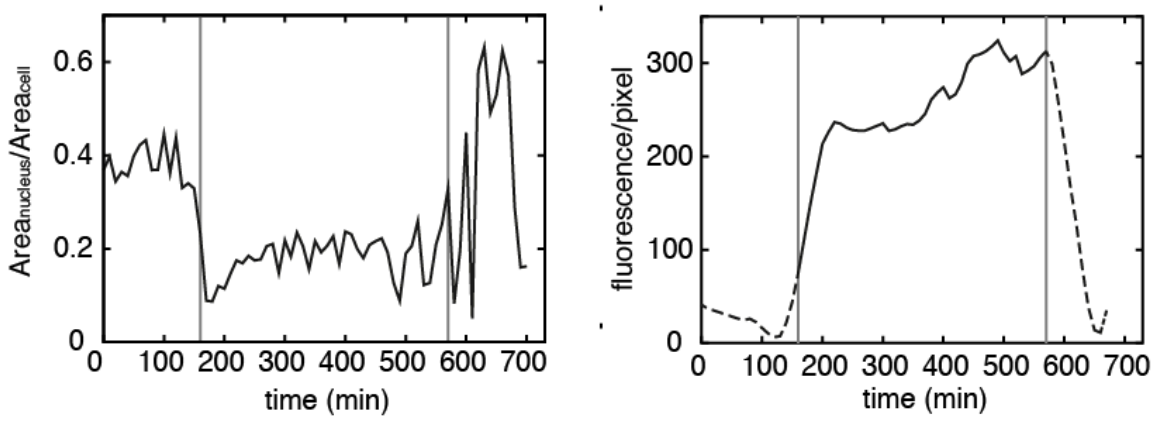

Figure 8. Estimation of the nuclear area from Clb2 clusters is reliable when the ratio $Area_{nucleus}/Area_{cell}$ is low. (Left panel) The ratio between the area of the nucleus, as estimated from the analysis of Clb2 signals through k-means method, and the whole cell area is plotted over time. The nuclear area is well defined when this ratio is lower than 0.3 (between the vertical lines). This corresponds to the time when Clb2 accumulates in the cell and is restricted to the nucleus (right panel). Adapted from Vernieri et al., 2013.

Adaptation time

In Mad2 overexpressing cells, adaptation time (Figure 22) was used as a measure of the duration of the SAC arrest before occurrence of adaptation. It was estimated as the time elapsed between the moment that Clb2 started to accumulate (20% increase relative to baseline levels) and the initial elongation of the mitotic spindle, indicative of adaptation and transition to anaphase. Given that transition from the metaphase to anaphase spindle typically occurred within one-two time points (10-20 minutes), in some cells it was quite difficult to visualize spindle elongation. In such cases, adaptation time was calculated on the basis of initial decrease of Clb2-GFP signal, which also was a good estimation of adaptation onset.

Clb2-GFP peak estimation

In cycling cells, Clb2-GFP peaks were the local maxima of the signal in each cell cycle. In Mad2 over-expressing cells, I considered the local maxima of smoothed Clb2-GFP signal during the first round of SAC arrest.

Mathematical model

The goal of this mathematical model was not to simulate the behavior of all species involved in the SAC and in the adaptation process. Its aim was to simplify the network and to schematically illustrate how the combination of Cdk1:Clb2→APC/C--|Cdk1:Clb2 negative feedback loop with $PP2A^{Cdc55}$--|APC/C--|$PP2A^{Cdc55}$ double negative feedback loop can cause continuous accumulation of Clb2 during SAC arrest and its rapid and irreversible degradation at adaptation time. In particular, equations and parameters have been chosen in such a way to obtain a switch-like oscillator, where transition from one phase (metaphase) to the next one (anaphase/G1) is rapid and irreversible. The following simplifications have been applied to build such a toy model:

1) the effect of the SAC on APC/C inactivation is included in the parameter kph, which summarizes the events that activate (high kph) or inactivate (low kph) APC/C;
2) it is assumed that at the metaphase-to-anaphase transition the only two elements that impact on APC/C activation status are Cdk1:Clb2 (positive regulator) and $PP2A^{Cdc55}$ (negative regulator);
3) the role of Cdh1 in APC/C activation is completely ignored in this model: APC/C is postulated to exist in just two forms: dephosphorylated, inactive APC/C (APC), and phosphorylated, active APC/C (APCP). APC is considered as sequestered by the MCC, APCP is considered as free from MCC and able to bind Cdc20 even in the presence of an active SAC.

Together with there major simplifications, I also assumed in this model that Cdk1 activation only depends on Clb2 and that $PP2A^{Cdc55}$ can only exist in two states: active $PP2A^{Cdc55}$ (Cdc55a in the model) and inactive $PP2A^{Cdc55}$ (Cdc55i in the model).

In the next sub-paragraphs I explicitly write down the reactions modeled and their conversion into Ordinary Differential Equations (ODEs).

Reactions

APC + Clb2 → APCP + Clb2 (kph = phosphorylation constant; Japc = Michaelis constant for APC phosphorylation)

APCP + Cdc55a (kdeph = dephosphorylation; Japci = Michaelis constant for APCP dephosphorylation)

Cdc55i → Cdc55a (ka = Cdc55 activation constant)

Cdc55a + APCP → Cdc55i + APCP (ki = Cdc55 inactivation)

→ Cdc20 (ksyn = Cdc20 synthesis)

Cdc20 + APCP → APCP (kdeg = Cdc20 degradation)

Algebraic equations.

APC = APCtot – APCP

Cdc55i = Cdc55tot – Cdc55a

Ordinary Differential Equations.

dAPCP/dt = (APC * kph * Clb2)/(Japc + APC) – [APCP * (kdeph * Cdc55a)]/(Japci + APCP)

dCdc55a/dt = ka * Cdc55i – (ki * APCP) * Cdc55a

dClb2/dt = ksyn – (kdeg * APCP) * Clb2

Parameters

For the following parameters, the units are 1/min.

APC/C phosphorylation: kph = 0.6 (checkpoint ON), kph = 1.2 (checkpoint OFF)

APC/C dephosphorylation: kdeph = 2.4

$PP2A^{Cdc55}$ activation: ka = 1.2

$PP2A^{Cdc55}$ inactivation:ki = 1.2

Cdc20 synthesis: ksyn = 0.02

Cdc20 degradation: kdeg = 0.055

For the following parameters, concentration units are arbitrary:

Michaelis constant for APC/C activation: Japc = 0.01

Michaelis constant for APC/C inactivation: Japci = 0.01

Total APC/C: APCtot = 1

Total Cdc55: Cdc55tot = 0.9

Initial conditions (arbitrary concentration units)

APCP = 0.012, Cdc55a = 0.875, Clb2 = 0.965

Simulations were produced through XPPAUT software. I chose parameters values to produce limit cycle solutions for our model (Figure 45). Any other choice of parameters that produces limit cycle solutions would produce qualitatively similar results. Similarly, both for the checkpoint ON condition (kph = 0.6) and for the checkpoint OFF condition (kph = 1.2), initial conditions were chosen from the limit cycle solution.

Results

Chapter 1

The struggle between Cdk1 and PP2A^{Cdc55} in adaptation

1.1 Phosphorylation of APC/C subunits is essential for adaptation to the SAC

As described in the introduction, the APC/C is activated through binding to its coactivators Cdc20 and Cdh1, and through phosphorylation of its Cdc16, Cdc27 and Cdc23 subunits by Cdk1 kinase. I therefore asked which one, if any, of these events are required for adaptation to the SAC.

Given that in our yeast media nocodazole effect starts to wear off after 5-6 hours, I could not use this depolymerizing agent to study SAC adaptation, which in wild-type cells begins 5-6 hours after the arrest and is completed at 8-10 hours (Rossio et al., 2010a). I therefore decided to perform adaptation experiments by inducing the SAC via Mad2 overexpression from the *GAL1* promoter. As explained in the Introduction (see Paragraph 4.4), Mad2 overexpression induces a genuine SAC arrest downstream of the kinetochores, with cell starting adaptation about 5 hours after being arrested in metaphase (Mariani et al., 2012; Rossio et al., 2010a). The checkpoint arrest resulting from Mad2 overexpression is characterized by accumulation of cells with metaphase

spindles (as detected by immunofluorescence (IF) analysis) and by stabilization of Pds1 and Clb2 (as detected by WB). Adaptation is instead marked by transition from metaphase to anaphase spindles and degradation of Pds1 and Clb2 (Rossio et al., 2010a).

I first tested the role of APC/C subunits phosphorylation in adaptation. As explained in the Introduction (see Paragraph 1.3), phosphorylation of Cdc16 and Cdc27 by Cdk1 is not essential to activate APC/C in normal conditions, but it may become essential upon the prolonged metaphase arrest imposed by the checkpoint, as suggested by (Rudner and Murray, 2000). I first verified that the non-phosphorylatable *cdc16-6A cdc27-5A* mutants of the APC/C complete an unperturbed cell cycle with only a small delay compared to APC/C wild-type cells, as previously described in literature (Figure 9A).

I then moved to testing the effect of cdc16-6A cdc27-5A mutant proteins in adaptation. I synchronized *GAL1-MAD2(3x)* and *GAL1-MAD2(3x) cdc16-6A cdc27-5A* cells in G1 phase and then I released them in galactose to activate the SAC via Mad2 overexpression. While wild-type cells started disassembling metaphase spindles and degrading Pds1 and Clb2 about 4 hours after the release as expected, mutant cells containing non-phosphorylatable APC/C subunits were permanently arrested in metaphase with stable Pds1 and Clb2 (Figures 8B and 8C).

I conclude from this first experiment that phosphorylation of APC/C subunits by Cdk1 is essential for adaptation to the SAC.

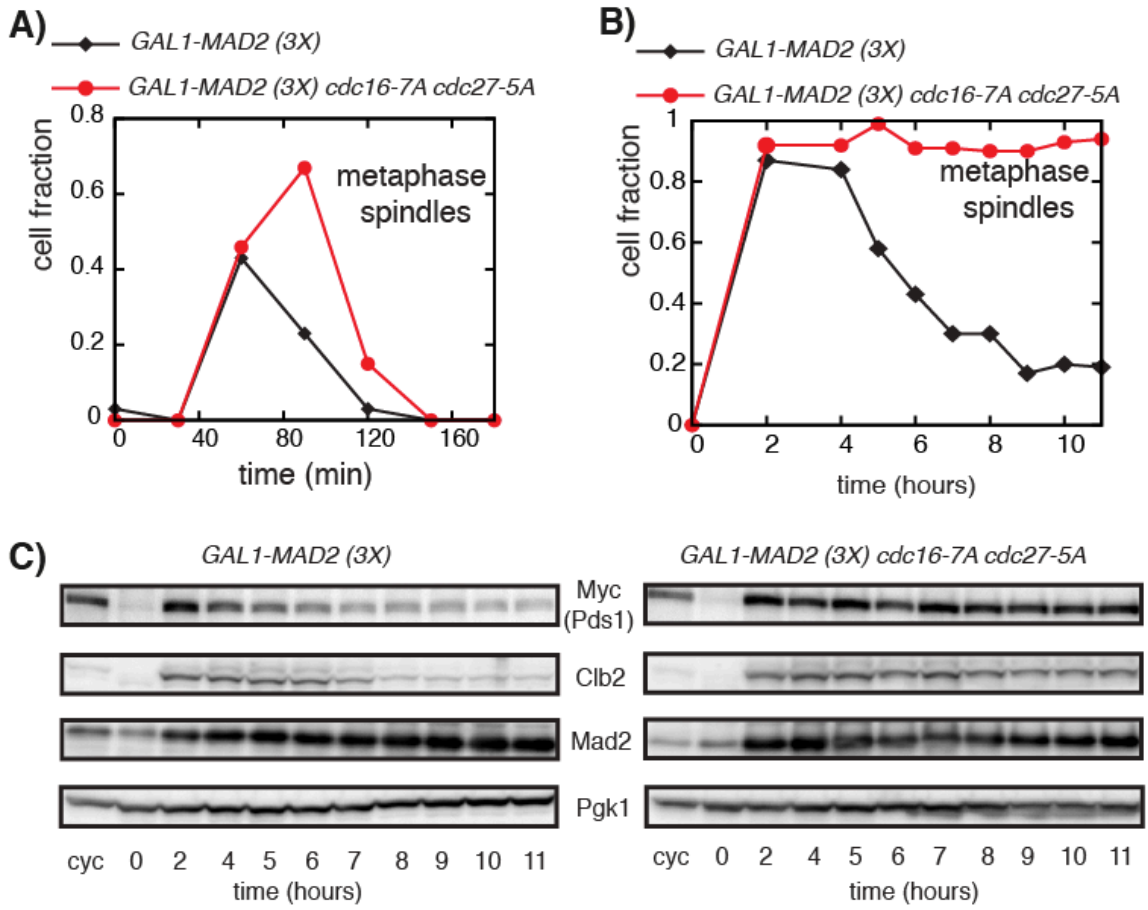

Figure 9. Phosphorylation of Cdc16 and Cdc27, two subunits of the APC/C, is essential for adaptation to the SAC. (A) *GAL1-MAD2(3x) PDS1-MYC18* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* cells were grown in raffinose medium (YEPR) at 30°C and arrested in G1 phase with α-factor for 2.5 hours. Then they were released from G1 into the cell cycle; α-factor was re-added after 80 minutes. Samples were taken for indirect immunofluorescence (IF) analysis of metaphase spindles. (B and C) *GAL1-MAD2(3x) PDS1-MYC18* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* cells were grown in raffinose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released in the presence of galactose to induce Mad2 overexpression from the *GAL1* promoter. 1.5 hours after release in galactose, α-factor was added again to resynchronize adapted cells in the next G1 phase. Samples were taken for immunofluorescence (IF) analysis of metaphase spindles (B) and for Western blotting (WB) analysis of Pds1, Clb2 , Mad2 and Pgk1 proteins (C). Adapted from Vernieri et al., 2013. This experiment is representative of three independent repeats.

I then addressed the requirement of APC/C coactivators, Cdc20 and Cdh1.

To inhibit Cdc20 in SAC arrested cells I used a strain in which Cdc20 expression is under the control of the methionine-repressible *MET3* promoter (i.e., addition of methionine to the culture medium represses Cdc20 synthesis). *GAL1-MAD2(3x) MET3-Cdc20-HA3* were released from G1-phase in galactose medium to induce the SAC through Mad2 overexpression. This medium lacked methionine to allow for Cdc20 expression from the *MET3* promoter. After 2 hours from the release the culture was spilt into two parts: the first one was kept in the medium lacking methionine; the second part was instead added methionine to repress Cdc20 expression. Cells grown in the absence of methionine expressed Cdc20 (Figure 10, right panel) and started to disassemble metaphase spindles about three hours after the release from G1. On the contrary, cells in which Cdc20 expression had been repressed by methionine (Figure 10, left panel) were unable to disassemble metaphase spindles and to adapt.

This experiment shows that Cdc20 is required for adaptation and that cells lacking this coactivator of APC/C are permanently arrested in mitosis. This result was highly expected, given the essential role of Cdc20 for the metaphase-to-anaphase transition during a regular cell cycle (Uhlmann et al., 1999).

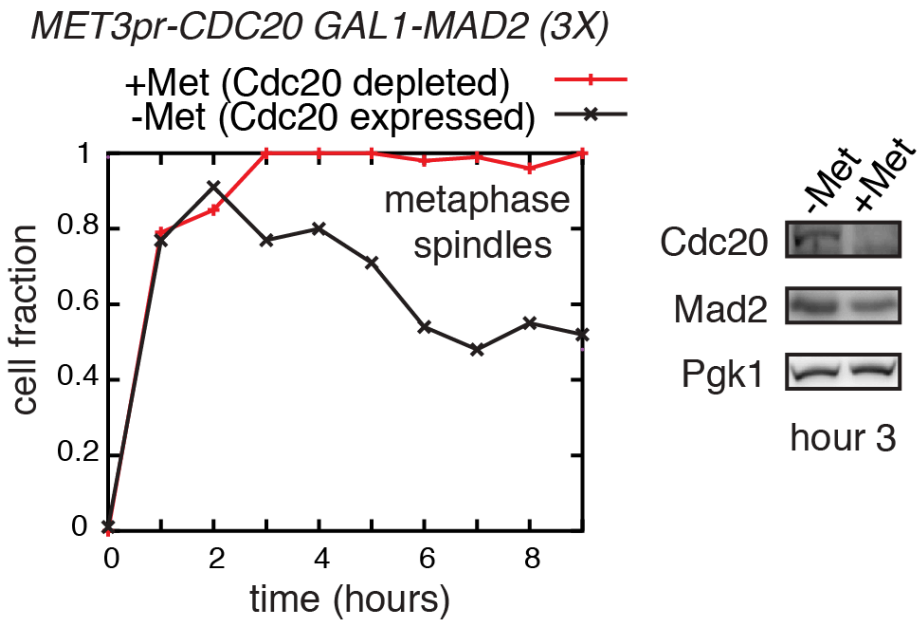

Figure 10. Cdc20 is essential for cells to adapt to the SAC. (A) *GAL1-MAD2(3X) PDS1-MYC18 MET3-CDC20-HA3* (yAC2138) cells were grown in minus-methionine synthetic medium at 30°C, synchronized in G1 with α-factor and released into galactose. 2 hours after the release α-factor was re-added and the culture was split into two halves: one half was kept in the same medium, the other one was added methionine to repress Cdc20 transcription Samples were taken at the indicated times for IF analysis (left) and at 3 hours after the release from α-factor for western blotting of Cdc20 (right). Adapted from Vernieri et al., 2013.

I then moved to test the requirement for the other coactivator of the APC/C, Cdh1, in the adaptation process. Cdh1 is non-essential during a regular cell cycle, but its active role in adaptation was proposed in the particular genetic context of Bub2 deletion (Toda et al., 2012). I decided to test Cdh1 involvement in adaptation in wild-type cells by comparing adaptation kinetics in cells expressing Cdh1 or not. I also used the double *cdc16-6A cdc27-5A* mutant as a positive control for an adaptation-defective strain. Given that *cdh1Δ* mutants can hardly be arrested in G1 by α-factor, in this experiment Mad2 overexpression was induced in cycling, asynchronous populations. *GAL1-MAD2(3x)*, *GAL1-MAD2(3x) cdh1Δ* and *GAL1-MAD2(3x) cdc16-6A cdc27-5A* cells

in log phase were grown in galactose to overexpress Mad2 and the kinetics of metaphase spindles assembly and disassembly were detected by immunofluorescence. As can be seen in Figure 11, *cdh1Δ* cells adapted with exactly the same kinetics as wild-type *CDH1* cells, while *cdc16-6A cdc27-5A* cells were permanently arrested in metaphase, as expected. This result excludes any essential requirement for Cdh1 in adaptation to the SAC.

I conclude that adaptation to the SAC requires the presence of Cdc20 and phosphorylation of Cdc16 and Cdc27 subunits of the APC/C; Cdh1 does not play any role in this phenomenon.

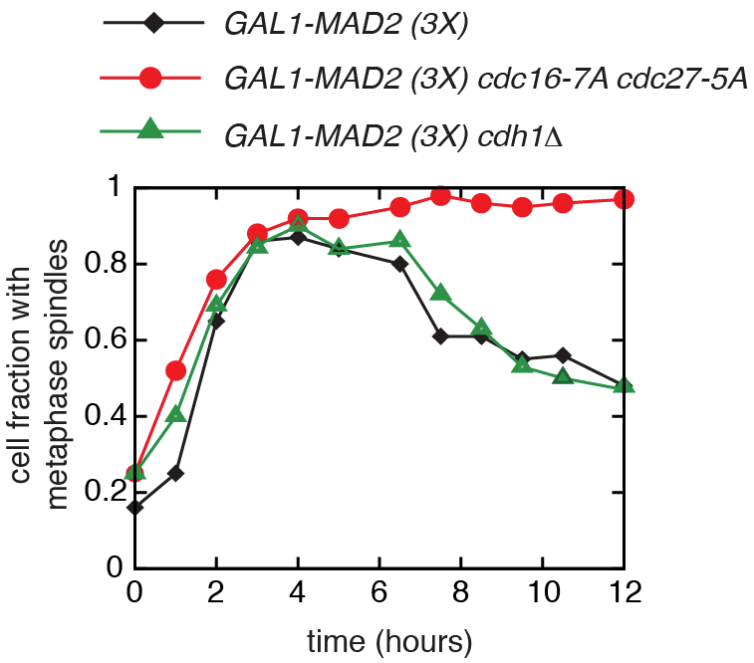

Figure 11. Cdh1 is dispensable for adaptation to the SAC. *GAL1-MAD2(3X) PDS1-MYC18*, *GAL1-MAD2(3X) PDS1-MYC18 cdh1Δ* and *GAL1-MAD2(3X) PDS1-MYC18 cdc16-6A cdc27-5A* were grown in YPER at 30°C; when in log phase, galactose 2% was added to the cultures. Samples were taken for IF analysis of metaphase spindles at indicated times. Adapted from Vernieri et al., 2013. This experiment is representative of two independent repeats.

1.2 Cdk1 inhibition does not inactivate the SAC

In the previous experiments, I demonstrated the requirement of Cdc20 for adaptation. The main question that now needs to be answered is how cells can activate APC/C^{Cdc20} during adaptation if Cdc20 is sequestered within the MCC.

Cdk1 has been proposed to have a role in keeping the SAC active both in yeast and in mammals (Amon, 1997; D'Angiolella et al., 2003; Kitazono et al., 2003). Coherently with this notion, adaptation to the SAC could be driven by the slow and progressive degradation of cyclin B, until a certain level of Cdk1 activity is attained, which is no more compatible with maintenance of the mitotic status (Brito and Rieder, 2006b). If this were true, ectopically inhibiting Cdk1 during a nocodazole arrest should switch off the checkpoint, accelerate adaptation and induce mitotic exit. To test this prediction, I decided to inhibit Cdk1 activity in cells arrested by the SAC and to see whether progressive inhibition of the kinase was associated with progressive decrease of adaptation time.

To modulate Cdk1 activity, I used a mutant version of Cdk1, cdc28-as1, which is inhibited by the ATP analog, 1NM-PP1 (Bishop et al., 2000). Increasing concentrations of 1NM-PP1 progressively inhibits cdc28-as1 kinase activity (Liang et al., 2012) and also progressively delays cell cycle progression (Bishop et al., 2000). For instance, 50nM 1NM-PP1 simply delay the cell cycle, 500nM completely inhibit mitosis, while 5μM are even incompatible with DNA replication.

I aimed at verifying that in my hands this mutant was behaving coherently with data reported in the literature: I synchronized *cdc28-as1* cells in G1 through α-factor and then I released them into the cell cycle in the presence of the following concentrations of 1NM-PP1: 0nM (only DMSO), 500nM, or 5μM. I monitored cell cycle progression by following DNA content (by FACS analysis) and metaphase spindles (by immunofluorescence analysis) kinetics. As expected, 500nM of 1NM-PP1 allowed

DNA replication but not metaphase spindle formation, while 5μM inhibited also DNA replication (Figure 12A and 11B).

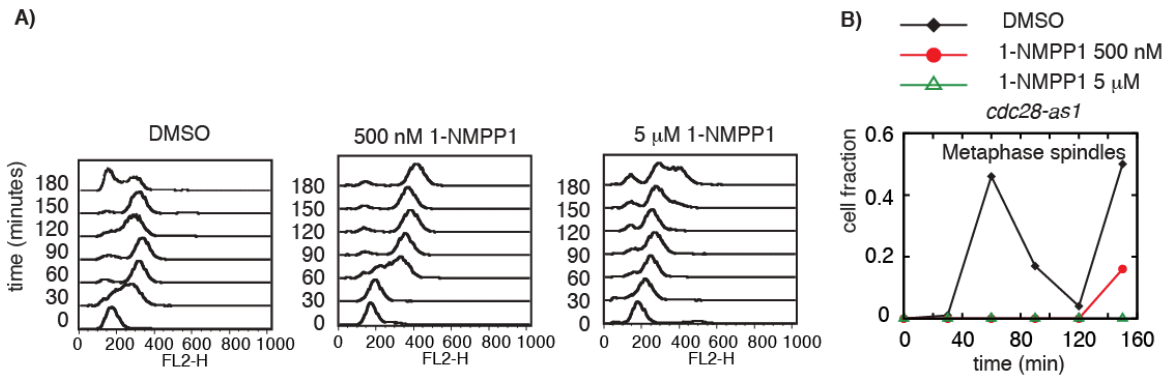

Figure 12. In *cdc28*-as1 cells 500nM of 1NM-PP1 inhibit metaphase; 5μM also block DNA replication. *cdc28-as1 PDS1-MYC18* cells were grown at 30°C in YEPD, synchronized in G1 phase with α-factor and then released from α-factor in YEPD plus DMSO, 500nM or 5μM 1NM-PP1. Samples were taken at the α-factor arrest and then every 30 minutes for FACS (A) and IF (B)analysis. Adapted from Vernieri et al., 2013.

To test the involvement of Cdk1 in the SAC, I used two high concentrations of 1NM-PP1, 500nM and 5μM, because I reasoned that if Cdk1 has any role in maintaining the SAC, strong inhibition of the kinase would surely inactivate the checkpoint.

First, I analyzed the role of Cdk1 in keeping the SAC cascade active at the kinetochores. As explained in the Introduction (see Paragraph 4.1), when the SAC is activated by disrupting the mitotic spindle, Mad2 localizes at the kinetochores; when the checkpoint is switched off, Mad2 disperses again within the nucleus and clusters at the nuclear pores. In budding yeast, kinetochores cluster in mitosis, and, upon SAC activation, GFP-tagged Mad2 can be visualized as a single fluorescent dot localized at the site of kinetochores clustering. When the checkpoint is inactivated, the dot disappears and Mad2 can be visualized at the nuclear membrane. I followed Mad2-3GFP localization to monitor the activation status of the SAC at the kinetochores. To

be sure that the GFP fluorescent dot actually corresponded to Mad2 localization at the kinetochores, I followed Mad2-3GFP colocalization with Nuf2, a protein that is constitutively localized at kinetochores during mitosis (Musacchio and Salmon, 2007).

I arrested *cdc28-as1 MAD2-3GFP NUF2-mCherry* cells in nocodazole for 3 hours, and then I treated them with DMSO alone, DMSO plus 500nM 1NM-PP1, or DMSO plus 5μM 1NM-PP1 while keeping nocodazole in the medium. A fourth portion of cells was released from nocodazole as a control for SAC inactivation and Mad2-3GFP delocalization from the kinetochores. 2 hours after treatment with 1NM-PP1 or nocodazole release, I counted the percentages of cells in which Mad2-3GFP colocalized with Nuf2-mCherry.

As expected, cells released from the SAC efficiently delocalized Mad2 from kinetochores. Quite surprisingly, in both 500nM and 5μM 1NM-PP1 treated cells, Mad2 remained at the kinetochores in percentages that were comparable to nocodazole-arrested cells treated with only DMSO. This result shows that even strong Cdk1 inactivation does not switch off the SAC cascade at the kinetochores (figure 13).

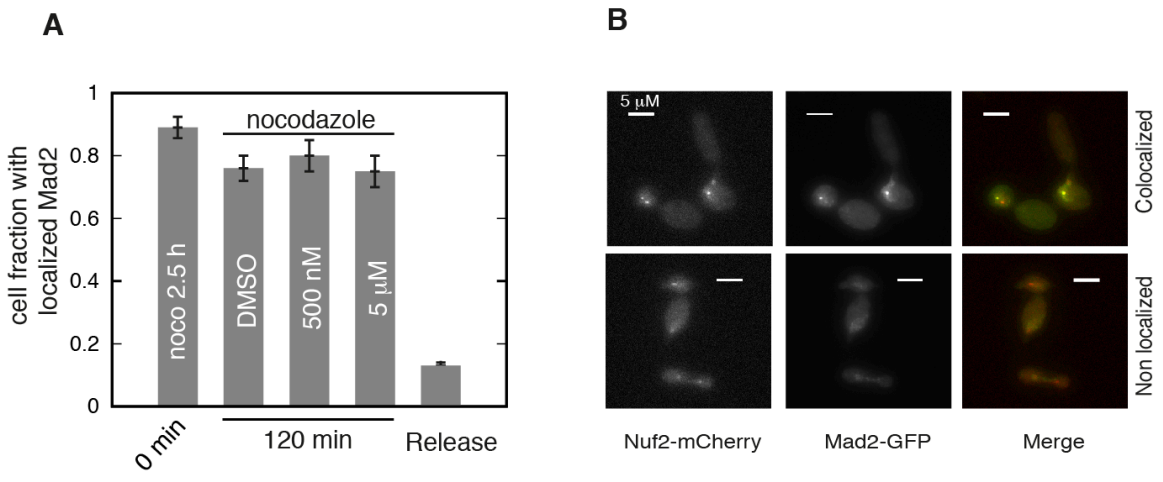

Figure 13. Inhibition of cdc28-as1 with 500nM and 5μM of 1NM-PP1 does not induce delocalization of Mad2 from kinetochores. (A and B) *cdc28-as1MAD2-3GFP NUF2-mCherry* cells were grown in YEPD at 30°C and then arrested in nocodazole for 2.5 hours. Then the culture was split into four parts: three parts of the four were given DMSO, 500nM and 5μM of 1NM-PP1 respectively,

while keeping nocodazole in the medium. The fourth part was released from nocodazole into fresh medium. Samples were taken at nocodazole arrest (2.5 hours) and 2 hours after culture split into the four indicated conditions for analysis of colocalization between Mad2-3GFP and Nuf2-Cherry. Histograms are reported that show colocalization percentages in the different conditions (A). One example of colocalization (B, upper panels) and one of non-colocalization (B, lower panels) between Mad2-3GFP and Nuf2-mCherry is shown. Adapted from Vernieri et al., 2013. This experiment is representative of three independent repeats.

At different time points during the experiment I verified by immunofluorescence that cells in nocodazole were lacking polymerized microtubules and that, on the contrary, cells released from nocodazole formed mitotic spindles again (data not shown).

The fact that Mad2 is not delocalized from kinetochores upon Cdk1 inhibition does not exclude that other essential SAC components actually are, or that Cdk1 maintains the checkpoint signal downstram of the kinetochores.

To test these possibilities, I looked at the effects of Cdk1 inhibition directly on APC/C^{Cdc20} activation. During a SAC arrest, Cdc20 levels are more or less constant (due to balanced synthesis and degradation), and the key substates of APC/C^{Cdc20}, Pds1 and Clb2, are stable (Musacchio and Salmon, 2007; Pan and Chen, 2004). Therefore, the activation status of APC/C^{Cdc20} can be monitored by looking at the stability/instability of Pds1 and Clb2. If Cdk1 has a role in stabilizing the MCC and maintaining the SAC, its inhibition during a SAC arrest should lead to Pds1 and Clb2 degradation.

I arrested *cdc28-as1* cells in nocodazole for 3 hours, and then I treated them with DMSO, 500nM 1NM-PP1, or 5μM 1NM-PP1, while keeping nocodazole in the medium.

Lack of mitotic spindles by IF analysis confirmed that nocodazole was properly working in depolymeryzing microtubules. Moreover, FACS analysis of DNA content confirmed that my cells in all indicated conditions arrested in mitosis (figure 14).

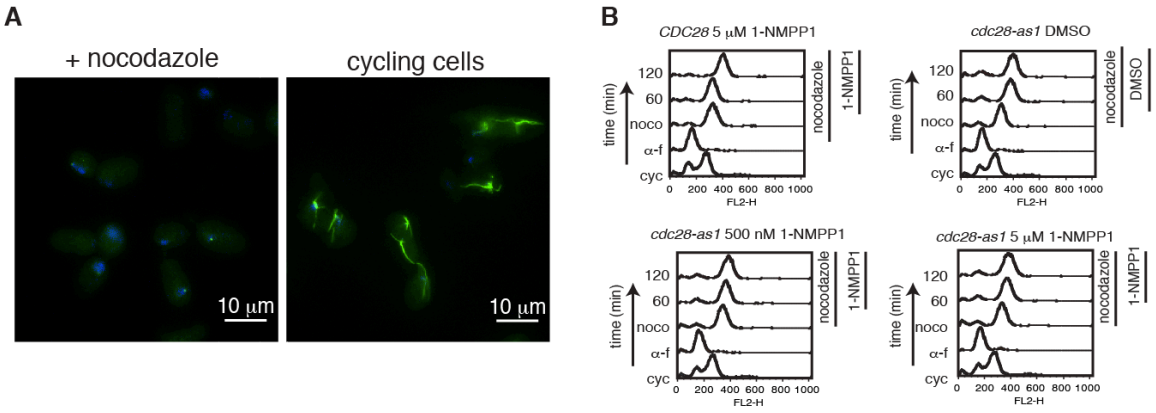

Figure 14. Cdk1 inhibition does not induce APC/C^{Cdc20} reactivation and SAC override (see also Figure 15). *PDS1-MYC18* and *cdc28-as1 PDS1-MYC18* cells were grown at 30°C in YEPD, synchronized in G1 phase with α-factor and then released from α-factor in nocodazole medium. After 3 hours in nocodazole, *CDC28* cells were given 5μM 1NM-PP1, while *cdc28-as1* cells were added DMSO, 500nM 1NM-PP1 or 5μM 1NM-PP1. In left panels I show two IF fields: one of nocodazole-treated cells (left), which clearly lack spindles, the other one belonging to a cycling population (right), which contains interphasic, (no spindles), metaphasic (short spindles) and anaphasic (elongated spindles) cells. In right panels I report FACS analysis showing the G2/M arrest of the two mutants in all treatment conditions. Adapted from Vernieri et al., 2013.

When I looked at the stability of the substrates of APC/C^{cdc20}, I verified that Pds1 and Clb2 were stable in cells treated with DMSO only just as in wild type cells (figure 15, A and B). Quite surprisingly, also cells treated with 500nM 1NM-PP1 did not degrade Pds1 and Clb2, suggesting that the SAC was not switched off and the APC/C was not re-activated under conditions of substantial Cdk1 inhibition (figure 15C). On the contrary, Cdc20 levels were significantly reduced upon Cdk1 inhibition, coherently with the role of Cdk1 in promoting Cdc20 synthesis in mitosis (Liang et al., 2012) (figure 15C). The fact that cdc28-as1 inhibition with 500nM kept Pds1 and Clb2 stable

while inducing the degradation of the main APC/C activator, Cdc20, prompted us to reconsider the role of Cdk1 in SAC maintenance:

1) Cdk1 role in inducing Cdc20 synthesis (and APC/C activation) suggests an anti-SAc activity of Cdk1;
2) it is unlikely that adaptation to the SAC occurs via degradation of Clb2, at least in budding yeast: 500nM of 1NM-PP1 reduce cdc28-as1 activity to pre-mitotic levels (figure 12), which could be sufficient to force SAC inactvation and mitotic exit, but these events did not occur in my experiment.

With higher concentrations of ATP analog (5μM), Cdc20 degradation was even faster than with 500nM, again in accordance with the essential role of Cdk1 in promoting Cdc20 synthesis. However, in these conditions, also APC/C substrates Pds1 and Clb2 were degraded, although with significant delay compared to Cdc20 (Figure 15D). This results is coherent with published data where strong Cdk1 inhibition was obtained via Sic1 overexpression (Amon, 1997).

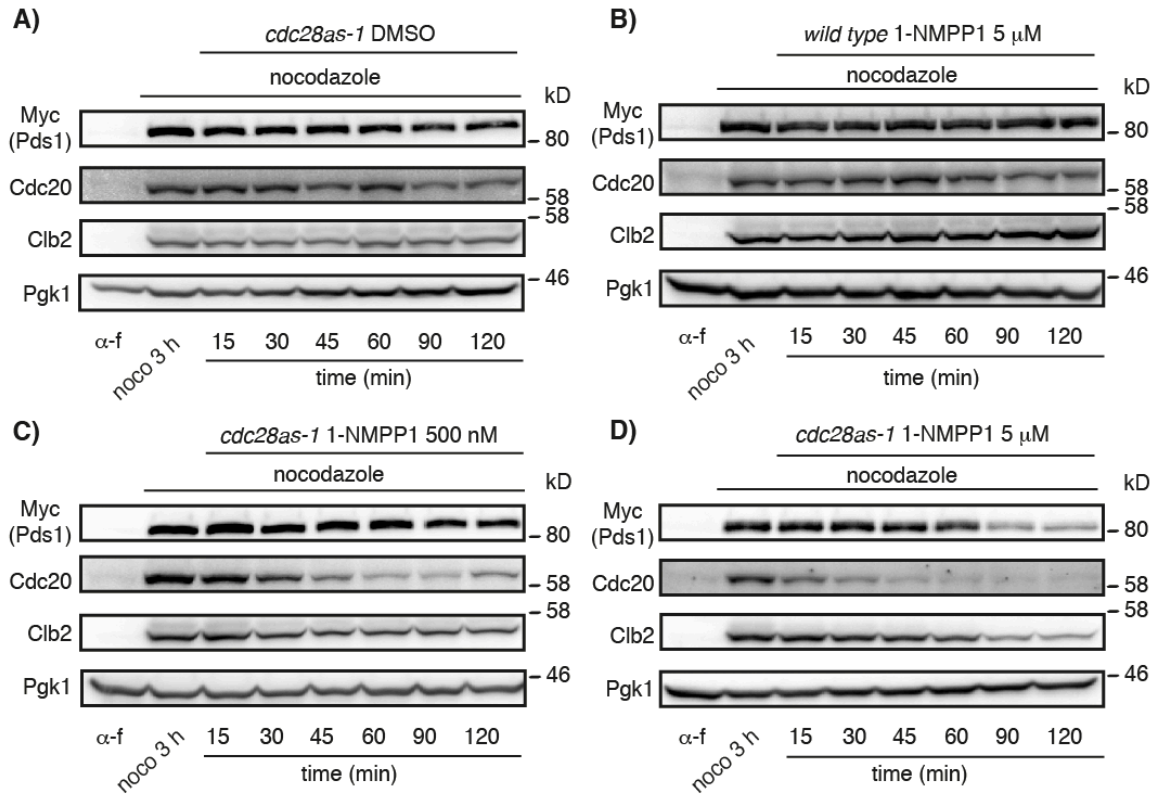

Figure 15. Cdk1 inhibition does not induce APC/C^{Cdc20} reactivation and SAC override. *PDS1-MYC18* and *cdc28-as1 PDS1-MYC18* cells were grown at 30°C in YEPD, synchronized in G1 phase with α-factor and then released from α-factor in nocodazole medium. After 3 hours in nocodazole, *CDC28* cells were given 5μM 1NM-PP1 (B), which *cdc28-as1* were added DMSO (A), 500nM 1NM-PP1 (C) or 5μM 1NM-PP1(D). At the same time, α-factor was also re-added to all cultures. Kinetics of Pds1, Cdc20 and Clb2 proteins in different conditions were analyzed by WB analysis. Adapted from Vernieri et al., 2013. The results shown in this panels are representative of five independent experiments.

One could be tempted to conclude from this experiment that Cdk1 has some role in SAC maintenance, because strong inhibition of its kinase activity with 5μM of 1NM-PP1 is associated to degradation of APC/C substrates. I believe that this interpretation is wrong, because degradation of Pds1 and Clb2 occurs when Cdc20, the main target of the SAC and main activator of the APC/C, has been degraded. It is rather more reasonable to believe that powerful, ectopic inhibition of Cdk1 activates parallel pathways that lead to activation of APC/C independently from Cdc20.

One good candidate for such an ectopic activator of APC/C is Cdh1, which is inhibited by Cdk1 phosphorylation (Zachariae et al., 1998): almost complete inactivation of cdc28-as1 with 5μ of 1NM-PP1 could relieve Cdk1 inhibitory effect on Cdh1 and lead to its massive activation against Pds1 and Clb2.

Before directly testing this hypothesis, I wanted to be sure that cdc28-as1 inhibition does not override the SAC also when Cdc20 is still synthetized. To test it, I needed to uncouple Cdc20 synthesis from cdc28-as1 activity. It is known from the literature that Cdk1 promotes Cdc20 transcription by phosphorylating and consequently inhibiting Cdc20 transcriptional repressor, Yox1 (see Introduction paragraph 4.4). I reasoned that deletion of *YOX1* would allow us to motitor the effects of Cdk1 inhibition on APC/C activity in the presence of Cdc20.

Given that with maximal cdc28-as1 inhibition (5μM of 1NM-PP1) Cdc20 synthesis is not rescued by *YOX1* deletion (data not shown), in this experiment I used 500nM of 1NM-PP1 to inhibit cdc28-as1. For my purposes this concentration is high enough, because it inhibits cdc28-as1 to pre-mitotic levels and allows us understand if mitotic Cdk1 activity is necessary to keep the SAC active in the presence of Cdc20.

I arrested G1-released *cdc28-as1 yox1Δ* cells in nocodazole for 3 hours; then I either added DMSO or 500nM of 1NM-PP1 to the media and followed the cells for additional two hours. Arrest in mitosis for the whole duration of the experiment was confirmed by IF and FACS analysis (data not shown). I again monitored Pds1 and Clb2 levels by Western blot as indicators of APC/C inhibition. As expected, deletion of YOX1 stabilized the levels of Cdc20 even after cdc28-as1 inhibition (figure 16B). Importantly, even with stable levels of Cdc20, inhibition of cdc28-as1 did not lead to degradation of Pds1 and Clb2, which were as stable as in untreated cells (figure 16, A and B). These data further support the claim against a role of Cdk1 in maintaining the SAC and reinforce my conclusion that the main role of Cdk1 in the SAC is to guarantee a continuous stimulation of Cdc20 synthesis, and not to inhibit APC/C.

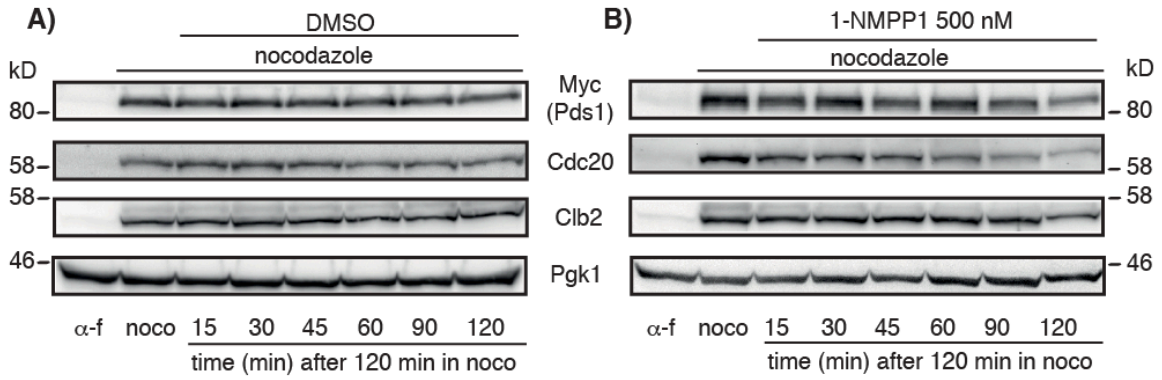

Figure 16. Even in the presence of Cdc20, Cdk1 inhibition does not override the SAC by activating APC/C. (A and B) *cdc28-as1 PDS1-MYC18 yox1Δ* cells were grown at 30°C in YEPD, synchronized in G1 phase with α-factor and then released from α-factor in nocodazole medium. After 3 hours in nocodazole, either DMSO (A) or 500nM of 1NM-PP1 (B) were added to the medium together with α-factor. Adapted from Vernieri et al., 2013. This experiment is representative of three repeats.

1.3 Cdh1 is required for ectopically bypassing the SAC upon Cdk1 inhibition

I then decided to directly test the hypothesis that complete inhibition of Cdk1 leads to degradation of Pds1 and Clb2 by inducing strong, ectopic activation of APC/C^{Cdh1}. If my hypothesis were true, deletion of *CDH1* should stabilize Pds1 and Clb2 even after cdc28-as1 inhibition with 5μM of 1NM-PP1.

Unfortunately, when I crossed the two haploid strains with *cdc28-as1* and *cdh1Δ* genotypes to produce the double mutant *cdc28-as1 cdh1Δ*, I discovered that the two mutations are synthetic lethal. I was then forced to use another approach to answer my question: I decided to inhibit Cdk1 activity by overexpressing its physiological inhibitor, Sic1, from the *GAL1* promoter (Amon, 1997). I first verified that Sic1 overexpression affected the cell cycle of *wild-type* cells similarly to 5μM of 1NM-PP1: *GAL1-SIC1* and *GAL1-SIC1 cdh1Δ* cells were syhchronized in G1 phase and then released into the cell cycle in glucose or galactose-containing media. Glucose represses expression from the *GAL1* promoter and only allows transcription of endogenous Sic1: as a consequence, cells released from G1 in glucose replicated DNA and completed the cell cycle in about 2.5 hours (figure 17, left panels). On the contrary, Sic1 overexpression in galactose completely inhibited DNA replication, similarly to 5μM of 1NM-PP1 (figure 17, right panels).

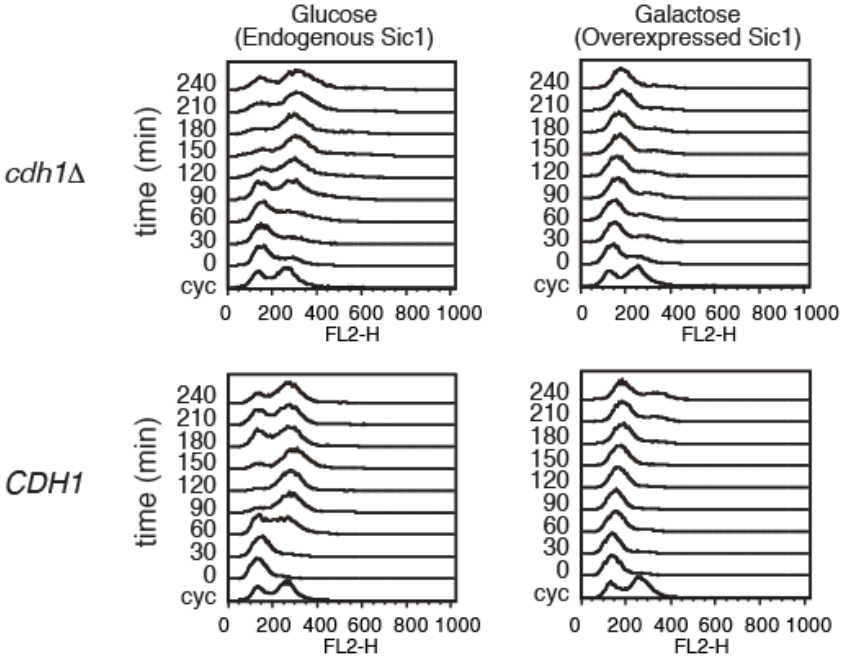

Figure 17. Sic1 overexpression inhibits DNA replication in both *CDH1* and *cdh1Δ* cells. *GAL-SIC1 PDS1-MYC18* and *GAL-SIC1 PDS1-MYC18 cdh1Δ* cells were grown at 23°C in raffinose-containing medium and arrested in α-factor for 3 hours. Then they were released into either glucose (left panels) or galactose (right panels) to respectively repress or induce Sic1 overexpression. Samples were taken for FACS analysis at indicated times. Adapted from Vernieri et al., 2013.

Having confirmed that Sic1 overexpression inhibits cells carrying wt Cdk1 similarly to what 5μM of 1NM-PP1 do in *cdc28-as1* mutants , I tested the role of APC/C^{Cdh1} ectopic activation on Pds1 and Clb2 degradation after Cdk1 inhibition in the SAC.

GAL1-SIC1 and *GAL1-SIC1 cdh1Δ* cells were arrested in nocodazole for 2.5 hours, and then either glucose (to inhibit Sic1 overexpression) or galactose (to induce Sic1 overexpression) were added to the culture. The levels of Pds1, Clb2 and Cdc20 were then monitored for 2.5 hours. In cells treated with glucose, Pds1 and Clb2 remained stable both in the presence and in the absence of Cdh1, as expected (figure 18A, upper panel); strikingly, cells overexpressing Sic1 (thus inhibiting Cdk1) due to galactose addition degraded Pds1 and Clb2 specifically in the presence of Cdh1, but not in its absence (figure 18B, upper panel). In both strains, Cdc20 was degraded

upon as a result of Cdk1 inhibition. This confirms that, upon strong Cdk1 inhibition, the SAC is not switched off via APC/C^{Cdc20} reactivation, but it is bypassed via the unphysiological activation of APC/C^{Cdh1} against Pds1 and Clb2.

FACS analysis of DNA content confirmed that both mutants were arrested in G2/M (double DNA content) after 2.5 hours in nocodazole and also after addition of either glucose or galactose (figure 18, A and B).

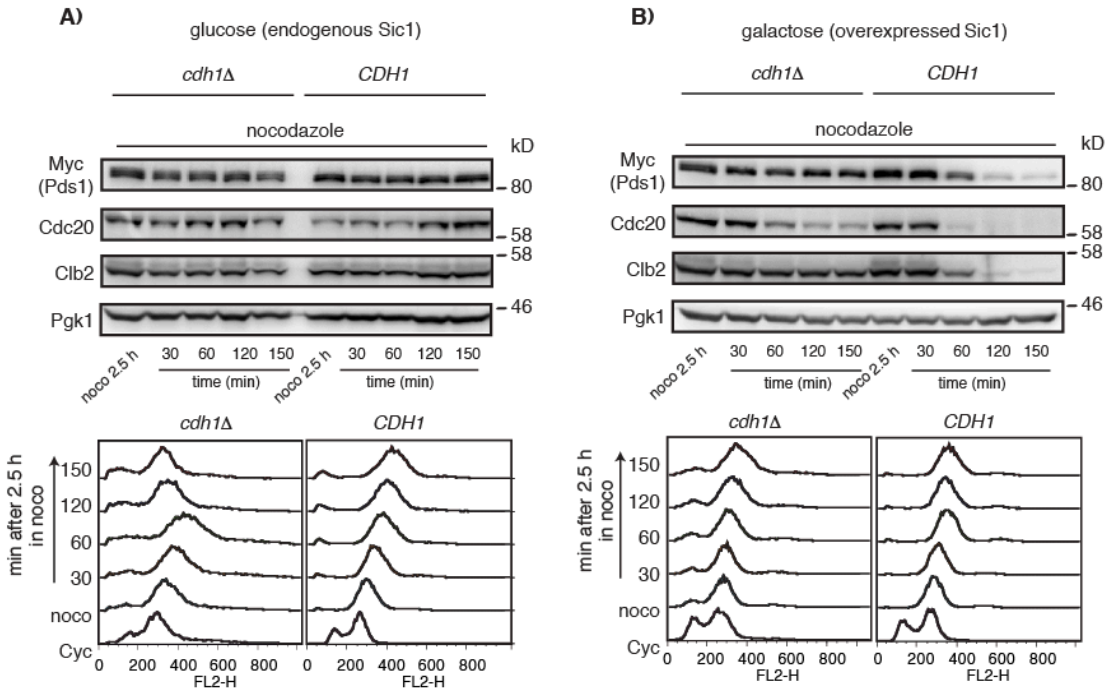

Figure 17. Pds1 and Clb2 degradation upon Cdk1 inhibition in nocodazole depends on Cdh1. *GAL-SIC1 PDS1-MYC18* and *GAL-SIC1 PDS1-MYC18 cdh1Δ* cells were grown at 23°C in raffinose-containing medium and arrested in nocodazole for 2.5 hours. Then they were either given glucose to repress Sic1 expression (A), or galactose to induce Sic1 overexpression (B) together with α-factor. They were then followed for other 2.5 hours. Samples were taken for WB (upper panels) and FACS (lower panels) analysis at indicated times. Adapted from Vernieri et al., 2013. This experiment is representative of two independent repeats.

One could argue that massive APC/C^{Cdh1} activation could take part in Pds1 and Clb2 degradation during adaptation. I believe this is very unlikely for the following reasons:

1) I have previously shown that Cdh1 is completely dispensable in adaptation (see figure 12);
2) APC/C^{Cdh1} activation in nocodazole is obtained only upon very strong inhibition of Cdk1 which lowers its activity to levels typical of the G1 phase (figure 17, right panels). It is very hard to believe that such low levels of Cdk1 activity can be reached before adaptation, when cells are in prometaphase/metaphase;
3) APC/C^{Cdh1} is active when Cdk1 activity is still significant, but only if DNA masses are physically separated and Cdc14 is released from the nucleolus (see Introduction, paragraph 2). This is not the case of a SAC arrest, when cohesin is stable, DNA masses unseparated and Cdc14 inactive.

1.4 Cdk1 is essential for adaptation to the SAC

Previous experiments suggest that Cdk1 activity is required during a SAC arrest to continuously induce synthesis of Cdc20, which is required for adaptation (see figures 9 and 14). Moreover, Cdk1-mediated phosphorylation of Cdc16 and Cdc27 subunits of the APC/C is also required to adapt (Figure 9). These two pieces of data suggest that the main role of Cdk1 in the SAC is not to promote checkpoint maintenance, but to stimulate adaptation to the checkpoint. This presumptive role would be more in line with the physiological role of Cdk1 in the cell cycle: to promote transitions from one phase to the next one. In the case of SAC adaptation, Cdk1 would promote transition into anaphase after a prolonged arrest in prometaphase/metaphase.

If my conclusion is correct, even partial inhibition of Cdk1 should strongly delay adaptation. I decided to test this hypothesis by using the *cdc28-as1* mutant again, but with low concentrations of 1NM-PP1 (50nM), which only induce a mild delay in an unperturbed metaphase-to-anaphase transition (figure 19, A and B).

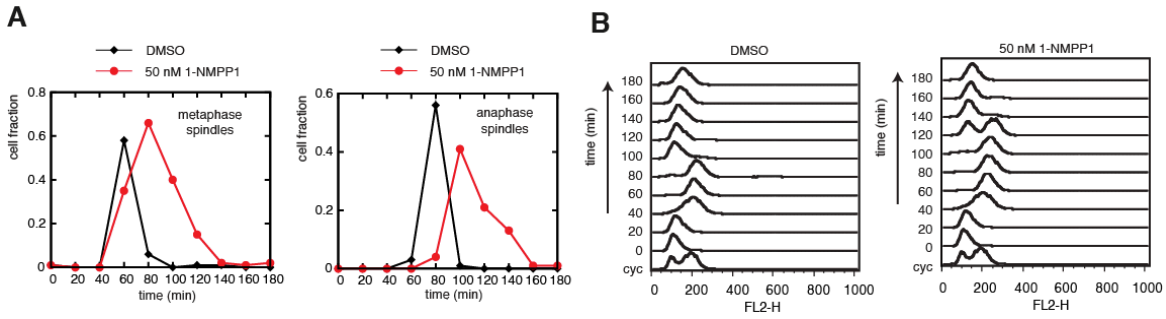

Figure 19. 50nM of 1NM-PP1 only mildly delay the metaphase-to-anaphase transition in unperturbed cell cycle in *cdc28-as1* cells. *GAL1-MAD2(3x) PDS1-MYC18 cdc28-as1* cells were grown in glucose medium at 30°C, arrested in G1 phase with α-factor for 2 hours and then released from α-factor into the cell cycle. 65 minutes after the release, when 90% of cells were small budded, either DMSO or 50nM of 1NM-PP1 were added to the medium, together with α-factor. Samples were taken for IF (A) and FACS (B) analysis at indicated time points. Adapted from Vernieri et al., 2013.

GAL1-MAD2(3x) cdc28-as1 cells were released from a synchronous arrest in G1 in the presence of galactose to induce Mad2 overexpression and SAC activtion. After 75 minutes, when all cells were presumably mitotic (with large buds), the culture was split into two: one half was given DMSO, the other 50nM 1NM-PP1 to partially inactivate cdc28-as1. The effect of 50nM 1NM-PP1 was striking: while untreated cells started adaptation about 3 hours after release from G1, cells treated with the ATP analog were still arrested in metaphase after 8 hours, with stable Pds1 and Clb2, and a 2C DNA content (Figure 20, A, B anc C).

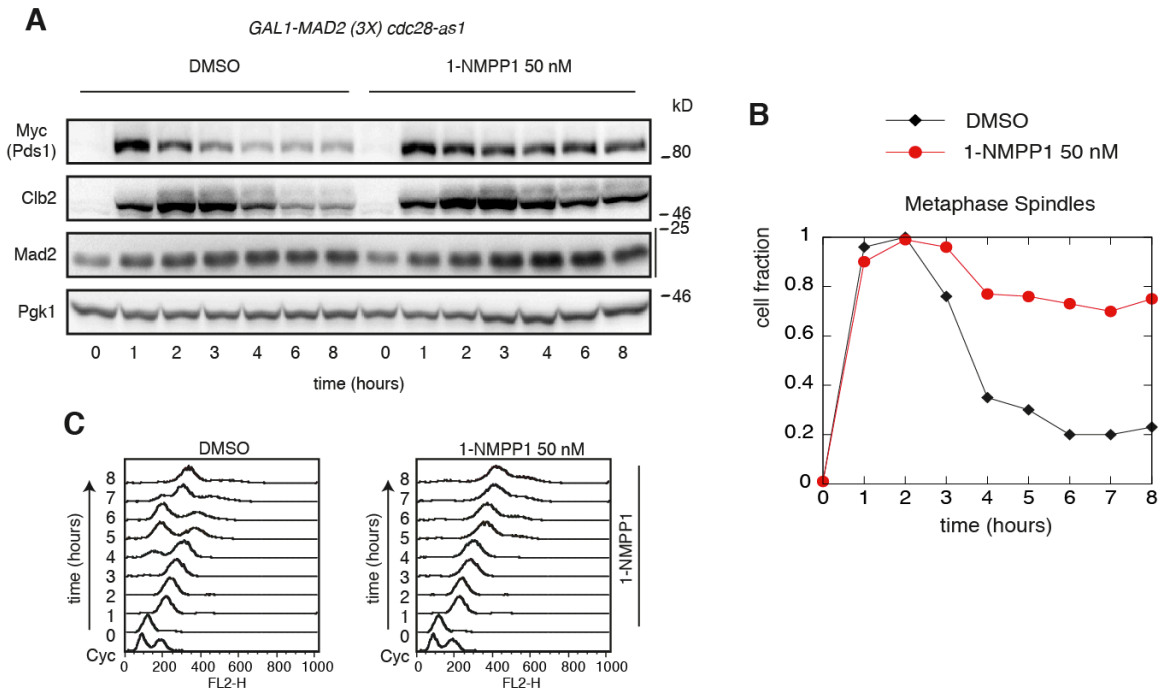

Figure 20. 50nM of 1NM-PP1 strongly inhibits adaptation in *cdc28-as1* cells. *GAL1-MAD2(3x) PDS1-MYC18 cdc28-as1* cells were grown in raffinose medium at 30°C, arrested in G1 phase with α-factor for 2 hours and then released from α-factor in the presence of galactose. After 2 hours, α-factor was re-added to the cells, together with either DMSO or 50nM of 1NM-PP1. Samples were taken for WB (A), IF (B) and FACS (C) analysis at indicated time points. This experiment is representative of five independent repeats.

I also confirmed that the activity of the ATP analog was specific for cdc28-as1 mutant: when I repeated the same experiment on *CDC28* cells, no delays in adaptation were observed in the population treated with 50nM 1NM-PP1 similarly to the control (Figure 21, A, B and C).

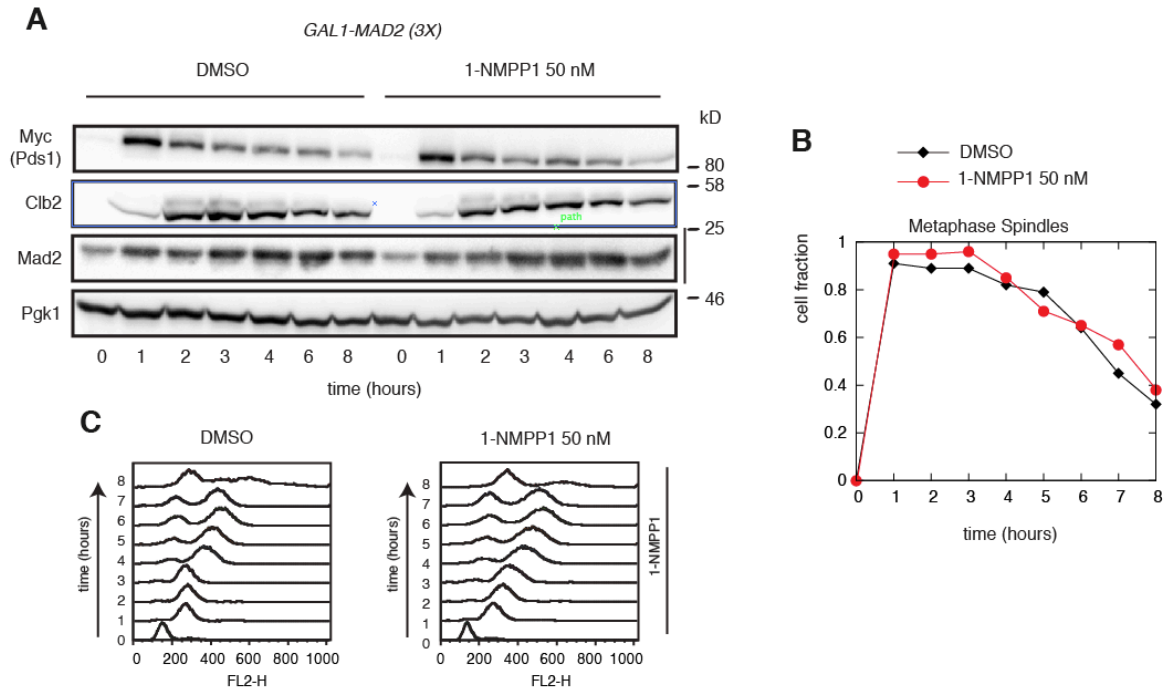

Figure 21. 50nM of 1NM-PP1 do not delay adaptation in cells containing wild-type Cdc28 (Cdk1). *GAL1-MAD2(3x) PDS1-MYC18* cells were grown in raffinose medium at 30°C, arrested in G1 phase with α-factor for 2 hours and then released from α-factor in the presence of galactose. After 2 hours, α-factor was re-added to the cells, together with either DMSO or 50nM of 1NM-PP1. Samples were taken for WB (upper left panel), IF (right panel) and FACS (lower left panel) analysis at indicated time points. Adapted from Vernieri et al., 2013.

This experiment shows that even partial inactivation of Cdk1 strongly inhibits adaptation to the SAC, and arrests cells in metaphase for a very long time.

I don't know if the effect of small 1NM-PP1 concentrations is mainly due to partial inhibition of Cdc20 synthesis or to reduced phosphorylation of APC/C subunits . I tend to favour the second hypothesis, because in an independent experiment I have observed that cdc28-as1 inhibition with 50nM of 1NM-PP1 does not significantly alter the steady state levels of Cdc20 during a SAC arrest (data not shown).

1.5 The APC/C is rapidly (and irreversibly) activated just before adaptation

The most accepted model of adaptation to the SAC is the slippage model (see Introduction, paragraph 5.2), which assumes that APC/C^{Cdc20} is incompletely inhibited by the SAC: some residual activity would be responsible for the progressive degradation the mitotic cyclin, until until cells slip out of mitosis.

This model seems to be confirmed by kinetics of slow Pds1 and Clb2 degradation in a population of yeast cells adapting to Mad2 overexpression (Figure 9C). However, such slow degradation at the population level could also be caused by the high asynchrony that characterizes adaptation in these cells (Figure 9B), and thus it could be rather an artifact.

I therefore moved to single cell analysis to better investigate how Clb2 degradation is temporally associated with adaptation. *GAL1-MAD2(3x) Clb2-GFP Tub2-Cherry* cells were grown in flow chambers in synthetic medium containing raffinose. Then galactose was added to the medium to induce Mad2 overexpression; GFP and Cherry signals were acquired every 10 minutes to follow Clb2 kinetics and progression through and out of mitosis, respectively. Quantification of Clb2-GFP nuclear signals in single cells revealed two interesting and quite surprising facts: Clb2 progressively accumulated in cells arrested in metaphase by the SAC (characterized by a thick mitotic spindle) and was then rapidly (within about 20 minutes) degraded concomitantly with adaptation (marked by transition from thick metaphase to thin, elongated, anaphase spindle) (Figure 22, left upper panel). Clb2 peak levels in SAC arrested cells were twice as much those of cycling cells, as a result of progressive Clb2 accumulation caused by APC/C inhibition by the SAC (Figure 22, lower panels).

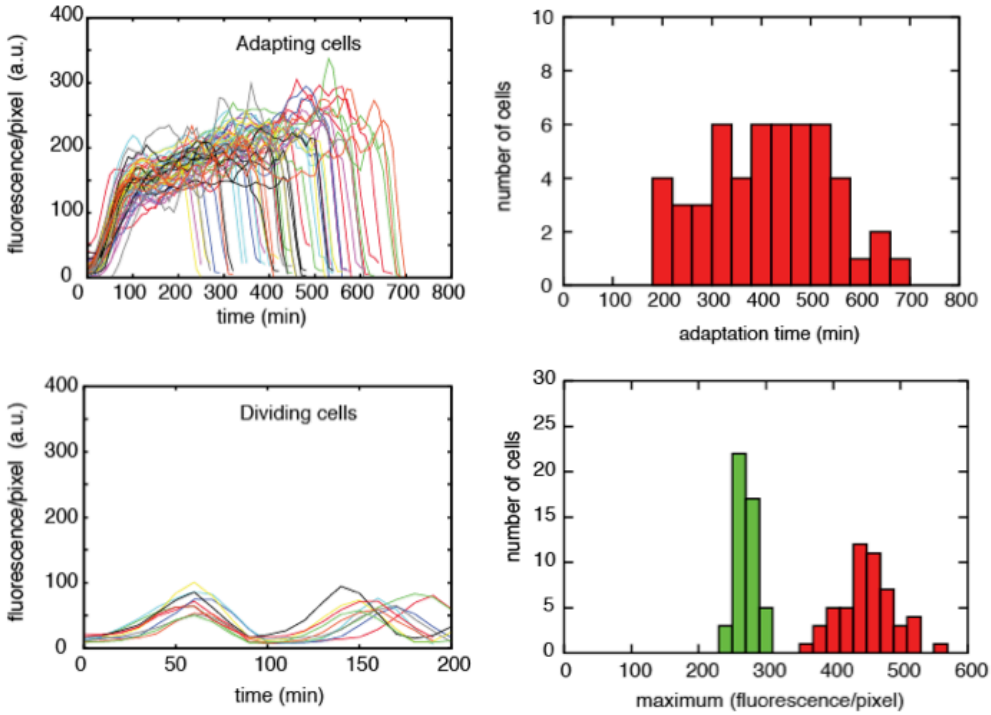

Figure 22. SAC arrested cells progressively accumulate nuclear Cdb2, which is then rapidly degraded at adaptation onset. *GAL1-MAD2(3x) CLB2-GFP TUB2-Cherry* cells were grown in raffinose-containing synthetic medium at 30°C. Then they were either added galactose to activate the checkpoint (upper left panel) or kept in raffinose to follow unperturbed cell cycle (lower left panel). In left panels, quantification of Clb2-GFP fluorescence intensity in single SAC-arrested (upper) and cycling (lower) cells is shown. In right panels, I show the distribution of adaptation times in SAC–arrested cells (upper) and distributions of Clb2-GFP fluorescence peak in both arrested and cycling cells (lower). Adapted from Vernieri et al., 2013.

I was very interested in confirming that such unexpected dynamics of Clb2 accumulation/ degradation during SAC arrest/adaptation were not specific for the Mad2 overexpression system, but could be also extended to other, and most commonly used, ways of inducing the SAC. As previously said, I had decided to resort to Mad2 overexpression instead of using nocodazole for both scientific reasons (it allowed us to focus on adaptation events downstream of the kinetochores) and for technical ones (at the concentrations used in yeast experiments, nocodazole wears off after about 5-6 hours). I observed at the population level that nocodazole inactivation

could be postponed by replenishing culture medium with fresh nocodazole. Since in flow chambers replenishment of the medium is continuous, I reasoned that maybe I would be able to observe adaptation even in nocodazole. To be sure about the effectiveness of nocodazole, I followed tubulin dynamics through Tub2-mCherry in single cells, which allowed us to select cells in which tubulin polymerization was still inhibited at the time of adaptation.

Clb2-GFP Tub2-Cherry cells were grown in flow chambers at 30°C in YEPD. Then nocodazole medium containing nocodazole was given to the cells in a regime of continuous flux. Even in these experimental setting, I confirmed that in most cells the mitotic spindle was reassembled before adaptation, due to nocodazole failure. Nevertheless, I was able to select a fraction of cells in which Clb2-GFP degradation and subsequent re-budding occurred when the mitotic spindle was clearly lacking. When I analyzed Clb2-GFP signal in these cells, I observed kinetics that were very similar to those observed when the SAC was induced via Mad2 overexpression: cells accumulated Clb2 in the nucleus, and then they rapidly degraded it (Figure 23, upper panel) before occurrence of re-budding (indicative of mitotic exit and entry into a new cell cycle) and yet in the absence of Tubulin clusters (Figure 23, lower panel).

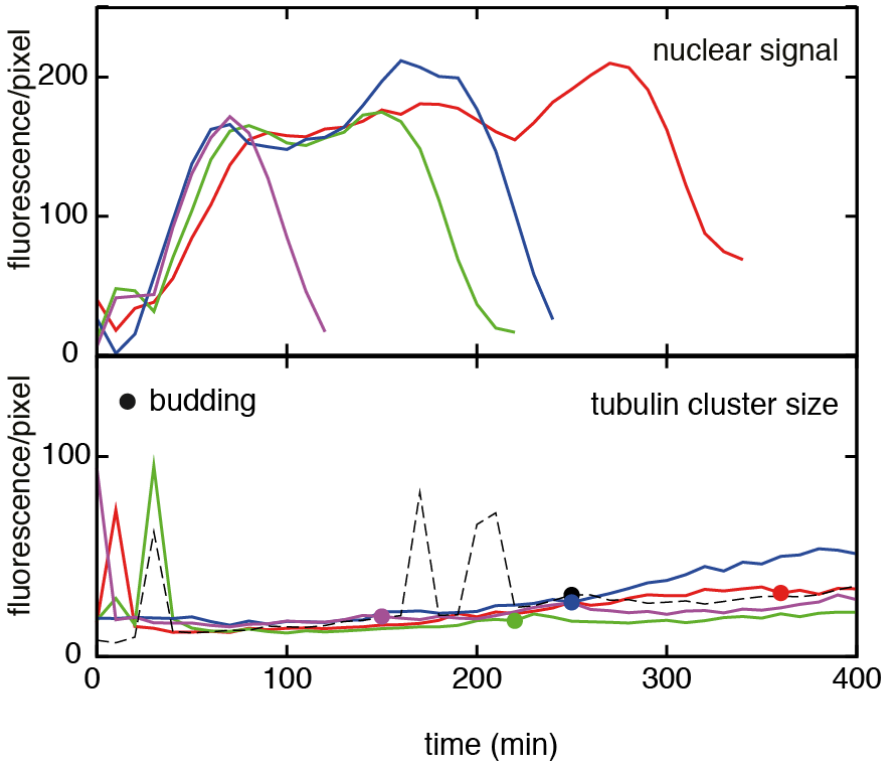

Figure 23. Cells treated with nocodazole accumulate Clb2 in the nucleus until adaptation, which coincides with its rapid degradation. *CLB2-GFP TUB2-Cherry* cells were grown in YEPD at 30ºC. Then nocodazole was added to activate the checkpoint. Kinetics of Clb2-GFP accumulation and degradation are shown in the upper panel for 4 adapting representative cells (upper panel). Absence of tubulin clusters (mitotic spindles) is confirmed for the same cells (lower panel). Dots indicate the time when cells that have adaptated to the SAC rebud (lower panel). Adapted from Vernieri et al., 2013.

In summary, these results show that the slow decay of Clb2 signal in western blots is the result of the tremendous asynchrony of the adaptation phenomenon (Figure 22, right, upper panel): Clb2 appears stable in western blots during the first hours of metaphase arrest because most cells are still accumulating the cyclin, while others have already degraded it. In the next hours, Clb2 signal appears as slowly, and not rapidly, declining because most cells have already degraded it, while a minority of them is still accumulating Clb2.

These experiments were very informative because they showed that:

1) the SAC is very effective in inhibiting APC/C^{Cdc20} because Clb2 progressively accumulates until cells remain arrested;
2) Clb2 degradation is very rapid and complete during adaptation, which suggests that APC/C^{Cdc20} is suddenly and irreversibly activated against its substrates.

Both these points are clearly in conflict with the slippage model, and suggest that in budding yeast APC/C^{Cdc20} activation is restrained during the whole SAC arrest, until an unknown event occurs, which activates APC/C^{Cdc20} in a switch-like manner.

1.6 PP2A^{Cdc55} dephosphorylates Cdc16 and thus inhibits adaptation

The increase in Clb2 levels observed at the single cell level is coherent with the positive role of Cdk1 in promoting adaptation to the SAC: increasing Clb2 is expected to result in increasing Cdk1 activity and APC/C phosphorylation, which is essential for adaptation.

If this were true, APC/C subunits should be progressively phosphorylated before adaptation, and then dephosphorylated again, concomitantly with cells re-entering the next G1 phase. I tested this prediction by synchronizing *GAL1-MAD2(3x)* cells in G1 phase and then releasing them in galactose medium to activate the SAC: the levels of Cdc16 phosphorylation were monitored by WB analysis with Phos-Tag reagent (P-Tag)-containing gels (Kinoshita et al., 2008). As can be seen from Figure 24 (left panel), some phosphorylation-specific bands appeared for Cdc16 during the SAC arrest, and they disappeared when cells started to adapt (as marked by metaphase spindle disassembly and Clb2 degradation) (right panel).

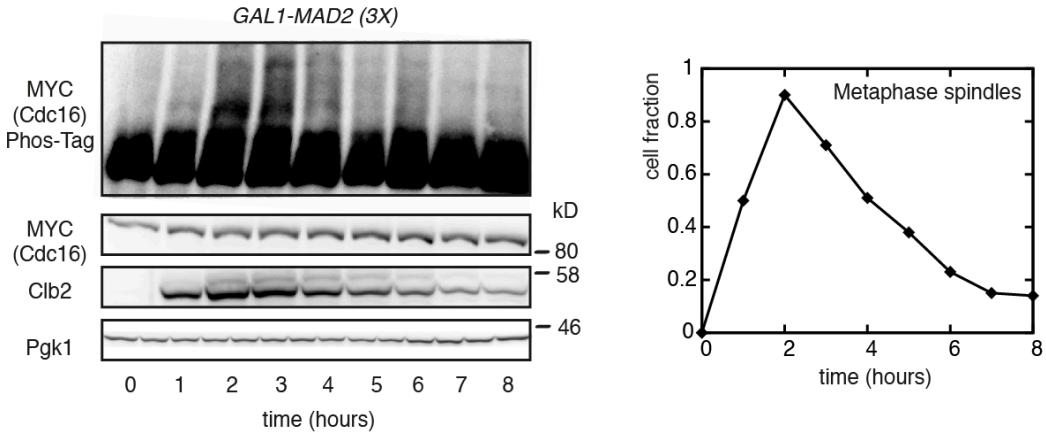

Figure 24. Cdc16 phosphorylation progressively increases during a SAC arrest and then it is reverted concomitantly with adaptation. *GAL1-MAD2(3x) CDC16-MYC6* cells were grown in raffinose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose. After 2 hours α-factor was re-added to the medium. Samples were taken at time 0 (α-factor arrest) and then every hour for Western blotting analysis of Cdc16 phosphorylation through Phos-Tag reagent (P-tag) acrylamide/bisacrylamide gels (left panel, upper part). The same extracts were also loaded on normal 10% acrylamide gels to check the total amount of Cdc16, Clb2 and Pgk1 (left panel, lower parts). Samples were also taken for IF analysis of metaphase spindles (right panel). Adapted from Vernieri et al., 2013.

I then asked why cells need to accumulate Clb2 for such a long time before Cdk1:Clb2 manages to phosphorylate APC/C subunits to a level that is sufficient to initiate adaptation. One possible hypothesis is that a phosphatase is active during the SAC arrest, continuously removing phosphate groups from APC/C subunits and thus opposing Cdk1 phosphorylation of APC/C subunits. Such a phosphatase would be therefore important in maintaining the SAC active, and its inhibition should accelerate adaptation or even make yeast cells SAC-defective.

$PP2A^{Cdc55}$ phosphatase is a good candidate: as explained in the Introduction (see Paragraph 3), cells lacking Cdc55 are unable to stabilize Pds1 in nocodazole. Moreover, deletion of *CDC55* partially rescues the temperature sensitivity of a mutant form of Cdc20, cdc20-1.

I first confirmed that *cdc55Δ* cells are SAC deficient also in the context of Mad2 overexpression: *GAL1-MAD2(3x)* and *GAL1-MAD2(3x) cdc55Δ* cells were released from G1 in the presence of overexpressed Mad2. While wild-type cells were arrested by the SAC for some hours before adaptation with stabilized Pds1 and Clb2, *cdc55Δ* cells failed to arrest in metaphase upon Mad2 overexpression and degraded most Pds1 and Clb2 within 3-4 hours after the release from G1 (Figure 25).

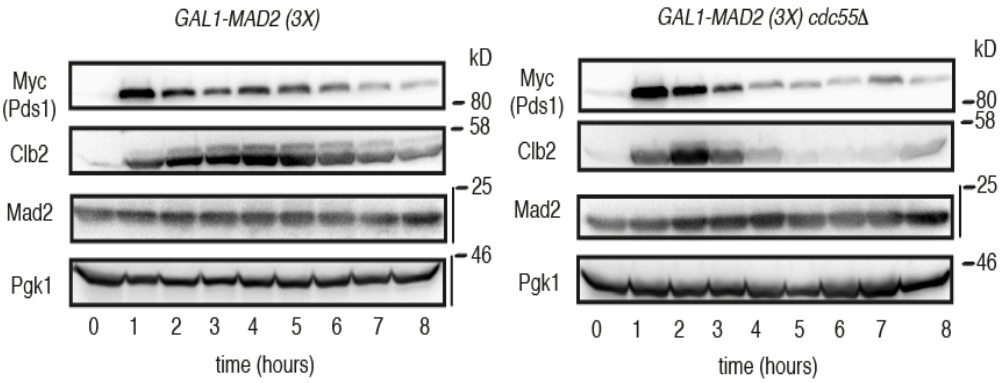

Figure 25. *cdc55Δ* cells are resistant to high levels of Mad2. *GAL1-MAD2(3x) PDS1-MYC18* and *GAL1-MAD2(3x) PDS1-MYC18 cdc55Δ* cells were grown in raffinose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose. After 80 minutes, when >90% cells were budded, α-factor was re-added to the medium. Samples Ire taken at time 0 (α-factor arrest) and then every hour for WB analysis of indicated proteins. Adapted from Vernieri et al., 2013.

Then I tested the hypothesis that $PP2A^{Cdc55}$ plays a role in the dephosphorylation of the Cdc16 and Cdc27 subunits of the APC/C during a SAC arrest. If this hypothesis were correct, Cdc16 and Cdc27 should be hyper-phosphorylated in *cdc55Δ* cells compared to wild-type upon release in nocodazole. Wild-type and *cdc55Δ* cells were released from G1 synchronization in nocodazole-containing medium and samples were taken for WB analysis. Protein extracts were run on P-Tag gels to separate phosphorylated from unphosphorylated bands of Cdc16 and Cdc27. Through IF analysis I verified that nocodazole had depolymerized microtubules throughout the

experiment (not shown). At 2 and 3 hours after the release, *cdc55Δ* mutants accumulated clear phospho-specific bands of Cdc16 that were absent in wild-type cells (Figure 26, left panel, see arrows). Also the quantification of the whole phosphorylation shift, normalized over total amount of Cdc16 signal, confirmed that, in *cdc55Δ* cells, Cdc16 is more phosphorylated than in wild-type cells (Figure 26, right panel).

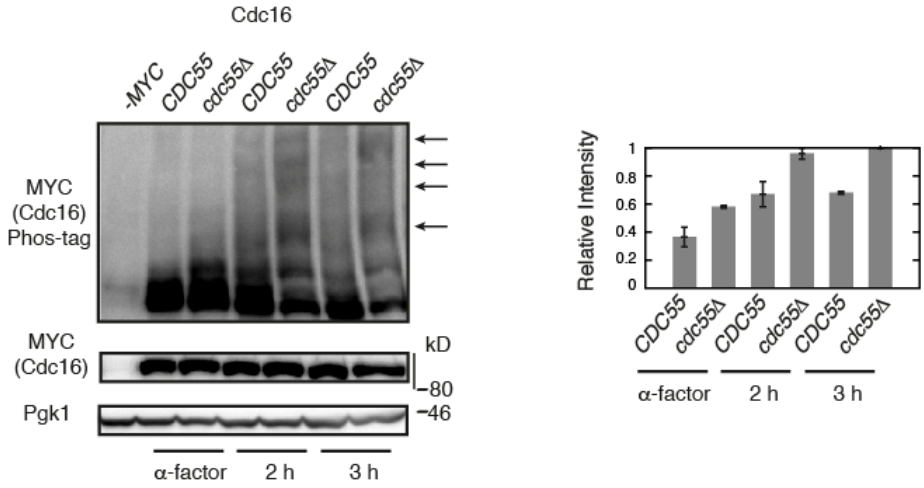

Figure 26. Deletion of Cdc55 increases the number and total amount of hyper-phosphorylated bands of Cdc16 in nocodazole. *CDC16-MYC6* and *CDC16-MYC6 cdc55Δ* cells were grown in YEPD medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of nocodazole. Samples were taken during the G1 arrest and after 2 and 3 hours in nocodazole for Western blotting analysis of Cdc16 phosphorylation using Phos-Tag reagent-containing polyacrylamide gels (left panel, upper part). The same extracts were loaded on normal 10% acrylamide gels to assess the total amount of Cdc16 and Pgk1 (left panel, lower part). Slowly migrating bands (phospho-specific shifts) were quantified over the total amount of protein at each time point for the two mutants, and relative signals were plotted in the form of histograms (right panel). Adapted from Vernieri et al., 2013. This experiment is representative of four repeats.

A difference in the phosphorylation status of Cdc16 between wild-type and *cdc55Δ* cells could also be visualized through normal polyacrylamide gels, although with

much inferior definition and without discriminating between individual phosphorylation bands (Figure 27, left panel).

Slowly migrating protein bands (shifts) on P-Tag gels are considered specific for phosphorylation isoforms of the protein (Kinoshita et al., 2008); nevertheless I verified through Calf-Intestine-Phosphatase (CIP) assay that the shift observed in Cdc16 WB signals of *cdc55Δ* cells was caused by phosphorylation (Figure 27, right panel).

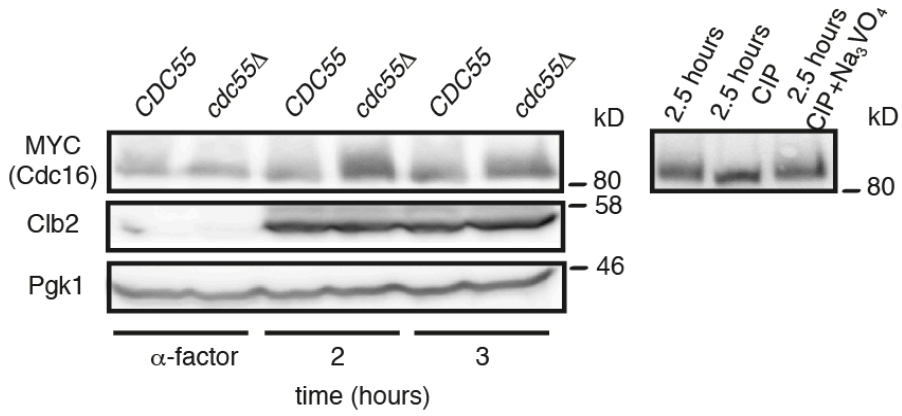

Figure 27. Deletion of Cdc55 increases the amount of slowly migrating Cdc16, which corresponds to phosphorylated protein. *CDC16-MYC6* and *CDC16-MYC6 cdc55Δ* cells were grown in YEPD medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of nocodazole. Samples were taken during the G1 arrest and after 2 and 3 hours in nocodazole for Western blotting analysis of Cdc16 phosphorylation using standard 7.5% acrylamide/bisacrylamide (80/1) gels (left panel, upper band). The same extracts were also loaded on normal 10% polyacrylamide gels to assess Clb2 and Pgk1 protein levels (left panel, lower bands). Calf-Intestine-Phosphatase (CIP) assay was performed on *cdc55Δ* extracts at 2 hours (right panel). Adapted from Vernieri et al., 2013. This experiment is representative of three repeats.

The clear difference in the phosphorylation status of Cdc16 between *CDC55* wild-type and *cdc55Δ* mutants could not be reproduced for Cdc27. While phosphorylation of Cdc27 increased from G1 phase to mitosis in both strains as expected, no differences could be observed in *cdc55Δ* mutant relative to the wild-type (Figure 28).

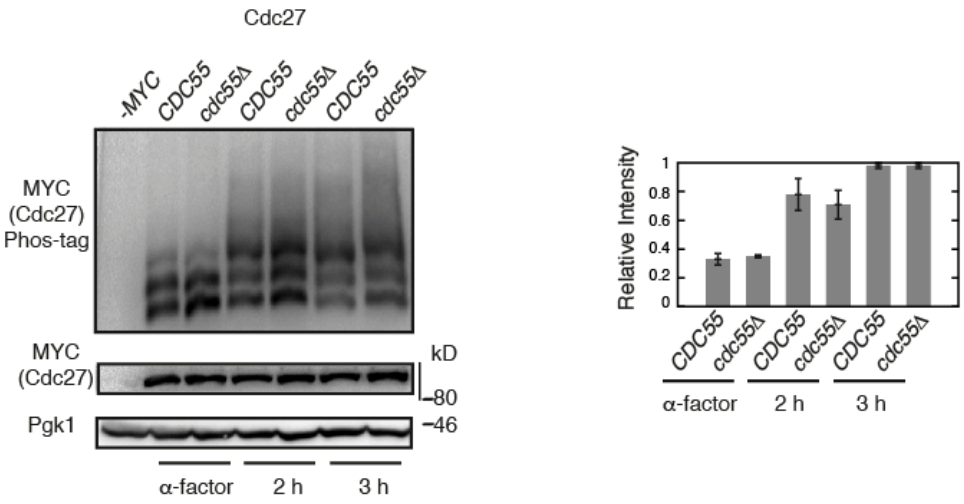

Figure 28. Deletion of Cdc55 does not enhance Cdc27 phosphorylation in nocodazole. *CDC27-MYC9* and *CDC27-MYC9 cdc55Δ* cells were grown in YEPD medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of nocodazole. Samples were taken during the G1 arrest and after 2 and 3 hours in nocodazole for Western blotting analysis of Cdc27 phosphorylation status using P-Tag reagent (left panel, upper part). The same extracts were loaded on normal 10% polyacrylamide gels to asses the total amount of Cdc27 and Pgk1 (left panel, lower part). Slowly migrating bands (phospho-specific shifts) were quantified over the total amount of protein at each time point for the two mutants, and relative signals were plotted in the form of histograms (right panel). Adapted from Vernieri et al., 2013. This experiment is representative of three independent repeats.

In summary, $PP2A^{Cdc55}$ directly or indirectly induces the dephosphorylation of Cdc16, which likely delays the onset of adaptation until Cdk1 activity is sufficiently high to overcome the effect of the phosphatase.

According to my model, *cdc55Δ* cells have hyper-phosphorylated Cdc16 and therefore adapt before wild-type cells; on the contrary, *cdc16-6A cdc27-5A* mutants never phosphorylate Cdc16 and Cdc27 and consequently they never adapt. If the main role of $PP2A^{Cdc55}$ in inhibiting adaptation is via Cdc16 dephosphorylation, Cdc55 deletion should not be able to rescue the adaptation-impaired double mutant *cdc16-6A cdc27-5A*; indeed, even in the absence of the phosphatase, this mutant cannot be

phosphorylated in Cdc27 and Cdc16. I decided to test this clear prediction to validate my model.

GAL1-MAD2(3x) cdc55Δ, *GAL1-MAD2(3x) cdc16-6A cdc27-5A* and *GAL1-MAD2(3x) cdc55Δ cdc16-6A cdc27-5A cdc55Δ* cells were released from G1 phase in the presence of galactose to induce Mad2 overexpression. *cdc55Δ* cells were not arrested in metaphase and *cdc16-6A cdc27-5A* cells were unable to adapt, as expected (Figure 29A and B). Importantly and coherently with my model, the combined *cdc55Δ cdc16-6A cdc27-5A* mutant had a phenotype that closely resembled *cdc16-6A cdc27-5A*: most cells were arrested in metaphase for at least 8 hours with stable levels of Pds1 (Figure 29A, C and D). The fact that the adaptation impairment phenotype was a bit less strong in *cdc55Δ cdc16-6A cdc27-5A* cells compared to *cdc16-6A cdc27-5A* cells can be due to the fact that APC/C subunits other than Cdc16 can be dephosphorylated by $PP2A^{Cdc55}$, and/or to other mechanisms by which the phosphatase inhibits adaptation.

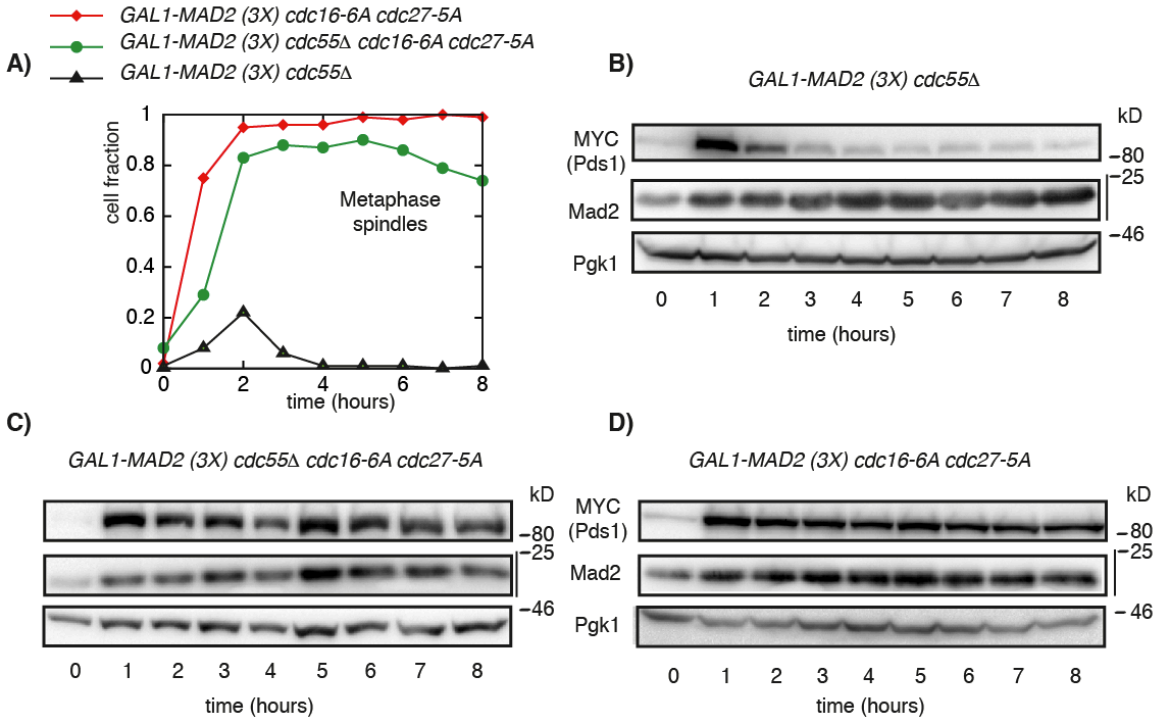

Figure 29. Deletion of Cdc55 does not rescue the adaptation-impaired phenotype of *cdc16-6A cdc27-5A* mutants. *GAL1-MAD2(3x) PDS1-MYC18 cdc55Δ*, *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A cdc55Δ* cells were grown in raffinose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose. After 80 minutes, when >90% cells were budded, α-factor was re-added to the medium. Samples were taken at time 0 (α-factor arrest) and then every hour for IF (upper panel) and WB (lower panels) analysis. Adapted from Vernieri et al., 2013. This experiment is representative of two independent repeats.

As a control for this experiment, I verified that in an unperturbed cell cycle the three mutants behave similarly: the result ensured that differences in adaptation kinetics are specific to SAC activation and excluded that they derive from significant differences in cell cycle duration (Figure 30).

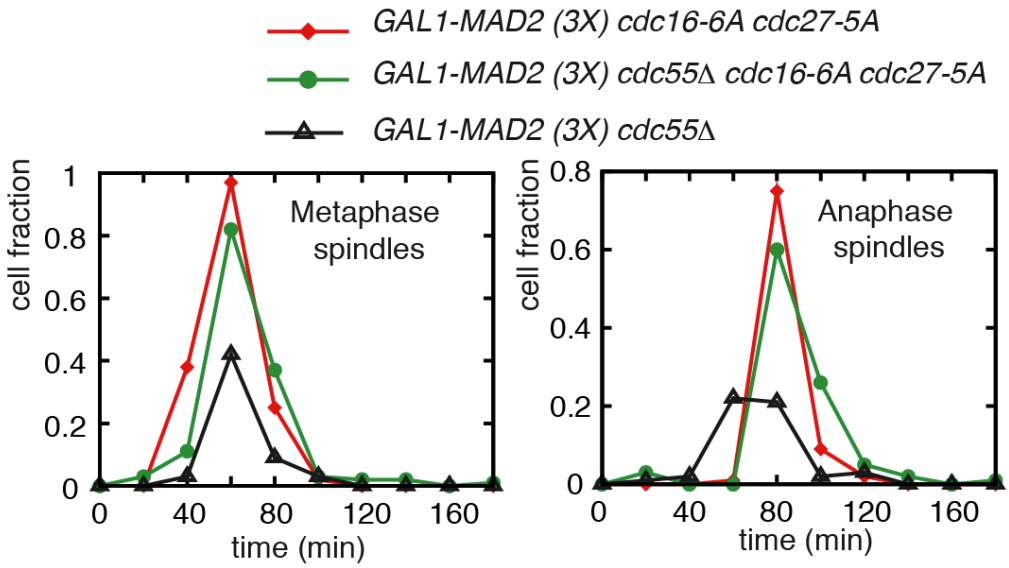

Figure 30. Deletion of Cdc55 does not rescue the adaptation-impaired phenotype of *cdc16-6A cdc27-5A* mutants. *GAL1-MAD2(3x) PDS1-MYC18 cdc55Δ*, *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A cdc55Δ* cells were grown in glucose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor and, after 80 minutes, when >90% cells were budded, α-factor was re-added to the medium. Samples were taken for IF analysis of metaphase (left panel) and anaphase (right panel) spindles at indicated time points. Adapted from Vernieri et al., 2013.

1.7 PP2A^{Cdc55} inhibits adaptation independently from Cdc14

In my experiments I showed that *cdc55Δ* mutants hyper-phosphorylate Cdc16, and this is likely the mechanism that allows these cells to bypass the prometaphase arrest. However, in the literature, SAC deficiency of *cdc55Δ* cells has been ascribed to precocious Cdc14 activation (via release from the nucleolus) (Wang and Ng, 2006a; Wang and Ng, 2006b; Yellman and Burke, 2006). Putting all these data together, we may conclude that PP2A^{Cdc55} inhibits the metaphase-to-anaphase transitions through two parallel pathways: by dephosphorylating APC/C subunits and by restraining Cdc14 release from the nucleolus.

If part of the SAC deficiency shown by *cdc55Δ* mutants were due to precocious release of Cdc14 from the nucleolus, yeast mutants which constitutively release Cdc14 should be SAC defective just as *cdc55Δ* cells.

It is known from the literature that deletion of Net1, the competitive inhibitor of Cdc14, causes constitutive release of the phosphatase from the nucleolus (Visintin et al., 1999). I then decided to compare the ability of *cdc55Δ* and *net1Δ* mutants to stabilize Pds1 in the presence of nocodazole as a readout of their SAC proficiency. I arrested *cdc55Δ* and *net1Δ* cells in G1 phase and then I released them in medium with or without nocodazole. FACS analysis was used to compare cell cycle progression of the two mutants in the absence of nocodazole and to confirm accumulation of double DNA content in nocodazole-treated cells.

In unperturbed conditions, *cdc55Δ* and *net1Δ* cells proceeded along the cell cycle with comparable kinetics, as judged by FACS profiles and Pds1 degradation (Figure 31, upper panels). Strikingly, in the presence of nocodazole, *cdc55Δ* mutants degraded Pds1 after 2-2.5 hours as expected, while *net1Δ* stabilized Pds1 for the whole duration of the experiment (5 hours) (Figure 31, lower panels).

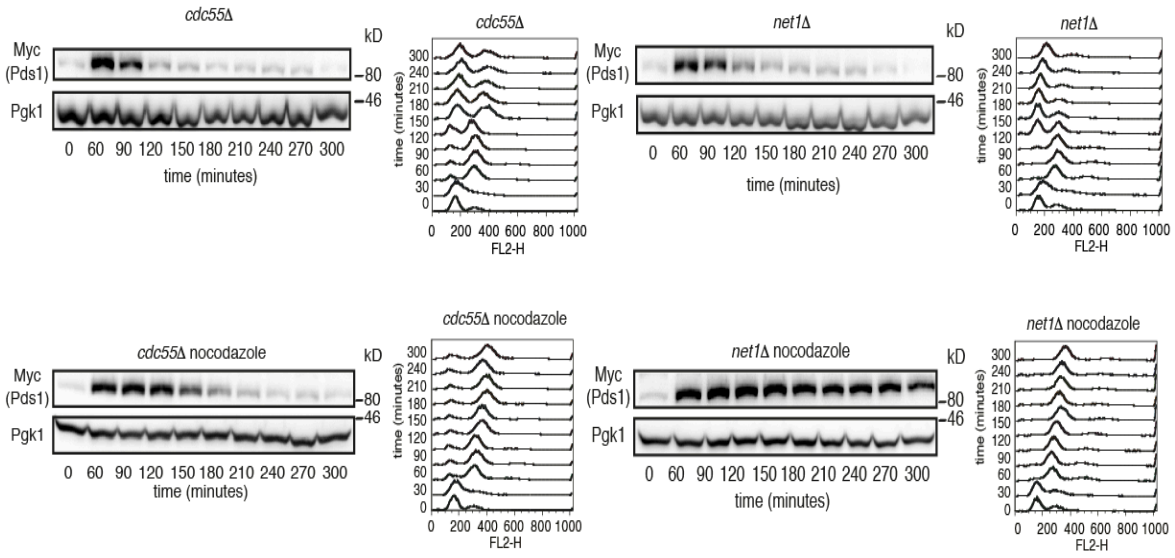

Figure 31. Contrarily to *cdc55Δ* cells, *net1Δ* mutants are SAC proficient. *cdc55Δ PDS1-MYC18* and *net1Δ PDS1-MYC18* cells were grown in glucose medium at 25°C, arrested in α-factor for 2.5 hours, and then released into the cell cycle without (upper panels) or with (lower panels) nocodazole. 80 minutes after the release, when most (>90%) cells were budded, α-factor was added again, to resynchronize cells completing one cell cycle in the next G1 phase. Samples were taken for WB and FACS analysis at the indicated times. Adapted from Vernieri et al., 2013. This experiment is representative of two repeats.

These results show that ectopic release of Cdc14 from the nucleolus is not sufficient to activate the APC/C in nocodazole and to bypass the SAC; for this reason *cdc55Δ* cells, which also precociously release Cdc14, are SAC deficient for causes that are independent from Cdc14. This result reinforces my conclusion that the main role of $PP2A^{Cdc55}$ in the SAC is via Cdc16 dephosphorylation, and not Cdc14 inhibition.

The fact that ectopic release of Cdc14 is not sufficient *per se* to bypass the SAC does not mean that this phosphatase is absolutely dispensable for adaptation. I decided to test the requirement for Cdc14 in this process by using a temperature-sensitive mutant of Cdc14, cdc14-1, which is inactivated at high temperatures (37° C). I also

used a temperature-sensitive mutant of Cdc15 (cdc15-2), an essential component of the MEN cascade that leads to Cdc14 activation (see Introduction paragraph 2.2). Both *cdc14-1* and *cdc15-2* mutants cannot exit mitosis at 37° C and thus permanently accumulate with elongated spindles (Rock and Amon, 2009) (figure 32).

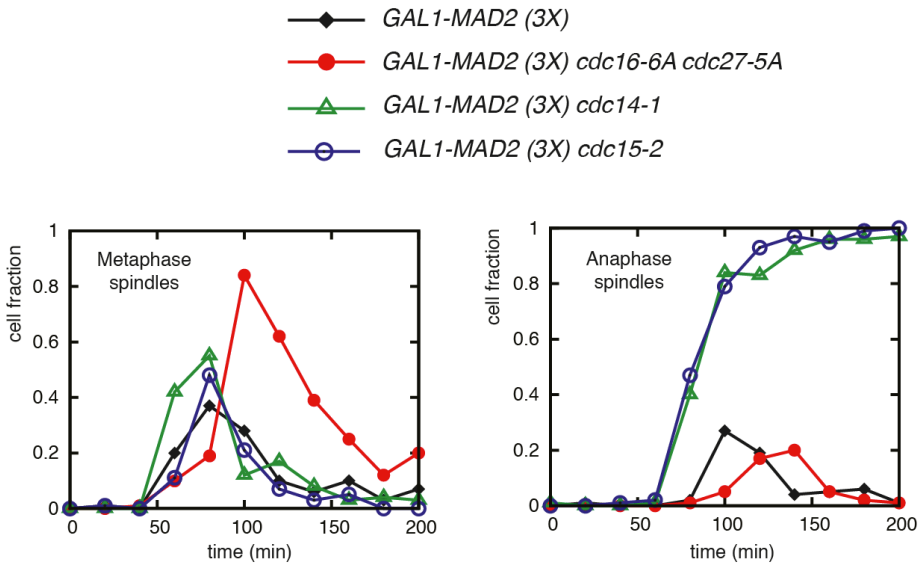

Figure 32. *cdc14-1* and *cdc15-2* transit into anaphase similarly to wild-type and *cdc16-6A cdc27-5A* mutants and then they remain arrested. *GAL1-MAD2(3x) PDS1-MYC18*, *GAL1-MAD2(3x) PDS1-MYC18 cdc14-1*, *GAL1-MAD2(3x) PDS1-MYC18 cdc15-2* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* cells were grown in glucose at 23°C and arrested in G1 phase with α-factor. After 3 hours they were released from α-factor in glucose medium at 37°C to inactivate cdc14-1 and cdc15-2, and α-factor was added to eventually resynchronize cells in the next G1 phase. Samples were taken at indicated time points for IF (right panels) analysis. Adapted from Vernieri et al., 2013.

I arrested wild-type, *cdc14-1*, *cdc15-2* and adaptation impaired *cdc16-6A cdc27-5A* cells in G1 phase at 23°C, and then I released them in galactose to activate the SAC. After 2 hours, when all cells were mitotic, I raised the temperature to 37° C to inactivate cdc14-1 and cdc15-2. If Cdc14 had an essential role in adaptation, *cdc14-1* and *cdc15-2* mutants would be expected to arrest in metaphase with high Pds1. What I observed instead was that *cdc14-1* and *cdc15-2* mutants degraded Pds1 and proceeded into anaphase, where they arrested due to impairment in mitotic exit. The

metaphase-to-anaphase transition occurred in these mutants with only a mild delay compared to wild-type cells (Figure 33). As expected, *cdc16-6A cdc27-5A* cells arrested in metaphase with high Pds1 for the whole experiment (Figure 33).

I conclude from this experiment that Cdc14 is not necessary for adaptation. In case, it could play only a minimal role in accelerating the process, as suggested by the mild delay observed in the metaphase-to-anaphase transition in both *cdc14-1* and *cdc15-2* cells compared to the wild-type.

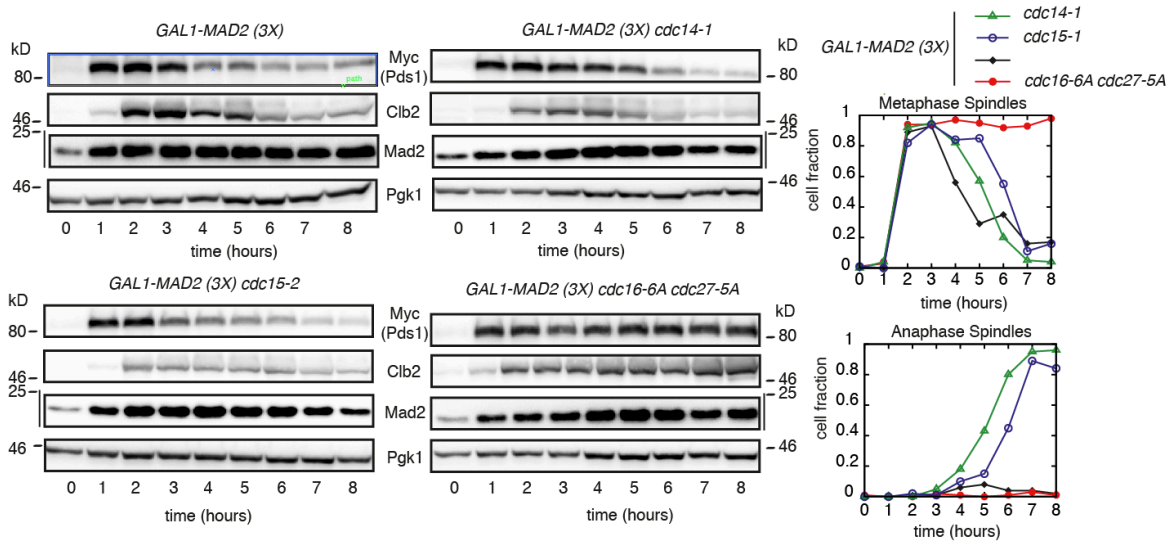

Figure 33. Cdc14 is not required for adaptation to the SAC. *GAL1-MAD2(3x) PDS1-MYC18*, *GAL1-MAD2(3x) PDS1-MYC18 cdc14-1*, *GAL1-MAD2(3x) PDS1-MYC18 cdc15-2* and *GAL1-MAD2(3x) PDS1-MYC18 cdc16-6A cdc27-5A* cells were grown in raffinose medium at 23°C and arrested in G1 phase with α-factor. After 3 hours they were released from α-factor in the presence of galactose for additional two hours. Then the temperature was increased to 37°C to inactivate cdc14-1 and cdc15-2, and α-factor was added to resynchronize cells exiting mitosis in the next G1 phase. Samples were taken at time 0 (α-factor arrest) and then every hour for WB (left panels) and IF (right panels) analysis. Adapted from Vernieri et al., 2013. This experiment is representative of two independent repeats.

Chapter 2

The role of Cdc20 levels in adaptation

2.1 Cdc20 levels increase before adaptation

Phosphorylation of APC/C subunits by Cdk1 is necessary for adaptation. It likely increases APC/C binding affinity for Cdc20, which might compete with MCC for APC/C and eventually trigger adaptation.

Together with APC/C phosphorylation, other events may favor APC/C binding to Cdc20. Given the role of Cdk1:Clb2 in promoting Cdc20 synthesis, increasing Clb2 levels during a SAC arrest (figures 21 and 22) could increase transcription of *CDC20*. Progressive increase of Cdc20 synthesis, coupled with constant degradation, would lead to increasing protein levels, eventually exceeding the buffering capability of the SAC and leading to formation of a critical amount of APC/C^{Cdc20}, which can trigger transition into anaphase.

I then decided to test whether Cdc20 accumulates during the SAC arrest and before adaptation. *GAL1-MAD2(3x) PDS1-MYC* cells were synchronized in G1 phase with α-factor and released in galactose to activate the SAC. Cdc20 levels were monitored by WB. As shown in Figure 34, Cdc20 levels progressively accumulated during the metaphase arrest, and then they declined concomitantly with the early stages of adaptation, detected by Pds1 and Clb2 degradation, and by disassembly of metaphase spindles.

Although the peak of Cdc20 temporally correlates with the beginning of adaptation, this does not prove the causal relationship between the two events. A formal proof in this direction would require the identification of a threshold level of Cdc20 above which cells adapt and below which they do not. Such an experiment would require to stabilize Cdc20 levels and modulate their value, an experiment that I am planning to do soon.

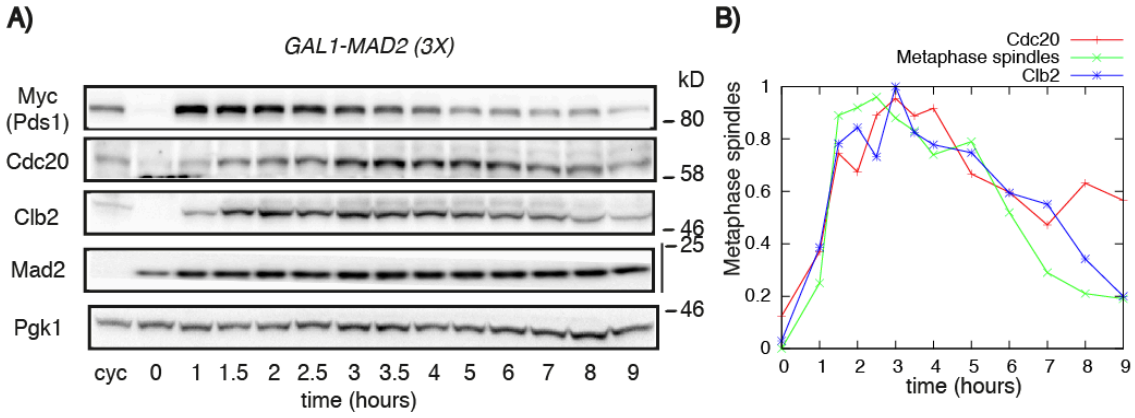

Figure 34. Cdc20 progressively accumulates during the SAC arrest before adaptation. (A and B) *GAL1-MAD2(3x) PDS1-MYC18* cells were grown in raffinose medium at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose. After 2 hours α-factor was readded to the medium. Samples were taken from cycling cells (cyc), at time 0 (α-factor arrest) and then every hour for WB (A) and IF analysis of metaphase spindles (B). In (B) I also plot quantifications Clb2 and Cdc20 Western blot signals normalized to Pgk1, and averaged over 5 quantifications of different gels. Note that Cdc20 peaks just before Clb2, and then their decrease progresses in parallel with metaphase spindles disassembly.

2.2 Mnd2 deletion accelerates adaptation

Although I have not managed so far to keep Cdc20 levels under control, it is reasonable to test the possible role of Cdc20 in adaptation using mutants lacking proteins required for Cdc20 degradation. One of them is the non-essential APC/C component Mnd2 whose deletion significantly reduces the ubiquitination and degradation rates of Cdc20 during a SAC arrest (Introduction, Paragraph 4.5 and (Foster and Morgan, 2012)). In nocodazole, indeed, *mnd2Δ* cells have increased Cdc20 levels and the dissociation rate of APC/C:MCC is reduced. Although Cdc20 levels increase, *mnd2Δ* cells are checkpoint proficient, and in fact they are released from the SAC with slower kinetics compared to wild-type cells (Foster and Morgan, 2012), because of the slow dissociation of APC/C:MCC complex which delays APC/C release and thus its binding to free Cdc20 (see Introduction, paragraph 4.5).

Given that both Cdc20 accumulation and APC/C release due to APC/C:MCC turnover can be important in driving adaptation to the SAC, and that these two events are regulated in opposite ways in *mnd2Δ* mutants, I asked which of them is predominant during a prolonged arrest. If Cdc20 accumulation plays an important role in adaptation, significantly higher levels of Cdc20 in these mutant cells could compensate for the reduced dynamics of APC/C:MCC and therefore anticipate adaptation. If, on the other hand, APC/C release from the MCC is the limiting step required for APC/C^{Cdc20} reactivation in adaptation, we would expect that *mnd2Δ* adapt later than wild-type cells, just as they are delayed in the release from the SAC.

GAL1-MAD2(3x) and *GAL1-MAD2(3x) mnd2Δ* cells were synchronized in G1 and released in galactose to activate the SAC. As usual, I monitored securin/Pds1 and Clb2 levels by WB and metaphase spindles by IF analysis to study adaptation kinetics. Interestingly, I found that adaptation was anticipated by about 1-2 hours in *mnd2Δ* cells relative to wild-type cells (Figure 35, A and B). On average, Cdc20 levels in

mnd2Δ cells were about twice the levels observed in wild-type cells at different time points (Figure 35 C).

In summary, although *mnd2Δ* cells release APC/C from APC/C:MCC with reduced ability, they adapt first than wild-type cells, therefore reinforcing the hypothesis that Cdc20 levels are an important determinant in adaptation.

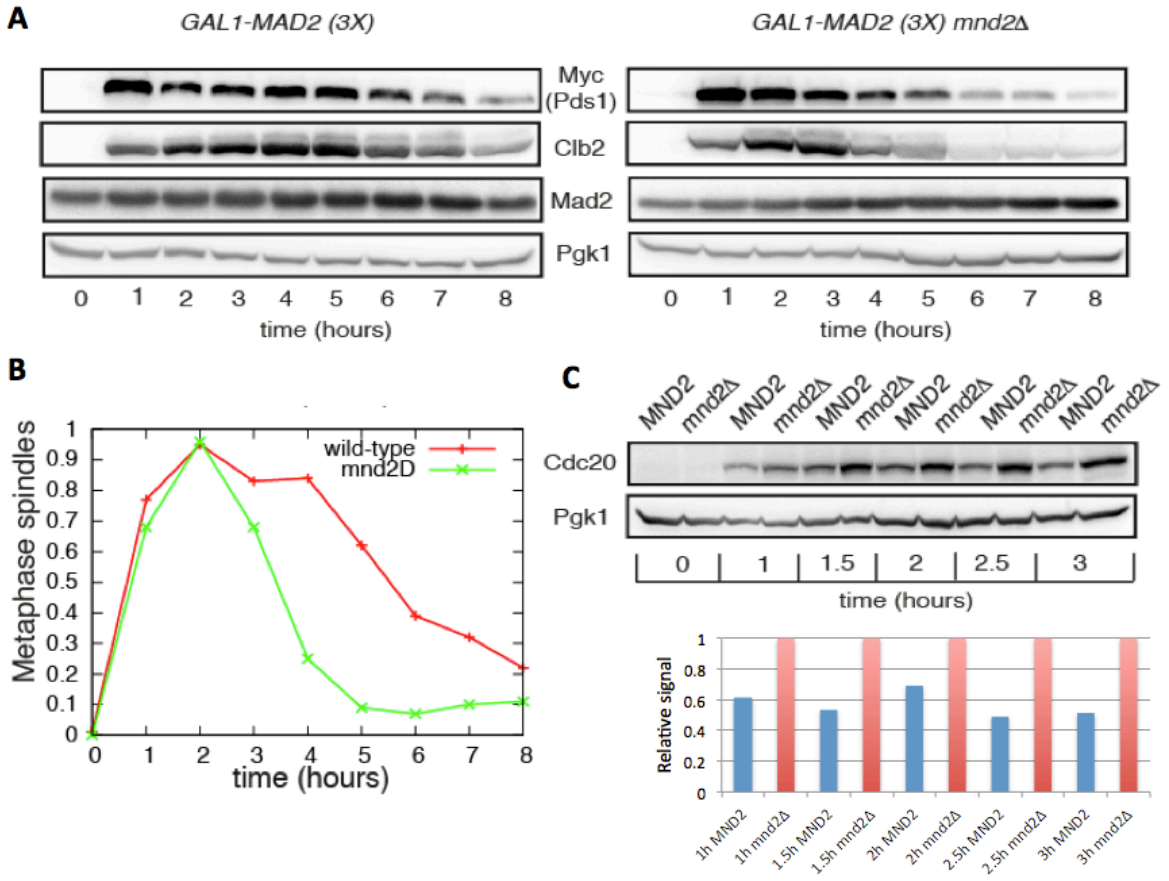

Figure 35. Deletion of Mnd2 accelarates adaptation to the SAC. (A,B and C) *GAL1-MAD2(3x) PDS1-MYC18* and *GAL1-MAD2(3x) PDS1-MYC18 mnd2Δ* cells were grown in raffinose medium at 30ºC and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose. After 2hours α-factor was re-added to the medium. Samples were taken at time 0 (α-factor arrest) and then every hour for WB (A) and IF (B) analysis. Cdc20 levels in *MND2* and *mnd2Δ* cells were analyzed by WB at indicated times (C, upper panel), and quantitatively compared after background subtraction and normalization for Pgk1 levels (C, lower panel). This experiment is representative of two independent repeats.

If the increased Cdc20 levels in *mnd2Δ* cells are responsible for the observed anticipated adaptation, these mutants would be closer to the threshold of Cdc20 that is required to adapt. One prediction of this model is that *mnd2Δ* cells not only adapt with faster kinetics compared to *wild-type* cells, as observed, but also that the adaptation phenomenon is more synchronous in these cells. To test this hypothesis, I compared the variability of the adaptation phenomenon in *MND2* and *mnd2Δ* cells. To do this, I moved to single cell analysis of time lapse movies. *GAL1-MAD2(3x) Clb2-GFP Tub2-Cherry* and *GAL1-MAD2(3x) mnd2Δ Clb2-GFP Tub2-Cherry* cells were grown in flow chambers in synthetic medium containing raffinose. Then galactose was added to the medium to induce Mad2 overexpression; GFP and Cherry signals were acquired every 10 minutes to follow Clb2 kinetics and progression through and out of mitosis, respectively. I analyzed 44 *MND2* and 48 *mnd2Δ* cells. I calculated the time occurring between budding and disassembly of the mitotic spindle (Time from budding to mitotic exit) and the time from Clb2 accumulation to disassembly of the mitotic spindle (Adaptation time) as two measures of the duration of the metaphase arrest before cells adapt.

I confirmed that *mnd2Δ* cells on average adapt much before than *MND2* ones (on average, 231 minutes and 352 minutes, respectively; $p<0.00000001$ at t-test). I also found that adaptation is much more synchronous in *mnd2Δ* cells than in *MND2* ones (standard deviations: 69 and 100 minutes, respectively; $p=0.011$ at Levene test of variance analysis) (Figure 36, right panel). Measurement of the time from budding to mitotic exit was coherent with measurement of adaptation times, with *mnd2Δ* cells exiting mitosis in reduced and less variable times compared to *MND2* cells (on average 292 and 424 minutes, respectively; standard deviations: 77 and 114 minutes, respectively) (Figure 36, left panel).

These results suggests that indeed Cdc20 accumulation can be a key factor for adaptation. I can still not conclude that this is the case in wild type cells as well.

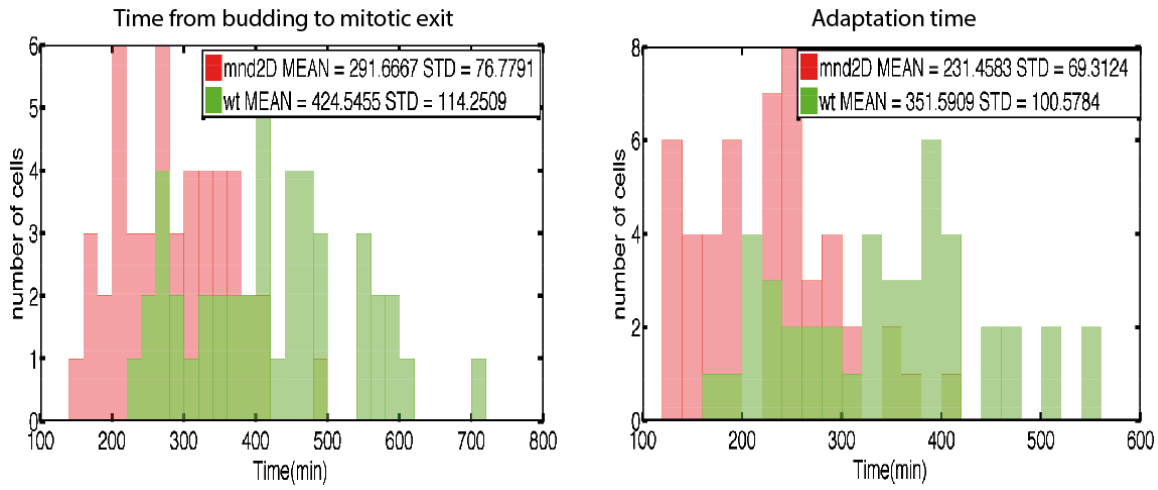

Figure 36. Adaptation is faster and more synchronous in *mnd2Δ* than in *MND2* cells. *GAL1-MAD2(3x) CLB2-GFP TUB2-Cherry* and *GAL1-MAD2(3x) mnd2Δ CLB2-GFP TUB2-Cherry* cells were grown in raffinose-containing synthetic medium at 30°C. Then they were added galactose to activate the checkpoint and followed during the mitotic arrest until adaptation. Histograms of the time from budding to mitotic exit (left panel) and of the adaptation time (right panel) in both strains are reported, together with their means (MEAN) and standard deviations (STD).

2.3 Mad3 and Mnd2 promotes Cdc20 degradation by the SAC in an additive way

My analysis of Cdc20 levels described above lead us to further clarify the mechanisms of Cdc20 degradation during a SAC-induced arrest. I report these preliminary data in the next two sections.

Based on data from literature (see Introduction, paragraph 4.6), Mad3 is the crucial component of the MCC that induces Cdc20 turnover during a SAC arrest. Mad3 likely exposes Cdc20 ubiquitination sites to the APC/C within the MCC, and in particular to Mnd2. If this were true, Mad3 and Mnd2 would act in series in the same complex, and

deletion of either component should stabilize Cdc20 steady state levels when the SAC is induced.

I decided to test this prediction by measuring Cdc20 levels during a synchronous arrest induced by nocodazole in wild-type, *mnd2Δ*, *mad3Δ*, and *mnd2Δ mad3Δ* cells. Given that *mad3Δ* mutants are SAC defective, they are expected to asynchronously bypass the metaphase arrest and exit mitosis in the presence of nocodazole; this level of asynchrony would probably preclude any reliable comparison of Cdc20 levels in prometaphase between the different mutants. For this reason I decided to overexpress an undegradable mutant of securin (*Pds1Δdb*) from the *GAL1* promoter to synchronize nocodazole-treated cells in prometaphase by inhibiting separase activation (Cohen-Fix and Koshland, 1999).

GAL-Pds1Δdb, GAL-Pds1Δdb mnd2Δ, *GAL-Pds1Δdb mad3Δ* and *GAL-Pds1Δdb mnd2Δ mad3Δ* cells were released from G1 in galactose to induce Pds1Δdb, either with or without nocodazole. Samples were taken for WB analysis 100 minutes after the release and Cdc20 levels were compared in the different mutants.

In the absence of nocodazole, cells arrested in metaphase by Pds1Δdb expression, as confirmed by IF analysis of spindles (data not shown). Cdc20 levels in the three mutants were more or less comparable with those of the wild-type strain, even if both *mnd2Δ* and *mnd2Δ mad3Δ* mutants showed slightly higher Cdc20 levels (Figure 37, four left lanes).

In nocodazole-treated cells, deletion of both Mad3 and Mnd2 produced increased Cdc20 levels, as expected. Surprisingly, combination of the two mutations had additive effects on the steady state levels of Cdc20. This suggests that Mad3 and Mnd2 not only act in series but also in parallel within the MCC in stimulating Cdc20 ubiquitination and degradation (Figure 37, four right lanes).

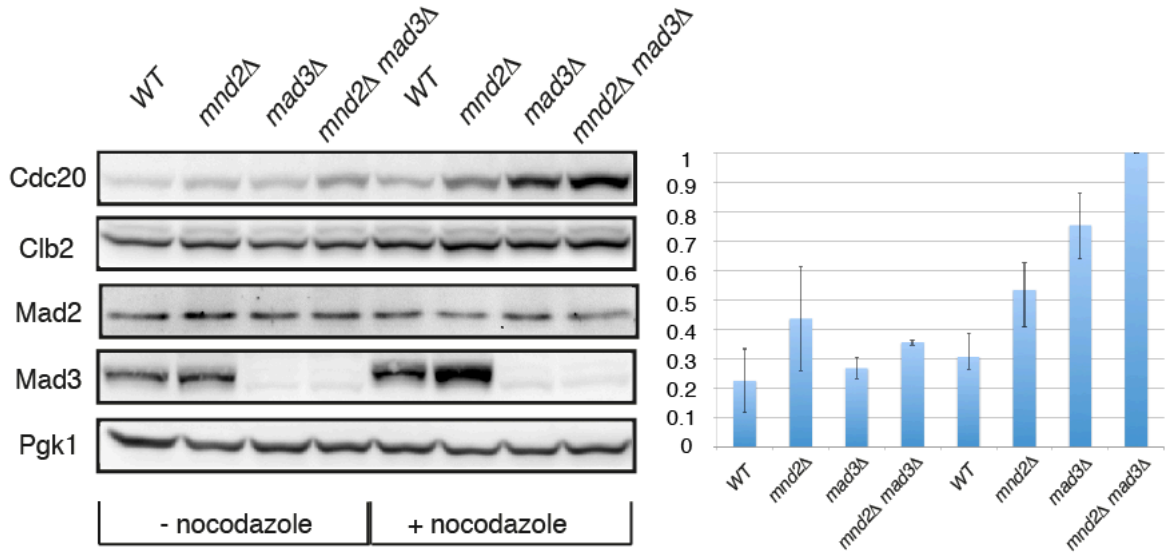

Figure 37. Mnd2 and Mad3 have a SAC-specific synergy in promoting Cdc20 degradation. *GAL-Pds1Δdb*, *GAL-Pds1Δdb mnd2Δ*, *GAL-Pds1Δdb mad3Δ* and *GAL-Pds1Δdb mnd2Δ mad3Δ* cells were grown in YEPR at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose, with or without nocodazole. Samples were taken at 100' after release for Western blotting analysis of the indicated proteins (left panel). In the right panel I show quantification of Cdc20 levels, normalized over Pgk1 signals, and then further normalized to the maximum value (corresponding to the *mnd2Δ mad3Δ* mutant). Histograms represent average of normalized Cdc20 levels coming from three independent experiments. Error bars represent the maximum and minimum level of normalized Cdc20 in these three experiments.

To confirm that the additive effect of *MAD3* and *MND2* deletions on Cdc20 levels is specifically due to their effect on Cdc20 degradation, I arrested *GAL-Pds1Δdb, GAL-Pds1Δdb mnd2Δ*, *GAL-Pds1Δdb mad3Δ*, and *GAL-Pds1Δdb mnd2Δ mad3Δ* cells in prometaphase with nocodazole and galactose, and then I added cycloexhimide to inhibit protein synthesis. In this condition, protein dynamics only reflect degradation. Western blots in Figure 38 show that single deletions of *MND2* and *MAD3* reduced Cdc20 degradation compared to the wild-type, and the combination of the two had an even stronger effect. I conclude that Mad3 and Mnd2 cooperate in promoting Cdc20 degradation during a SAC arrest.

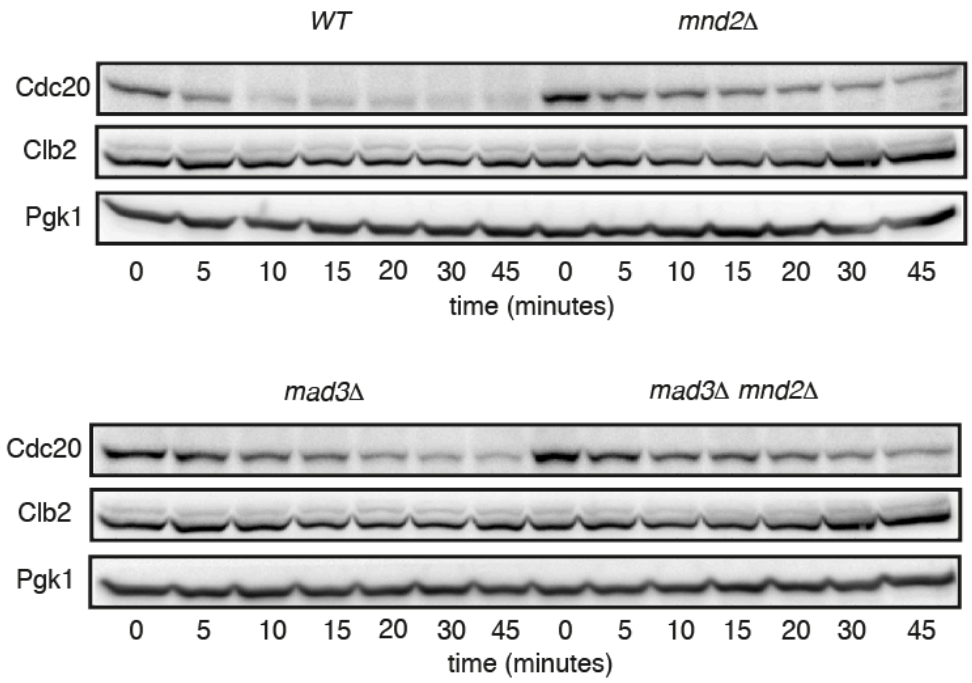

Figure 38. Combination of Mnd2 and Mad3 deletions increases Cdc20 stability during a checkpoint arrest. *GAL-Pds1Δdb*, *GAL-Pds1Δdb mnd2Δ*, *GAL-Pds1Δdb mad3Δ* and *GAL-Pds1Δdb mnd2Δ mad3Δ* cells were grown in YEPR at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose and nocodazole. After 100 minutes, cycloheximide was added to the cultures to inhibit protein synthesis. Samples were taken for Western blot analysis of Cdc20 kinetics at indicated time points after cycloheximide addition (time 0). This experiment is representative of three repeats.

If higher Cdc20 levels accelerate the transition into anaphase of SAC-arrested cells, we would expect that the double *mnd2Δ mad3Δ* mutant proceedes into anaphase with faster kinetics compared to *mad3Δ* cells. I looked at the kinetics of SAC bypass by analyzing DNA reduplication by FACS in cells treated with nocodazole. Wild-type, *mnd2Δ*, *mad3Δ*, and *mnd2Δ mad3Δ* cells were synchronized in G1 phase and then released into the cell cycle with or without nocodazole. In the absence of nocodazole, the four strains showed comparable cell cycle times, therefore showing that deletion of these MCC components does not significantly affect an unperturbed cell cycle (Figure 39).

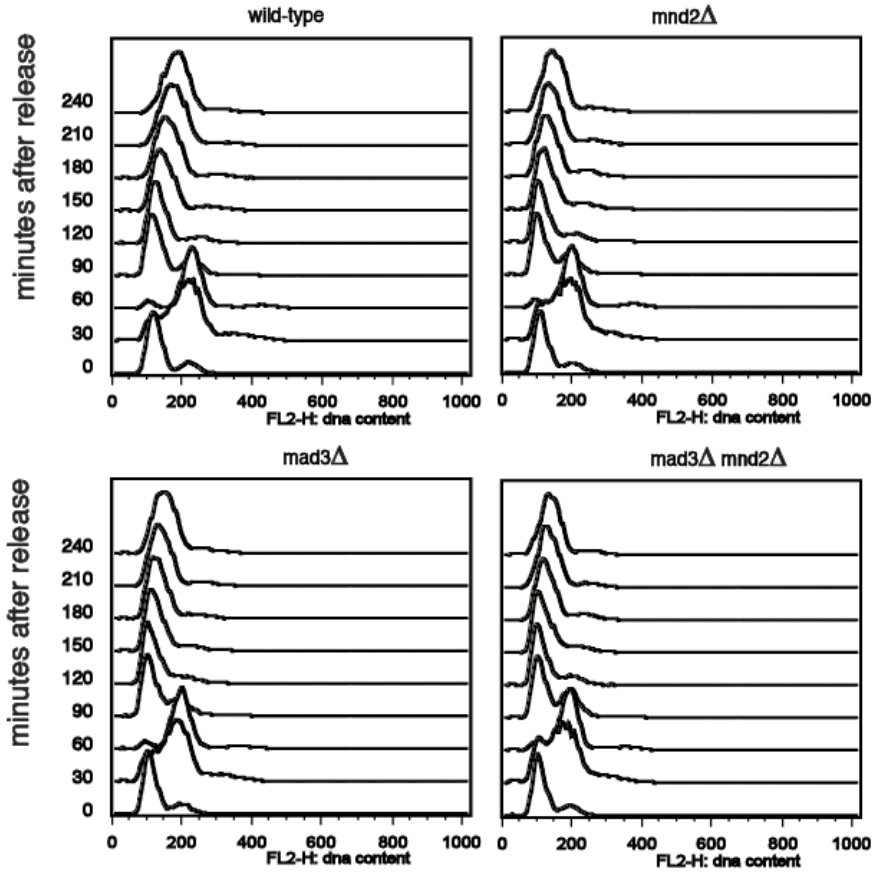

Figure 39. Deletion of Mad3 and Mnd2 does not impact on cell cycle dynamics in unperturbed conditions. Wild-type, *mad3Δ*, *mnd2Δ* and *mad3Δ mnd2Δ* cells were grown in YEPD at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released into the cell cycle; α-factor was re-added after 60 minutes. Samples were taken for FACS analysis of DNA content at indicated time points.

In the presence of nocodazole, wild-type and *mnd2Δ* cells arrested with a double (2C) DNA content until 5 hours, as expected. This arrest is compatible with the SAC proficiency of these two strains (Foster and Morgan, 2012). *mad3Δ* mutants progressively accumulated with a quadruple (4C) DNA content, which reflects their inability to inhibit anaphase and mitotic exit in the presence of nocodazole.

Interestingly, double *mnd2Δ mad3Δ* mutants reduplicated their DNA with faster kinetics (more than one hour before) relative to single *mad3Δ* mutants, although they had entered in the cycle with a small delay (Figure 40).

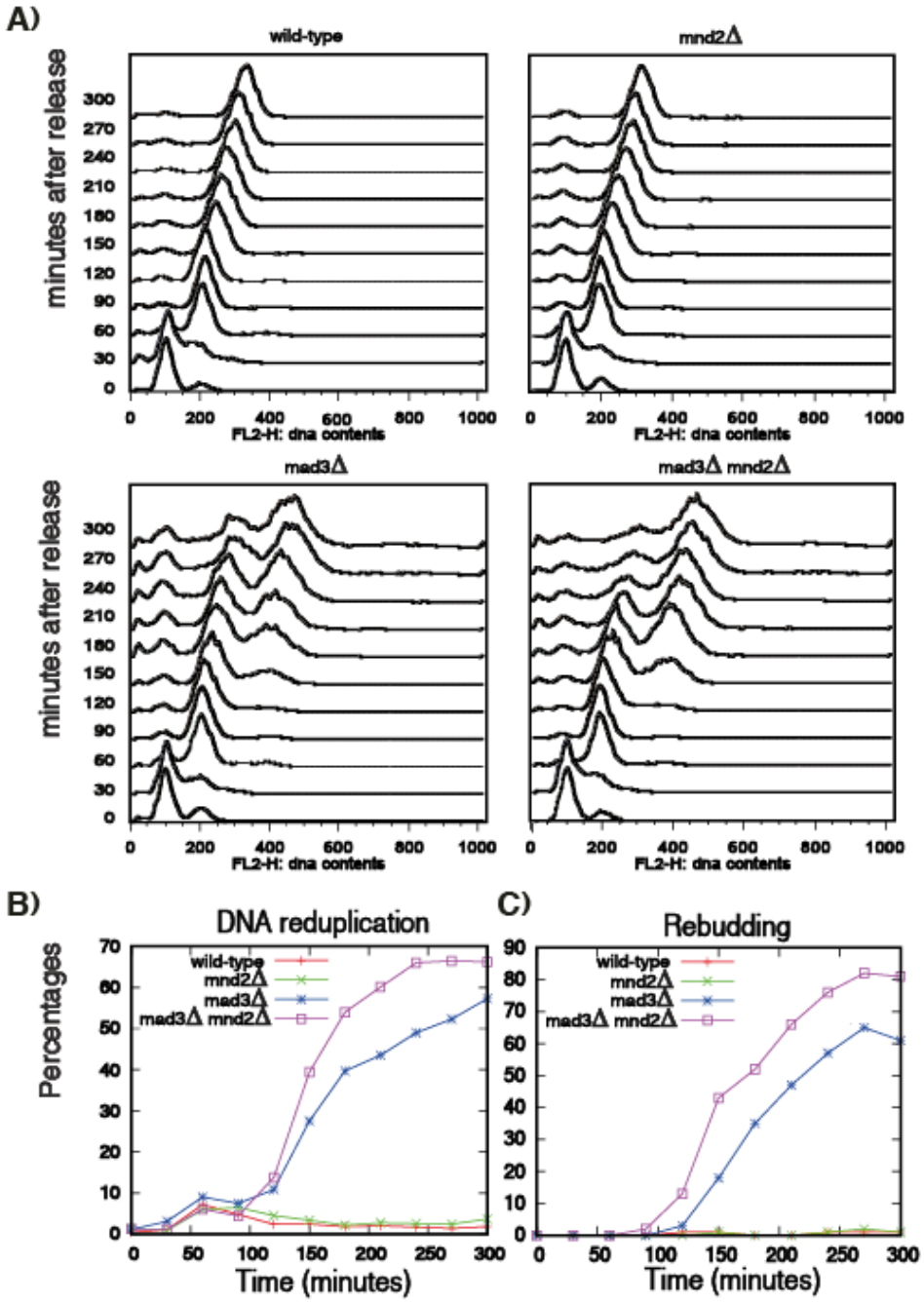

Figure 40. Deletion of Mad3 and Mnd2 synergistically accelerates SAC override. (A, B and C) Wild-type, *mad3Δ*, *mnd2Δ* and *mad3Δ mnd2Δ* cells were grown in YEPD at 30ºC and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of nocodazole. Samples were taken for FACS analysis of DNA content at indicated time points (A). The percentage of cells with a DNA content greater than double (250 arbitrary units on the x-axis) at FACS analysis (B) and the percentage of rebudded cells (C) was measured for each strain and at each time point and plotted. The results shown in these panels are representative of three independent experiments.

This synergy between Mad3 and Mnd2 deletions in escaping from the SAC arrest is not obvious: in the *mad3Δ* background, the deletion of *MND2* could have locked APC/C on APC/C:Mad2:Cdc20 complex, thus reducing its turnover and APC/C activation. The significantly faster DNA reduplication in the double *mnd2Δ mad3Δ* mutant excludes this possibility and shows that combining the two deletions produces synergistic effects on APC/C activation, likely via the increase of Cdc20.

2.4 Mad3 promotes Cdc20 degradation by the SAC, whereas Mad2 inhibits it

It has been shown that deletion of one among *MAD1*, *MAD2* and *MAD3* -- essential components of the SAC pathway -- leads to increased steady state levels of Cdc20 in nocodazole (see Introduction: paragraph 4.6) (Pan and Chen, 2004). This result suggests that when the SAC cascade is activated, Cdc20 is ubiquitinated and primed for degradation by APC/C:MCC. In the absence of one of the essential components of the checkpoint, MCC cannot be formed, Cdc20 cannot be ubiquitinated, and its steady state levels increase. The fact that the *mad3Δ* mutants showed higher Cdc20 levels, compared to *mad1Δ* and *mad2Δ* (Pan and Chen, 2004), suggests that Mad3 could favor Cdc20 degradation also independently from Mad1 and Mad2, and thus from the MCC. This conclusion is supported by the finding that also in an unperturbed mitosis the deletion of *MAD3* leads to increased Cdc20 half-life (Pan and Chen, 2004).

I was intrigued by the fact that *mad3Δ* cells accumulate more Cdc20 than the other *mad* mutants. Published data show that Mad3 does not bind Cdc20 in the absence of either Mad1 or Mad2 (Hardwick et al., 2000); it is therefore unlikely that Mad3 has a role in Cdc20 ubiquitination independently from Mad2 and Mad1. A possible explanation for the stabilization of Cdc20 in *mad3Δ* cells is that, without Mad3, Mad2

sequesters Cdc20 and prevents its ubiquitination by the APC/C (in agreement with (Foster and Morgan, 2012)). As a consequence, in *mad3Δ* cells the combined effect of reduced Cdc20 ubiquitination by APC/C:MCC and the protective effect by Mad2 may lead to an increase of Cdc20 levels that is higher than what observed in *mad1Δ* and *mad2Δ* mutants. Here, reduced ubiquitination due to lack of MCC is partially compensated by the lack of the protective effect due to Mad2.

If the hypothesis that Mad2 inhibits Cdc20 ubiquitination in the absence of Mad3 were correct, we would expect the double *mad2Δ mad3Δ* mutant to show Cdc20 levels that are similar to those of the single *mad2Δ* mutant, and lower than the *mad3Δ* cells.

On the contrary, if Mad2 and Mad3 play similar roles in Cc20 ubiquitination, as suggested by Pan and Chen, *mad2Δ mad3Δ* cells should contain levels that are at least as high as in the single *mad3Δ* mutant.

To test these alternative predictions, I synchronized *GAL-Pds1Δdb*, *GAL-Pds1Δdb mad2Δ*, *GAL-Pds1Δdb mad3Δ* and *GAL-Pds1Δdb mad2Δ mad3Δ* cells in G1 for 2 hours and then I released them into the cell cycle with or without nocodazole. Galactose was also added to the medium to overexpress undegradable securin (Pds1Δdb) from the *GAL1* promoter and guarantee synchronous prometaphase arrest also in the SAC mutants. 100' after release from G1, samples were taken for WB analysis of Cdc20 levels. In cells that were not treated with nocodazole, the synchronous arrest in metaphase was confirmed by IF analysis of mitotic spindles. In the populations treated with nocodazole, immunofluorescence confirmed absence of microtubules. The arrest in mitosis was confirmed by high levels of Clb2 in all cases, together with the 2C-DNA content at FACS analysis.

The results in nocodazole-treated cells were quite interesting: while as expected *mad3Δ* cells accumulated Cdc20 which was about three times higher than in wild-

type cells, *mad2Δ* mutants contained the same Cdc20 levels as wild-type cells. Moreover, the double *mad2Δ mad3Δ* mutant also showed Cdc20 protein amount that was comparable to wild-type and *mad2Δ* cells (Figure 41, four right lanes).

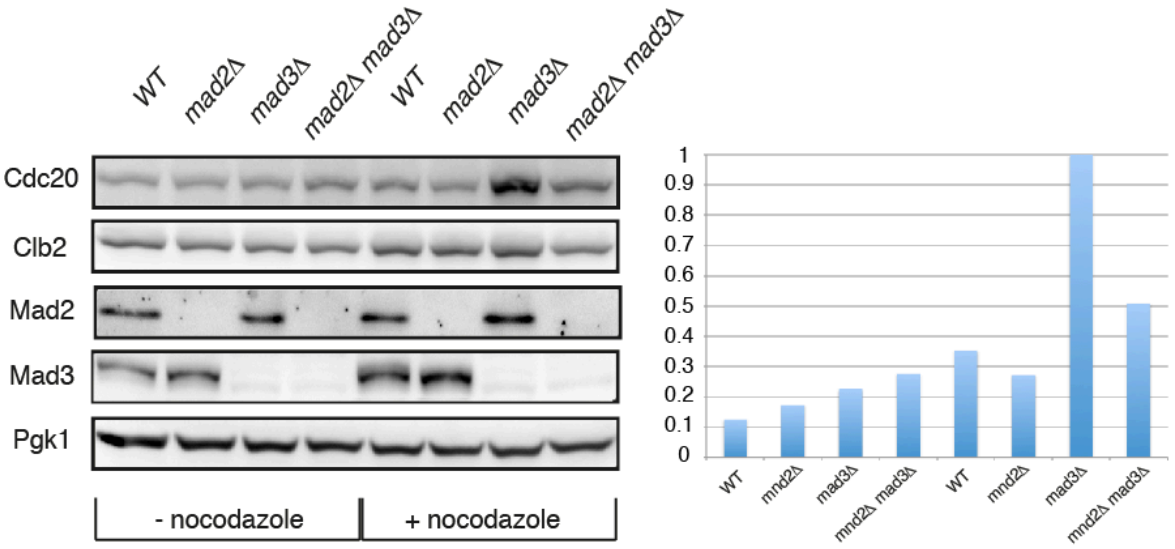

Figure 41. Mad3 stimulates Cdc20 degradation during a SAC arrest, while Mad2 inhibits it. *GAL-Pds1Δdb*, *GAL-Pds1Δdb mad2Δ*, *GAL-Pds1Δdb mad3Δ* and *GAL-Pds1Δdb mad2Δ mad3Δ* cells were grown in YEPR at 30°C and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of galactose and with or without nocodazole. Samples were taken at 100' after release for Western blotting analysis of the indicated proteins (left panel). Cdc20 signals in minus/plus nocodazole and in the four strains were quantified, normalized to respective Pgk1 signals, and the resulting numbers were again normalized to their maximum, corresponding to *mad3Δ* mutant (right panel). This experiment is representative of three repeats.

Both these results are in line with the idea that Mad3 is the main actor of Cdc20 uquitination by the SAC; Mad2 is essential for Mad3 binding to Cdc20 (thus for Cdc20 ubiquitination by APC:MCC), but, in absence of Mad3, Mad2 essential role is to inhibit Cdc20 ubiquitination and to protect it from degradation.

My experiment contradicts Pan and Chen data showing that also single deletion of *MAD2* leads to increased Cdc20 steady state levels: this difference could be due to the

fact that in their experiments a Myc-tagged version of Cdc20 was used, as opposed to my endogenous Cdc20. Myc tag could alter turnover of the protein, leading to erroneous conclusions.

Another interesting result from my experiment is that, in the absence of nocodazole, the three mutant strains showed mitotic Cdc20 levels that are comparable to those of the wild type. This again contradicts Pan and Chen and suggests that regulation of Cdc20 ubiquitination by SAC proteins (stimulation by Mad3 and inhibition by Mad2) only occurs when the SAC cascade is active, and not during an unperturbed mitosis (Figure 41, four left lanes). This in turn can be due to the absence of significant binding of Mad2 and Mad3 to Cdc20 during an unperturbed mitosis.

From this experiment I can conclude that lowering Cdc20 levels is necessary but not sufficient to keep the SAC arrest: *mad2Δ* and *mad2Δ mad3Δ* cells do not accumulate Cdc20 but they are SAC deficient just as *mad3Δ* cells, which accumulate Cdc20. In fact, the rate of death on benomyl plates and the timing of DNA reduplication in the presence of nocodazole (two indicators of SAC deficiency) are comparable in *mad3Δ, mad2Δ* and *mad2Δ mad3Δ* mutants (see Figure 42). Therefore cells not only need to degrade Cdc20 during the SAC arrest; they also need to keep most of Cdc20 sequestered within the MCC. The already recognized importance of the MCC in sequestering Cdc20 is further strengthened by these results.

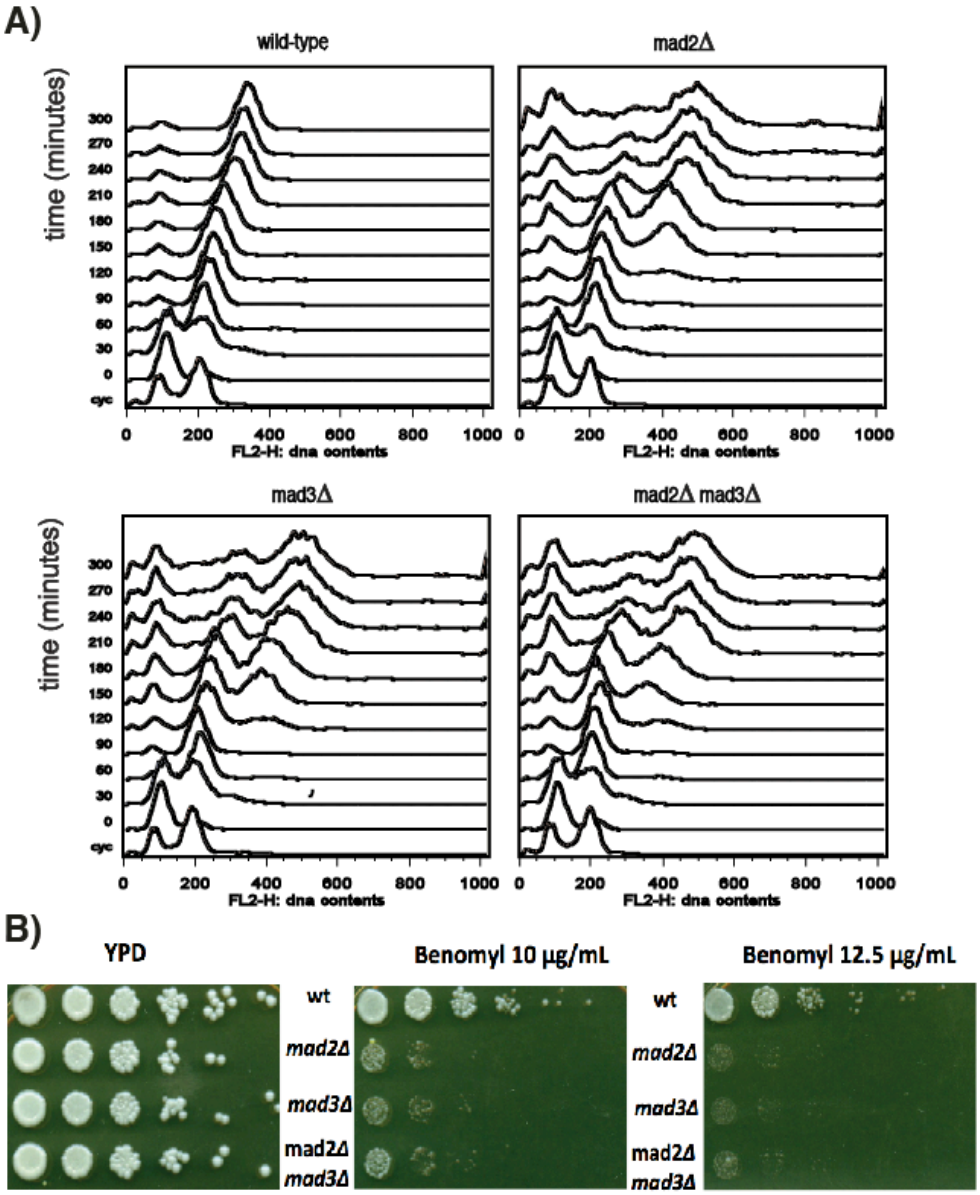

Figure 42. Combination of Mad2 and Mad3 deletion does not synergize in overriding the SAC.

(A) Wild-type, *mad2Δ*, *mad3Δ* and *mad2Δ mad3Δ* cells were grown in YEPD at 30ºC and arrested in G1 phase with α-factor for 2 hours. Then they were released from α-factor in the presence of nocodazole. Samples were taken for FACS analysis of DNA content at indicated time points. (B) Cycling cells of the indicated mutants were spotted on only-YPD plates (left panel) or on YPD plates containing either 10 µg/ml (central panel) or 15 µg/ml (right panel) benomyl. Serial diluitions (1/10 from right to left) were made starting from $3x10^{-7}$ cells. This experiment is representative of two repeats.

Discussion

Cdk1 is not required to maintain the SAC active in budding yeast

Previous works have implicated Cdk1:Clb2 in SAC activation/maintenance in budding yeast (Amon, 1997; Li and Cai, 1997); the data accumulated in favor of this hypothesis were largely indirect and not definitive, because there was no clear demonstration that Cdk1 inhibition underlies silencing of the checkpoint at some level of the SAC cascade. In particular, Angelika Amon showed that almost complete inhibition of Cdk1 kinase activity causes sister chromatid separation in the presence of nocodazole, but the same Amon didn't prove, and neither she claimed, that this was due to MCC disassembly and re-activation of APC/C^{Cdc20} (Amon, 1997).

In this work I have demonstrated that maximal inhibition of Cdk1 causes degradation of securin/Pds1 and Clb2 in nocodazole (which confirms Amon's results), and that this is due to the unphysiological overactivation of APC/C^{Cdh1} and not to APC/C^{Cdc20} reactivation resulting from MCC disassembly.

I came to this conclusion based on the following data:

1) deletion of *CDH1* prevents Pds1 and Clb2 degradation upon Cdk1 inhibition in nocodazole;
2) Mad2 remains localized at unattached kinetochores even when Cdk1 activity is strongly inhibited. This suggests that Cdk1 is not required to keep the SAC active upstream in the cascade;
3) Cdc20 is rapidly and completely degraded upon Cdk1 inhibition, much before the occurrence of Pds1 and Clb2 degradation. This last point conclusively excludes that APC/C^{Cdc20} reactivation is implicated in the degradation of Pds1

and Clb2 observed by Amon and confirmed by us. In our view, this result supports the notion that APC/C^{Cdh1} is the only responsible for overcoming the SAC arrest when Cdk1 is inhibited.

The requirement of Cdk1 activity for maintaining Cdc20 synthesis and mitotic levels of Cdc20 suggests that Cdk1:Clb2 is implicated not in SAC activation, but rather in adaptation to the SAC (see next paragraph).

In *Xenopus laevis* and mammalian cells, inhibition of Cdk1:cyclin B does not induce Cdc20 degradation but MCC disassembly and APC/C^{Cdc20} reactivation; this suggests that APC/C regulation by Cdk1 is quite different between lower (e.g., budding yeast) and higher eukaryotes (e.g., mammalian cells) (D'Angiolella et al., 2003).

In budding yeast, my results indicate that, during a checkpoint arrest, Cdk1 and APC/C^{Cdc20} form a negative feedback loop (NFL), with Cdk1:Clb2 activating the APC/C through stimulation of Cdc20 synthesis and phosphorylation of APC/C subunits, and APC/C^{Cdc20} inducing Clb2 degradation and therefore Cdk1 inactivation (Figure 43, left panel).

In vertebrates, Cdk1 not only promotes APC/C^{Cdc20} activation by phosphorylating APC/C subunits (Hegemann et al., 2011; Kraft et al., 2003; Kramer et al., 2000), but it is also clearly implicated in APC/C^{Cdc20} inhibition, at least in conditions of SAC activation (D'Angiolella et al., 2003). This generates the incoherent regulation of the APC/C by Cdk1:cyclin B that has been discussed in paragraph 4.3 of the Introduction (Figure 43, right panel).

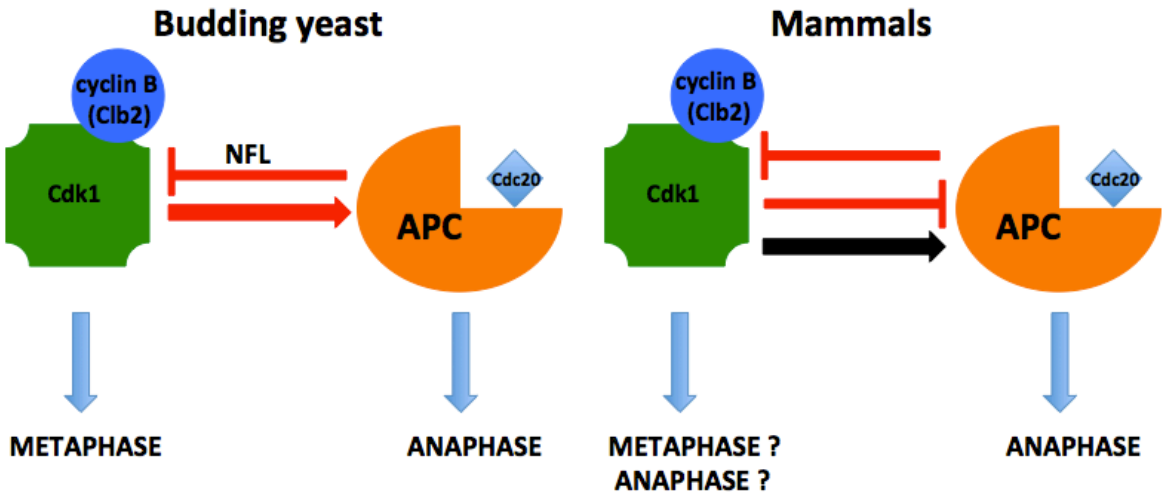

Figure 43. Differences of APC/C^{Cdc20} regulation by Cdk1:cyclin B between yeast and mammals. In budding yeast, Cdk1:cyclin B is implicated in APC/C^{Cdc20} activation by stimulating Cdc20 synthesis and by phosphorylating APC/C subunits; once activated, APC/C^{Cdc20} ubiquitinates cyclin B and induces transition into anaphase by inhibiting Cdk1 activity. The resulting topological connection between Cdk1:cyclin B is a negative feedback loop (NFL), with high Cdk1 activity being typical of metaphase, and high APC/C^{Cdc20} activity characterizing anaphase (left panel). In mammalian cells, Cdk1:cyclin B is also associated to APC/C^{Cdc20} inhibition, at least in conditions of SAC activation; this generates an incoherent regulation of the APC/C by Cdk1:cyclin B, with the result that the output of the system (metaphase, anaphase) cannot be simply predicted on the basis of Cdk1 activity (low, high) (right panel).

Multiple mechanisms of adaptation in yeast cells: what is the truth?

Several mechanisms have been proposed to play a role in adaptation to the SAC in budding yeast cells (see Introduction, paragraph 5.2). Most of them can be conceptually included in two groups:

1) mechanisms of ectopic APC/C activation that bypass the requirement for Cdc20;
2) mechanisms that switch off the SAC cascade at the level of the kinetochores.

My results contradict or exclude most of these mechanisms, as discussed in the next two paragraphs.

1) Adaptation to the SAC requires APC/C^{Cdc20}, which cannot be bypassed by other APC/C activating mechanisms

The idea that adaptation occurs while Cdc20 is still completely sequestered by the MCC assumes that the MCC is a very stable complex and that the rate of Cdc20 sequestration in the MCC is not significantly altered during the SAC arrest: in this view, cells would adapt activating APC/C against Pds1 and Clb2 via Cdc20-independent mechanisms. The most obvious candidate for such an activation of the APC/C is Cdh1, the other known coactivator. Dephosphorylation of Cdh1 by Cdc14 strongly induces APC/C^{Cdh1} activity (Visintin et al., 2008). This is why Cdh1 and Cdc14 have been considered as the most relevant molecular actors for bypassing Cdc20 during adaptation (Rossio et al., 2010a; Toda et al., 2012).

The fact that inhibition of Cdc14 is lethal for SAC-arrested cells has been interpreted as a requirement for Cdc14 to adapt to the SAC (Rossio et al., 2010a). In my experiments, however, I clearly showed that cells in which Cdc14 was inactivated (cdc14-1 mutants at 37^{o}C) adapt to the SAC and enter anaphase similarly to wild-type cells. It is true that after adaptation they are permanently arrested in anaphase/telophase, but this is because Cdc14 is required for mitotic exit, in adapted as well as in unperturbed cells (see Introduction, Paragraph 2.2). It is thus not surprising that Cdc14 inhibition is lethal for SAC arrested cells just as it is lethal for normally cycling cells.

I also excluded directly any essential role for Cdh1 by showing that cells lacking this coactivator of the APC/C adapt with kinetics that are practically identical to those of wild-type cells (see Figure 12). I interpret the previously reported involvement of Cdh1 in adaptation as the consequence of the specific genetic background (deletion of

the MEN inhibitor Bub2) in which those experiments were performed: in fact, *bub2Δ* cells are known to bypass the SAC arrest by ectopically activating APC/C^{Cdh1} via precocious release of Cdc14 (see Introduction, Paragraph 5.2, and (Toda et al., 2012)).

In summary, my results suggest that adaptation occurs via reactivation of APC/C^{Cdc20} and no other parallel mechanisms of the APC/C activation are essentially involved in the process.

2) Adaptation is induced by a downstream event that influences the ability of the MCC to completely sequester Cdc20

In my experiments I activated the SAC by overexpressing Mad2. This has been shown to be a reliable system to induce MCC assembly and APC/C^{Cdc20} inhibition independently from Mad1, Bub1 localization at kinetochores, the kinetochore component Ndc10 (Mariani et al., 2012), and Mps1 (data not shown from our lab). I can thus conclude that adaptation to the SAC, at least in conditions of Mad2 overexpression, cannot be due to the negative regulation of either Mad1 or Bub1 or Mps1. This argument excludes several mechanisms that have been proposed to explain adaptation to the SAC.

In particular, some studies suggested that adaptation in yeast is driven by degradation of Bub1 (Toda et al., 2012). Similarly, Mps1 degradation could be potentially, slowly degraded during a SAC arrest, until its activity becomes insufficient to keep the arrest, just as occurs during the silencing of the checkpoint (Palframan et al., 2006). Both Bub1 and Mps1 are essentially required to localize Mad1 and Mad2 at kinetochores upon spindle disruption; their inactivation would switch off the SAC cascade and consequently induce disassembly of the MCC, similarly to SAC silencing (see Introduction, Paragraph 4.6). In another work, it was shown

that loss of Mad1 binding to Bub3 temporally corresponds to the time of adaptation, just as if Mad1 inactivation played a role in this event (Rossio et al., 2010a).

For the reasons outlined above, I believe that these hypotheses cannot be correct. Instead, Mad2 overexpressing cells adapt with kinetics that are similar to those observed in the presence of nocodazole, which activates the full SAC cascade at the level of unattached kinetochores (Rossio et al., 2010a). This fact suggests that the event that initiates adaptation occurs downstream in the cascade, at the level of the MCC, and I hypothesize that this mechanism competes for Cdc20 with MCC itself. According to this hypothesis, in my study I show that reactivation of APC/C^{Cdc20} is necessary for adaptation and that this likely occurs through phosphorylation-induced stimulation of APC/C binding to Cdc20. Along this line, I believe that, when cells adapt, a certain amount of Cdc20 binds and activates phosphorylated APC/C (APCP), instead of being sequestered within the MCC.

It is worth mentioning that the concept that adaptation to the SAC occurs independently from kinetochores signaling and instead implies a downstream event of the SAC cascade is also corroborated by data coming from studies on mammalian cells (Brito and Rieder, 2006b).

How can then we make sense of the previously cited results which suggest that adaptation requires the silencing of the SAC cascade upstream of the MCC? Degradation of Bub1 and Mps1 at the anaphase onset depends on APC/C^{Cdc20} (Goto et al., 2011; Palframan et al., 2006). I thus tend to believe that the degradation of these proteins, together with the loss of Mad1-Bub3 binding, are the consequence, and not the cause, of APC/C^{Cdc20} reactivation that underlies adaptation.

A model of SAC adaptation in yeast cells

APC/C phosphorylation by Cdk1 is essential to adapt

The main question then becomes: how is it possible that cells activate APC/C^{Cdc20} during adaptation if Cdc20 is sequestered within the MCC?

The simplest model that could explain such APC/C^{Cdc20} activation is the so-called ''Slippage model", which was proposed by Brito and Rieder to explain adaptation to the SAC in animal cells (Brito and Rieder, 2006b). According to this model, the SAC is not 100% effective in sequestering Cdc20: a little activity of APC/C^{Cdc20} is always present and induces slow degradation of APC/C substrates, until cyclin B levels become so low that cells cannot sustain the mitotic state and thus slip out of mitosis. In this case cells do not need to reactivate APC/C^{Cdc20} because it is always partially active.

In contrast with the slippage model, I show that the SAC completely inhibits APC/C^{Cdc20} in yeast, as demonstrated by complete Clb2 stabilization during the several hours of checkpoint-induced arrest. Then, concomitantly with adaptation, APC/C is rapidly activated and induces the fast degradation of Clb2. These dynamics of Clb2 accumulation and degradation, which reflect dynamics of APC/C^{Cdc20} inhibition and reactivation, suggest that at a certain point an unknown molecular mechanism leads to fast and irreversible reactivation of APC/C^{Cdc20}.

In this work I investigated the nature of this mechanisms. I provided evidence that Cdk1-induced phosphorylation of Cdc16 and Cdc27 subunits of the APC/C is necessary to reactivate APC/C^{Cdc20}. In doing so, Cdk1 is counteracted by the $PP2A^{Cdc55}$ phosphatase, which, directly or indirectly, promotes dephosphorylation of Cdc16 (Figure 44).

How can phosphorylated APC/C (i.e., APCP) remove Cdc20 from the MCC?

In previous studies, the phosphorylation state of Cdc16 and Cdc27 was shown to have a strong impact on the ability of the APC/C to bind Cdc20 (Rudner and Murray, 2000). A plausible idea that can explain the requirement for APC/C phosphorylation in adaptation is that Cdc20 binds APCP better than Mad2 and Mad3. As a consequence, as long as APC/C subunits remain in an unphosphorylated/poorly phosphorylated state, Cdc20 is sequestered with high efficiency by Mad2 and Mad3 within APC:MCC. Once Cdc16 and Cdc27 become progressively phosphorylated, APCP starts to compete with Mad2 and Mad3 for binding Cdc20.

My data suggest that APC/C phosphorylation might be helped by other mechanisms to drive adaptation to the SAC. Progressive accumulation of Cdc20 (Figure 34), due to an unbalance between synthesis and degradation over the several hours that precede adaptation, could increase the chance of Cdc20 to bind APCP and to reach the threshold of APC/C^{Cdc20} activity that is required to induce degradation of securin/Pds1 and the metaphase-to-anaphase transition.

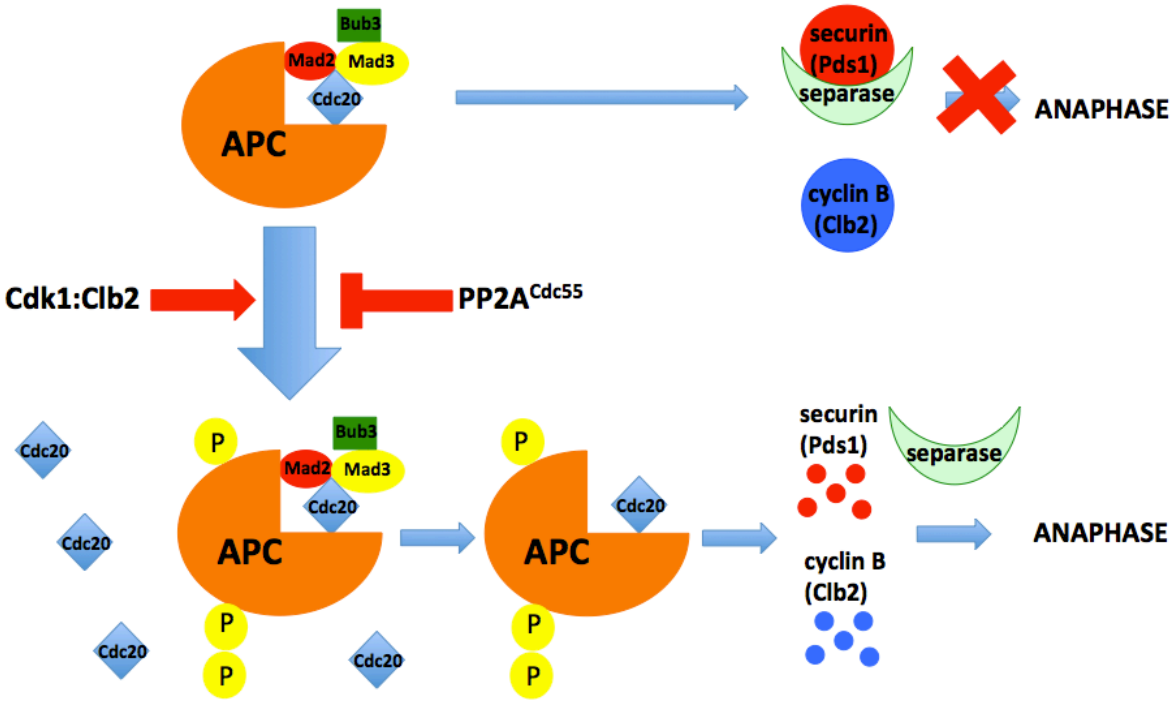

Figure 44. Schematic representation of my model of adaptation to the SAC. Progressive phosphorylation of the APC/C by Cdk1, which is counteracted by PP2A^{Cdc55}, increases the binding

affinity of APCP for free Cdc20. This, together with the increase in free Cdc20 due to intrinsic, long-term unbalance between its synthesis and degradation, increases the chance for APC/C to bind one free Cdc20 molecule, instead of being sequestered by APC:MCC. Once formed, even a low amount of $APCP^{Cdc20}$ can finally trigger ubiquitination of securin/Pds1 and cyclin B, leading to their degradation and to transition into anaphase.

A positive feedback (PFL) loop can explain the fast APC/C activation during adaptation

The struggle between Cdk1 kinase and $PP2A^{Cdc55}$ phosphatase for determining the phosphorylation state of APC/C during a SAC arrest can explain the progressive increase in APC/C phosphorylation and activation that precedes adaptation as a result of the increasing amount of Clb2 (and likely of Cdk1 activity)(Figure 24). Nevertheless, it cannot satisfactorily explain the fast and complete activation of the APC/C against Clb2 (and Pds1) during adaptation (Figure 22). Indeed, the fact that Cdk1 activity progressively increases in time with the accumulation of Clb2 would suggest a progressive increase of APCP, and thus eventually to a smooth degradation of Clb2. In my single-cell experiments, instead, I observe an all-or-none degradation of Clb2.

In the simplest hypothesis, rapid and irreversible degradation of Clb2 could be guaranteed if the APC/C and $PP2A^{Cdc55}$ were involved in a double negative loop of reciprocal inhibition (APC/C--| $PP2A^{Cdc55}$ --| APC/C). In this case, the initial activation of APC/C would strongly and irreversibly potentiate itself by inhibiting its inhibitor, PP2A. This would result in a fast and irreversible degradation of Clb2 as a result of the rapid and irreversible activation of the APC/C.

I therefore looked for the presence of such a double negative feedback loop. Putting together data from the literature, I found that such a loop is guaranteed by the following molecular connections: APC/C inhibits securin (Yamamoto et al., 1996a;

Yamamoto et al., 1996b), which inhibits separase (Tinker-Kulberg and Morgan, 1999), which inhibits $PP2A^{Cdc55}$ (Queralt et al., 2006), which inhibits APC/C via dephosphorylation (Figure 26). This is a closed loop consisting of four negative connections (APC/C--|securin--|separase--|$PP2A^{Cdc55}$--|APC/C), which can be schematically visualized as a double negative feedback loop between APC/C and PP2A (APC/C--|$PP2A^{Cdc55}$--|APC/C) (Figure 45, A and B). Such a topological structure conceptually corresponds to a positive feedback loop (PFL), which can guarantee existence of two alternative states of the system: one with high PP2A and low APC/C activity (the metaphase state), and the opposite one with high APC/C and low PP2A activity (the anaphase state) -- similarly to what explained for Mps1 (Introduction, Paragraph 4.6). Transition from the former to the latter state corresponds to adaptation and is characterized by the fact that initial activation of the APC/C is reinforced and speeded up by the inhibition of its inhibitor, $PP2A^{Cdc55}$; in this way, full activation of the APC/C corresponds to inhibition of $PP2A^{Cdc55}$ (Figure 45, A and B). At this point, even if for some reasons (e.g. partial re-sequestration of Cdc20 or reduced Cdc20 levels) APC/C^{Cdc20} activity is partially reduced, the fact that $PP2A^{Cdc55}$ is completely inhibited keeps the APC/C in an active state and avoids stabilization of Clb2.

In this view, the event that initiates adaptation might consist in the progressive increase of Cdk1 activity (driven by progressive increase of Clb2 levels (Figure 45B)), which would induce in turn an increase in APCP sufficient to trigger the PFL and to switch the system from the $PP2A^{Cdc55}$ activated-APC/C inhibited state to the APC/C activated-$PP2A^{Cdc55}$ inhibited one.

The, yet uncertain, origin of variability in adaptation times

Nevertheless, while the antagonism between Cdk1 and $PP2A^{Cdc55}$ can satisfactorily explain the fast and irreversible activation of APC/C that initiates adaptation, it cannot account for the wide variability of the adaptation time between individual cells (Figure 22, right upper panel).

According to my model, this variability should stem from stochastically different initial levels of Cdk1 and $PP2A^{Cdc55}$ among different cells that would result in different initial levels of APC/C activation and, consequently, in highly variable times required to reach the threshold of APC/C phosphorylation (and activation) required to trigger the PFL that initiates adaptation.

However, my data demonstrate that initial levels of Clb2 are uniformly low in cells entering mitosis after background subtraction (Figure 22, left panels); moreover, Clb2 (and, likely, Cdk1:Clb2 activity) increases with very similar rates (see the slopes of Clb2 accumulation curves in Figure 22, left panels) in the several cells analyzed. If there is a strong link between Clb2 levels measured and Cdk1:Clb2 activity as expected, these results potentially exclude that the observed variability in the adaptation times originates from stochastically different levels of Cdk1. The concept that the origin of variability lies in the different levels of APCP is nevertheless possible: it would require that adaptation times correlate with the activation status of $PP2A^{Cdc55}$. As I don't have any single-cell indicator of the potentially variable activity of $PP2A^{Cdc55}$, I cannot corroborate this hypothesis yet.

Another stochastic element that could account for the highly different adaptation times is represented by free Cdc20: initial activation of APC/C could be due to casual binding of a free molecule of Cdc20 to APC/C. As a consequence, cells bearing higher

levels of free Cdc20 would have increased chances to activate the APC/C and to transit into anaphase. As yet, I haven't managed to visualize functional, fluorescently-tagged Cdc20 at the single cell level; as a consequence, I don't know if there actually exist a positive correlation between higher Cdc20 levels and shorter adaptation times.

Whatever the event that initiates the adaptation process, once the transition to the APC/C-activated state ($APCP^{Cdc20}$) has finally occurred, the positive feedback loop between Cdk1:Clb2 and $APCP^{Cdc20}$ ensures fast and complete degradation of Clb2 (Figure 45B).

According to this view, coexistence of stochastic elements that initiate APC/C activation with system-level properties (the positive feedback loop) that guarantee irreversibility of transitions is perfectly compatible with observed data and maybe is the best way to explain them.

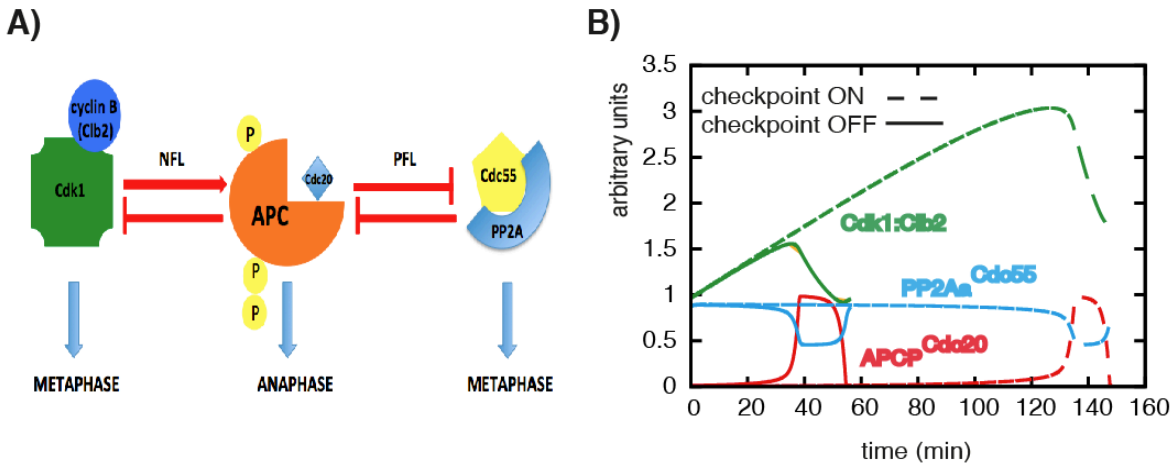

Figure 45. PFL between active APC/C and $PP2A^{Cdc55}$ allows switch-like transition into anaphase. (A) Schematization of the network at the basis of the metaphase-to-anaphase transition: negative feedback loop (NFL) between Cdk1:Clb2 and active APC/C^{Cdc20} ($APCP^{Cdc20}$) is associated to positive feedback loop (PFL) between $APCP^{Cdc20}$ and $PP2A^{Cdc55}$. (B) The PFL in (A) guarantees that $APCP^{Cdc20}$ activation coincides with $PP2A^{Cdc55}$ inhibition and fast Clb2 degradation at the metaphase-to-anaphase transition. Adapted from Vernieri et al., 2013.

The role of Cdc20 accumulation

I don't know as yet if Cdc20 accumulation that precedes adaptation plays any role in increasing the reactivation rate of APC/C^{Cdc20}. Based on the following indirect indications I believe that this could be the case:

a) yeast mutants expressing slightly increased Cdc20 levels are SAC-defective even in the presence of a fully active SAC machinery (Pan and Chen, 2004);
b) when I deleted the non-essential *MND2* subunit of the APC/C, I observed a two-fold increase in Cdc20 levels and faster adaptation kinetics (Figure 35);
c) simultaneous deletion of Mnd2 and Mad3, which are both implicated in Cdc20 destabilization during a SAC arrest, has an additive effect on Cdc20 levels and speeds up rebudding and DNA-reduplication kinetics in the presence of nocodazole (Figure 40).

Adaptation in mammalian cells: any relationship with budding yeast?

My single-cell experiments in budding yeast indicate that adaptation to the SAC is caused by an event that induces fast and strong APC/C^{Cdc20} reactivation. Until this event occurs, the APC/C is very efficiently inhibited by the checkpoint, as confirmed by stable Clb2 levels during the time of arrest in metaphase (Figures 21 and 22).

Strikingly, single cell experiments performed on human cell lines, both transformed and not, reached the opposite conclusion: the APC/C is not completely inhibited during a SAC arrest, which results in a constant, slow degradation of cyclin B, until residual activity of Cdk1:cyclin B is insufficient to maintain the mitotic state and cells slip out of mitosis (Brito and Rieder, 2006b; Gascoigne and Taylor, 2008). As predicted by the slippage model, mild cyclin B overexpression prolong the mitotic

arrest and increase the chance for cancer cells to activate the apoptotic pathways and consequently die in mitosis (Gascoigne and Taylor, 2008)..

In my opinion, one weak point of the slippage model is that it is difficult to reconcile it with the role of Cdk1 in activating the SAC (D'Angiolella et al., 2003): the double negative feedback loop between Cdk1 and APC/C^{Cdc20} in human cells (see Figure 6) would predict that at a certain point cyclin B degradation strongly potentiates APC/C^{Cdc20} activity, thus speeding up the last phase of cyclin B degradation. The fact that cyclin B is progressively, and not abruptly, degraded before adaptation does not fit with this prediction. Moreover, it remains unproven that degradation of cyclin B is the cause and not the consequence of the slippage: indeed, inhibition of mitotic slippage that is caused by expression of undegradable cyclin B could be simply due to the fact that stable cyclin B in mammals inhibits transition into anaphase also during an unperturbed cell cycle (Chang et al., 2003).

Another criticism that I move to the slippage model originates from quite a technical argument. It has been shown that mitotic translation of cyclin B in embryonic and non-embryonic mammalian cells strongly depends on cytoplasmic polyadenylation elements (CPEs) that are present in the 3'UTR of cyclin B mRNA (Groisman et al., 2002; Malureanu et al., 2010). These CPE elements are recognized by specific binding proteins that start initiation of translation. In the absence of 3'UTR sequences, transcription of cyclin B, and, consequently, its protein levels in metaphase, are strongly reduced. The relevance of these regulatory elements is not minor: differences in cyclin B degradation kinetics between normal and Cdc20 hypomorphic-cells are obscured by the classical cyclin B-GFP reporter, but reemerge once 3'UTR are reintroduced (Malureanu et al., 2010).

In works that proposed and validated the slippage model, cells were transformed with plasmids containing fusion genes of cyclin B-GFP where, as far as we know, the

CPE elements were lacking. If so, the real kinetics of cyclin B degradation may have been obscured by unrealistically low, initial levels of cyclin B, and the slow activity of APC/C^{Cdc20} might have been enough to get rid of the poorly translated cyclin B messenger. In our lab we are now testing this hypothesis by analyzing kinetics of cyclin B degradation in SAC-arrested cells in which a GFP-coding sequence follows the 3' of the endogenous gene of cyclin B which was inserted from a Bacterial Artifical Chromosome (BAC) which contains 70kb downstream of the ATG and as such is likely to contain all regulatory elements.

If slow degradation of cyclin B during adaptation to the checkpoint will be confirmed also in these cell population, then we will definitively believe that the mechanisms of SAC adaptation in yeast are very different from those in mammals. If, on the contrary, expression of fully regulated cyclin B induced higher levels of stable protein during the arrest, followed by rapid degradation during adaptation as observed in yeast, this could be a strong argument against the slippage model. Such a result would require a profound rethinking of the molecular mechanisms of adaptation also in mammalian cells.

In the case that the slippage model should be confirmed, the main open question would be the following: why should lower eukaryotes, such as budding yeasts, mount a very efficient SAC response with completely inhibited APC/C^{Cdc20}, while higher eukaryotes, such as mammalian cells, are not able to do so?

The reason why vertebrate cells don't actually need a completely efficient SAC can be likely understood by looking at the physiological role of the checkpoint in these cells.

In vertebrates, the SAC ensures accurate segregation of DNA and avoids occurrence of aneuploidy during any unperturbed cell cycle.

When vertebrate cells activate the SAC in prometaphase, the checkpoint usually remains active for a short time; within minutes cells properly biorient sister

chromatids and can safely proceed into anaphase. In these physiological conditions it is sufficient for these cells to simply delay the onset of anaphase until all chromosomes are attached to microtubules.

The inhibition of the APC/C provided by the SAC, even if not complete, is more than sufficient to assure such a delay (Meraldi et al., 2004). Prolonged arrests, such as those caused by treatment with high doses of nocodazole and taxol, are not physiological at all: it is difficult to imagine a moment in the life of an eukaryotic cell, when microtubules cannot attach kinetochores for hours. Maybe evolution of vertebrate organisms was not forced to further improve the efficiency of the SAC response, so to provide 100% inhibition of APC/C^{Cdc20} upon SAC activation. Why should have evolution encouraged such a strong efficiency in yeast, that uses the SAC even less than higher eukaryotes remains a great mystery.

References

Amon, A. 1997. Regulation of B-type cyclin proteolysis by Cdc28-associated kinases in budding yeast. *EMBO J*. 16:2693-2702.

Azzam, R., S.L. Chen, W. Shou, A.S. Mah, G. Alexandru, K. Nasmyth, R.S. Annan, S.A. Carr, and R.J. Deshaies. 2004. Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. *Science*. 305:516-519.

Bardin, A.J., M.G. Boselli, and A. Amon. 2003. Mitotic exit regulation through distinct domains within the protein kinase Cdc15. *Molecular and cellular biology*. 23:5018-5030.

Bardin, A.J., R. Visintin, and A. Amon. 2000. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell*. 102:21-31.

Bishop, A.C., J.A. Ubersax, D.T. Petsch, D.P. Matheos, N.S. Gray, J. Blethrow, E. Shimizu, J.Z. Tsien, P.G. Schultz, M.D. Rose, J.L. Wood, D.O. Morgan, and K.M. Shokat. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature*. 407:395-401.

Bizzari, F., and A.L. Marston. 2011. Cdc55 coordinates spindle assembly and chromosome disjunction during meiosis. *The Journal of cell biology*. 193:1213-1228.

Brady, D.M., and K.G. Hardwick. 2000. Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. *Current biology : CB*. 10:675-678.

Brito, D.A., and C.L. Rieder. 2006a. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr Biol*. 16:1194-1200.

Brito, D.A., and C.L. Rieder. 2006b. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Current biology : CB*. 16:1194-1200.

Caydasi, A.K., B. Ibrahim, and G. Pereira. 2010. Monitoring spindle orientation: Spindle position checkpoint in charge. *Cell division*. 5:28.

Chang, D.C., N. Xu, and K.Q. Luo. 2003. Degradation of cyclin B is required for the onset of anaphase in Mammalian cells. *The Journal of biological chemistry*. 278:37865-37873.

Chao, W.C., K. Kulkarni, Z. Zhang, E.H. Kong, and D. Barford. 2012. Structure of the mitotic checkpoint complex. *Nature*. 484:208-213.

Chiroli, E., V. Rossio, G. Lucchini, and S. Piatti. 2007. The budding yeast PP2ACdc55 protein phosphatase prevents the onset of anaphase in response to morphogenetic defects. *The Journal of cell biology*. 177:599-611.

Chu, Q., M. Vincent, D. Logan, J.A. Mackay, and W.K. Evans. 2005. Taxanes as first-line therapy for advanced non-small cell lung cancer: a systematic review and practice guideline. *Lung cancer*. 50:355-374.

Clift, D., F. Bizzari, and A.L. Marston. 2009. Shugoshin prevents cohesin cleavage by PP2A(Cdc55)-dependent inhibition of separase. *Genes Dev*. 23:766-780.

Cohen-Fix, O., and D. Koshland. 1999. Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. *Genes & development*. 13:1950-1959.

Collin, P., O. Nashchekina, R. Walker, and J. Pines. 2013. The spindle assembly checkpoint works like a rheostat rather than a toggle switch. *Nature cell biology*. 15:1378-1385.

D'Angiolella, V., C. Mari, D. Nocera, L. Rametti, and D. Grieco. 2003. The spindle checkpoint requires cyclin-dependent kinase activity. *Genes Dev*. 17:2520-2525.

De Antoni, A., C.G. Pearson, D. Cimini, J.C. Canman, V. Sala, L. Nezi, M. Mapelli, L. Sironi, M. Faretta, E.D. Salmon, and A. Musacchio. 2005a. The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. *Current biology : CB*. 15:214-225.

De Antoni, A., C.G. Pearson, D. Cimini, J.C. Canman, V. Sala, L. Nezi, M. Mapelli, L. Sironi, M. Faretta, E.D. Salmon, and A. Musacchio. 2005b. The mad1/mad2 complex as a template for mad2 activation in the spindle assembly checkpoint. *Curr Biol*. 15:214-225.

Ditchfield, C., V.L. Johnson, A. Tighe, R. Ellston, C. Haworth, T. Johnson, A. Mortlock, N. Keen, and S.S. Taylor. 2003. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *The Journal of cell biology*. 161:267-280.

Drapkin, B.J., Y. Lu, A.L. Procko, B.L. Timney, and F.R. Cross. 2009. Analysis of the mitotic exit control system using locked levels of stable mitotic cyclin. *Mol Syst Biol*. 5:328.

Fesquet, D., P.J. Fitzpatrick, A.L. Johnson, K.M. Kramer, J.H. Toyn, and L.H. Johnston. 1999. A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. *The EMBO journal*. 18:2424-2434.

Foe, I.T., S.A. Foster, S.K. Cheung, S.Z. DeLuca, D.O. Morgan, and D.P. Toczyski. 2011. Ubiquitination of Cdc20 by the APC occurs through an intramolecular mechanism. *Current biology : CB*. 21:1870-1877.

Fossella, F.V., R. DeVore, R.N. Kerr, J. Crawford, R.R. Natale, F. Dunphy, L. Kalman, V. Miller, J.S. Lee, M. Moore, D. Gandara, D. Karp, E. Vokes, M. Kris, Y. Kim, F. Gamza, and L. Hammershaimb. 2000. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 18:2354-2362.

Foster, S.A., and D.O. Morgan. 2012. The APC/C subunit Mnd2/Apc15 promotes Cdc20 autoubiquitination and spindle assembly checkpoint inactivation. *Molecular cell*. 47:921-932.

Fraschini, R., E. Formenti, G. Lucchini, and S. Piatti. 1999. Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2. *The Journal of cell biology*. 145:979-991.

Gascoigne, K.E., and S.S. Taylor. 2008. Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell*. 14:111-122.

Gascoigne, K.E., and S.S. Taylor. 2009. How do anti-mitotic drugs kill cancer cells? *J Cell Sci*. 122:2579-2585.

Geymonat, M., A. Spanos, G. de Bettignies, and S.G. Sedgwick. 2009. Lte1 contributes to Bfa1 localization rather than stimulating nucleotide exchange by Tem1. *The Journal of cell biology*. 187:497-511.

Ghersi, D., N. Wilcken, and R.J. Simes. 2005. A systematic review of taxane-containing regimens for metastatic breast cancer. *British journal of cancer*. 93:293-301.

Gordon, D.J., B. Resio, and D. Pellman. 2012. Causes and consequences of aneuploidy in cancer. *Nature reviews. Genetics*. 13:189-203.

Goto, G.H., A. Mishra, R. Abdulle, C.A. Slaughter, and K. Kitagawa. 2011. Bub1-mediated adaptation of the spindle checkpoint. *PLoS Genet*. 7:e1001282.

Groisman, I., M.Y. Jung, M. Sarkissian, Q. Cao, and J.D. Richter. 2002. Translational control of the embryonic cell cycle. *Cell*. 109:473-483.

Hardwick, K.G., R.C. Johnston, D.L. Smith, and A.W. Murray. 2000. MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p. *J Cell Biol*. 148:871-882.

Hardwick, K.G., and A.W. Murray. 1995. Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *The Journal of cell biology*. 131:709-720.

Hardwick, K.G., E. Weiss, F.C. Luca, M. Winey, and A.W. Murray. 1996. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science*. 273:953-956.

He, E., O. Kapuy, R.A. Oliveira, F. Uhlmann, J.J. Tyson, and B. Novak. 2011. System-level feedbacks make the anaphase switch irreversible. *Proceedings of the National Academy of Sciences of the United States of America*. 108:10016-10021.

Hegemann, B., J.R. Hutchins, O. Hudecz, M. Novatchkova, J. Rameseder, M.M. Sykora, S. Liu, M. Mazanek, P. Lenart, J.K. Heriche, I. Poser, N. Kraut, A.A. Hyman, M.B. Yaffe, K. Mechtler, and J.M. Peters. 2011. Systematic phosphorylation analysis of human mitotic protein complexes. *Science signaling*. 4:rs12.

Holland, A.J., and D.W. Cleveland. 2009. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature reviews. Molecular cell biology*. 10:478-487.

Holt, L.J., A.N. Krutchinsky, and D.O. Morgan. 2008. Positive feedback sharpens the anaphase switch. *Nature*. 454:353-357.

Howell, B.J., B.F. McEwen, J.C. Canman, D.B. Hoffman, E.M. Farrar, C.L. Rieder, and E.D. Salmon. 2001. Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *The Journal of cell biology*. 155:1159-1172.

Hoyt, M.A., L. Totis, and B.T. Roberts. 1991. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell*. 66:507-517.

Huang, B., and T.C. Huffaker. 2006. Dynamic microtubules are essential for efficient chromosome capture and biorientation in S. cerevisiae. *The Journal of cell biology*. 175:17-23.

Huang, H.C., J. Shi, J.D. Orth, and T.J. Mitchison. 2009. Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly. *Cancer cell*. 16:347-358.

Huang, J.N., I. Park, E. Ellingson, L.E. Littlepage, and D. Pellman. 2001. Activity of the APC(Cdh1) form of the anaphase-promoting complex persists until S phase and prevents the premature expression of Cdc20p. *The Journal of cell biology*. 154:85-94.

Hwang, L.H., L.F. Lau, D.L. Smith, C.A. Mistrot, K.G. Hardwick, E.S. Hwang, A. Amon, and A.W. Murray. 1998. Budding yeast Cdc20: a target of the spindle checkpoint. *Science*. 279:1041-1044.

Jaspersen, S.L., and D.O. Morgan. 2000. Cdc14 activates cdc15 to promote mitotic exit in budding yeast. *Current biology : CB*. 10:615-618.

Jiang, Y. 2006. Regulation of the cell cycle by protein phosphatase 2A in Saccharomyces cerevisiae. *Microbiology and molecular biology reviews : MMBR*. 70:440-449.

Johnson, V.L., M.I. Scott, S.V. Holt, D. Hussein, and S.S. Taylor. 2004. Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. *Journal of cell science*. 117:1577-1589.

Jordan, M.A., D. Thrower, and L. Wilson. 1991. Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer research*. 51:2212-2222.

Jordan, M.A., and L. Wilson. 2004. Microtubules as a target for anticancer drugs. *Nature reviews. Cancer*. 4:253-265.

Kelling, J., K. Sullivan, L. Wilson, and M.A. Jordan. 2003. Suppression of centromere dynamics by Taxol in living osteosarcoma cells. *Cancer research*. 63:2794-2801.

Kimata, Y., J.E. Baxter, A.M. Fry, and H. Yamano. 2008. A role for the Fizzy/Cdc20 family of proteins in activation of the APC/C distinct from substrate recruitment. *Molecular cell*. 32:576-583.

Kinoshita, E., E. Kinoshita-Kikuta, M. Matsubara, S. Yamada, H. Nakamura, Y. Shiro, Y. Aoki, K. Okita, and T. Koike. 2008. Separation of phosphoprotein isotypes having the same number of phosphate groups using phosphate-affinity SDS-PAGE. *Proteomics*. 8:2994-3003.

Kitazono, A.A., D.A. Garza, and S.J. Kron. 2003. Mutations in the yeast cyclin-dependent kinase Cdc28 reveal a role in the spindle assembly checkpoint. *Mol Genet Genomics*. 269:672-684.

Kops, G.J., and J.V. Shah. 2012. Connecting up and clearing out: how kinetochore attachment silences the spindle assembly checkpoint. *Chromosoma*. 121:509-525.

Kraft, C., F. Herzog, C. Gieffers, K. Mechtler, A. Hagting, J. Pines, and J.M. Peters. 2003. Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *The EMBO journal*. 22:6598-6609.

Kramer, E.R., N. Scheuringer, A.V. Podtelejnikov, M. Mann, and J.M. Peters. 2000. Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Molecular biology of the cell*. 11:1555-1569.

Kulukian, A., J.S. Han, and D.W. Cleveland. 2009. Unattached kinetochores catalyze production of an anaphase inhibitor that requires a Mad2 template to prime Cdc20 for BubR1 binding. *Developmental cell*. 16:105-117.

Kwiatkowski, N., N. Jelluma, P. Filippakopoulos, M. Soundararajan, M.S. Manak, M. Kwon, H.G. Choi, T. Sim, Q.L. Deveraux, S. Rottmann, D. Pellman, J.V. Shah, G.J. Kops, S. Knapp, and N.S. Gray. 2010. Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function. *Nature chemical biology*. 6:359-368.

Lau, D.T., and A.W. Murray. 2012b. Mad2 and Mad3 cooperate to arrest budding yeast in mitosis. *Current biology : CB*. 22:180-190.

Lee, S.E., L.M. Frenz, N.J. Wells, A.L. Johnson, and L.H. Johnston. 2001. Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. *Current biology : CB*. 11:784-788.

Lengauer, C., K.W. Kinzler, and B. Vogelstein. 1997. Genetic instability in colorectal cancers. *Nature*. 386:623-627.

Li, R., and A.W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell*. 66:519-531.

Li, X., and M. Cai. 1997. Inactivation of the cyclin-dependent kinase Cdc28 abrogates cell cycle arrest induced by DNA damage and disassembly of mitotic spindles in Saccharomyces cerevisiae. *Molecular and cellular biology*. 17:2723-2734.

Liang, H., H.H. Lim, A. Venkitaraman, and U. Surana. 2012. Cdk1 promotes kinetochore bi-orientation and regulates Cdc20 expression during recovery from spindle checkpoint arrest. *The EMBO journal*. 31:403-416.

Lim, H.H., P.Y. Goh, and U. Surana. 1998. Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Current biology : CB*. 8:231-234.

Maldonado, M., and T.M. Kapoor. 2011. Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation. *Nature cell biology*. 13:475-482.

Malureanu, L., K.B. Jeganathan, F. Jin, D.J. Baker, J.H. van Ree, O. Gullon, Z. Chen, J.R. Henley, and J.M. van Deursen. 2010. Cdc20 hypomorphic mice fail to counteract de novo synthesis of cyclin B1 in mitosis. *The Journal of cell biology*. 191:313-329.

Mansfeld, J., P. Collin, M.O. Collins, J.S. Choudhary, and J. Pines. 2011. APC15 drives the turnover of MCC-CDC20 to make the spindle assembly checkpoint responsive to kinetochore attachment. *Nature cell biology*. 13:1234-1243.

Mapelli, M., L. Massimiliano, S. Santaguida, and A. Musacchio. 2007. The Mad2 conformational dimer: structure and implications for the spindle assembly checkpoint. *Cell*. 131:730-743.

Marcus, R., K. Imrie, P. Solal-Celigny, J.V. Catalano, A. Dmoszynska, J.C. Raposo, F.C. Offner, J. Gomez-Codina, A. Belch, D. Cunningham, E. Wassner-Fritsch, and G. Stein. 2008. Phase III study of R-CVP compared with cyclophosphamide, vincristine, and prednisone alone in patients with previously untreated advanced follicular lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 26:4579-4586.

Mariani, L., E. Chiroli, L. Nezi, H. Muller, S. Piatti, A. Musacchio, and A. Ciliberto. 2012. Role of the Mad2 dimerization interface in the spindle assembly checkpoint independent of kinetochores. *Curr Biol*. 22:1900-1908.

Matyskiela, M.E., and D.O. Morgan. 2009. Analysis of activator-binding sites on the APC/C supports a cooperative substrate-binding mechanism. *Molecular cell*. 34:68-80.

Meraldi, P., V.M. Draviam, and P.K. Sorger. 2004. Timing and checkpoints in the regulation of mitotic progression. *Developmental cell*. 7:45-60.

Michel, L.S., V. Liberal, A. Chatterjee, R. Kirchwegger, B. Pasche, W. Gerald, M. Dobles, P.K. Sorger, V.V. Murty, and R. Benezra. 2001. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature*. 409:355-359.

Millband, D.N., and K.G. Hardwick. 2002. Fission yeast Mad3p is required for Mad2p to inhibit the anaphase-promoting complex and localizes to kinetochores in a Bub1p-, Bub3p-, and Mph1p-dependent manner. *Mol Cell Biol*. 22:2728-2742.

Minshull, J., A. Straight, A.D. Rudner, A.F. Dernburg, A. Belmont, and A.W. Murray. 1996. Protein phosphatase 2A regulates MPF activity and sister chromatid cohesion in budding yeast. *Current biology : CB*. 6:1609-1620.

Mirchenko, L., and F. Uhlmann. 2010. Sli15(INCENP) dephosphorylation prevents mitotic checkpoint reengagement due to loss of tension at anaphase onset. *Curr Biol*. 20:1396-1401.

Morin, V., S. Prieto, S. Melines, S. Hem, M. Rossignol, T. Lorca, J. Espeut, N. Morin, and A. Abrieu. 2012. CDK-dependent potentiation of MPS1 kinase activity is essential to the mitotic checkpoint. *Curr Biol*. 22:289-295.

Murata-Hori, M., M. Tatsuka, and Y.L. Wang. 2002. Probing the dynamics and functions of aurora B kinase in living cells during mitosis and cytokinesis. *Molecular biology of the cell*. 13:1099-1108.

Musacchio, A., and E.D. Salmon. 2007. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol*. 8:379-393.

Nasmyth, K., and C.H. Haering. 2009. Cohesin: its roles and mechanisms. *Annual review of genetics*. 43:525-558.

Niitani, H. 1999. [Navelbine (vinorelbine): a review of its antitumor activity and toxicity in clinical studies]. *Gan to kagaku ryoho. Cancer & chemotherapy*. 26:1495-1507.

Nilsson, J., M. Yekezare, J. Minshull, and J. Pines. 2008. The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat Cell Biol*. 10:1411-1420.

Oromendia, A.B., S.E. Dodgson, and A. Amon. 2012. Aneuploidy causes proteotoxic stress in yeast. *Genes & development*. 26:2696-2708.

Palframan, W.J., J.B. Meehl, S.L. Jaspersen, M. Winey, and A.W. Murray. 2006. Anaphase inactivation of the spindle checkpoint. *Science*. 313:680-684.

Pan, J., and R.H. Chen. 2004. Spindle checkpoint regulates Cdc20p stability in Saccharomyces cerevisiae. *Genes Dev*. 18:1439-1451.

Pfleger, C.M., and M.W. Kirschner. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes & development*. 14:655-665.

Piatti, S., M. Venturetti, E. Chiroli, and R. Fraschini. 2006. The spindle position checkpoint in budding yeast: the motherly care of MEN. *Cell division*. 1:2.

Pinsky, B.A., C.R. Nelson, and S. Biggins. 2009. Protein phosphatase 1 regulates exit from the spindle checkpoint in budding yeast. *Current biology : CB*. 19:1182-1187.

Primorac, I., and A. Musacchio. 2013. Panta rhei: the APC/C at steady state. *The Journal of cell biology*. 201:177-189.

Primorac, I., J.R. Weir, E. Chiroli, F. Gross, I. Hoffmann, S. van Gerwen, A. Ciliberto, and A. Musacchio. 2013. Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling. *eLife*. 2:e01030.

Queralt, E., C. Lehane, B. Novak, and F. Uhlmann. 2006. Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. *Cell*. 125:719-732.

Rahal, R., and A. Amon. 2008. Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation. *Genes Dev*. 22:1534-1548.

Rajagopalan, H., M.A. Nowak, B. Vogelstein, and C. Lengauer. 2003. The significance of unstable chromosomes in colorectal cancer. *Nature reviews. Cancer*. 3:695-701.

Rieder, C.L., A. Schultz, R. Cole, and G. Sluder. 1994. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *The Journal of cell biology*. 127:1301-1310.

Robbins, J.A., and F.R. Cross. 2010a. Regulated degradation of the APC coactivator Cdc20. *Cell division*. 5:23.

Rock, J.M., and A. Amon. 2009. The FEAR network. *Current biology : CB*. 19:R1063-1068.

Rossio, V., E. Galati, M. Ferrari, A. Pellicioli, T. Sutani, K. Shirahige, G. Lucchini, and S. Piatti. 2010a. The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdc14 phosphatase. *J Cell Biol*. 191:981-997.

Rossio, V., E. Galati, and S. Piatti. 2010b. Adapt or die: how eukaryotic cells respond to prolonged activation of the spindle assembly checkpoint. *Biochemical Society transactions*. 38:1645-1649.

Rossio, V., and S. Yoshida. 2011. Spatial regulation of Cdc55-PP2A by Zds1/Zds2 controls mitotic entry and mitotic exit in budding yeast. *The Journal of cell biology*. 193:445-454.

Rudner, A.D., K.G. Hardwick, and A.W. Murray. 2000. Cdc28 activates exit from mitosis in budding yeast. *J Cell Biol*. 149:1361-1376.

Rudner, A.D., and A.W. Murray. 2000. Phosphorylation by Cdc28 activates the Cdc20-dependent activity of the anaphase-promoting complex. *J Cell Biol*. 149:1377-1390.

Santaguida, S., and A. Musacchio. 2009. The life and miracles of kinetochores. *The EMBO journal*. 28:2511-2531.

Santaguida, S., C. Vernieri, F. Villa, A. Ciliberto, and A. Musacchio. 2011. Evidence that Aurora B is implicated in spindle checkpoint signalling independently of error correction. *The EMBO journal*. 30:1508-1519.

Schreiber, A., F. Stengel, Z. Zhang, R.I. Enchev, E.H. Kong, E.P. Morris, C.V. Robinson, P.C. da Fonseca, and D. Barford. 2011. Structural basis for the subunit assembly of the anaphase-promoting complex. *Nature*. 470:227-232.

Schwab, M., M. Neutzner, D. Mocker, and W. Seufert. 2001. Yeast Hct1 recognizes the mitotic cyclin Clb2 and other substrates of the ubiquitin ligase APC. *The EMBO journal*. 20:5165-5175.

Sethi, N., M.C. Monteagudo, D. Koshland, E. Hogan, and D.J. Burke. 1991. The CDC20 gene product of Saccharomyces cerevisiae, a beta-transducin homolog, is required for a subset of microtubule-dependent cellular processes. *Molecular and cellular biology*. 11:5592-5602.

Shepperd, L.A., J.C. Meadows, A.M. Sochaj, T.C. Lancaster, J. Zou, G.J. Buttrick, J. Rappsilber, K.G. Hardwick, and J.B. Millar. 2012. Phosphodependent recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 kinase maintains the spindle checkpoint. *Current biology : CB*. 22:891-899.

Shirayama, M., A. Toth, M. Galova, and K. Nasmyth. 1999. APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*. 402:203-207.

Shou, W., J.H. Seol, A. Shevchenko, C. Baskerville, D. Moazed, Z.W. Chen, J. Jang, H. Charbonneau, and R.J. Deshaies. 1999. Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell*. 97:233-244.

Simonetta, M., R. Manzoni, R. Mosca, M. Mapelli, L. Massimiliano, M. Vink, B. Novak, A. Musacchio, and A. Ciliberto. 2009. The influence of catalysis on mad2 activation dynamics. *PLoS Biol*. 7:e10.

Steen, J.A., H. Steen, A. Georgi, K. Parker, M. Springer, M. Kirchner, F. Hamprecht, and M.W. Kirschner. 2008. Different phosphorylation states of the anaphase promoting complex in response to antimitotic drugs: a quantitative proteomic analysis. *Proceedings of the National Academy of Sciences of the United States of America*. 105:6069-6074.

Stegmeier, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annual review of genetics*. 38:203-232.

Stegmeier, F., J. Huang, R. Rahal, J. Zmolik, D. Moazed, and A. Amon. 2004. The replication fork block protein Fob1 functions as a negative regulator of the FEAR network. *Current biology : CB*. 14:467-480.

Stegmeier, F., R. Visintin, and A. Amon. 2002. Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell*. 108:207-220.

Stemmann, O., H. Zou, S.A. Gerber, S.P. Gygi, and M.W. Kirschner. 2001. Dual inhibition of sister chromatid separation at metaphase. *Cell*. 107:715-726.

Sullivan, M., T. Higuchi, V.L. Katis, and F. Uhlmann. 2004. Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. *Cell*. 117:471-482.

Sullivan, M., and D.O. Morgan. 2007. Finishing mitosis, one step at a time. *Nature reviews. Molecular cell biology*. 8:894-903.

Tang, Y.C., and A. Amon. 2013. Gene copy-number alterations: a cost-benefit analysis. *Cell*. 152:394-405.

Thornton, B.R., T.M. Ng, M.E. Matyskiela, C.W. Carroll, D.O. Morgan, and D.P. Toczyski. 2006. An architectural map of the anaphase-promoting complex. *Genes & development*. 20:449-460.

Tinker-Kulberg, R.L., and D.O. Morgan. 1999. Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. *Genes & development*. 13:1936-1949.

Toda, K., K. Naito, S. Mase, M. Ueno, M. Uritani, A. Yamamoto, and T. Ushimaru. 2012. APC/C-Cdh1-dependent anaphase and telophase progression during mitotic slippage. *Cell division*. 7:4.

Uhlmann, F., F. Lottspeich, and K. Nasmyth. 1999. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*. 400:37-42.

Varetti, G., C. Guida, S. Santaguida, E. Chiroli, and A. Musacchio. 2011a. Homeostatic control of mitotic arrest. *Mol Cell*. 44:710-720.

Varetti, G., C. Guida, S. Santaguida, E. Chiroli, and A. Musacchio. 2011b. Homeostatic control of mitotic arrest. *Molecular cell*. 44:710-720.

Vernieri, C., E. Chiroli, V. Francia, F. Gross, and A. Ciliberto. 2013. Adaptation to the spindle checkpoint is regulated by the interplay between Cdc28/Clbs and PP2ACdc55. *The Journal of cell biology*. 202:765-778.

Vigneron, S., S. Prieto, C. Bernis, J.C. Labbe, A. Castro, and T. Lorca. 2004. Kinetochore localization of spindle checkpoint proteins: who controls whom? *Molecular biology of the cell*. 15:4584-4596.

Visintin, C., B.N. Tomson, R. Rahal, J. Paulson, M. Cohen, J. Taunton, A. Amon, and R. Visintin. 2008. APC/C-Cdh1-mediated degradation of the Polo kinase Cdc5 promotes the return of Cdc14 into the nucleolus. *Genes & development*. 22:79-90.

Visintin, R., and A. Amon. 2001. Regulation of the mitotic exit protein kinases Cdc15 and Dbf2. *Molecular biology of the cell*. 12:2961-2974.

Visintin, R., K. Craig, E.S. Hwang, S. Prinz, M. Tyers, and A. Amon. 1998. The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Molecular cell*. 2:709-718.

Visintin, R., E.S. Hwang, and A. Amon. 1999. Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature*. 398:818-823.

Visintin, R., F. Stegmeier, and A. Amon. 2003. The role of the polo kinase Cdc5 in controlling Cdc14 localization. *Molecular biology of the cell*. 14:4486-4498.

Vitre, B.D., and D.W. Cleveland. 2012. Centrosomes, chromosome instability (CIN) and aneuploidy. *Current opinion in cell biology*. 24:809-815.

Vodermaier, H.C., C. Gieffers, S. Maurer-Stroh, F. Eisenhaber, and J.M. Peters. 2003. TPR subunits of the anaphase-promoting complex mediate binding to the activator protein CDH1. *Current biology : CB*. 13:1459-1468.

Wang, Y., and D.J. Burke. 1997. Cdc55p, the B-type regulatory subunit of protein phosphatase 2A, has multiple functions in mitosis and is required for the kinetochore/spindle checkpoint in Saccharomyces cerevisiae. *Molecular and cellular biology*. 17:620-626.

Wang, Y., and T.Y. Ng. 2006a. Phosphatase 2A negatively regulates mitotic exit in Saccharomyces cerevisiae. *Mol Biol Cell*. 17:80-89.

Wang, Y., and T.Y. Ng. 2006b. Phosphatase 2A negatively regulates mitotic exit in Saccharomyces cerevisiae. *Molecular biology of the cell*. 17:80-89.

Warren, C.D., D.M. Brady, R.C. Johnston, J.S. Hanna, K.G. Hardwick, and F.A. Spencer. 2002. Distinct chromosome segregation roles for spindle checkpoint proteins. *Molecular biology of the cell*. 13:3029-3041.

Wasch, R., and F.R. Cross. 2002. APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. *Nature*. 418:556-562.

Weaver, B.A., and D.W. Cleveland. 2005. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer cell*. 8:7-12.

Winton, T., R. Livingston, D. Johnson, J. Rigas, M. Johnston, C. Butts, Y. Cormier, G. Goss, R. Inculet, E. Vallieres, W. Fry, D. Bethune, J. Ayoub, K. Ding, L. Seymour, B. Graham, M.S. Tsao, D. Gandara, K. Kesler, T. Demmy, and F. Shepherd. 2005. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *The New England journal of medicine*. 352:2589-2597.

Wojcik, E., R. Basto, M. Serr, F. Scaerou, R. Karess, and T. Hays. 2001. Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. *Nature cell biology*. 3:1001-1007.

Yamagishi, Y., C.H. Yang, Y. Tanno, and Y. Watanabe. 2012. MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components. *Nature cell biology*. 14:746-752.

Yamaguchi, S., A. Decottignies, and P. Nurse. 2003. Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. *The EMBO journal*. 22:1075-1087.

Yamamoto, A., V. Guacci, and D. Koshland. 1996a. Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *The Journal of cell biology*. 133:85-97.

Yamamoto, A., V. Guacci, and D. Koshland. 1996b. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *The Journal of cell biology*. 133:99-110.

Yellman, C.M., and D.J. Burke. 2006. The role of Cdc55 in the spindle checkpoint is through regulation of mitotic exit in Saccharomyces cerevisiae. *Molecular biology of the cell*. 17:658-666.

Yeong, F.M., H.H. Lim, C.G. Padmashree, and U. Surana. 2000. Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. *Molecular cell*. 5:501-511.

Yu, H.G., and D. Koshland. 2007. The Aurora kinase Ipl1 maintains the centromeric localization of PP2A to protect cohesin during meiosis. *The Journal of cell biology*. 176:911-918.

Zachariae, W., M. Schwab, K. Nasmyth, and W. Seufert. 1998. Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science*. 282:1721-1724.

Declaration of contributions

I personally performed all the experiments reported in this thesis, with the following exceptions:

- experiments described in Figures 9, 16, 17, 29 and 31 were done in collaboration with Francia Valentina;
- experiments described in Figures 18 and 26 were performed together with Chiroli Elena:
- the analyses of single-cell experiments reported in Figures 21 and 22 were performed by Gross Fridolin.

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