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XXIX CYCLE

Evidence that Maintenance and Resolution of the Spindle Assembly Checkpoint does not require Activity of Protein Phosphatase 2A.

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I ABBREVIATIONS

APC/C: Anaphase promoting complex/cyclosome.
APC^{Cdc20}: Anaphase promoting complex/cyclosome, activated by Cdc20.
APC/C^{MCC}: Anaphase promoting complex/cyclosome, bound with MCC.
ARPP19: cAMP-regulated phosphoprotein 19.
BSA: bovine serum albumin.
BUB: Budding uninhibited by benzimidazole.
C-Mad2: closed mitotic arrest deficient 2.
Cdk1: Cyclin dependent kinase 1.
CENP: Centromere protein.
CPC: Chromosomal passenger complex.
dsDNA: Double strand DNA.
DSPs: Dual specificity phosphatases.
ENSA: Endosulfine.
FBS: Fetal calf serum.
G_0 phase: Gap 0 phase.
G_1 phase: Gap 1 phase.
G_2 phase: Gap 2 phase.
GWL: Greatwall kinase.
FCP1: RNA polymerase II C-terminal domain phosphatase.
Mad1: mitotic arrest deficient 1.
Mad2: mitotic arrest deficient 2.
MAPK: Mitogen activated protein kinase.
MCC: Mitotic checkpoint complex.
MPS1: Multipolar spindle-1.
OA: Okadaic acid.
O-Mad2: opened mitotic arrest deficient 2.
NEK2: Never-in-mitosis-A-related kinase 2.
PLK1: Polo like kinase.
PP1: Protein phosphatase 1.
PP2A: Protein phosphatase 2A.
PP2A-C: Protein phosphatase 2A catalytic subunity.
PPMs: Metal-dependent protein phosphatases.
PPPs: Phosphoprotein phosphatases.
PRC1: Protein regulator of cytokinesis 1.
PSPs: Serine/threonine phosphatases.
PTPs: Tyrosine phosphatases.
RNAi: RNA interference.
ROD: Rough deal.
S phase: Synthesis phase.
SAC: Spindle assembly checkpoint.
SGO: Shugoshin.
TPR: Tetratricopeptide repeat.
ZW10: Zeste white-10.

II ABSTRACT

Mitosis is the critical stage of the cell cycle deputed to form two daughter cells by the faithful separation of replicated chromosomes of a mother cell into two identical sets, daughter cells genomes. Inappropriate resolution of sister chromatids, prior to proper bipolar attachments, during mitosis, leads to random chromosomes segregation and aneuploidy, a deleterious condition often associated with various pathological conditions (Weaver & Cleveland, 2006). To prevent errors in chromosome segregation, a safeguard pathway, the spindle assembly checkpoint (SAC), is activated by improper kinetochore-spindle microtubule attachments and prevents activation of the mitosis exit-promoting ubiquitin-ligase Anaphase Promoting Complex/Cyclosome (APC/C) to delay mitosis exit until all the chromosomes become correctly bi-orientated at the equator of the metaphase plate (Lampson *et al.*, 2004).

Several protein kinases and phosphatases play essential roles in these processes presumably by controlling the phosphorylation status of a number of proteins involved in SAC control. There is a balance between kinase and phosphatase activities and this balance is constantly changing during the time required for spindle assembly completion (Visconti *et al.*, 2013).

Protein phosphatases as protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), are well known to be required for mitosis completion, however, whether and how activity of these phosphatases is involved in SAC control is still incompletely understood.

PP2A, in particular, is a holoenzyme composed by a scaffolding (A), a catalytic (C) and a regulatory (B) subunit. The B subunit consists of four distinct subfamilies (B, B', B" and B"') acting as target modulators to provide substrate specificity; holoenzyme formation, in all its three subunits, is fundamental for PP2A action (Wurzenberger & Gerlich, 2011; Rogers *et al.*, 2016).

PP2A has been suggested to contribute to SAC resolution and to the metaphase-to-anaphase transition, namely through experiments based on small interfering

RNAs-mediated genetic knockdown of the various PP2A subunits (Schmitz *et al.*, 2010; Wurzenberger & Gerlich, 2011; Kruse *et al*., 2013; Nijlenhuis *et al*., 2014). However, the contribution of the catalytic activity of PP2A in SAC control has not been directly investigated.

In this work we investigated the role of PP2A activity in SAC regulation by using inhibitors of PP2A catalytic activity, like Okadaic acid (OA) and LB 100, without interfering with holoenzyme formation. We found compelling evidence that the catalytic activity of PP2A does not substantially affect SAC maintenance or resolution in human cells.

III BACKGROUND

1 THE CELL CYCLE

The biological history of any cell, either prokaryotic or eukaryotic, begins with its birth as a result of the progenitor cell division and continues with the transmission of its genetic content when it, in turn, divides into two daughter cells. The stage through which the cell passes between a cell division and the next constitutes the cell cycle.

Even though the cell cycle is considered to be a continuous process, cell growth, DNA duplication and chromosomes segregation happen during specific phases of the cell cycle. Progression through the cell cycle is led by a meticulous regulatory system that coordinates intracellular with extracellular signals to control cell growth and division.

The cell cycle is mainly divided into two basic parts: interphase and mitosis (or M phase). During interphase, the chromosomes are distributed throughout the nucleus in a non-condensed form. While the cell grows steadily during all the interphase, DNA synthesis is limited to a specific portion.

The eukaryotic cell cycle is subdivided into four sequential phases: G_1, S, G_2 and M. The G_1 phase, S, G_2 form the interphase.

The G_1 phase (gap 1) takes place at the cell cycle beginning. During G_1, the cell grows in dimension and is metabolically active and produces new enzymes and organelles. After growth, cells replicate their genome during S phase (synthesis) and after DNA synthesis, cells prepare for mitosis in the G_2 phase (gap 2), during which duplicated centrioles are positioned externally to the nuclear membrane.

In M phase, mitosis, duplicated chromosomes are segregated and cells divide through cytokinesis (Cooper, 2000).

Mitosis is further divided into five phases: prophase, prometaphase, metaphase, anaphase and telophase. During prophase the chromatin filaments spiralize and fold, organizing themselves into thin and long chromosomes. Each chromosome appears to be composed of two identical chromatids, associated longitudinally between them.

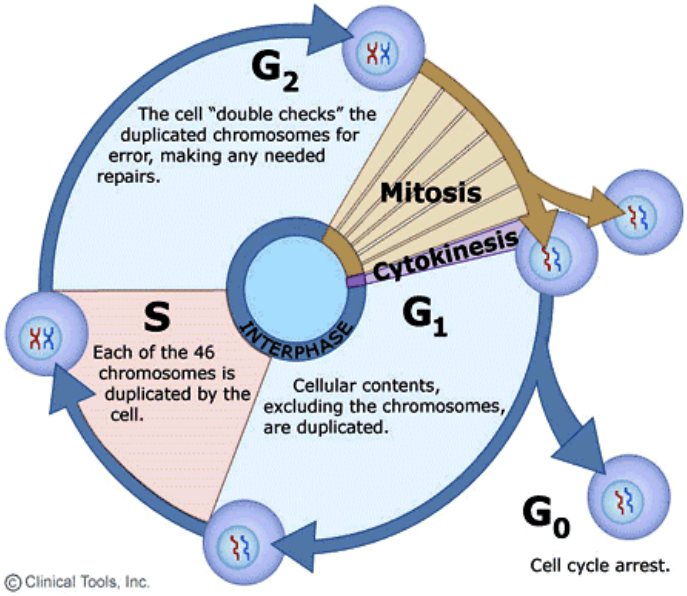
Chromosomes draw near to the nuclear membrane, at the same time the mitotic apparatus is organizing, aster and the microtubules of the mitotic spindle are forming, through each pair of centrioles duplicated during interphase.

Prometaphase is characterized by the nuclear envelope breakdown and the disappearance of the nucleoli while the mitotic spindle is organizing.

Metaphase is characterized by mitotic spindle formation with chromosomes arranged neatly on the spindle equator. Each chromosome presents the arms open towards the surface of the cell and each centromere of sister chromatids is in contact with the spindle microtubules through the kinetochores.

The sister chromatids separation occurs during anaphase, which is further divided into two stages: anaphase A (migration of chromatids toward the poles) and anaphase B (further separation of the two poles for stretching and sliding of microtubules), while during telophase chromosomes decondense and nuclear membrane and the nucleoli reappear. Daughter cells separate in two distinct cells during cytokinesis (Fig.1) (Hartwell & Weinert, 1989; De Souza & Osmani, 2007).

A



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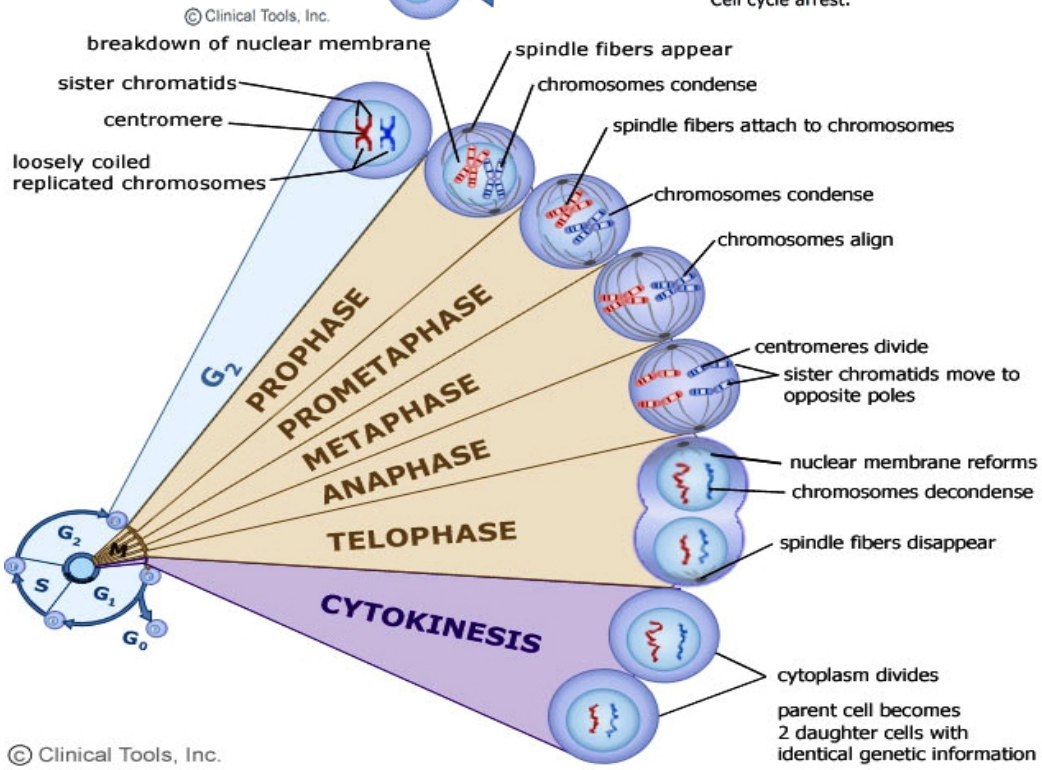

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Figure 1: Cell cycle is divided into interphase and mitosis. A During interphase the cell can be quiescent (G_0), metabolically active and preparing (G_1) for the DNA replication (S). The completion of DNA synthesis is followed by cell control of DNA replication and further growing (G_2). **B** Mitosis takes up about 1/24 of the cell cycle, during this step the cell is subjected to many changes. Mitosis is divided in: prophase, prometaphase, metaphase, anaphase and telophase; during phases progression sister chromatids align to mitotic spindle, to be divided during anaphase and migrate to opposite spindle poles. After nuclear membranes reformations daughter cells cytoplasm division occurs during cytokinesis.

2 CELL CYCLE CHECKPOINTS

In order for the duplication process and cell division to occur correctly there are 4 checkpoints, the control points where the cell cycle can only proceed if some previous events have been correctly accomplished. These are:

-G_1 checkpoint: it controls the cell size and is regulated by extracellular signals from the environment, as well as by internal signals that monitor and coordinate the various processes that take place during this phase.

-G_1/S checkpoint: that assesses the absence of genetic damage. Genetic damages like fractures in double strand DNA (dsDNA) cause the activation of ATM kinase which in turn, activates p53 through phosphorylation at Ser 15. Phosphorylation of p53 induces the transcription of the p21 gene, and subsequent translation; p21 than inhibits the complex CycE / Cdk2, important for cell cycle progression from G_1 to S phase (Sherr, 1996).

-G_2 checkpoint: it prevents mitosis initiation until DNA replication completion. This checkpoint is caused by not replicated DNA that acts as a signal for cell cycle arrest. This checkpoint preventing the M phase initiation prevents cells to go beyond G_2 phase. Only when genome is completely replicated G_2 progression is concluded and the cell is able to divide during mitosis to distribute to daughter cells a complete and equal genome (Kastan & Bartek, 2004).

-M checkpoint or spindle assembly checkpoint (SAC): this checkpoint monitors the correct alignment of chromosomes on the mitotic spindle. The SAC effector branch is the mitotic checkpoint complex (MCC), which binds and inhibits the APC/C (the E3 ubiquitin ligase that is responsible for chromosome segregation and anaphase onset) and is assembled on unattached chromosomes (Weaver & Cleveland 2005).

3. THE SPINDLE ASSEMBLY CHECKPOINT

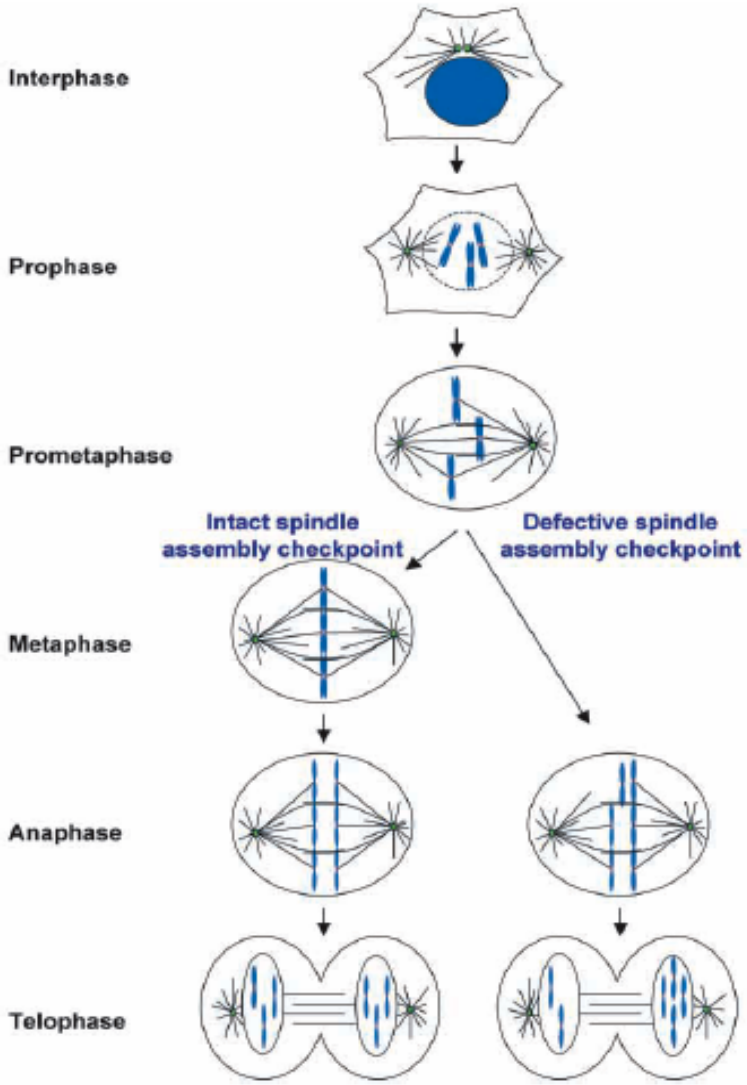
Mitosis is the critical stage of the cell cycle in which the genome daughter cells forms and this process must be the result of the accurate separation of replicated chromosomes (sister chromatids) of the mother cell into two identical sets. During mitosis progression equal segregation of each replicated pair of sister chromatids must be ensured (Fig. 2).

Resolution of sister chromatids or cell division prior to proper bipolar attachment (in which sister kinetochores are attached to the microtubules of the two opposite spindle poles) leads to chromosomes mis-segregation causing aneuploidy (and consequent cancers and birth defects) (Weaver & Cleveland, 2006). To prevent this condition, the SAC pathway is activated by unattached kinetochores to inhibit the premature metaphase-to-anaphase transition, while erroneous kinetochore-microtubule attachments are corrected at the same time (Lampson *et al.*, 2004).

SAC delays chromosome segregation until all the chromosomes are attached by kinetochore microtubules from two opposite spindle poles and proper tension is placed on the paired kinetochores.

During mitotic progression, crucial steps to ensure correct chromosome segregation are:

1) Proper alignment of sister chromatids at the mitotic spindle.
2) Correct activation of the APC/C, to allow the entry into anaphase and eventually cell division (cytokinesis) (Ciliberto & Shah, 2009).



From Zhou *et al.*, 2002

Figure 2: Chromosome segregation under intact or defective SAC: Intact spindle assembly checkpoint leads to correct sister chromatids alignment, during metaphase, and consequent correct cell division (left arrow between prometaphase and metaphase). When SAC is defective cells could be able to divide incorrectly with random chromosome segregation, leading to aneuploid daughter cells progeny (right arrow).

3.1 MICROTUBLE SPINDLE ATTACHMENTS

Accurate chromosome segregation occurs when sister kinetochores are properly attached to microtubules of the two opposite spindle poles: opposite kinetochores must be attached to the microtubule of opposite spindle poles (bipolar attachments).

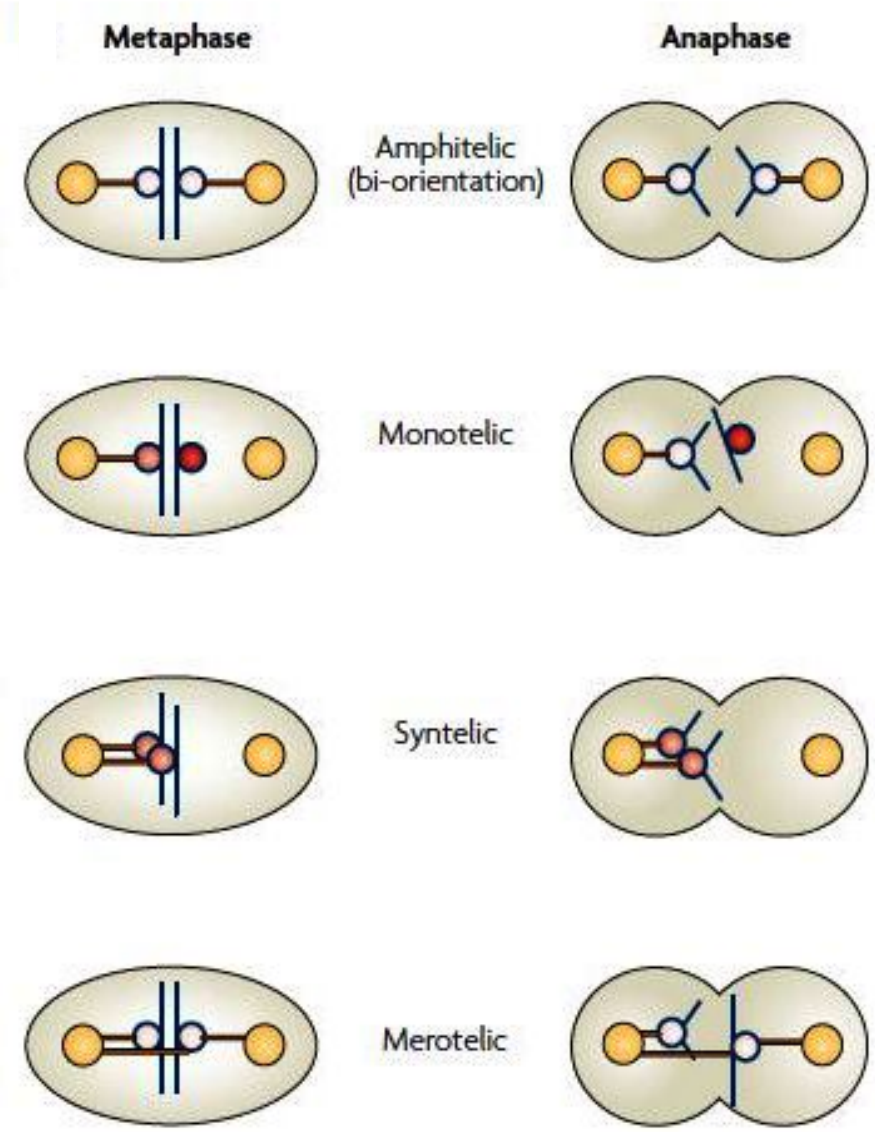
At beginning of mitosis, kinetochore–microtubule interactions are usually stochastic. Although sister kinetochores are arranged in opposing directions, a situation that brings statistically more likely to bipolar attachment, also erroneous attachments could form (Sakuno *et al.*, 2009).

Three types of erroneous attachments can occur during spindle assembly:

-Monotelic attachment: sister kinetochores bind microtubules from only one spindle pole.
-Syntelic attachment: where both sister kinetochores interact with microtubules of the same pole.
-Merotelic attachment: where a single kinetochore is connected to both spindle poles (Fig. 3).

If not corrected, these erroneous attachments might result in the mis-segregation of chromosomes during anaphase, leading to aneuploid progeny (Funabiki & Wynne, 2013).

Correction of these erroneous configurations is mediated by kinases such as Aurora B, a subunit of the chromosomal passenger complex (CPC), which also contains INCENP, Survivin and Borealin, whose phosphorylation facilitates the destabilization of kinetochore-microtubule attachments (Cimini *et al.,* 2006). Aurora B is able directly modulate kinetochore proteins by phosphorylation, such as the microtubule binding components of the KMN network (KNL1/Mis12 complex/Ndc80 complex) and microtubule depolymerizing kinesins (MCAK, Kif2b), and in this way it provides to detach incorrect kinetochore-microtubules attachments (Gregan *et al.*, 2011).



From Musacchio & Salmon, 2007

Figure 3: Correct and incorrect attachments during metaphase. Correct (amphitelic) and incorrect (monotelic, syntelic and merotelic) attachments can both occur during mitosis. Incorrect attachments lead to chromosome incorrect segregation, during anaphase, causing aneuploidy and mitotic defects.

Aurora B-dependent phosphorylation of substrates at the kinetochore is sensitive to kinetochore-microtubule attachment status; in particular Aurora B can create a gradient of phosphorylation depending on microtubule tension across sister kinetochores. In erroneous alignment Aurora B is able to phosphorylate its kinetochore substrates, leading to destabilization of the microtubules. In bipolar attachments at contrary, kinetochores and microtubules are stably attached, leading to a strong reduction of Aurora B phosphorylation to its substrates.

The destabilization of erroneous attachments by Aurora B phosphorylation could stimulate MCC activation, creating unattached kinetochores; this underlines the complexity of SAC (Tanaka *et al.,* 2002; Funabiki & Wynne, 2013).

3.2 SPINDLE ASSEMBLY CHECKPOINT FUNCTIONS

SAC is activated in early mitosis (prometaphase), when Cdk1/Cyclin B1 complex is fully working and sister chromatids are held together by cohesin complex.

The SAC generates a cell cycle arrest by inhibiting the APC/C, an E3 ubiquitin ligase complex that ubiquitinates mitotic cyclins and securin to target them for 26S proteosomal degradation.

Securin is a stoichiometric inhibitor of the protease separase. Separase is essential to cleave the cohesin complex, that holds sister chromatids together; cohesin cleavage firstly allows anaphase execution (Peters, 2006).

On the other hand, the proteolysis of Cyclin B1 inactivates the master mitotic kinase Cdk1, promoting mitosis exit. The SAC goal is to block APC/C activity by formation of the mitotic checkpoint complex (MCC).

MCC functions as a pseudosubstrate inhibitor of the APC/C and is made of several proteins. Among these, crucial are Cdc20, an APC/C co-factor, Mad2 and BuBR1. When MCC is formed, APC/C can’t be activated by Cdc20 (Fig. 4). In this way APC/C mediated polyubiquitination and Cyclin B and securin degradation are prevented.

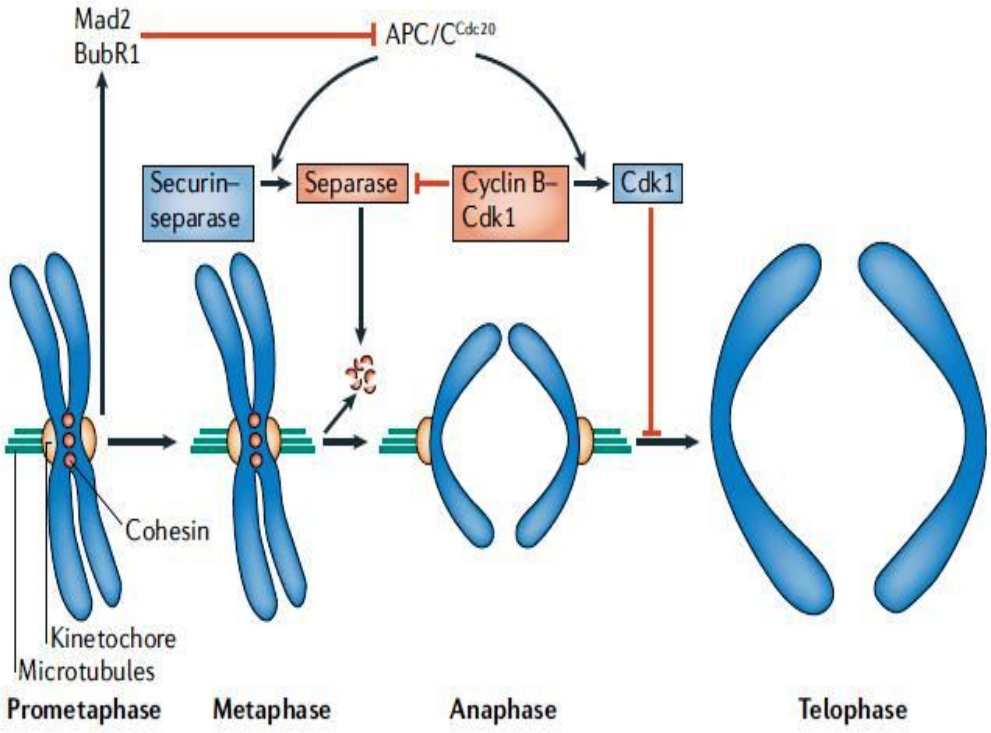
SAC blocks mitosis exit until chromosomes have accomplished bi-orientation and the metaphase plate has correctly formed.

Chromosome bi-orientation allows cells to go beyond metaphase, extinguishing the checkpoint. SAC silencing lead to irreversible sister chromatids segragation, if chromosomes were not correctly attached and SAC is inappropriately inactivated, there will be mis-segregations and consequently formation of daughter cells with aneuploid genomes (Musacchio & Salmon, 2007; Funabiki & Wynne, 2013).

Several protein kinases and phosphatases play essential roles in these processes by controlling the phosphorylation of a number of kinetochore proteins, all these proteins work in a very sophisticated way. There is a balance between the kinases and phosphatases activities and this balance is constantly changing depending on the moment of the checkpoint and the spatial configuration of all the participating proteins in the mitotic spindle (Visconti *et al.,* 2013).

SAC can be summarized as follows:

-Mad2, BuBR1, BUB3 and Cdc20 form the MCC.
-MCC binds the APC/C as a pseudosubstrate inhibitor, blocking the APC/C activity and the anaphase onset.
- Correct bipolar chromosome attachments lead to MCC switch off.
-Cdc20, devoid of SAC components, functions as an APC/C coactivator.
-APC/C triggers poliubiquitination cascade to degrade securin and Cyclin B to proteasome.
-All the checkpoint substrates are progressively dephosporylated, Cdk1 is inhibited by phosphorylation and the cell continues mitosis, entering into anaphase.



From Peters, 2006

Figure 4: Regulation of APC/C and metaphase to anaphase transition. In prometaphase chromosomes are not fully bi-orientated on the metaphase plate. MCC components, as Mad2 and BuBR1, are recruited at kinethochores unattached or incorrectly attached with microtubules (in green) and APC/C can't be activated by Cdc20. Chromosome bi-orientation extinguishes the checkpoint; Cdc20 activates APC/C (APC/C^{Cdc20}), that polyubiquitilates Cyclin B (allowing consequent Cdk1 inactivation) and securin (thereby activating the protease separase). Separase then cleaves cohesin complexes (shown as red circles) while Cdk1 substrates are dephosphorylated by protein phosphatases, enabling metaphase – to anaphase transition and exit from mitosis.

3.2.1 MCC ACTIVATION, RESOLUTION AND INACTIVATION

SAC is made of several "core" components that, during metaphase plate formation, work on incorrectly attached kinetochores. One of the principal SAC complexes is the MCC, responsible APC/C inactivation. MCC contains Cdc20 and three SAC proteins: Mad2 (mitotic arrest deficient), BuBR1/Mad3 and BUB3 (budding uninhibited by benzimidazole).

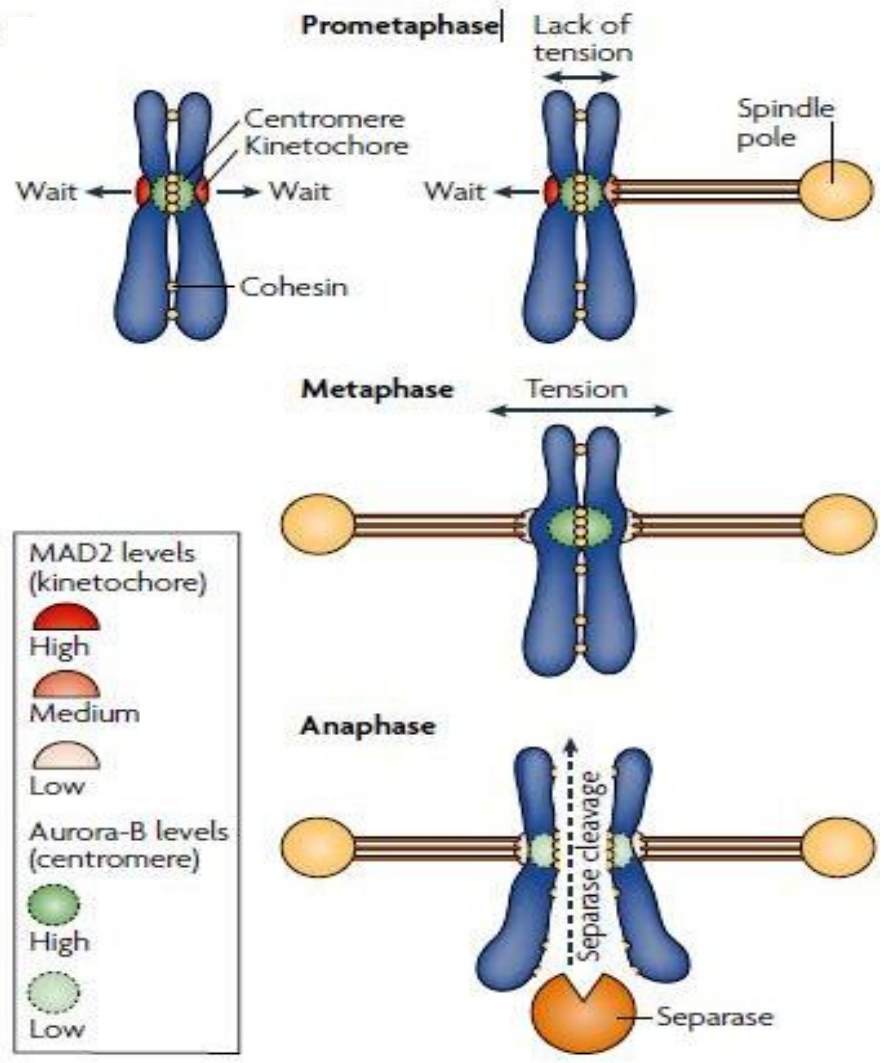
Beside MCC, other SAC components are required to amplify the SAC signal, including Mad1 and the kinases BUB1, MPS1 (multipolar spindle-1) and Aurora B (Musacchio & Salmon, 2007).

Additional proteins that regulate SAC activity are:

- $p31^{comet}$ (Xia *et al*, 2004).
-Kinases as the mitogen activated protein kinase (MAPK), Cdk1, NEK2 and polo-like kinase-1 (PLK1) (Mishull *et al.*, 1994; D'Angiolella *et al.*, 2003; Lou *et al.*, 2004; van Vugt & Medema, 2005).
-Microtubule motors centromere protein (CENP)-E, dynein and dynein-associated proteins (Abrieu *et al.*, 2000).
-Constituents of the ROD (rough deal)–ZW10 (zeste white-10)–ZWILCH (RZZ) complex (Karess, 2005).

In the presence of unattached kinetochores, Cdc20 is seized by SAC components to form the MCC complex; the entire MCC complex binds the APC/C as a pseudosubstrate (APC^{MCC}), blocking its activity. The lack of tension, that characterizes the unattached and uncorrected attached kinetochores, can be compared to a "wait" signal that creates the SAC arrest (Fig. 5)**.**

There are different models about SAC formation and function, of which the most experimentally reliable is the *Mad2 template model*. In this model the first event during the formation of MCC and SAC activation is a conformational change of the protein Mad2.



From Musacchio & Salmon, 2007

Figure 5: Sister chromatids tension leads to SAC ending and chromatids segregation. Untill sister chromatids bi-orientation unattached kinetochores generate a wait signal that prevents the entry into anaphase. Under these conditions Mad2 is high at unattached and lower to the attached kinetochores, while Aurora B kinase concentrates at centromeres. Bi-orientation depletes Mad2 from kinetochores and when this condition is achieved by all the chromosomes SAC signal extinguishes and sister chromatids segregate, during anaphase, thanks to separase cleavage.

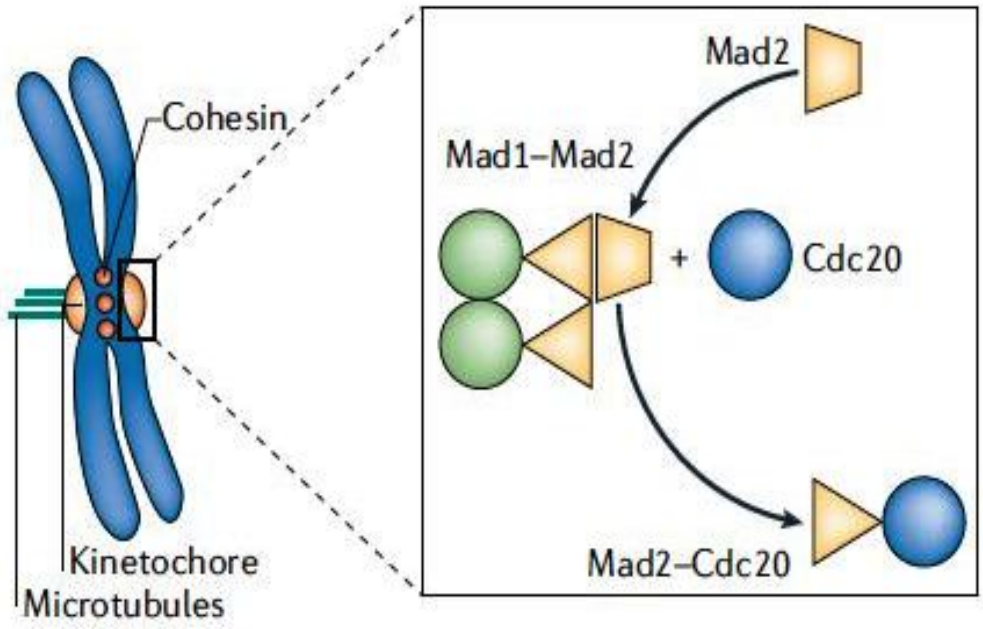
Mad2 has two forms approximately equal in amount: an open-Mad2 form (O-Mad2) and a closed form (C-Mad2). O-Mad2 is cytosolic and has an high turnover, while C-Mad2 is bound to kinetochores and is more stable and active

Mad1 binds O-Mad2 in order to change Mad2 into the more stable and active form C-Mad2, by inducing a Mad1-C-Mad2 (the template) complex formation. Once Mad1-C-Mad2 binds kinetochore it becomes a receptor for the O-Mad2 favoring the binding of it to Cdc20, consequently promoting the APC/C^{MCC} formation.

Cdc20 leads to the same conformational change in Mad2, like the one triggered by Mad1 (Fig. 6). Mad2 is able to form a heterodimer with closed Mad2, at kinetochores; this heterodimer facilitates the binding of Cdc20 with Mad2 and consequent APC/C activation delay. In the same way also Cdc20-C-Mad2 might facilitates the binding of O-Mad2 and Cdc20 to kinetochore, establishing a positive feedback loop (Lou *et al.*, 2002; Lou *et al.*, 2004; De Antoni *et al.*, 2005).

Immediately after the last kinetochore is hooked, and the last couple of sister chromatids is well aligned on the metaphase plate, MCC is disassembled, APC/C^{Cdc20} is activated and the consequent degradation of securin and mitotic cyclin. Thus, MCC dissociation leads to SAC switch off and APC/C reactivation.

The protein $p31^{comet}$ acts as a negative regulator of the SAC by binding Mad2 protein. Protein $p31^{comet}$ is able to bind C-Mad2 with high affinity preventing O-Mad2-C-Mad2 dimerization hindering Cdc20 recruitment to MCC complex and leading to MCC disassembly. Kinetochores might negatively regulate the ability of $p31^{comet}$ to bind C-Mad2 resulting in the accumulation of Mad2–Cdc20 and SAC activation. The reactivation of $p31^{comet}$ at metaphase would then trigger SAC inactivation (Xia *et al.*, 2004; Chao *et al.,* 2012).



From Peters, 2006

Figure 6: Mad2 activation and conformational change to kinetochores, according to the *Mad2 template model*. Mad2 has an open conformation (yellow rhomboid), in the cytosol, when it is not active. During SAC Mad2 changes to a closed conformation (yellow triangle), by binding the SAC protein Mad1 (green circle). According to *Mad2 template model* open Mad2 is able to form a heterodimer with closed Mad2, at kinetochores, this heterodimer facilitates the binding of Cdc20 with Mad2 and consequent APC/C^{Cdc20} activation delay.

3.2.2 THE ROLE OF KINASES DURING SAC

During SAC mitotic kinases are activated and phosphorylate several substrates, kinases activity are indispensable to promote strong SAC activity and avoid kinetochore-microtubule erroneous attachments. Although the precise identity and targets of all these kinases is not totally known, studies mainly from RNA interference (RNAi) have identified some kinases: MPS1, BUB1, Cdk1, Greatwall kinase, BuBR1, PLK1, NEK2, MAPK and Aurora B kinase (Rogers *et al.*, 2016).

Mitotic kinases work simultaneously with synergistic effects to block the APC/C, in a sophisticated and complex manner: At least three kinases, BUB1, MAPK and Cdk1 phosphorylate Cdc20 to modulate its binding by Mad2 or BuBR1. BuBR1 binds Cdc20 through its N-terminal region and this binding (in human cells) requires previous binding of Mad2 and Cdc20. BuBR1 phosphorylates CENP-E, a microtubule plus-end motor that contributes to chromosome alignment, and interacts with it (Musacchio & Salmon, 2007).

Furthermore the checkpoint protein BUB1, a protein kinase known to phosphorylate the histone H2A at Thr120 in human, allows the shugoshin proteins (SGO1 and SGO2) recruitment at the inner centromere and consequent recruitment the CPC by interacting with Survivin (in fission yeast) or Borealin (in human) by SGO1 and SGO2 (Kawashima *et al.*, 2010).

Another SAC key regulator kinase is MPS1, a dual specificity kinase required for kinetochore localization of Mad1, Mad2, PLK1 and CENP-E. Inhibition of MPS1 causes displacement of SAC proteins from mitotic kinetochores, and also dissociates Cdc20 from Mad2 and BuBR1 during interphase and mitosis, indicating that MPS1 activity is constitutively required for both kinetochore-independent and –dependent mechanisms of MCC assembly.

Aurora B phosphoregulation has a fundamental role in chromosome alignment, it is required to arrest cell cycle until all sister chromatids are correctly aligned on the spindle equator. The major targets of Aurora B are the components of the KMN network (KNL1, Mis12 complex and Ndc80 complex), which is critical for microtubule-kinetochores correct attachments, and the Ska complex, that further supports these attachments (Cheeseman *et al.*, 2006).

Aurora B phosphorylation negatively regulates: Ndc80 and KNL1 microtubule binding activity, the recruitment, by Mis12 complex, of KNM network and other kinetochore components, the Ndc80 complex recruitment, by Ska complex, and the consequent stabilization of kinetochore-microtubule attachments (Chan *et al.*, 2012).

3.2.3 THE ROLE OF PHOSPHATASES DURING SAC

Completion of mitosis requires the action of several protein phosphatases. Major phosphatases, like PP1 and PP2A, dephosphorylate, in a specific temporal order, thousands of mitotically phosphorylated proteins at mitosis exit.

Eukaryotic phosphatases can be divided into three super families: serine/threonine phosphatases (PSPs), tyrosine phosphatases (PTPs) and dual specificity phosphatases (DSPs).

During mitosis exit most of dephosphorylations are exerted by the PSPs phosphatase family (Rogers *et al.*, 2016).

Some of the PSPs form a large number of diverse oligomeric complexes. In particular, PP2A forms ~100 heterotrimeric holoenzymes and PP1 forms ~400 heterodimeric holoenzymes. The PSPs are further divided into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and aspartate-based phosphatases. The PPP family is the largest family of phosphatases, and many PPPs are involved in cell cycle regulation, in particular PP1 and PP2A. The PPP phosphatase family has a structurally conserved active site configuration (Hunter, 1995; Wlodarchack & Xing, 2016).

This specific configuration consists of six conserved residues, two aspartate (D), one asparagine (N) and three histidine (H) residues that coordinate two catalytic metal ions with a catalytic water molecule. One conserved histidine and two arginine (C) residues control the phosphate binding. The dephosphorylation reaction proceeds *via* an SN2 mechanism with the water molecule serving as a nucleophile to attack the phosphate group on the serine (S) or treonine (T) residues.

The PPP family consists of PP1, PP2A, PP3, PP4, PP5, PP6 and PP7. PP1 and PP2A are two of the most abundant enzymes in many cell types, regulating different physiological and cellular processes as well as the cell cycle (Shi, 2009).

PP1 is a highly characterized serine/threonine phosphatase. In mammalian genomes there are three different genes encoding for four distinct catalytic

subunits of PP1: PP1α, PP1β/δ and the splice variants PP1γ1 and PP1γ2. PP1α, PP1β/δ and PP1γ1 are ubiquitous, while PP1γ2 is present only in the testis.

PP1 is made of a catalytic core (C subunit), which can bind more than 200 proteins and creating over 650 distinct complexes. The catalytic site of PP1 consists of three regions: hydrophobic, acidic and C-terminal grooves. All these regions have a potential substrate-binding activity.

Most PP1 regulatory proteins and some PP1 substrates interact with PP1 via primary PP1-binding motifs. These motifs are essential for substrate binding, specificity and binding regulation, but generally they are dispensable for PP1 catalytic activity. RVxF motif is the best characterized, it is generally conformed to the consensus sequence [K/R][K/R][V/I][x][F/W] (x is any residue other than Phe, Ile, Met, Tyr, Asp, or Pro). Additional, recently identified docking sites, such as the SSILK and MyPhoNE motifs, also play important roles in the regulation of PP1 activity and substrate specificity (Bollen *et al.*, 2010).

Each PP2A holoenzyme is a heterotrimer formed by three different subunits: a common catalytic (C) subunit containing the active site, a regulatory (B) subunit which confers substrate specificity and a common scaffolding (A) subunit that takes holoenzyme joint together, holding B and C subunits. In the majority of cases, the C-subunit binds to the A subunit to form a stable base complex, which then recruits a regulatory B-subunit providing specificity. There are two isoforms (α and β) for both A and C, and they share high sequence homology; the isoform α is the predominant one.

PP2A catalytic subunit (PP2A-C) has a globular structure; it is ubiquitously expressed in almost every tissue but being more abundant in the heart and brain. PP2A-C has two isoforms: Cα and Cβ. The isoform Cα is predominantly expressed in the plasma membrane and Cβ in the cytoplasm and nucleus. Furthermore Cα is expressed in higher abundance than Cβ due to its strong promoter activity and to the differences in the rate of mRNA turnover. PP2A-C C-terminal tail is uniquely conserved (304TPDYEL309) and binds to the scaffold and regulatory subunits.

The PP2A regulatory subunit (PP2A-B) is structurally different, it contains four distinct subfamilies (B, B', B" and B"') encoded by 15 distinct genes in the human genome, with over 26 potential isoforms and splice variants (Fig.7).

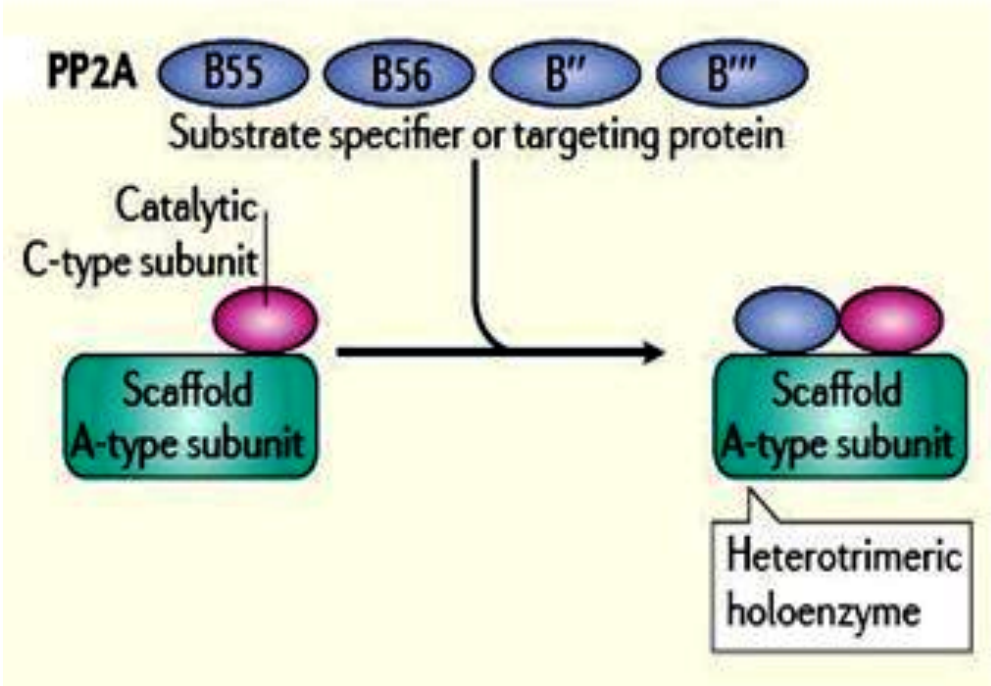
The B and B' subunits (also known as B55 and B56, respectively) are the only ones associated with mitotic exit regulation in higher eukaryotes and human cells (Cho *et al.*, 2007; Wurzenberger & Gerlich, 2011; Wlodarchak & Xing, 2016).

PP2A-B55 is structurally characterized by a principal protein-protein interaction motif; this motif consists of tryptophan-aspartate repeats (40-WD repeats) and facilitates substrate binding. B55 subunit has four different isoforms (α, β, γ and δ). It is observed that the expression of B55γ increases and B55β decreases gradually after birth and they are developmentally regulated.

PP2A-B56 subunit has five different isoforms (α, β, γ, δ, and ε). These isoforms show 80% identical sequences in their central region but differ in their N and C terminals, leading to tissues different expression levels. Intracellular localization of B56 isoforms vary, as B56γ is expressed in the nucleus while B56α, B56β, and B56ε are expressed in the cytoplasm, B56δ seems to be expressed in both the nucleus and cytoplasm.

PP2A scaffold subunit is encoded by two distinct genes, for this reason it has two isoforms: Aα and Aβ. Both isoforms are ubiquitously expressed, sharing at least 86% sequence similarity. PP2A-A has a scaffold function, allowing the escort of the C subunit and facilitating the interaction with the regulatory subunit and with other substrates (Seshacharyulu *et al.*, 2013).

PP1 and PP2A play crucial roles in G_2-M and metaphase to anaphase transition; in particular PP1 and PP2A dephosphorylate substrates for mitotic onset, though the activities of other phosphatases are also needed during mitosis.



From Wurzenberger & Gerlich, 2011

Figure 7: PP2A has different regulatory subunit to control phosphatase specificity. PP2A has three subunits: a common scaffolding (A) subunit, a regulatory (B) subunit, which confers substrate specificity and a common catalytic (C) subunit. The PP2A B-subunit has four distinct subfamilies (B, B', B" and B"'). Potentially, more than 70 heterotrimeric PP2A complexes can be generated by combining these A-, B- and C-type subunits.

PP2A holoenzymes is important in regulating cellular structures reorganization during mitosis, including nuclear envelope breakdown, rearrangement of intracellular organelles (such as the endoplasmic reticulum and the Golgi apparatus), attachment of cytoplasmic microtubules to kinetochores, assembly of mitotic chromosomes and of the mitotic spindle.

PP1 is considered the major phosphatase counteracting Aurora B, a crucial kinase for spindle assembly and SAC; chromatin decondensation requires PP1 and its

regulatory subunits Repo-Man and PNUTS (phosphatase 1 nuclear targeting subunit).

At mitosis exit, PP2A-B55 is activated following Cdk1 and Greatwall kinase (GWL) inactivation and is required for return to the interphase state. PP2A-B55 activation is also helped by another phosphatase, Fcp1, the RNA polymerase II C-terminal domain phosphatase, in a transcription-independent manner. Indeed, at mitosis exit Fcp1 binds and dephosphorylates GWL, this way phosphorylation of ENSA/ARPP19, two potent PP2A inhibitor when phosphorylated by GWL, is lessened and ENSA/ARPP19 (Endosulfine/cAMP-regulated phosphoprotein 19)-dependent PP2A-B55 inhibition is reversed (Della Monica *et al.*, 2015).

PP2A-B56, instead, has been involved in earlier stages of mitosis exit, like spindle assembly and SAC control. Although the role of PP2A catalytic activity in these events is less characterized, data obtained through RNA interference-mediated knockdown of PP2A, in particular PP2A-B56, indicate a mitotic role for this phosphatase, in particular for kinetochore–microtubule attachments and SAC inactivation (Seeling *et al.*, 1999; Okamoto *et al.*, 2002; Chen *et al.*, 2004; Arnold & Sears, 2006; Hégarat *et al.*, 2016; Wlodarchack & Xing, 2016).

IV AIM OF THE STUDY

The SAC is regulated by numerous proteins and kinases and phosphatases are the primary regulators of this complex pathway. Recently phosphatases are increasingly appreciated for their tight regulation during mitotic progression.

Protein phosphatases like PP1 and PP2A have been suggested to dephosphorylate several substrates involved in cell cycle regulation. PP2A has been suggested to dephosphorylate over than 300 substrates, most of them involved in cell cycle regulation. However, the role of PP2A in SAC control is only beginning to be investigated and the contribution of its catalytic activity in this regulation is still not completely understood (Wlodarchack & Xing, 2011).

Evidence from precedent studies showed that the knockdown of PP2A heterotrimeric complex subunits, by RNA interference, lead to several mitotic defects including post mitotic reassembly of the nuclear envelope, Golgi apparatus and chromatin decondensation, in particular by B55α gene expression knock-down, but also defects in the metaphase-to-anaphase transition and SAC control, in particular by B56 gene expression knock-down (Schmitz *et al.*, 2010; Kruse *et al.*, 2013; Nijenhuis *et al.*, 2014). In addition, recent data about phosphatase activity during SAC, using phosphoprotein phosphatases chemical inhibitors (OA and calyculin A), showed that phosphatase activity is required for SAC maintenance and function (Foss *et al.*, 2016).

In this study we have aimed at defining the role for PP2A activity in SAC regulation. To this end, we did not attempt to interfere with PP2A holoenzyme formation, that may have important structural relevance in SAC control, but rather we selectively inhibited PP2A catalytic activity, using two chemical inhibitors: Okadaic acid (OA; selective for PP2A at low concentrations) and LB 100, and assessed the ability of transformed and non-transformed human cells to maintain SAC-dependent arrest and to silence the SAC upon spindle assembly under PP2A-inhibited conditions.

V RESULTS

1 SAC MAINTENANCE AND RESOLUTION IN HeLa CELLS TREATED WITH THE PP2A INHIBITOR LB 100

To study the possible effects of PP2A catalytic activity inhibition on SAC maintenance and resolution, we set out to determine the effects of LB 100, a rather selective PP2A competitive inhibitor (Hong *et al.*; 2015) on SAC maintenance and resolution in HeLa cells. Cells were first arrested in prometaphase by a double thymidine block followed by release into fresh medium containing nocodazole, a reversible tubulin polymerization inhibitor. After 12 hours incubation in the presence of nocodazole, mitotic HeLa cells were harvested and nocodazole was washed out. Immediately after release into fresh medium, time 0 of the experiment, cells were divided into two sets, one received vehicle (H_2O) as control and the other LB 100 (10 μM). Cells were then taken at the indicated time points of further incubation (Fig. 8). Two cell samples in the control set also received again nocodazole (Control; Noco; Fig. 8) and nocodazole plus LB 100 (Control; Noco LB 100; Fig. 8) respectively, and then taken the indicated time points of further incubation (Fig. 8). To evaluate the effective inhibition of PP2A activity by LB 100 treatment, we monitored the loss of phosphorylated Cdk1 substrates (Cdk1 p-subs; with an anti phoshospecific antibody recognizing the K/R−pS−P−X−K/R sequence; where X = any residue and pS = phosphorylated Ser) and loss of phosphorylated PRC1 T481 (pT481-PRC1) during SAC resolution in control and LB 100 treated cells. Both Cdk1 p-subs pT481-PRC1 have been shown to be dephosphorylated during mitosis exit in PP2A-dependen manner (Schmitz *et al.*, 2010; Cundell *et al.*, 2013).

Thus, total samples were separated on SDS-PAGE and immunoblotted for phosphorylated Cdk1 substrates (Cdk1 p-subs), pT481-PRC1 and PRC1 as well as Cyclin B1 (Cyc B1) and Cdk1 to monitor SAC silencing. The data show that in

control cells, as SAC was silenced upon nocodazole wash out, Cyclin B1 was degraded and Cdk1-P-subs and pT481-PRC1 dephosphorylation ensued. In cells released into LB 100-containing medium, Cyclin B1 was degraded with similar kinetics to control cells, however, dephosphorylation of Cdk1-P-subs and pT481-PRC1 were substantially hampered for the duration of the experiment (Fig. 8). Indeed, quantitation of pT481-PRC1 signal intensity showed that, from time 0 from nocodazole release to 80 minutes incubation, the signal intensity dropped by more than 80% in control cells, while, in the same temporal range, the signal dropped by less 10% in cells released in the presence of LB 100 (Fig. 8; graph). Quantitation of the Cyclin B1 signal intensity confirmed that the protein was degraded with similar kinetics in control and LB 100 treated cells (Fig. 8; graph). Control cells that were treated from time 0 with nocodazole or nocodazole plus LB 100 showed similar levels of phosphorylated proteins as well as of Cyclin B1 abundance after 100 minutes incubation, indicating that LB 100 did not induce override of the SAC-dependent mitotic arrest.

Together these data indicate that treatment of HeLa cells during SAC resolution with the PP2A inhibitor LB 100 substantially inhibited PP2A-dependent dephosphorylations, otherwise observed during mitosis exit, but did not affect the degradation of Cyclin B1, suggesting that PP2A activity is dispensable for SAC resolution. In addition, LB 100 treatment did not appear to substantially impair maintenance of, or override, SAC-dependent block to Cyclin B1 degradation.

Figure 8

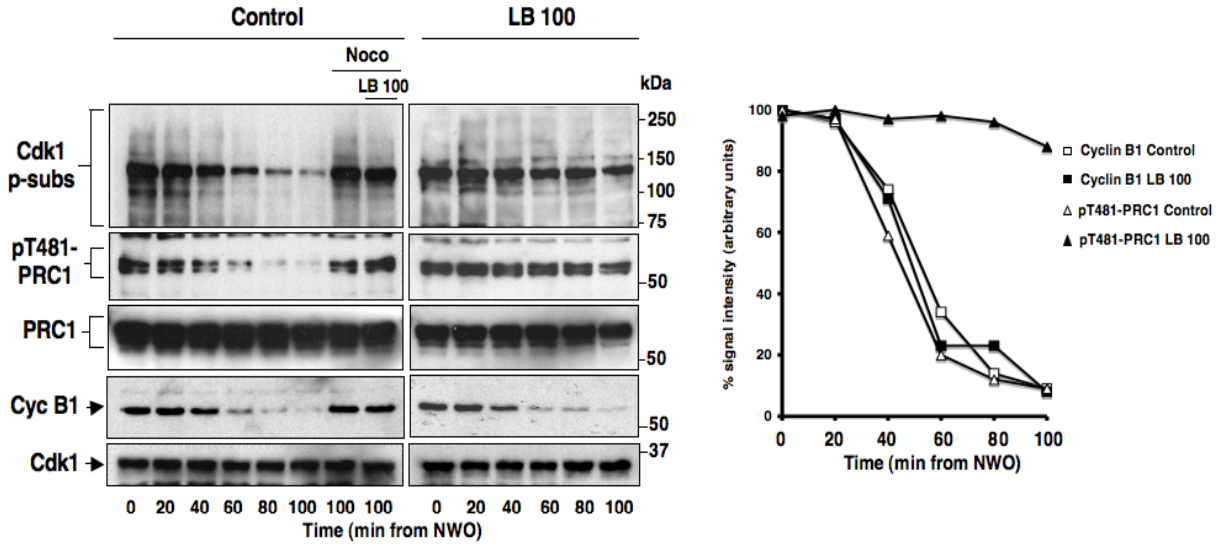


Figure 8. Effects of the PP2A inhibitor LB 100 on SAC maintenance and resolution in HeLa cells. HeLa cells were divided into two sets, one received vehicle (DMSO) as control and the other LB 100 (10 μM). Cells were then taken at the indicated time points of further incubation. Two cell samples in the control set also received again nocodazole (Noco) and one of the two LB 100 (LB 100) and taken the indicated time points of further incubation. Left panels: total samples were separated on SDS-PAGE and immunoblotted for phosphorylated Cdk1 substrates (Cdk1 p-subs), pT481-PRC1, PRC1, Cyclin B1 (Cyc B1) and Cdk1. Right graph: optical pT481-PRC1 (triangles) and Cyclin B1 (squares) signal density were plotted as % of time 0 samples from control (open symbols) and LB 100-treated (filled symbols) cells. The data shown are representative of four independent experiments performed under identical conditions and giving similar results.

2 SAC MAINTENANCE AND RESOLUTION IN hTERT-RPE1 CELLS TREATED WITH THE PP2A INHIBITOR LB 100

We next asked whether the lack of effects of LB 100 on SAC control observed in HeLa cells was also replicable in non-transformed cells. To this end, we used ne non-transformed, telomerase-immortalized, human retinal epithelium cells hTERT-RPE1. HTERT-RPE1 cells were prometaphase arrested with nocodazole following a similar protocol to that used for HeLa cells. Upon nocodazole wash out, cells were further incubated for 90 minutes in: fresh medium plus nocodazole

(Fig. 9; Noco +), just fresh medium (Fig. 9; Noco -), fresh medium plus LB 100 (Fig. 9; Noco – LB 100 +), or fresh medium plus nocodazole and LB 100 (Fig. 9; Noco + LB 100 +). Total samples were separated on SDS-PAGE and immunoblotted for Cdk1 p-subs, pT481-PRC1, PRC1, Cyc B1 and Cdk1 (Fig. 9). Release of hTERT-RPE1 cells from nocodazole arrest into fresh medium resulted in Cyclin B1 degradation and Cdk1 p-subs and pT481-PRC1 dephosphorylations after further 90 minutes incubation (Fig. 9; Noco -), while, release of these cells into LB 100-containing medium blocked Cdk1 p-subs and pT481-PRC1 dephosphorylation without affecting Cyclin B1 degradation (Fig. 9; compare Noco – with Noco – LB 100 +), much like what observed in HeLa cells under similar conditions. In addition, LB 100 did not induce Cyclin B1 degradation in the presence of nocodazole, indicating that PP2A inhibition did not cause SAC-dependent arrest override (Fig. 9; Noco + LB 100 +; OD = optical density values of Cyc B1 signals, arbitrary units).

Thus, also in non-transformed hTERT-RPE1, LB 100 appears to strongly inhibit PP2A-dependent dephosphorylation but does not affect Cyclin B1 degradation upon SAC inactivation and appears as well unable to override the SAC-dependent cell cycle arrest as Cyclin B1 remained stable under active SAC conditions (Fig. 9).

Figure 9

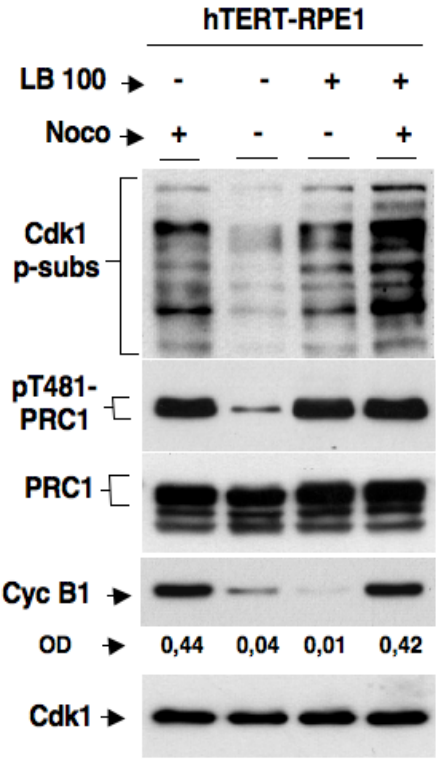


Figure 9. Effects of the PP2A inhibitor LB 100 on SAC maintenance and resolution in hTERT-RPE1 cells. Nocodazole-treated, prometaphase-arrested, hTERT-RPE1 cells were collected. Upon nocodazole wash out, cells were further incubated for 90 min in: fresh medium plus nocodazole (Noco +), just fresh medium (Noco -), fresh medium plus LB 100 (Noco – LB 100 +) or fresh medium plus nocodazole and LB 100 (Noco + LB 100 +). Total samples were separated on SDS-PAGE and immunoblotted for the indicated antigens. The data shown are representative of three independent experiments performed under identical conditions and giving similar results.

3 EFFECTS OF THE PHOSPHATASE INHIBITOR OKADAIC ACID ON SAC MAINYENANCE AND RESOLUTION IN HeLa AND hTERT-RPE1 CELLS

Okadaic acid (OA) is a potent PP2A inhibitor. In a relatively high concentration range (1 μM and higher), OA inhibits also other phosphatases including PP1. Indeed, in a recent report it has been shown that HeLa cells treated with 1 μM OA are unable to maintain SAC-dependent block to Cyclin B1 degradation, thus

indicating that protein phosphatases are indeed involved in SAC control (Foss *et al.*, 2016). However, our data using LB 100 to selectively inhibit PP2A indicate that PP2A activity is irrelevant for SAC maintenance and silencing.

To determine whether we could achieve rather selective inhibition of PP2A activity with OA, to analyze if and how it affected SAC inactivation and maintenance, we treated both HeLa and hTERT-RPE1 cells with two doses of OA under the following conditions. Nocodazole-treated, prometaphase-arrested, HeLa and hTERT-RPE1 were wash out nocodazole and further incubated for 90 minutes in: fresh medium plus nocodazole (Fig. 10; Noco +), just fresh medium (Fig. 10; Noco -), fresh medium plus OA at 0.5 μM (Fig. 10; Noco - OA 0.5), fresh medium plus OA at 1 μM (Fig. 10; Noco - OA 1), fresh medium plus nocodazole and OA at 0.5 μM (Fig. 10; Noco + OA 0.5) and fresh medium plus nocodazole and OA at 1 μM (Fig. 10; Noco + OA 1). Total samples were separated on SDS-PAGE and immunoblotted for Cdk1 p-subs, pT481-PRC1, PRC1, Cyc B1 and Cdk1 (Fig. 10). In both cell lines released form nocodazole, OA at both concentrations substantially blocked Cdk1 p-subs and pT481-PRC1 dephosphorylation without affecting Cyclin B1 degradation (Fig. 10; compare Noco – with Noco – OA 0.5 and Noco – OA 1).

In the cell samples containing nocodazole and OA, Cyclin B1 remained stable in the presence of OA at 0.5 μM but was destabilized in the presence of OA at 1 μM (Fig. 10; compare Noco + with Noco + OA 0.5 and Noco + OA 1).

Our data confirmed that high OA doses, presumably inhibiting PP1 and other phosphatases, induces Cyclin B 1 degradation, by overriding the SAC as recently proposed (Foss *et al.*, 2016). Nevertheless, our data showed that lower concentrations of OA (0.5 μM) substantially blocked PP2A-dependent dephosphorylations but did not affect Cyclin B1 degradation upon SAC resolution nor induced Cyclin B1 degradation during SAC-dependent arrest.

Thus, OA at concentrations that rather selectively inhibit activity of PP2A is unable to override SAC-dependent arrest and does not affect SAC resolution,

further supporting the conclusion that PP2A activity is dispensable for both SAC maintenance and resolution.

Figure 10

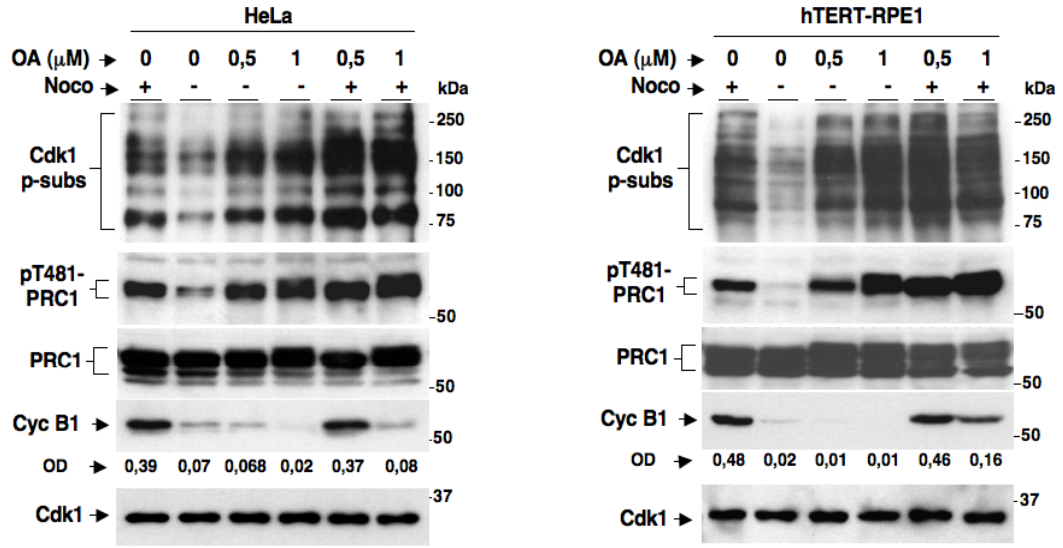


Figure 10. SAC maintenance and resolution in HeLa and hTERT-RPE1 cells treated with the phosphatase inhibitor Okadaic Acid. Nocodazole-treated, prometaphase-arrested, HeLa and hTERT-RPE1 cells were collected. Upon nocodazole wash out cells were further incubated for 90 min in: fresh medium plus nocodazole (Noco +), just fresh medium (Noco -), fresh medium plus OA at 0.5 μM (Noco - OA 0.5), fresh medium plus OA at 1 μM (Noco - OA 1), fresh medium plus nocodazole and OA at 0.5 μM (Noco + OA 0.5) and fresh medium plus nocodazole and OA at 1 μM (Fig. 10; Noco + OA 1). Total samples were separated on SDS-PAGE and immunoblotted for the indicated antigens. The data shown are representative of four independent experiments performed under identical conditions and giving similar results.

4 PP2A ACTIVITY DOES NOT SUBSTANTIALLY AFFECT MITOTIC CHECKPOINT COMPLEX (MCC) DYNAMICS DURING SAC MAINTENANCE AND RESOLUTION

The SAC-dependent arrest of mitosis exit is granted by the formation of the SAC effector branch in which the MCC plays a crucial role in inhibiting APC/C ubiquiting ligase action. The core proteins forming the MCC are Cdc20, the

APC/C coactivator, bound to Mad2 and BuBR1, two major SAC effectors.

We, thus, set out to determine directly whether PP2A activity affected MCC maintenance under SAC conditions and disassembly upon SAC silencing during mitosis exit by coimmunorecipitation (CoIP) experiments. Nocodazole-treated, prometaphase-arrested, HeLa cells were wash out nocodazole and further incubated for 60 min in: fresh medium plus nocodazole (Fig. 11; Noco +), just fresh medium (Fig. 11; Noco -), fresh medium plus LB 100 (10 μM; Fig. 11; Noco - LB 100 +), fresh medium plus OA (0.5 μM; Fig. 11; Noco - OA +), fresh medium plus nocodazole and LB 100 (10 μM; Fig. 11; Noco + LB 100 +) and fresh medium plus nocodazole and OA (0.5 μM; Fig. 11; Noco + OA +). Cdc20 was immunoprecipitated (Ip) from cell lysates. Cdc20 and CoIp BuBR1 and Mad2 were visualized by immunoblotting Cdc20 Ip, resolved on SDS/PAGE (Fig.11). Total lysates were also probed for Cdc20, BuBR1 and Mad2. In SAC arrested cells (Fig. 11; Noco +), significant amounts of Mad2 and BuBR1 were found bound to Cdc20, marking and assembled MCC. In cells released from nocodazole (Fig. 11; Noco -), that were able to assembly mitotic spindles and inactivate the SAC, Mad2 and BuBR1 substantially dissociated from Cdc20, marking MCC disassembly, and these events happened regardless of the presence of LB 100 or OA (0.5 μM; Fig. 11; Noco - LB 100 + and Noco - OA +).

These data directly indicate that PP2A activity is dispensable for SAC resolution and MCC disassembly. In addition, we found that LB 100 or OA (0.5 μM) did not lead to SAC override since the MCC remained stable when cells were incubated with either PP2A inhibitor in the presence of nocodazole (Fig. 11; compare Noco + with Noco + LB 100 + and Noco + OA +).

Together these findings indicate that PP2A activity is neither required for MCC disassembly and SAC silencing nor for SAC and MCC maintenance.

Figure 11

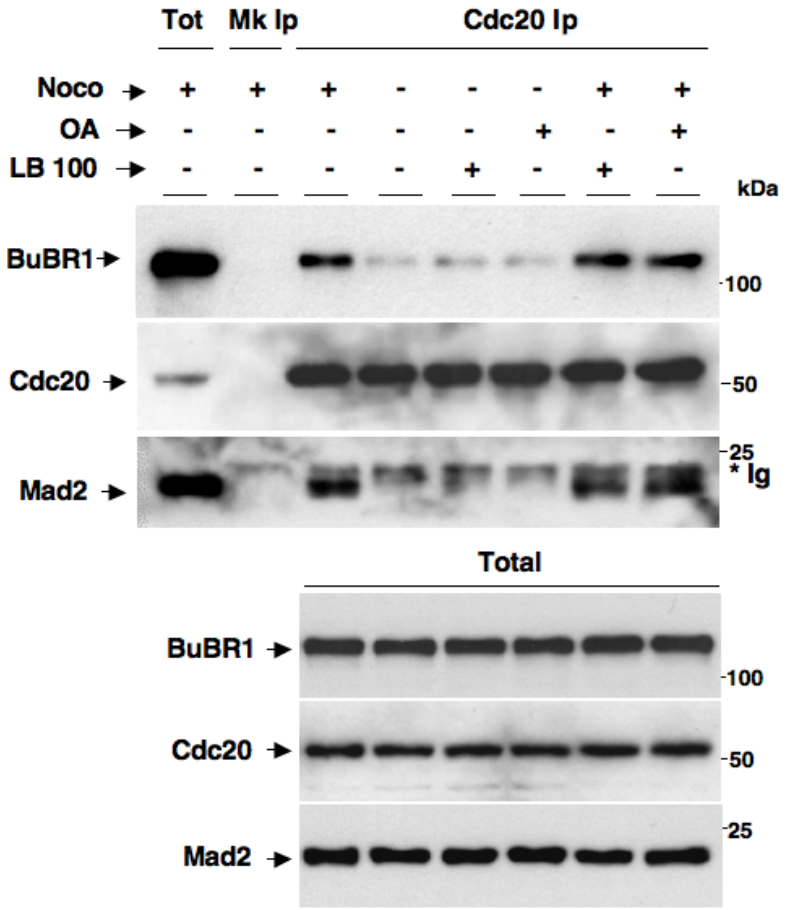


Figure 11: PP2A activity does not affect MCC maintenance and disassembly. Nocodazole-treated, prometaphase-arrested, HeLa cells were wash out nocodazole and further incubated for 60 min in: fresh medium plus nocodazole (Noco +), just fresh medium (Fig. 11; Noco -), fresh medium plus LB 100 (10 µM; Noco - LB 100 +), fresh medium plus OA (0.5 µM; Noco - OA +), fresh medium plus nocodazole and LB 100 (10 µM; Noco + LB 100 +) and fresh medium plus nocodazole and OA (0.5 µM; Noco + OA +). Cdc20 was immunoprecipitated (Ip) from cell lysates. Top panels, Cdc20 Ips (Cdc20 Ip) were resolved on SDS/PAGE and subsequently probed by immunoblotting for the indicated antigens (a total cell lysate, Tot; and mock Ip, Mk Ip, were also included as control in the SDS/PAGE; the asterisk marks immunoglobulin signal, *Ig). Lower panels, total lysates were also probed for the indicated antigens. The data shown are representative of three independent experiments performed under identical conditions and giving similar results.

VI DISCUSSION

1 THE COMPLEXITY OF PHOSPHATASE REQUIREMENTS AT MITOSIS EXIT

SAC has evolved to ensure accurate chromosome segregation; this occurs by inhibiting the APC/C (and the consequent Cyclin B and securin degradation by APC/C polyubiquitination), until all the chromosomes reach the bipolar attachment between the two opposite spindle poles and proper tension is placed on the paired kinetochores (Peters, 2006). In fact, sister chromatids segregation or cell division prior to proper bipolar attachment leads to random segregation of chromosomes causing aneuploidy, which is closely associated with cancers and birth defects (Weaver & Cleveland, 2006).

Protein phosphatases play essential roles in these processes by controlling the phosphorylation state of a number of proteins involved in mitotic control; furthermore phosphatase activity has been implicated in regulating APC/C activity (Rogers *et al.*, 2016). In original works in yeast, PP1 activity was described to have a crucial role in silencing the SAC upon spindle assembly (Pinsky *et al.*, 2009; Vanoosthuyse & Hardwick, 2009). On the other hand, PP2A has also been involved in the control of mitosis progression. Like PP1, PP2A is a rather heterogeneous holoenzyme and its functions depend on the subunit composition that constitutes the heterotrimeric holoenzyme. The B subunits, in particular, have been the subject of several studies because of their role in determining substrates specificity of the PP2A holoenzyme. Indeed, several studies on the various PP2A-B subunits helped us to better under understand this complex enzyme.

Activity of PP2A-B55, for instance, has been shown to be crucially involved in mitosis completion by dephosphorylating Cdk1 substrates at the end of mitosis and to be necessary to control several aspects of post-anaphase mitosis completion like cytokinesis and reformation of the interphase nucleus (Schmitz *et al.*, 2010; Cundell *et al.*, 2013; Della Monica *et al.*, 2015).

PP2A-B56, instead, has been involved in chromosome alignment at metaphase and SAC control. Indeed, the SAC effector protein BuBR1 has been shown to physically interact with the PP2A B56 subunit while small interfering RNAs-mediated B56 downregulation has been shown to affect chromosome alignment and segregation (Kruse *et al*., 2013; Nijenhuis *et al*., 2014). In addition, these studies indicated that PP2A-B56 could be required to recruit PP1 at kinetochores, and that the activity of PP1 would ultimately be required to silence the SAC (Nijenhuis *et al*., 2014).

Despite all these studies, a direct role for PP2A phosphatase activity in SAC regulation has been inferred but never directly demonstrated (Wlodarchack & Xing, 2011).

2 ACTIVITY OF PP2A IS DISPENSABLE FOR SAC MAINTENANCE AND RESOLUTION

In this work we asked whether PP2A catalytic activity was directly involved in SAC control. Recently Foss *et al.* have approached the topic of the role of protein phosphatase activity in SAC control and have reached the conclusion that phosphatase activity is required to maintain SAC action (Foss *et al.*, 2016). They found that treating SAC arrested cells with the protein phosphatase inhibitor OA lead to APC/C activation and override of SAC-dependent arrest. In their work they used doses of OA that likely inhibit several protein phosphatases including PP1. We, instead, asked specifically the relevance for PP2A activity in SAC control. To this end, we achieved a rather selective PP2A inhibition by using the chemical inhibitors LB 100 and OA at doses that selectively inhibited PP2A activity. Indeed, under our conditions, these inhibitors substantially inhibited PP2A-dependnet dephosphorylations that mark mitosis exit upon SAC silencing but did not block SAC silencing itself as demonstrated by the fact that Cyclin B1 degradation was unaffected as well as disassembly of the MCC (see Figures 8 to 11). In addition, cells maintained a rather consistent mitotic arrest when cells were

incubated in the presence of both PP2A inhibitors and nocodazole to maintain an active SAC (see Figures 10 and 11). Thus, we concluded that the catalytic activity of PP2A is dispensable for SAC maintenance and silencing.

VII CONCLUSIONS AND FUTURE PERSPECTIVES

Protein phosphatase 2A has been called into regulation of mitosis exit and SAC control. While the role concerning PP2A-B55 activity in promoting mitosis exit by stimulating dephosphorylations required for post-anaphase events (like cytokinesis, reformation of the nuclear envelop etc.) has been well documented (Schmitz *et al.*, 2010; Cundell *et al.*, 2013; Della Monica *et al.*, 2015), the requirement for activity of PP2A, in particular that of PP2A-B56, in pre-anaphase events like spindle assembly and SAC control, have been the result of deduction from data obtained through genetic downregulation of PP2A subunits (Kruse *et al.*, 2013; Nijenhuis *et al.*, 2014). We have now investigated specifically the role for PP2A activity in SAC maintenance and resolution and found that inhibition of PP2A activity did not significantly affect these controls in both transformed and non-transformed human cells. Together with the previous observations obtained through genetic downregulation of PP2A subunits, our data suggest a different role for PP2A in SAC control that is independent of its catalytic activity. In this perspective the PP2A holoenzyme could be envisaged as the basis of multiprotein hubs required to structurally link specific structures, like kitetochores, to signaling molecules, like the SAC protein BuBR1, to control spindle assembly and SAC, a role that is independent of PP2A catalytic activity. Finally, our results cast doubt on the possibility that targeting PP2A activity may offer a therapeutic advantage to improve efficacy of widely used anticancer drugs that target the mitotic spindle like taxanes and vinca alkaloids.

VIII MATERIALS AND METHODS

1 CELL CULTURES AND TREATMENTS

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), hTERT-RPE1 were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Thermo Fischer Scientific, Waltham, MA, USA), both supplemented with 10% fetal calf serum (FBS; GE Healthcare Hyclone, Little Chalfont, Buckinghamshire, UK) and 1% 10000 U/ml penicillin–10 mg/ml streptomycin (Sigma-Aldrich). Cells were incubated during at 37°C with a 5% CO_2 partial pressure.

Prometaphase arrested cells were obtained by performing a double thymidine (4mM; Sigma-Aldrich) block (18 hours each, separated by a 6 hours incubation in fresh medium) followed by release into fresh medium containing nocodazole (500 nM; Calbiochem, Billerica, MA, USA) and incubated for 12 or 14 hours for HeLa and hTERT-RPE1, respectively. Cells were detached from substrate, recovered by shake-off and released from prometaphase arrest by washing detached cells twice with phosphate-buffered saline (PBS; EuroClone, Milan, IT) and twice with fresh medium, followed by incubation in fresh medium.

PP2A catalytic activity inhibition was performed by using OA (500 nM or 1 μM; Calbiochem) or LB100 (10 μM; Selleckchem, Houston, TX, USA).

Cells were collected at different time points and lysed in NP40/EB (1:1) lysis buffer (NP40: 0.2% IGEPAL; EB: 80 mM 2-glycerophosphate, 10 mM $MgCl_2$ and 20 mM EGTA, Sigma-Aldrich; solutions were made in PBS, EuroClone). Lysates were incubated 30 minutes on ice and spun for 20 minutes at 13,200 rpm, in a refrigerated centrifuge.

2 IMMUNOPRECIPITATIONS

For immunoprecipitations (IPs) experiments cell pellets were lysed in NP40/EB lysis buffer; lysates were incubated 30 minutes on ice and spun for 20 minutes at 13,200 rpm, in a refrigerated centrifuge.

Lysates were used to immunoprecipitate Cdc20, using agarose beads conjugated with P55 CDC (SC13162AC, 500 µg/mL, 25% agarose) mouse monoclonal IgG (Santa Cruz Biotecnology, Dallas, TX, US).

IPs were incubated on a rotator wheel over night at 4°C and washed three times abundantly in NP40/EB.

3 ANTIBODIES AND IMMUNOBLOTTING

Total samples and IPs were separated in SDS-PAGE and blotted onto nitrocellulose membrane (Amersham-GE Healthcare, Milan, Italy). After blotting membranes were blocked with 5% not fat dried milk (NFDM; BioRad, Milan, Italy) in PBS for 1 hour and incubated with primary antibodies over night.

The primary antibodies used for the immunoblots are as follows: rabbit polyclonal anti-Cyclin B1 (1:1000), mouse monoclonal anti-CDC2 (Cdk1; 1:1000), rabbit polyclonal anti-PRC1 (1:250), mouse monoclonal anti-p-PRC1 (recognizing phospho-Treonine 481; 1:500), mouse monoclonal anti-Mad2 (1:300), rabbit polyclonal anti-P55 CDC (Cdc20; 1:500); all these antibodies were purchased from Santa Cruz Biotecnology (Dallas, TX, US). Rabbit monoclonal anti-phospho-serine Cdk1 Substrates (P-S2-100; recognizing K/HpSP; 1:1000) was purchased from Cell Signaling Technology (Danvers, MA, USA) while mouse monoclonal anti-BUBR1 (1:1000) was purchased from BD Biosciences (San Jose, CA, USA).

Then after eliminating the excess of primary antibodies, by washing twice in PBS, primary antibodies on immunoblots were revealed by horseradish peroxidase-conjugated donkey anti-rabbit (1:5000) or anti-mouse (1:3000) IgG antibodies (GE Healthcare, Milan, IT) and using an ECL kit (Cyanagen, Bologna, IT).

The intensity of the western blot bands was measured by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

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