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Polo/Plk1-mediated inhibition of PP1 defines a
molecular switch to control SAC signalling

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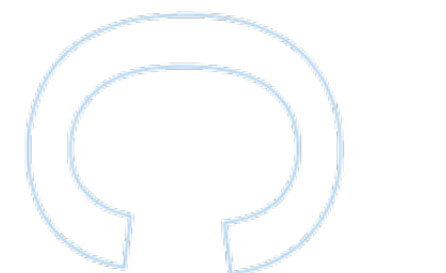
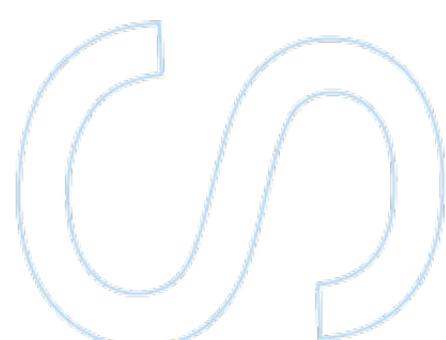
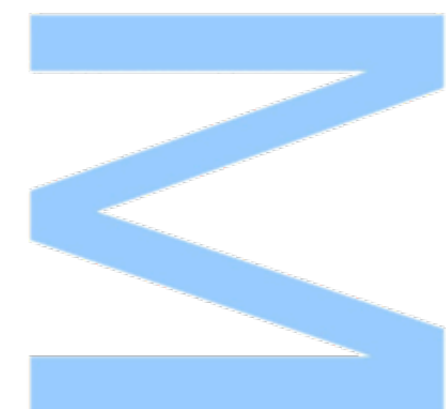
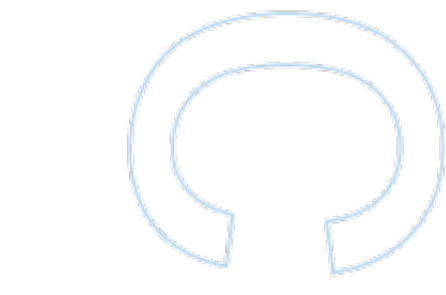
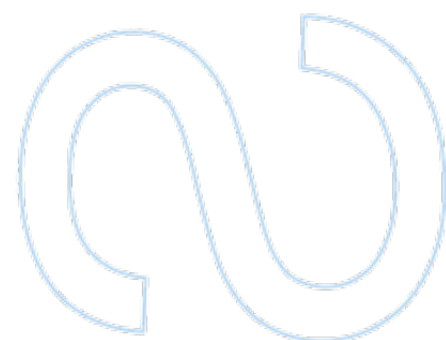
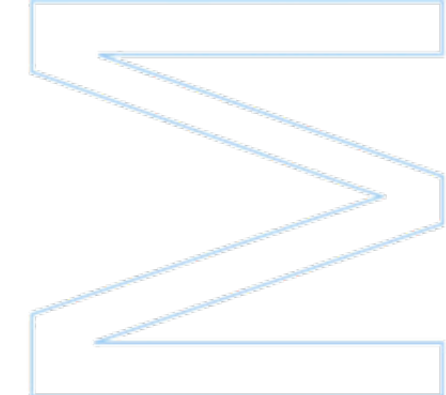
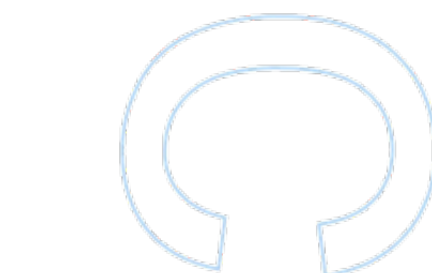
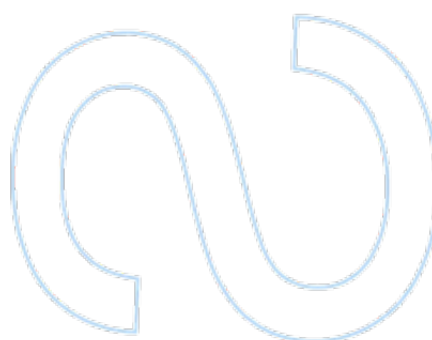
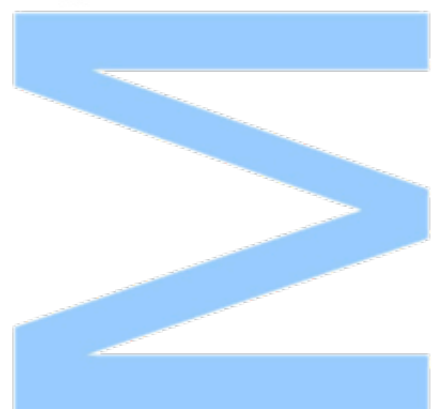
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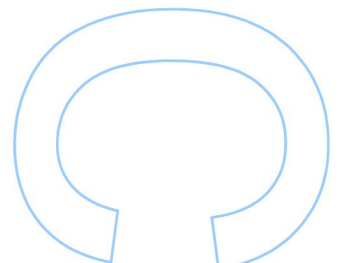
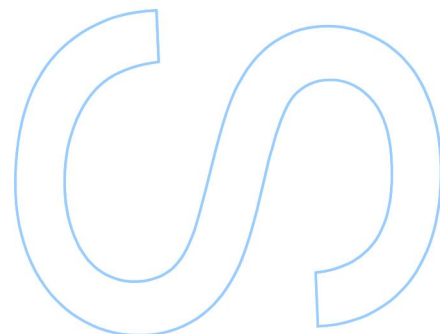
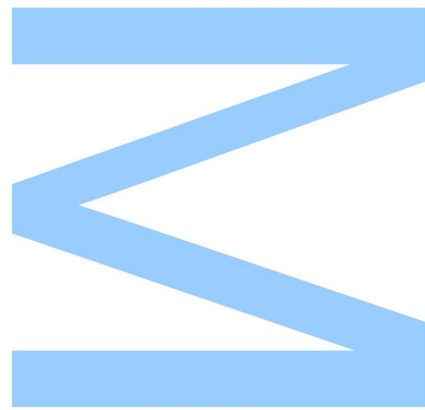
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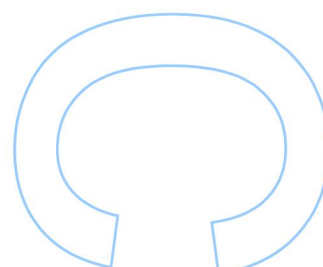
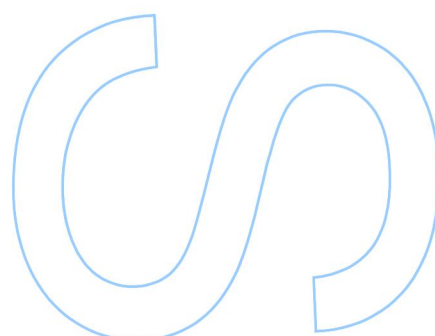
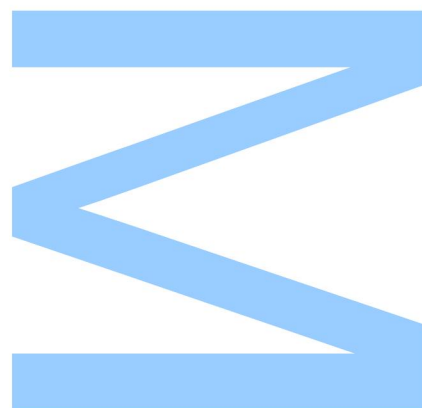




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O Presidente do Júri,

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Abstract

Accurate genome partitioning during cell division relies on the Spindle Assembly Checkpoint (SAC), a conserved signalling pathway that delays anaphase onset until all chromosomes are correctly attached to the mitotic spindle (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). Mps1 kinase is an upstream regulator of pre-mitotic and mitotic anaphase inhibitors (Liu and Winey, 2012; Rodriguez-Bravo et al., 2014) but the mechanisms underlying timely control of its activity remain unknown. We show that PP1 dephosphorylates Mps1 T-loop to promote its inactivation and consequently prompt SAC silencing. We find that Polo in its active conformation structurally inhibits PP1 activity allowing Mps1 activation, which is further potentiated by Polo phosphorylation of Mps1 N-terminal. Because Polo activating phosphorylation is also antagonized by PP1 (Yamashiro et al., 2008), this two-pronged regulation of Mps1 by Polo defines a molecular switch that ensures robust Mps1 activation on mitotic commitment and swift inactivation following chromosome biorientation. Thus, our findings uncover a new mechanism that contributes to establish strength and responsiveness, traits of the SAC required to prevent unequal segregation of chromosomes and genetic imbalance. Furthermore, the negative feedback loop between Polo and PP1 might be important to control extensive changes in overall protein phosphorylation that underlie the transition between interphase and mitosis.

Keywords: Polo, Mps1, PP1, SAC, Kinetochore, Mitosis, Regulation.

Resumo

A manutenção da estabilidade genómica durante a divisão celular depende do *Spindle Assembly Checkpoint* (SAC), o qual evoluiu como um mecanismo de vigilância que controla a ligação de microtubulos a cinetocoros e impede a entrada em anafase até que todos os cromossomas estejam corretamente ligados ao fuso mitótico (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). A cinase Mps1 atua como regulador de inibidores da anafase pré-mitóticos e mitóticos (Liu and Winey, 2012; Rodriguez-Bravo et al., 2014) mas os mecanismos envolvidos no controlo da sua atividade permanecem desconhecidos.

Os resultados apresentados mostram que a PP1 desfosforila o *T-loop* do Mps1 permitindo a sua inativação e, consequentemente, o rápido silenciamento do SAC. O Polo, na sua conformação ativa inibe estruturalmente a PP1 permitindo a ativação do Mps1, que é ainda potenciada por fosforilação pelo Polo no N-terminal do Mps1. Sendo a fosforilação ativadora do Polo antagonizada pela PP1 (Yamashiro et al., 2008), esta dupla regulação do Mps1 pelo Polo define um *switch* molecular que assegura uma ativação robusta do Mps1 em mitose e a rápida inativação após bi-orientação dos cromossomas. Assim, estes resultados revelam um novo mecanismo que contribui para o estabelecimento da força e responsividade, características do SAC necessárias para evitar uma segregação desigual dos cromossomas e um desequilíbrio genético. Além disso, o *loop* de *feedback* negativo entre Polo e PP1 deverá ser importante para controlar alterações extensas na fosforilação total de proteínas que estão na base da transição entre interfase e mitose.

Palavras-chave: Polo, Mps1, PP1, SAC, Cinetocoro, Mitose, Regulação.

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List of Abbreviations

APC/C	Anaphase Promoting Complex / Cyclosome
ATP	Adenosine Triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
Bub	Budding uninhibited by benzimidazole
BubR1	Bub1-Related protein kinase
C-Mad2	Closed Mad2
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
Cdc	Cell division cycle
Cdh	Cdc20 homolog
Cdk	Cyclin-dependent kinase
cDNA	complementary DNA
CDS	Coding Sequence
Cenp	Centromeric protein
CID	Centromere Identifier
CPC	Chromosomal Passenger Complex
D-box	Destruction box
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
ds	double stranded
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid

EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethyleneglycoltetraacetic Acid
FBS	Fetal Bovine Serum
FRAP	Fluorescence Recovery After Photobleaching
H3T3	Tyr3 on histone H3
Hec1	Highly expressed in cancer 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
IB	Immunoblotting
IF	Immunofluorescence
INCENP	Inner Centromere Protein
IP	Immunoprecipitation
KD	Kinase-Dead
KEN box	Lys-Glu-Asn motif
KMN	Kn1-1/Mis12 complex/Ndc80 complex
KNL-1	Kinetochore null-1
LB	Luria Bertani medium
Mad	Mitotic arrest deficient
MBP	Maltose Binding Protein
MCC	Mitotic Checkpoint Complex
mCherry	Monomer red fluorescent protein
MELT	Met-Glu-Leu-Thr motif
MOPS	3-(N-Morpholino)propanesulfonic acid
Mps1	Monopolar spindle 1
Mps1 ^{KD}	Kinase-dead version of Mps1
Mps1 ^{T185A}	Mps1 mutated on Threonine 185 to Alanine
Mps1 ^{T185D}	Mps1 mutated on Threonine 185 Aspartate

Mps1 ^{T490Ph}	Mps1 phosphorylated on Threonine 490
Mps1 ^{WT}	Wild-type version of Mps1
mRNA	messenger RNA
Ndc80	Nuclear division cycle 80
NEB	Nuclear Envelope Breakdown
NTE	N-terminal extension
Nuf2	Nuclear filament-containing protein 2
OA	Okadaic Acid
O-Mad2	Open Mad2
PBD	Polo Box Domain
PBS	Phosphate-buffered saline
PBST	PBS 0.05% Tween20
PBSTF	PBS 0.05% Tween20 10% FBS
PCR	Polymerase Chain Reaction
Ph	Phosphorylated
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
Plk1	Polo-like kinase 1
Polo ^{T182A}	Polo mutated on Threonine 182 to Alanine
Polo ^{T182D}	Polo mutated on Threonine 182 to Aspartate
Polo ^{WT}	Wild-type version of Polo
PP	Protein Phosphatase
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Region of interest
RZZ	Rough Deal/Zeste White 10/Zwilch
S2 cells	Drosophila Schneider cells
SAC	Spindle Assembly Checkpoint

SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
Sgo	Shugoshin
SPB	Spindle Pole Body
Spc105	Spindle pole body component 105
TPR	Tetratricopeptide domain
tw	twins
UTR	Untranslated region
WB	Western blot
wdb	widerborst
WT	Wild-type
Zw10	Zeste White 10
Zwint-1	Zw10 interactor 1

CHAPTER 1

INTRODUCTION

1.1. Mitosis Overview

The idea that cells are the fundamental building blocks of life was postulated in 1839 by Schleiden and Schwann in what became known as the *Cell Theory*, and gained widespread acceptance after Rudolf Virchow published, in 1855, the now famous aphorism, *omnis cellula e cellula*, that means “all cells arise from other cells” (Nurse, 2000). The generation of new cells is a process that can occur either symmetrically, for proliferation of a certain cell type, or asymmetrically, for differentiation in specialized cells; either way it involves the equal separation of the genetic material to two daughter cells. The process of cell division in eukaryotes was named mitosis (from the Greek mitos: threads) by Walther Flemming in 1879, upon observing what looked like threads in dividing cells (Mitchison and Salmon, 2001).

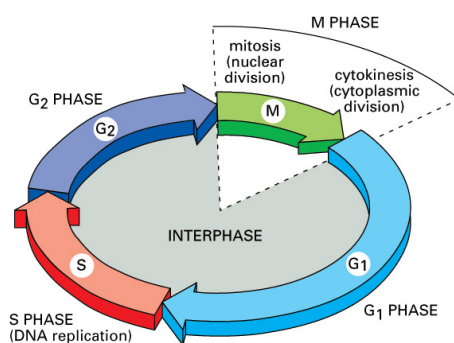


Figure 1.1 – Phases of Cell Cycle. Cell division includes G₁, S, G₂ and M phases, being G₁ and G₂ gap phases between S and M phases. M phase includes mitosis and cytokinesis (Alberts 2008).

The sequence of events, starting with a signal to begin cell division and ending in the actual division of the cell, is known as the cell cycle (figure 1.1). Before the generation of two daughter cells, the mother cell needs to have its DNA and organelles duplicated. These processes take place in G₁, S and G₂ phases (collectively termed interphase), which are sequentially initiated due to the activity of several Cyclin-dependent kinases (Cdk) in complex with Cyclins.

Mitosis encompasses five morphologically distinct stages – prophase, prometaphase, metaphase, anaphase and telophase (figure 1.2). The activation of Cyclin-dependent kinase1 (Cdk1)/Cyclin B, which is dependent on the removal of inhibitory phosphorylations by the phosphatase Cdc25 (Domingo-Sananes et al, 2011), signals mitotic commitment and leads to nuclear envelope breakdown (NEB; Gavet and Pines, 2010; Virshup and Kaldis, 2010). During prometaphase, microtubules of the mitotic spindle interact with chromosomes through specialized multi-protein structures assembled on centromeric DNA named

kinetochores (Cheeseman and Desai, 2008). Kinetochore-microtubule attachments lead to the progressive alignment of chromosomes at the metaphase plate, a condition in which all chromosomes are correctly attached to opposite spindle poles (biorientation). At this point, the Anaphase Promoting Complex/Cyclosome (APC/C) is activated by Cell division cycle 20 (Cdc20). APC/C is a multisubunit E3 ubiquitin protein-ligase, targeting proteins containing one of two targeting motifs, Lys-Glu-Asn (KEN)-box and Arg-x-x-Leu (RxxL)-box (also known as Destruction (D)-box) for degradation by the 26S proteasome, among which Cyclin B and Securin (figure 1.3; King et al, 1995; Hagting et al, 2002; Pines, 2011). Securin is a stoichiometric inhibitor of Separase, a protease, which cleaves the Cohesin ring that holds sister chromatids together. Its removal therefore allows sister chromatid separation and anaphase onset. Degradation of Cyclin B renders Cdk1 inactive allowing mitotic exit (Wolf et al, 2006; Lara-Gonzalez et al, 2012). During telophase, chromosomes decondense and nuclear envelope reforms around them, giving rise to two separate daughter nuclei. At the end of cell division a contractile ring forms midway between the two new nuclei and upon constriction pinches the cell in two at cytokinesis.

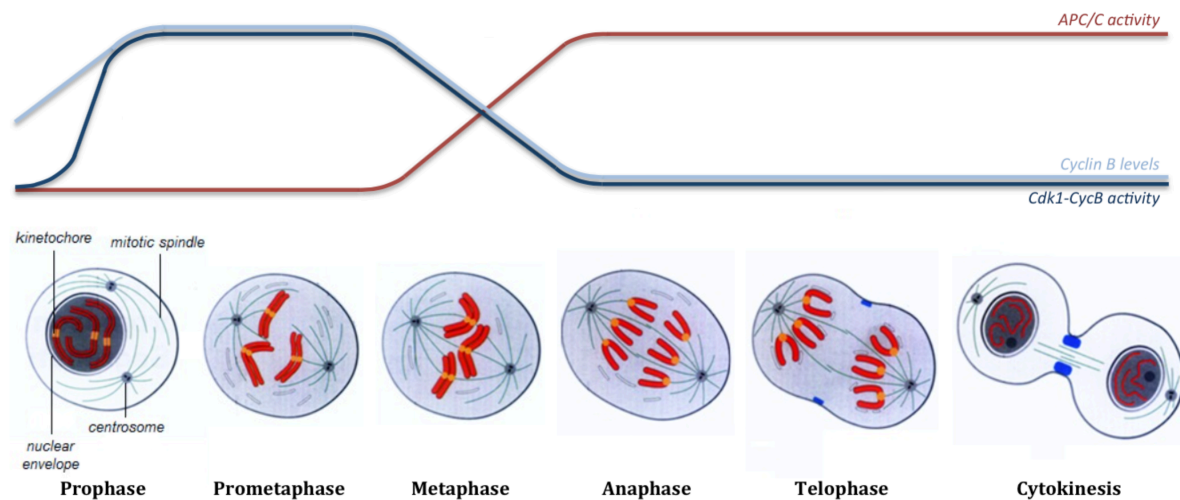


Figure 1.2 – Mitotic stages. DNA condensation becomes visible and centrosomes begin to separate in prophase. Rising Cyclin B levels activate Cdk1, which drives mitotic progression. Prometaphase is initiated with NEB, which allows bipolar spindle formation. In metaphase, all chromosomes align at the metaphase plate and kinetochore-microtubule are correctly attached. At this point APC/C activity rises, leading to Cdk1-CycB inactivation and beginning of anaphase. After segregation of sister chromatids to opposite poles, nuclear envelope reforms during telophase. The resulting two daughter cells emerge at cytokinesis after constriction of a contractile ring, which separates their cytoplasmic content (adapted from Morgan, 2007).

1.2. The Spindle Assembly Checkpoint

Abnormal chromosome segregation results in aneuploidy, a hallmark of most solid tumors and birth defects. To prevent segregation errors, eukaryotic cells have evolved a signal transduction pathway known as the Spindle Assembly Checkpoint (SAC). This quality control mechanism monitors the status of kinetochore-microtubule interactions and restrains the irreversible transition into anaphase until all chromosomes are correctly attached to the mitotic spindle (Lara-Gonzalez et al, 2012).

The SAC was first described, in 1991, as a pathway that is active in prometaphase and prevents the precocious sister chromatids separation in the presence of drugs affecting mitotic spindle assembly. Two different screens in *Saccharomyces cerevisiae* identified most of the key components, namely Mitotic-arrest deficient (Mad) 1, 2 and 3 and Budding uninhibited by benzimidazole (Bub) 1 and 3 (Li and Murray, 1991; Hoyt et al, 1991). These genes are conserved in all eukaryotes (Musacchio and Salmon, 2007), with exception for Mad3. Although in higher eukaryotes the N-terminal sequence of its counterpart Bub-related-1 (BubR1) kinase, does display homology with Mad3, it also contains a C-terminal kinase domain, which suggests a relation to Bub1 (Taylor et al, 1998; Suijkerbuijk et al, 2012a).

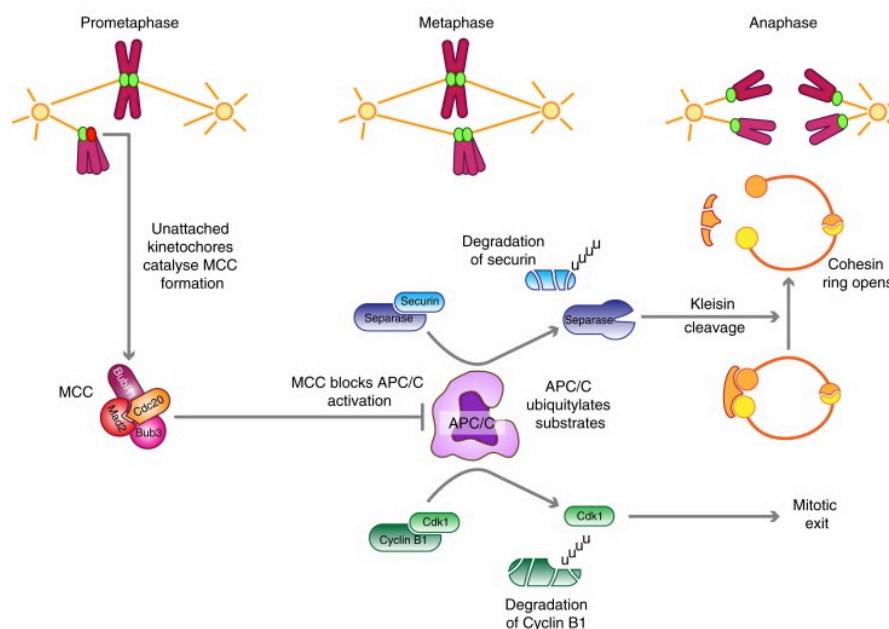


Figure 1.3 - APC/C regulation and mitotic progression. At prometaphase, in the presence of unattached kinetochores, MCC is formed and inhibits APC/C function. As kinetochores become properly attached to microtubules and chromosomes align, cells reach metaphase. At this stage kinetochores are unable to sustain efficient MCC formation and APC/C is activated. APC/C functions include ubiquitination of Cyclin B1 and Securin, which targets them for degradation by the 26S proteasome. Cyclin B1 degradation leads to Cdk1 inactivation and

allows mitotic exit, while Securin degradation frees Separase, which in turn cleaves Cohesin. (Lara-Gonzalez et al, 2012).

An important hallmark for the comprehension of the SAC mechanism was the discovery that anaphase onset in vertebrate cells is blocked by a single unattached kinetochore (Gorbsky and Ricketts, 1993; Rieder et al, 1994; Rieder et al, 1995; Nicklas et al, 1995). Furthermore, SAC proteins, as well as Cdc20, were found to be recruited to unattached kinetochores in a hierarchical way (Chen et al, 1996; Taylor and McKeon, 1997; Jin et al, 1998; Taylor et al, 1998; Vigneron et al, 2004), with Mad2, BubR1, Bub3 and Cdc20 displaying high turnover rates (Shah et al, 2004; Howell et al, 2004). These proteins were also identified as members of complexes that inhibit APC/C activity (Kallio et al, 1998; Hwang et al, 1998; Kim et al, 1998; Fang et al, 1998; Tang et al, 2001; Sudakin et al, 2001; Fang, 2002). All together, these findings suggested that the SAC monitors kinetochore-microtubule attachments, rather than spindle assembly, and led to the current SAC signalling paradigm – SAC proteins are recruited to unattached kinetochores, which serve as catalytic platforms for the production of a “wait-anaphase signal” (figure 1.3).

The anaphase inhibitory signal prevents APC/C activation by targeting its mitotic activator Cdc20 until all kinetochores are correctly attached. However, it is not consensual the exact composition of the inhibitory signal, since Mad2/Cdc20, Cdc20/BubR1/Bub3 and Mad2/Cdc20/BubR1/Bub3 complexes have been isolated (figure 1.4). Current models for SAC molecular framework suggest that Mad1/Mad2 heterotetramers associate with unattached kinetochores and catalyze the structural conversion of O-Mad2 to C-Mad2, that has higher affinity for APC/C activator Cdc20. (De Antoni et al, 2005). C-Mad2-Cdc20 was shown to inhibit the APC/C by sequestering its activator (Fang et al, 1998; Lau and Murray, 2012), however its inhibitory potential was determined to be inferior to the one of a complex that also includes BubR1 and Bub3, which was termed Mitotic Checkpoint Complex (MCC) (Sudakin et al, 2001; Fang, 2002). Although it remains unclear how C-Mad2/Cdc20 and BubR1/Bub3 intersect to form MCC, it was recently shown that C-Mad2-Cdc20 interaction primes Cdc20 to bind BubR1 through its N-terminal KEN box to generate the final MCC that efficiently inhibits APC/C activity (Kulukian et al, 2009). It was reported that Cdc20 associates predominantly with BubR1 and not Mad2 *in vivo* (Nilson et al, 2008; Kulukian et al, 2009); however, Mad2 is required for efficient BubR1-Cdc20 association and was proposed to act as a catalyst, promoting Cdc20 binding to BubR1/Bub3, without actually being part of the final inhibitory complex (figure 1.3; Kulukian et al, 2009).

Several studies have been conducted to understand how the MCC inhibits APC/C activity. It was found, by determination of APC/C-MCC structure, that when Cdc20 is bound to APC/C as part of MCC it is displaced from its usual subunit in APC/C (Herzog *et al*, 2009). Binding to MCC holds APC/C in a less flexible conformation, which may contribute to reduced substrate recruitment and decreased binding to ubiquitin E2 conjugating enzymes (Chao *et al*, 2012). Moreover, BubR1 interaction with Cdc20 sterically hinders access to Cdc20 destruction-box (D-box) binding site (Lara-Gonzalez *et al*, 2011).

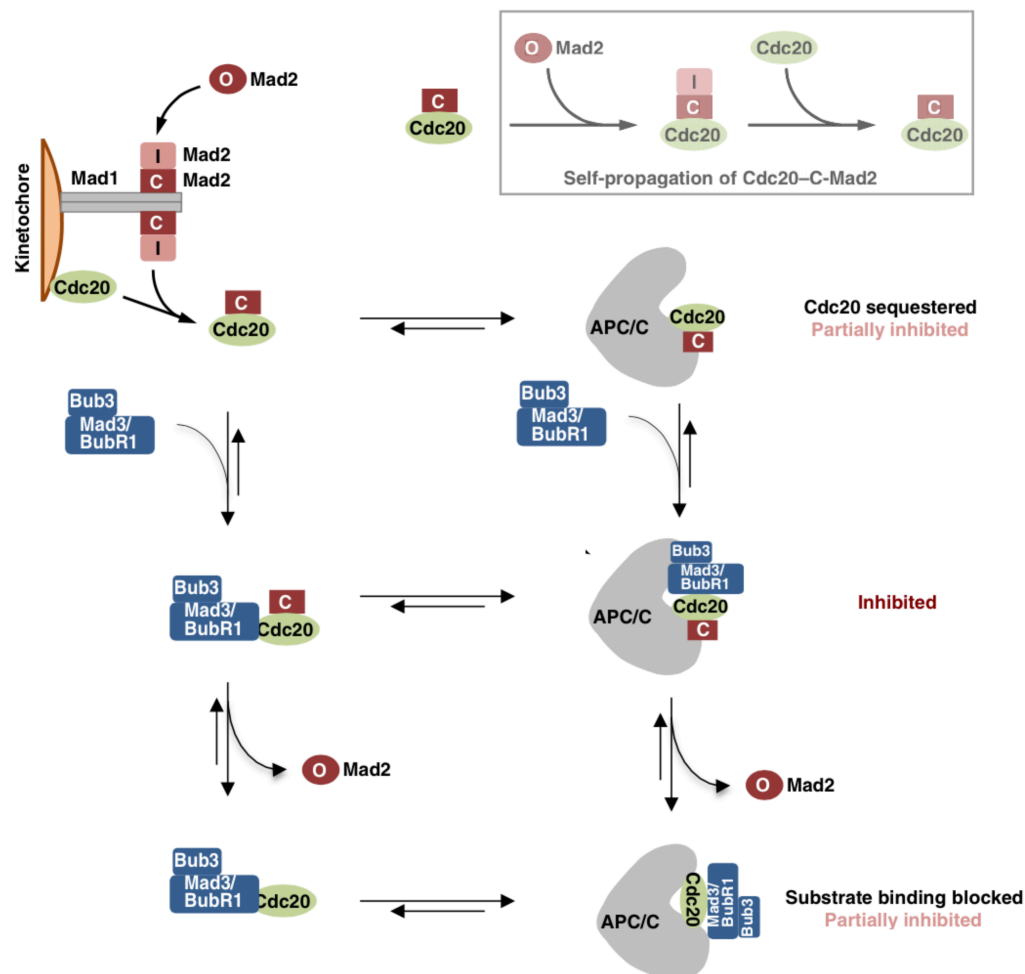


Figure 1.4 – Assembly of APC/C inhibitory complexes. C-Mad2-Mad1 bound to unattached kinetochores binds free O-Mad2 and promotes its conformational change to an intermediary state and binding to Cdc20. It has been proposed that C-Mad2-Cdc20 in turn is able to self-propagate through a similar mechanism. C-Mad2-Cdc20 binds to BubR1-Bub3, forming the MCC. Mad2 itself may, however, only act as a catalyst, leaving Cdc20 bound to BubR1. These three complexes are able to inhibit the APC/C. Mad2 acts through Cdc20 sequestration, while BubR1 blocks substrate recruitment by the APC/C, both resulting in partial inhibition of the APC/C. Full inhibition of the APC/C requires synergistic action of both Mad2 and BubR1 (adapted from Luo and Yu, 2012).

It has also been proposed that MCC targets Cdc20 for destruction in an ubiquitin-dependent manner (Nilsson *et al*, 2008; Mansfeld *et al*, 2011). Lowering Cdc20 levels could contribute to prevent APC/C activity (Nilsson, 2011); however, Cdc20 ubiquitination may serve other purposes, such as MCC dissociation and SAC silencing (Reddy *et al*, 2007).

1.3. Mitotic kinases in the spindle assembly checkpoint

Reversible phosphorylation of proteins by kinases represents a regulatory motif common to most intracellular signalling pathways, and the SAC is no exception. The discovery of the 3F3/2 phosphoepitope, present at kinetochores lacking tension (Gorbsky and Rickett, 1993), led to the initial suggestion that phosphoregulation of kinetochore components could have a major role in transduction and amplification of SAC signals.

There is now growing evidence that several mitotic kinases are required at multiple points along SAC pathway to generate the MCC. An intricate kinase network present at kinetochores connected by multiple positive feedback loops creates a robust SAC response that is promptly silenced upon microtubule attachments by the action of antagonizing phosphatases (Lara-Gonzalez *et al*, 2012). Nevertheless, knowledge of how several mitotic kinases act in concert within the signalling pathway to orchestrate SAC function is still emerging. In the following sections, the involvement of Mps1, Aurora B and Polo in the orchestration of SAC signalling will be discussed.

1.3.1. Aurora B kinase

The Chromosomal Passenger Complex (CPC) is a major mitotic regulator, which ensures faithful chromosome segregation through the regulation of different mitotic events (reviewed in Ruchaud *et al*, 2007; van der Waal *et al*, 2012a). CPC is a complex composed of Aurora B, a serine/threonine kinase, and three non-enzymatic subunits, Inner Centromere Protein (INCENP), Borealin and Survivin which function as a structural unit to control Aurora B localization, activity and stability (Jeyaprakash *et al*, 2007). From late prophase to metaphase, CPC localizes to centromeres and in the absence of centromeric tension accesses directly to kinetochore components that mediate the binding of microtubules. It has been proposed that Aurora B-mediated phosphorylation of the KNL1/Mis12 complex/ Nuclear division cycle 80 (Ndc80) complex (KMN) network reduces the affinity of these proteins for spindle microtubules, thus ensuring the correction of improper attachment

configurations (Cheeseman and Desai, 2008). This correction mechanism generates unattached kinetochores, which results in SAC activation, revealing an indirect contribution of Aurora B for SAC. Aurora B accumulates at centromeres through Haspin kinase phosphorylation of histone H3, which in turn promotes CPC localization through Survivin (Kelly et al, 2010; Wang et al, 2010). At the same time, Bub1 phosphorylation of histone H2A recruits Sgo to centromeres, which interacts, in a Cdk1/Cyclin B dependent way, with Borealin (Yamagishi et al, 2010; Tsukahara et al, 2010).

It was initially proposed that the role of Aurora B in SAC signalling was indirect as a result of its error correction mechanism, which generates transient unattached kinetochores that activate the SAC (Pinsky *et al*, 2006). However, Aurora B-dependent Mps1 kinetochore recruitment indicates a more direct role in SAC signalling (Santaguida *et al*, 2010; Saurin *et al*, 2011), consistent with the partial SAC response elicited by artificial retention of the CPC at the centromere during anaphase (Vázquez-Novelle and Petronczki, 2010). The relationship between Mps1 and Aurora B has proved to be much more complex than initially thought, as Mps1 also regulates Aurora B activity. This regulation occurs in, at least, two distinct ways: Direct phosphorylation of Borealin, that has been shown to enhance Aurora B activity in human cells (Jelluma *et al*, 2008a; Bourhis *et al*, 2009; Sliedrecht *et al*, 2010); and enhancement of Aurora B centromeric localization through Bub1 by Mps1 activity (van der Waal *et al*, 2012b). This represent a positive feedback loop between Aurora B and Mps1, which probably ensures quick establishment of both error correction mechanisms and SAC signalling, upon mitotic entry. The feedback loop could explain biorientation defects seen in the absence of Mps1 activity (Jelluma *et al*, 2008b; Sliedrecht *et al*, 2010; Kwiatkowski *et al*, 2010).

Aurora B also supports SAC function through direct phosphorylation of Zeste-White10 (Zw10) interactor (Zwint-1), which promotes de recruitment of Rough Deal (ROD)/Zw10/Zwilch (RZZ) to unattached kinetochores (Famulski and Chan, 2007; Kasuboski *et al*, 2011), and consequently the localization of Mad1 and Mad2 (Kops *et al*, 2005; Buffin *et al*, 2005). Aurora B activity was also shown to be required for Bub1 and BubR1 kinetochore localization and possibly for the stability of cytosolic MCC (Hauf *et al*, 2003; Ditchfield *et al*, 2003; Maldonado and Kapoor, 2011; van der Waal *et al*, 2012a). This may be due to the intricacies of the previously explained connections between Mps1, Aurora B and Bub1.

1.3.2. Mps1 kinase

Monopolar spindle 1 (Mps1) is a serine-threonine kinase present in eukaryotes fundamental for SAC signalling, which has been found overexpressed in different tumors and deemed a promising target for cancer therapy (Malumbres and Barbacid, 2007; Kwiatkowski *et al*, 2010; Colombo *et al*, 2010; Slidrecht *et al*, 2010; Daniel *et al*, 2011). It was firstly thought to be a kinase essential for the duplication of spindle pole body (SPB), in budding yeast, but rapidly, it was discovered that Mps1 is a critical regulator of SAC signalling (Liu and Winey, 2012). Mps1 is required for SAC function by acting at multiple points along the signalling pathway. Mps1 checkpoint function is attributed to its upstream role in the recruitment of Mad1, Mad2, BubR1, Bub1 and Bub3 to unattached kinetochores (Lan and Cleveland, 2010).

Upon mitotic entry, Mps1 is recruited to unattached kinetochores in an Ndc80/Hec1 and Aurora B dependent manner (Martin-Lluesma *et al*, 2002; Stucke *et al*, 2004; Vigneron *et al*, 2004; Santaguida *et al*, 2010; Saurin *et al*, 2011), which correlates with a peak in its kinase activity (Stucke *et al*, 2002). One possibility is that Ndc80 phosphorylation by Aurora B promotes Mps1 kinetochore accumulation, possibly in a tension-sensitive manner (Cheeseman *et al*, 2006). Mps1 recruitment to unattached kinetochores has been proposed to raise its local concentration, which favors dimerization and subsequent *trans*-autophosphorylation of its activating T-loop (Howell *et al*, 2004; Kang *et al*, 2007; Santaguida *et al*, 2010; Hewitt *et al*, 2010). Full kinase activity was shown to be required for efficient SAC function. At the onset of mitosis, Mps1 promotes Mad1/C-Mad2 recruitment to unattached kinetochores (Tighe *et al*, 2008; Hewitt *et al*, 2010). Afterwards, Mps1 kinase activity continuously promotes O-Mad2 recruitment to the Mad1/C-Mad2 complex at kinetochore, in order to promote the conformational change of Mad2 and subsequent Cdc20 binding to form the MCC (Hewitt *et al*, 2010; Kim and Yu, 2011). Important insights were recently provided regarding the role of Mps1 in the recruitment of Bub proteins to unattached kinetochores. Studies in human cell lines and yeast have shown that Mps1 promotes BubR1 and Bub1 recruitment to Spindle pole body component 105 (Sp105) / Kinetochore null protein 1 (KNL1) through phosphorylation of its Met–Glu–Leu–Thr (MELT) motifs, which are then able to bind to Bub3. (London *et al*, 2012; Shepperd *et al*, 2012; Yamagishi *et al*, 2012) and recruit the Mad1-Mad2 complex.

Interestingly, *Drosophila* Sp105 has specific MELT-like motifs that lack the phosphorylatable threonine, hence making Mps1 dispensable for kinetochore recruitment of Bub1, BubR1 or Bub3 in flies (Conde *et al*, 2013b). Nevertheless, Mps1 activity is still

required for BubR1 hyperphosphorylation. Mps1-dependent BubR1 phosphorylation results in the accumulation of the 3F3/2 phosphoepitope at unattached/tensionless kinetochores, which seems to facilitate BubR1/Cdc20 association and consequently efficient MCC formation (Conde *et al*, 2013a).

To accomplish this variety of functions, Mps1 must be tightly regulated. Mps1 kinases are expressed at low levels and the most important mechanisms of regulation are the ones that operate via phosphorylation and degradation. Mps1 specifically functions during mitosis, so that, its transcription is repressed in interphase and upregulated in G2 and mitosis (Liu and Winey, 2012), resulting in a peak in mitosis and a decline when cells enter anaphase, with Mps1 being target for proteasome degradation via Cdc20- or Cdh1- associated APC/C complexes (Pike and Fisk, 2011).

The aforementioned phosphoregulation of Mps1 is implied in kinetochore localization and turnover rate of the kinase, however, it is not very clear yet what are the real effects of phosphorylation of Mps1. Phosphodeficient mutations of nine autophosphorylation sites in the N-terminal of human Mps1 in SW480 cells caused a significant decrease in kinetochore targeting of Mps1. Consistent with this observation, a kinase-dead version of Mps1 expressed in SW480 cells exhibited reduced kinetochore recruitment upon depletion of endogenous Mps1 (Xu *et al*, 2009). Likewise, inhibition of endogenous Mps1 in US20S cells with the inhibitor NMS-P715 led to reduction of Mps1 levels at unattached kinetochores (Colombo *et al*, 2010). On the other hand, different studies showed that inhibition of Mps1 activity with AZ3146 or Mps1-IN-1 increases its half-time of recovery at unattached kinetochores and causes an increase of Mps1 levels at these sites indicating that Mps1 activity promotes its own release from kinetochores (Hewitt *et al*, 2010; Jelluma *et al*, 2010). These divergences are not fully understood although there might have some plausible reasons, such as differences on the cell type, degree of inhibition and experimental conditions. To evaluate whether the kinase activity is required for the kinetochore localization of *Drosophila* Mps1, Althoff and colleagues expressed a kinase-dead version of Mps1 *mps1aldb4* mutant embryos. This mutation results in a premature stop after the first 47 amino acids (Page *et al*, 2007) hence allowing the authors to assess kinetochore recruitment of the catalytic inactive Mps1 without the interference of endogenous Mps1. Under these conditions, Mps1KD levels at prometaphase kinetochores were dramatically reduced when compared with Mps1WT expressed analogously, hence clearly indicating that Mps1 kinase activity is required for its kinetochore localization in *Drosophila* (Althoff *et al*, 2012).

1.3.3. Polo kinase

Genetic screens led to the discovery of Cdc5 and Polo, the first members of the Polo-like kinase (Plk) family, in budding yeast and *Drosophila*, respectively (Hartwell *et al*, 1973; Sunkel and Glover, 1988). Although the original mutations in Polo induced abnormal spindle poles during mitosis (Sunkel and Glover, 1988), subsequent studies of different mutant alleles showed that Plks orchestrate multiple processes during mitosis (Zitouni *et al*, 2014).

The role of Polo kinases in SAC signalling remains elusive and controversial. Several reports of a SAC dependent prometaphase arrest with Mad2 and BubR1 accumulating at kinetochores upon Plk1 inhibition or RNAi-mediated depletion (Sumara *et al*, 2004; Lénárt *et al*, 2007; Petronczki *et al*, 2008) contrast with other studies demonstrating a significant decrease of Mad2 and Cdc20 levels at unattached kinetochores upon Plk1 depletion (Ahonen *et al*, 2005), as well as with its role in Mad1 phosphorylation, which has been shown to promote its recruitment to unattached kinetochores and consequently Mad2 accumulation and SAC activity (Chi *et al*, 2008).

Human Plk1 was initially linked to SAC function through the generation of the 3F3/2 phosphoepitope at kinetochores of tensionless chromosomes (Ahonen *et al*, 2005) and subsequent work revealed that phosphorylation of BubR1 by Plk1 generated the 3F3/2 phosphoepitope and was required for SAC arrest in *Xenopus* egg extracts (Wong and Fang, 2005). Recent studies in *Drosophila* provided important new insights into this controversy. Loss of Polo activity in S2 cells results in a SAC independent mitotic arrest. Interestingly, Polo depletion severely reduced APC/C mediated ubiquitination of Cyclin B in SAC impaired cells, suggesting that Polo is required for mitotic exit possibly by promoting APC/C activation (Conde *et al*, 2013b). This requirement masks a role for Polo in SAC signalling, clearly revealed by significantly decreased levels of MCC and failure to recruit Mad2 to unattached kinetochores in the absence of Polo activity (Conde *et al*, 2013a). Polo contributes for SAC function in *Drosophila* at least through two pathways: (i) by promoting proper loading of Ndc80, the receptor of Mps1 at kinetochores, and (ii) by controlling proper Aurora B centromeric localization and activity (Conde *et al*, 2013a; Moutinho-Santos *et al*, 2012), which synergizes with Ndc80 to potentiate Mps1 kinetochore localization in early prometaphase, required for robust SAC signalling. Interestingly, expression of a constitutively active form of Polo revealed that Polo might also directly regulate Mps1 kinetochore recruitment by a mechanism that remains unclear (Conde *et al*, 2013a; Conde *et al*, 2013b).

Polo kinases have an amino-terminal serine-threonine catalytic domain and a carboxyl-terminal region containing two conserved Polo boxes that contain the characteristic Polo Box Domain (PBD), which confers substrate specificity, controls subcellular localization and, in part, regulates the kinase activity. Polo kinases are additionally controlled by phosphorylation, proteolysis and transcription, depending on the biological context.

The expression of Plk1 is tightly regulated in time at both mRNA and protein levels, being low in interphase and high in mitosis. Expression of the *Plk1* gene is under the control of various transcriptional repressors in G1 and transcriptional activators in G2 (Zitouni *et al*, 2014). Plk1 is subjected to ubiquitin-dependent proteolysis by the proteasome. Degradation is initiated during anaphase triggered by APC/C-Cdh1 that recognizes a D-box conserved in Polo orthologues of higher eukaryotes. Paradoxically, Plk1 activity is required for cytokinesis and Cenp-A deposition at centromeres and has been found to be present at centromeres in G1 (McKinley and Cheeseman, 2014), suggesting a localized degradation of Plk1.

In addition to regulation of its expression, the subcellular localization of Plk1 is also dynamically regulated, which contributes to fine-tune its activity and is crucial for its multiple functions in mitosis. Critical for the spatial regulation of Polo activity is the PBD. The PBD regulates Plk1 kinase activity by binding to the kinase domain in a conformation that mutually inhibits its activity. Structural studies revealed that the kinase domain is inhibited in three ways: (i) by the PBD itself, which reduces the flexibility of the kinase domain hinge region; (ii) by the interdomain linker, which links the kinase domain with the PBD and sequesters the T-loop and (iii) by binding of the PBD to Map205 protein, which stabilizes the autoinhibited state and also sequesters the kinase from its substrates (Zitouni *et al*, 2014).

Plk1 activity increases at the G2/M transition, reaching a maximum during mitosis (Bruinsma *et al*, 2012). Phosphopeptide binding and/or phosphorylation of the conserved Thr210 in the T-loop (Thr182 in *Drosophila*) contribute to coordinate spatiotemporal Polo activation. Aurora A was shown to phosphorylate Plk1 on Thr210 before mitotic entry (Macurek *et al*, 2008; Seki *et al*, 2008; van de Weerd *et al*, 2005), and this is suggested to disrupt the intramolecular interaction between the kinase domain and the PBD that inhibits the functions of both domains (Jang *et al*, 2002; Lowery *et al*, 2005).

1.4. Protein Phosphatases in Spindle Assembly Checkpoint

The importance of protein kinases for the spatial and temporal control of the mitotic events has long been established. However, only more recently, the role of protein

phosphatases has come to limelight (reviewed in Bollen *et al* 2009; and Wieser and Pines, 2015). It is now recognized that this counteraction phosphatases-kinases produces switch-like responses, allowing the transitions through the mitotic stages.

Genetic and small interfering RNA (siRNA) screens, in fission yeast (Kinoshita *et al*, 1990) and *Drosophila* (Chen *et al*. 2007) led to the identification of the PP1 and PP2A protein phosphatase families and revealed their importance for control of mitotic progression. An interesting fact is that, despite the exquisite substrate selection of phosphatases, there are only few protein phosphatase catalytic subunits, and with very low substrate specificity. PP1 and PP2A are actually families of hundreds of protein serine/threonine phosphatases, as a result of the association of the few catalytic subunits with a wide range of regulators (figure 1.5; reviewed in Virshup and Shenolikar, 2009). These regulatory subunits confer specificity, selectivity, localization, and regulation of the phosphatase complex.

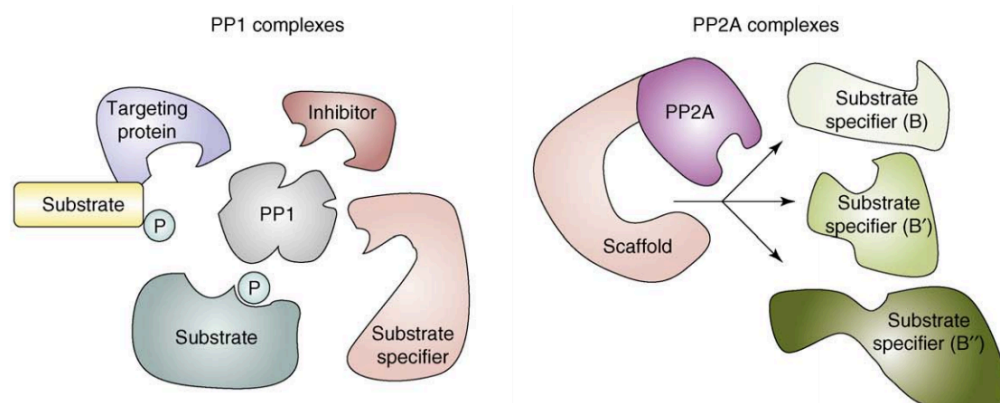


Figure 1.5 – Subunit structure of PP1 and PP2A holoenzymes. Mammalians have about 40 genes encoding protein Ser/Thr phosphatases (about 10 times less than Ser/Thr kinases); however, if considered the total of holoenzymes, kinases and phosphatases are equivalent. For mammalian PP1 isoforms, almost 200 interactors are identified (that function as inhibitors of the catalytic activity, substrate-specifying subunits, targeting subunits, or substrates); the majority of PP1 holoenzymes are composed of two subunits (one catalytic and one regulatory), but some others contain a third inhibitory subunit. PP2A family contains a catalytic subunit (C), a non-catalytic scaffolding subunit (A) and a third subunit associated (B); this B-subunit, that acts as a substrate specifier, can be divided into three subfamilies, termed B (PR55/B55), B' (PR61/B56) and B'' (PR72), each containing 4-9 isoforms; taken together with the isoforms of A and C subunits, PP2A family includes more than 70 phosphatase complexes. PP4 has, as PP2A, a trimeric structure, but the non-catalytic subunits are not well studied, yet (Bollen *et al*, 2009).

Phosphoprotein Phosphatases (PPPs) superfamily is divided in seven families: PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7, but only PP1, PP2A and PP4 have been implicated

in mitosis (Bollen *et al*, 2009). From all PPPs, PP1 and PP2A are some of the most well conserved enzymes across species and represent more than 90% of the protein phosphatase activity in eukaryotic cells. It's predicted that PP1 is involved in the majority of dephosphorylation events (around one third of all eukaryotic proteins) (Rebelo *et al*, 2015).

During mitosis, PP1 and PP2A feature three distinct functions: control of mitotic kinases, dephosphorylation of specific pools of mitotic kinase's substrates and mitotic exit through controlled mass dephosphorylation. (Bollen *et al*, 2009).

1.4.1. Protein Phosphatase 1 (PP1)

Several centromeric and kinetochore kinases regulate kinetochore-microtubule attachment and/or SAC activation; however, the mechanisms responsible for rapid SAC silencing upon chromosome bi-orientation remain unclear.

In mammals, PP1 is present in three isotypes, α , β and γ and their functions are modulated by interaction with several regulatory subunits, which often contain PP1-docking segments called SILK and RVxF motifs (Hendrickx *et al*, 2009). The binding of PP1 to its kinetochore receptor Spc105/KNL1 through SILK and RVxF motifs is essential for SAC silencing. Importantly, phosphorylation of Spc105/KNL1 by Aurora B compromises the PP1-Spc105 interaction (Liu *et al.*, 2010) thus contributing to maintain low levels of PP1 activity at kinetochores during prometaphase, when robust SAC signalling is required. Surprisingly, persistent Spc105/PP1 interaction does not affect chromosome segregation and is insufficient to silence the SAC in the absence of microtubules. (Rosenberg *et al*, 2011). It is proposed that binding of PP1 to Spc105 enables local regulation of dynamic phosphorylation and dephosphorylation events at kinetochores in order to combine microtubule attachment and SAC silencing (Rosenberg *et al*, 2011; Espeut *et al*, 2012). For example, the multisite phosphorylation of the KMN network has synergistic effects on microtubule binding (Welburn *et al*, 2010), so a partial dephosphorylation by PP1 may result in raising affinity of kinetochores for microtubules to achieve load-bearing attachments, but a most pronounced dephosphorylation may be required for SAC silencing, explaining the high amount of PP1 bound to kinetochores of metaphase chromosomes (Funabiki and Wynne, 2013).

Another hypothesis is that dephosphorylation by PP1 could occur preferentially in some substrates (important for SAC) in detriment of others (important for microtubules attachment) (Funabiki and Wynne, 2013). In agreement with this, some proteins are pointed as potentially important on SAC silencing through dephosphorylation by PP1: Spc105, which

Mps1-dependent phosphorylation allows Bub1 recruitment (however, Bub1 is found in bi-oriented chromosomes, so that it's not probable that Bub1 release from kinetochores inactivate SAC); BubR1, phosphorylated by Aurora B; and Ndc80, phosphorylated also by Mps1. Dynein is dephosphorylated by PP1 too, contributing to SAC inactivation through kinetochore removal of checkpoint proteins (Whyte et al, 2008).

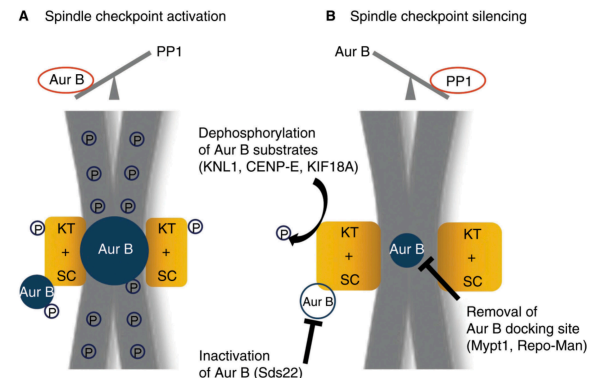
One of the most ancient and conserved PP1-regulatory subunits, Sds22, binds to PP1 through leucine-rich repeats and plays an essential role for mitotic progression. Sds22 and PP1 are mutually dependent for kinetochore recruitment, suggesting that Sds22 interaction with kinetochores requires its prior association with PP1 (Lesage *et al*, 2011; Funabiki and Wynne, 2013). Reduction of Sds22 in cells results in a metaphase delay and a slight chromosome misalignment; in agreement with the function of Sds22 in increasing PP1 activity, this reduction leads to enhanced levels of Aurora B autophosphorylation and Aurora B substrates phosphorylation (such as Dsn1, a member of Mis12 complex); however it was reported that phosphorylation of Ndc80 and MCAK decreased in this conditions. This phenotypic difference may be due to a role of Sds22 as inhibitor for specific substrates but not for others. (reviewed in Funabiki and Wynne, 2013). Sds22 has not a major contribution for kinetochore-microtubule attachment in metaphase, but it has during anaphase chromosome movement, along with Repo-Man, which in turn recruits PP1 to chromosomes during anaphase (Wurzenberger et al. 2012). In conclusion, the role of PP1/Sds22 at the kinetochore seems to be silencing SAC, with a secondary function in kinetochore-microtubule attachment.

Repo-Man is another PP1 interacting protein; the complex PP1/Repo-Man was shown to dephosphorylate de H3 histone on Tyr3 (H3T3), centromeric docking site for Aurora B complex, in mitosis (Qian *et al*, 2011; Vagnarelli *et al*, 2011). The dephosphorylation of H3T3 on the chromosome arms in prometaphase prevents the mislocalization of Aurora B outside the centromeres. In anaphase, the centromeric dephosphorylation of H3T3 is probably responsible for the translocation of Aurora B to the spindle midzone; which is important as it prevents the re-initiation of SAC signalling as soon as sister chromatids tension in anaphase onset is lost (reviewed in Lesage *et al*, 2011).

As described, different PP1 holoenzymes regulate Aurora B localization and activity: on one hand, they act retaining Aurora B at inner centromeres (PP1-Repo-Man) and, on the other hand, PP1 inactivates kinetochore-associated Aurora B and dephosphorylates some of its substrates (PP1/Sds22) (figure 1.6). It was suggested that the tight balance kinase-phosphatase during SAC signalling is disrupted for PP1's side as soon as all kinetochores are under tension, what results in SAC silencing. It is likely that PP1 associated to

kinetochores has a function in the downregulation of other checkpoint kinases, such as Bub1, BubR1 and Mps1 (Lesage *et al*, 2011).

Figure 1.6 – The balance between Aurora B and PP1 in checkpoint signalling. (a) In (pro)metaphase, Aurora B (Aur B) phosphorylates several kinetochore (KT) and spindle checkpoint (SC) proteins, in order to destabilize incorrect kinetochore–microtubule attachments and turn SAC on. (b) When sister chromatids come under tension, kinetochore-associated PP1 holoenzymes reverse Aurora B signalling and trigger spindle checkpoint silencing. PP1 dephosphorylates Aurora B, Aurora B substrates and a centromeric Aurora B docking site (Lesage *et al*, 2011).



1.4.2. Protein Phosphatase 2A (PP2A)

Unlike PP1, PP2A importance is essentially on stability of microtubule attachment, and not on SAC function (Foley *et al*, 2011). Depending on its regulatory subunits, PP2A is able to regulate spindle bipolarity, kinetochore function, and progression into anaphase (Chen *et al*, 2007).

The PP2A heterotrimeric phosphatase is constituted by a catalytic C subunit, a scaffolding A subunit and a variety of regulatory B subunits. In *Drosophila*, the catalytic subunit is PP2A microtubule star (mts), the scaffolding is PP2A-29A and the four different regulatory subunit are twins (tws)/B, widerborst (wdb)/B', PP2A-B' and PP2A-B'' (Chen *et al*, 2007). The name of PP2A catalytic subunit is due to its mutant phenotype: embryos present multiple individual centrosomes with disorganized astral arrays of microtubules. In S2 cells depleted for mts it is usual identify a high proportion of bipolar monoastral spindles or monopolar spindles emanating from a single centrosome, consequence of the spindle collapse rather than failure in centrosome duplication (Chen *et al*, 2007). In agreement with this phenotype, inhibition of PP2A with Okadaic Acid (OA) in *Xenopus* egg extracts leads to the formation of longer mitotic microtubules and the impossibility to form bipolar spindles (Tournebize *et al*, 1997).

Depletion of either PP2A-mts or PP2A-29A results in similar effects, what would be expected since depletion of the scaffolding subunit results in a reduction of mts protein

levels. Depletion of distinct regulatory subunits diverge phenotypically. RNAi-mediated depletion of *tw*s leads to lagging chromosomes whereas *wdb* depletion leads to dramatic scattering of chromosomes throughout the spindle (Chen *et al*, 2007). B56/B' family members localize at kinetochores during mitosis and its localization is most prominent on unattached kinetochores. This seems to be the major regulatory subunit controlling PP2A action on kinetochore-microtubule attachments (Funabiki and Wynne, 2013). When B56 subunit is depleted, the levels of KNL1 phosphorylation by Aurora B and Plk1-dependent phosphorylation of BubR1 increase and kinetochore attachment to microtubules is severely compromised. Upon inhibition of Aurora B activity, this situation is overcome, thus suggesting that PP2A has an antagonist action in relation to Aurora B activity at kinetochores (Foley *et al*, 2011).

Plk1-dependent phosphorylation of BubR1 promotes the recruitment of PP2A-B56 (Suijkerbuijk *et al*, 2012b), resulting in the generation of a negative feedback loop: PP2A-B56 dephosphorylates Plk1 substrates at the kinetochore, promoting dissociation of the kinase (Foley *et al*, 2011). It remains unclear if kinetochore-dissociation of PP2A-B56 has a functional effect, but it is possible that it helps to keep a low phosphorylation level, required for the integrity of kinetochore architecture, dynamic association of microtubules, and prevention of merotelic attachments; actually, Aurora B-dependent phosphorylation level decreases but does not necessarily disappear after bipolar attachment (DeLuca *et al*, 2011; Kops and Shah 2012; Welburn *et al*, 2010). On the other hand, excessive phosphorylation of kinetochore substrates must be avoided to support initial kinetochore–microtubule attachment. It is necessary to quantify kinetochore substrates phosphorylation levels during mitotic progression in the presence and absence of microtubule attachment to uncover the real function of this negative feedback.

PP2A-B56/PP2A-*wdb* has been found to interact with Shugoshin/MEI-S332, a dynamic centromeric marker, where they are thought to protect centromeric cohesion subunits from phosphorylation, what would result in premature sister-chromatid separation (Kitajima *et al*. 2006; Riedel *et al*. 2006; Tang *et al*. 2006; Chen *et al*, 2007). Even though, the phosphatase role in kinetochore–microtubule attachment seems to be distinct from its regulation of sister chromatid cohesion.

Taken together, these evidences suggest a division of labor between mitotic phosphatases in which PP2A-B56 promotes dephosphorylation of substrates at unattached kinetochores to promote kinetochore–microtubule attachment while PP1 acts at SAC level, turning it off, and in the maintenance of kinetochore–microtubule interaction in attached

kinetochores. However, the role of phosphatases remains unwell known (Funabiki and Wynne, 2013).

1.5. Objectives

Maintenance of genomic stability during eukaryotic cell division relies on the spindle assembly checkpoint (SAC), a surveillance mechanism that restrains anaphase onset until all chromosomes are correctly attached to microtubules from opposite spindle poles. A single unattached kinetochore generates a signal, in the form of the Mitotic Checkpoint Complex (MCC), strong enough to inhibit the anaphase promoting complex (APC/C) activity and consequently halt mitotic progression. Remarkably, anaphase takes place swiftly after all kinetochores stably attach to spindle microtubules, revealing that the SAC is not only extremely robust, but also highly responsive. How the SAC encompasses simultaneously both features remains unclear. Mps1 kinase is a key SAC regulator but the mechanisms underlying its kinetochore recruitment, activation and inactivation remain elusive.

The aim of this thesis is to further elucidate how mitotic kinases and phosphatases are integrated in SAC signalling. With the present project we are using *Drosophila* as a model system and resort to live-cell imaging, confocal microscopy, biochemistry and cell biology assays to elucidate the molecular mechanisms underlying Mps1 regulation and their relevance for the establishment of SAC robustness and responsiveness.

CHAPTER 2

MATERIALS AND METHODS

2.1. Rubidium Chloride competent cells generation and transformation

To produce competent *E. coli* TOP10 cells (Invitrogen), 5mL of a starting culture (grown overnight, at 37°C) was used to inoculate 500mL of LB medium, which were grown at 37°C until they reached an OD₅₉₅ of 0.5. At this time, cells were cooled down on ice, for 15 minutes. Afterwards, the culture was centrifuged for 10 minutes at 4500rpm and 4°C to collect the cell pellet, which was resuspended in 30mL of Tfb I (100mM RbCl, 50mM MnCl₂·4H₂O, 30mM Potassium Acetate, 10mM CaCl₂·2 H₂O and 15% Glycerol, prepared in deionized H₂O) and incubated for another 15 minutes on ice. Then, cells were centrifuged for 5 minutes at 4000rpm and 4°C and the cell pellet was resuspended in 6mL Tfb II (0.2M MOPS, 10mM RbCl, CaCl₂·2 H₂O and 15% Glycerol). Aliquots were frozen in dry ice and stored at -80°C.

For each transformation, one 50µL aliquot of TOP10 competent cells was defrosted on ice for a short period of time. About 100ng of the intended plasmid or 5µL of a ligation reaction were then added to the cells and the mixture was incubated on ice for 20 minutes. Transformation was induced through heat-shock at 42°C for 45-60 seconds. After 2 minutes on ice, 200µL of LB medium were added and the culture was incubated at 37°C for 1h. To select transformed bacteria, cells were plated on LB agar supplemented with specific antibiotics (ampicillin and kanamycin were used at the final concentrations of 100µg·mL⁻¹ and 50µg·mL⁻¹, respectively).

Plasmid extractions were done with Fast-n-Easy Plasmid Mini-Prep Kit (Jena Bioscience GmbH) or Plasmid Midi Kit (QIAGEN) according to the manufacturer's instructions.

2.2. RNA interference (RNAi) synthesis

To deplete Polo, CENP-C and PP1-87B from *Drosophila* S2 cells, double stranded (ds)RNA was synthesized, targeting Polo and Cenp-C and PP1-87B 5'UTR and 5'end of the coding region. RNAi synthesis was performed with T7 Megascript kit (Ambion), according to the manufacturer's instructions. The resulting single stranded RNA was denaturated at 96°C for 5 minutes, in order to eliminate secondary structures, and gradually cooled down (2°C every minute) to allow the formation of dsRNA duplex. The integrity,

concentration and purity degree of the synthesized dsRNA duplexes was evaluated by 1% agarose gel electrophoresis.

2.3. Constructs

Phosphomimetic and phosphodeficient versions of pMT-EGFP-Mps1 for putative Polo phosphorylation residue Thr185 were generated by site-directed mutagenesis, with the set of primers indicated in Table 2.4.2. For the PCR reaction with Phusion (New England Biolabs), 18 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 3 minutes were performed. Afterwards, 15.5µL of Mps1T185D/A PCR product was digested with DpnI restriction enzyme (New England Biolabs) at 37°C for three hours and used to transform competent DH5α bacteria.

To generate EGFP-PP1-87B coding sequence (CDS) was cloned in frame with N-terminal EGFP under regulation of a metallothionein promoter in the pMT-EGFP-C vector (Invitrogen). Full-length PP1-87B was amplified by PCR from *Drosophila* genomic DNA, using the set of primers indicated in Table 2.4.1, and Phusion Polymerase (New England Biolabs) according to the manufacturer's instructions with 35 cycles of denaturation at 95°C for 10 seconds, annealing at 70°C for 30 seconds and extension at 72°C for another 30 seconds. The resulting PP1-87B PCR product and pMT-EGFP-C were digested with KpnI and XmaI restriction enzymes (New England Biolabs) for two and a half hours, according to the manufacturer's instructions. PCR product and destination vector were purified (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel) and roughly 75ng of the insert were mixed with 25ng of the digested plasmid and incubated with T4 DNA ligase (New England Biolabs) at 16°C for four hours. Afterwards, the ligation reaction was used to transform competent DH5α bacteria. Individual colonies were grown overnight in 3 mL of LB medium supplemented with ampicillin.

To generate pMalc-PP1-87B for protein expression in the bacteria, PP1 PCR product and pMalc vector A were digested with StuI and XbaI restriction enzymes (New England Biolabs) at 37°C for 5 hours. Both digestions were purified (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel) and roughly 75 ng of the insert were mixed with 50 ng of the digested vector and incubated with T4 DNA ligase (New England Biolabs) for 4 hours at 16°C. The ligation reaction was used to transform competent DH5α bacteria.

To screen for insert-containing plasmids, individual colonies were grown overnight in 3mL of LB medium supplemented with ampicillin or kanamycin. At least 400µL of each

liquid culture were centrifuged and cells resuspended in 100µL of cracking buffer (0.1M NaOH, 0.25% SDS, 0.2% bromophenol blue, 5 mM EDTA pH 8.0, 10% sucrose, prepared in deionized water). After denaturation at 72°C for 5 minutes, lysed cells were run in a 1% agarose gel and positive clones identified through the distance migrated. After plasmid DNA extraction of the positive clones, construct integrity was evaluated by 1% agarose gel electrophoresis and concentration and purity degree were ascertained by measuring the absorbance in a nanodrop (Nanodrop Spectrophotometer ND-1000). Insertion was confirmed through restriction analysis or PCR and plasmids were sent for sequencing (GATC Biotech). All enzymes and kits were used according to the manufacturer's instructions.

Table 2.1 – Egfp-Mps1 T185A/D, Egfp-PP1 87B and MBP-PP1 primers. Set of primers used to clone Egfp-Mps1 T185A/D, Egfp-PP1 87B and MBP-PP1 87B into the respective vectors

Egfp-Mps1	T185A Forward: 5' – CCACTCCAAGAGACAGAGGCGCCACTG – 3'
	T185D Forward: 5' – CTCCAAGAGACAGAGGACCCACTGCGTAATG – 3'
	Reverse: 5' – CTCTGTCTCTTGGAGTGGGC – 3'
Egfp-PP1 87B	Forward: 5' – GACGGTACCATGGGCGACGTGATGAATATCGAC – 3'
	Reverse: 5' – GATCCCGGGTTACTTTTTACGCTTGTCGGCTGG – 3'
MBP-PP1 87B	Forward: 5' – GGTAGGCCTATGGGCGACGTGATGAATA – 3'
	Reverse: 5' – TCCTCTAGATTACTTTTTACGCTTGTCGGC – 3'

2.4. *Drosophila* S2 cell transfection

Transient or Stable transfection of recombinant plasmids into S2 cells was performed using Effectene Transfection Reagent (QIAGEN). For each transfection, 10⁶ *Drosophila* S2 cells were seeded in a twelve-well plate in 800µL Schneider's medium (Gibco, BRL) supplemented with 10% FBS. One hour later, the medium was replaced with 500µL of fresh Schneider's medium with 10% FBS and a plasmid mix (0.5µg of pMT-Egfp-PP1-87B and 0.5 µg of pAC-Tub-mCh, 0.5µg of pMT-Egfp-Mps1^{T185A} and 0.5 µg of pAC-Tub-mCh or 0.5µg of pMT-Egfp-Mps1^{T185D} and 0.5 µg of pAC-Tub-mCh in 75µL of EC Buffer, 8µL of Enhancer, 10µL of Effectene and 1mL of Schneider's medium with 10% FBS prepared according to the manufacturer's instructions). For stable transfections, after a three-day period was started the selection with blasticidin, at a final concentration of 25 µg/mL. For

transient transfections cells were not selected and harvested three or four days after transfection and processed for immunofluorescence analysis or time-lapse microscopy.

2.5. S2 cell culture, RNAi-mediated depletion and drug treatment

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS). For each depletion, 10⁶ S2 cells/mL of Schneider's medium (Gibco, BRL) were plated in twelve-well plates (0.5 mL) and 15 µg (for Cenp-C and Polo) or 30 µg (for PP1-87B) of the respective dsRNAi were added. After one hour at 25°C, cells were supplemented with 1mL of Schneider's medium (Gibco, BRL) 10% FBS. The incubation period was 120 hours. At the selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy, immunoprecipitation or immunoblotting. Protein depletion was monitored through Western blotting or immunofluorescence analysis. When required, cells were subjected to several drug treatments before being collected and processed. In order to depolymerize microtubules, cells were incubated with 30µM Colchicine (Sigma) for selected time periods. To prevent mitotic exit, in a checkpoint independent manner, cells were incubated with 20 µM of the proteasome inhibitor MG132 (Calbiochem). Polo and Aurora B were inhibited using BI2536 (Boehringer Ingelheim) at a final concentration of 100 nM and Binuclein (Bin2), at a final concentration of 40 µM, respectively.

Expression of EGFP-Mad1, EGFP-Mps1, EGFP-Mps1-T185A, EGFP-Mps1-T185D and Egfp-PP1-87B transgenes was induced by treatment of the S2 transfected cells with 100µM CuSO₄ at least 8 hours before analysis. To induce expression of Polo^{WT}-GFP, PoloT182D-GFP and PoloT182A-GFP, under regulation of Hsp70 promoter, cells were incubated for 30 minutes at 37°C 6 to 8 hours prior to cell processing.

2.6. Immunofluorescence analysis in *Drosophila* S2 cells

For immunofluorescence analysis, *Drosophila* S2 cells (10⁵ cells in 120 µL) were collected and centrifuged onto slides for 5 min, at 1000 rpm (Cytospin 2, Shandon). Cells were processed for simultaneous fixation and extraction in 3.7% Formaldehyde diluted in PBS Triton X-100 0.5% for 10 minutes followed by three washing steps in PBS-Tween20 0.05% (PBST20) for 5 minutes each. Alternatively, to reveal spindle morphology or

cytoplasmic localization of proteins, cells were fixed with 3.7% Formaldehyde in PHEM (240 mM PIPES, 100 mM HEPES pH 7.0, 40 mM EGTA and 16 mM MgSO₄) for 12 minutes and extracted with PBS Triton X-100 0.05% thrice for 5 minutes.

Fixed cells were blocked for one hour in PBST20 with 10% fetal bovine serum (PBSTF) at room temperature and then incubated overnight at 4°C with primary antibodies (prepared in blocking solution). After three five-minute washes in PBST20, cells were incubated with fluorescent-labeled secondary antibodies diluted in PBSTF for one hour at room temperature. Finally, cells were washed thrice with PBST20 for 5 minutes and slides were mounted with Vectashield mounting medium for fluorescence with 1 mg/mL of DAPI (Vector Laboratories, UK).

Images were collected in a Leica TCS SP5 II laser scanning confocal microscope (Leica Microsystems, Germany). Data stacks were analyzed using ImageJ 1.49k software (<http://rsb.info.nih.gov/ij/>).

For immunofluorescence quantification of proteins, the mean pixel intensity obtained from maximum projected raw images acquired with fixed exposure acquisition settings. Kinetochore location was defined manually based on CID staining within a specific predefined region of interest (ROI). After subtraction of background intensities, estimated from regions outside the cell, the intensity relative to CID signal was determined for each kinetochore.

2.7. Fluorescence Recovery After Photobleaching (FRAP) analysis

The FRAP experiments were performed in S2 cells expressing EGFP-Mps1WT, EGFP-Mps1-T185A and EGFP-Mps1-T185D. When required, 100 nM BI2536 were added 30 minutes prior FRAP measurement. Data sets were collected at 25°C with a Leica TCS SP5II scanning confocal microscope (Leica Microsystems, Germany).

The EGFP tag of Mps1 was excited using the 488 nm laser line set to 10% and bleached with the 405 nm laser line. Single kinetochores were bleached for three iterations once the fluorescence signal of EGFP-Mps1 had become stable. Fluorescence intensity in the bleached area was acquired every 757 ms before and after bleaching.

FRAP data analysis was performed with ImageJ. For each measurement, the average fluorescence intensity in the bleached area was corrected for background and the ratio to

average fluorescence in the cytoplasm determined. Average values before bleaching were set to 100%. The exponential kinetics of FRAP were analysed by calculating the normalized unrecovered fluorescence at each time point $(F_{inf}-F(t))/(F_{inf}-F(0))$ where F_{inf} is the value reached at the plateau, $F(0)$ is the value observed in the first frame after bleaching and $F(t)$ is the value at a given time point. FRAP kinetics parameters were determined by one-exponential curve fitting to normalized data using GraphPad Prism software.

2.8. Time-lapse microscopy

Live analysis of mitosis was done in *Drosophila* S2 cell lines co-expressing Tub-mCh with Mad1-EGFP, EGFP-Mps1, H2B-GFP, or EGFP-PP1-87B. After the desired treatment cells were plated in MatTek glass bottom dishes (MatTek Corporation) previously treated with Concanavalin A at 0,25 mg/mL (Sigma). Four-dimensional datasets were collected at 25°C with a spinning disc confocal system (Revolution; Andor) equipped with an electron multiplying charge-coupled device camera (iXonEM+; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus). Two laser lines (488 and 561 nm) were used for near-simultaneous excitation of EGFP and mCherry, respectively. The system was driven by iQ software (Andor). Time-lapse imaging of z stacks with 0.8 µm steps covering the entire volume of the cell were collected every 30, 60 or 120 seconds. Image sequence analysis and video assembly was done with ImageJ and Andor iQ software.

2.9. Production of recombinant proteins in bacteria

To generate pMalc-PP1-87B for protein expression in the bacteria, PP1 PCR product and pMalc were digested with *Stu*I and *Xba*I restriction enzymes (New England Biolabs) at 37°C for 5 hours. Both digestions were purified (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel) and roughly 75 ng of the insert were mixed with 50 ng of the digested vector and incubated with T4 DNA ligase (New England Biolabs) for 4 hours at 16°C. The ligation reaction was used to transform competent DH5α cells and positive clones were screened and confirmed as positive as described in 2.3.

The obtained recombinant pMalc-PP1 87B was then used to transform by heat-shock BL21-star competent cells in order to generate recombinant PP1 87B. Transformed

bacteria were selected on LB agar supplemented with 100 μ g mL⁻¹ ampicillin, at 37°C overnight. A single colony was picked and grown overnight at 37°C in 3 mL of LB medium supplemented with 100 μ g mL⁻¹ ampicillin, 250 μ L of the starting culture were used to inoculate 10mL of LB medium supplemented with ampicillin. After two and a half hours at 37°C the culture was cooled down at 4°C and the protein production was induced with 0.1M IPTG. Then, the culture was incubated overnight at 15°C.

Lysates were sonicated and clarified by centrifugation at 4°C. Recombinant MBP-PP1 87B was purified with magnetic beads (New England Biolabs). The protein was eluted with 10mM maltose. Protein expression was confirmed by SDS-PAGE.

2.10. Protein extraction from *Drosophila* S2 cells

Drosophila S2 cultured cells were harvested through centrifugation at 5000 rpm for 10 min at 4°C and afterwards washed twice with 2-4 mL of PBS supplemented with protease inhibitors (Complete Cocktail Tablets, Roche). Cell pellet was resuspended in Lysis Buffer (150mM KCl, 75 mM Hepes pH7.5, 1.5 mM EGTA, 1.5 mM MgCl₂, 15% glycerol, 0.1%NP-40), supplemented with protease (Complete Cocktail Tablets, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 3, Sigma-Aldrich)) and frozen in liquid nitrogen.

After an incubation period of at least 30 min on ice, cell lysates were clarified through centrifugation at 8000 rpm and 4°C for 10 min and quantified by measuring the absorbance in a nanodrop (Nanodrop Spectrophotometer ND-1000). Alternately, after washing, cell pellet was directly resuspended in laemmli buffer (4% SDS, 10% mercaptoetanol, 0.125 M Tris-HCl, 20% glycerol, 0.004% bromophenol blue). Protein extracts were then either used immediately or frozen in liquid nitrogen and stored at -80°C.

2.11. Pull-down Assays

Total cell lysates (0.5 mg) of protein extract were diluted in a final volume of 500 μ L of column buffer (200mM NaCl, 20mM Tris-HCl, 1mM EDTA, 1mM DTT; pH 7.4) supplemented with protease and phosphatase inhibitors for each immunoprecipitation assay. Lysates were then incubated with 5 μ L of beads with either MBP or MBP-PP1 87B bound and after one and a half hours at 4°C in a rotating wheel, the magnetic beads and bound protein fraction were collected and washed three times with 750 μ L of column buffer supplemented with 0.1% Tween20. Finally the magnetic beads and bound protein were

ressuspended in an adequate volume of laemmli buffer and boiled for 5 min at 95°C. After removal of the magnetic beads, samples were resolved by SDS-PAGE and probed for proteins of interest through western blotting.

2.12. Western blot

Protein extracts and immunoprecipitates were resolved in 8% or 10% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane, using the iBlot Dry Blotting System (Invitrogen) according to the manufacturer's instructions. Transferred proteins were confirmed by ponceau staining (0.25% Ponceau S in 40% methanol and 15% acetic acid). Membranes were blocked for 1 hour at room temperature with 5% powder milk prepared in PBST20. After an overnight incubation at 4°C with the primary antibodies diluted in blocking solution, membranes were washed three times for 10 minutes with PBST20 and incubated for one hour at room temperature with the HRP-conjugated secondary antibodies diluted in blocking solution. Blots were developed with ECL Chemiluminescent Detection System (Amersham) according to manufacturer's protocol and detected on X-ray film (Fuji Medical X-Ray Film).

2.13. Antibodies

The primary antibodies used were anti- α -tubulin mouse B512 (Sigma) at 1:4000 for immunofluorescence (IF), anti-CID rat (Martins et al, 2009) at 1:250 (IF), anti-Mps1 guinea pig (Gp15) (Scott Hawley) at 1:250 (IF), anti-Mps1T676Ph rabbit (gift from Geert Kops) at 1:5000 (IF) and 1:1000 (IB), anti-Ndc80 rabbit (Rb272; Michael Goldberg) at 1:1000 (IF), anti-PhosphoT210 Plk1 mouse (ab39068; Abcam) at 1:5000 (IF), anti-Phospho-Erkin (Thr567)/Radixin (Thr564)/ Moesin (Thr558) (3141, Cell Signaling Technology) at 1:100 (IF), anti-MBP mouse (gift from Eurico Sá) at 1:5000 and anti PP1-87B Sheep (Dundee university) at 1:1000.

Secondary antibodies conjugated with horseradish peroxidase (HRP, Santa Cruz) or fluorescent dyes from the Alexa series (Invitrogen) were used according to the manufacturer's instructions. Anti-rabbit HRP was used at 1:5000 and anti-mouse HRP at 1:2500. Fluorescent secondary antibodies were used at 1:2000 with the exception of Alexa 647 conjugated ones, which were used at 1:1000.

2.14. Kinase Assay

For in vitro phosphorylation assays, His6-DmMps1WT, His6-DmMps1KD, His6-DmMps1150-379 or Casein were incubated with active Polo (a gift from Bill Sullivan), His6-DmMps1WT or HsMps1 (SignalChem) for 30 minutes in a total volume of 30 μ L of kinase reaction buffer (5 mM MOPS pH 7.4, 2.5 mM β -glycerol-phosphate, 5 mM MgCl₂, 1 mM EGTA, 0.4 mM EDTA, 0.25 mM DTT, 100 μ M ATP and 10 μ Ci [g-32P]ATP [3000 Ci/mmol, 10 mCi/mL]). Kinase reactions were carried out at 25°C for Polo or His6-DmMps1WT and at 30°C when using HsMps1. For inhibition of HsMps1, 10 μ M MPS1-IN-1 (a gift from Helder Maiato) were added in a final volume of 1 mL DMSO. Reactions were stopped by addition of Laemmli sample buffer (4% SDS, 10% mercaptoetanol, 0.125 M Tris-HCl, 20% glycerol, 0.004% bromophenol blue) and heated for 5 min at 95°C. The labeled proteins were resolved in an 8% SDS-PAGE, stained with Coomassie Blue and radioactive phosphate incorporation was detected on X-Ray film (Fuji Medical X-Ray Film).

Alternatively, some kinase assays were performed without radioactive ATP and the phosphorylation on Mps1 T-loop was detected by Western blotting with anti-Mps1^{T767Ph} antibody. In these reactions HsMps1 (SignalChem) were incubated with active Plk1 and/or PP1 at different concentrations and different timepoints.

2.15. Phosphatase Assay

For in vitro phosphatase assays, PP1- γ was incubated with active Plk1, Cdk1, Mps1, Aurora B (all from SignalChem) or Okadaic Acid (O.A.) for 30 minutes in a total volume of 50 μ L of adequate buffer (containing kinase buffer (kinase assay buffer I from SignalChem) and phosphatase buffer (PMP Buffer from New England Biolabs supplemented with MnCl₂)). Reactions were carried out at 30°C. Then, this reactions were added to 50 μ L of a solution containing 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) in phosphatase buffer), in a 96-well plate and then fluorescence was detected with a multi-mode plate reader (Synergy 2). The reactions were performed according to manufacturer's (EnzChek Phosphatase Assay Kit, Molecular PROBES) instructions.

2.16. Surface Plasmon Resonance (SPR)

SPR experiments were performed at 25°C using a BIACORE X100 (GE Healthcare, Little Chalfont, UK) equipped with a research-grade CM5 sensor chip. Plk1 (70 kDa) was used as ligand and immobilized using amine-coupling chemistry. The surface of the flow cell 2 was activated for 7 minutes with a 1:1 mixture of 0.1M NHS (N-hydroxysuccinimide) and 0.1M EDC (3-(N,N-dimethylamino) propyl-N-ethylcarbodiimide) at a flow rate of 5µL/min. Plk1 (10µg/mL in 10mM sodium acetate pH 4.5) was immobilized at a density of 800 RU followed by blocking of the surface with a 7 minute injection of 1M ethanolamine pH 8.5. Reference flow cell 1 was activated and inactivated following the same procedure without immobilization of any protein. GST-PP1 (63.88 kDa) or GST (control, 26.9 kDa) were diluted in 10mM HEPES pH 7.4, 150mM NaCl, 0.005% (vol/vol) P20 and injected over the two flow cells at a concentration of 3.9µM and at a flow rate of 30µL/min. The complex was allowed to associate and dissociate for 120 and 200s, respectively.

CHAPTER 3

RESULTS AND DISCUSSIONN

3.1. Polo is required for Mps1 activation during prophase and controls Mps1 recruitment to unattached kinetochores.

Accurate chromosome segregation depends on the Spindle Assembly Checkpoint (SAC), a conserved signalling pathway that monitors the attachment of kinetochores to microtubules and prevents sister chromatid separation until all chromosomes are correctly attached to opposite spindle poles (biorientation) (Musacchio and Salmon, 2007; Foley and Kapoor, 2013).

Mps1 kinase is a master regulator of SAC function, acting upstream of other SAC components to promote the assembly of anaphase inhibitory complexes (Abrieu et al., 2001; London et al., 2012; Yamagishi et al., 2012). Mps1 is recruited to unattached kinetochores and this local accumulation has been proposed to potentiate trans-autophosphorylation of its T loop, which ensures full kinase activity and robust SAC signalling (Kang et al., 2007; Sacristan and Kops, 2015). Microtubule attachments decrease the affinity of Mps1 for kinetochores, hence directly preventing its recruitment to kinetochores of bioriented chromosomes (Ji et al., Sacristan et al., 2015). However, precluding Mps1 dissociation from metaphase kinetochores still allows SAC silencing in fission yeast (Aravamudhan et al., 2015) and its forced expression in anaphase is sufficient to induce SAC signalling in budding yeast (Palframan et al., 2006).

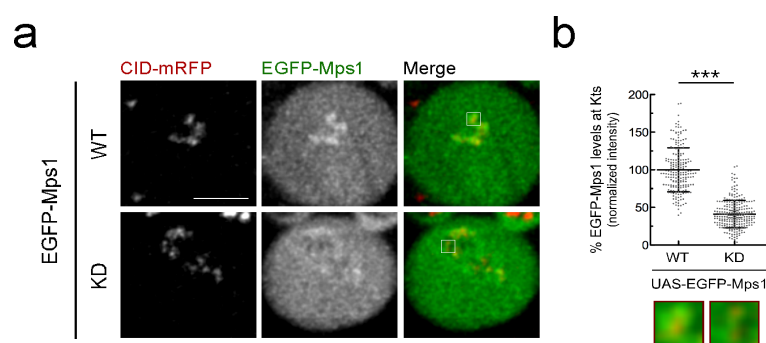


Figure 3.1 – Mps1 kinetochore localization requires its kinase activity. (a) Representative images of aldD4422 neuroblasts expressing CID-mRFP and Egfp-Mps1^{WT} or Egfp-Mps1^{KD}. Neuroblasts were incubated with 50μM colchicine for 2 hours. (b) Quantification of kinetochore intensities of Egfp-Mps1^{WT} or Egfp-Mps1^{KD} relative to CID-mRFP signal intensities. The insets in (b) display magnifications of the outlined regions from (a) Data represent means ± s.d. Mean values for EGFP-Mps1^{WT} were set to 100%. Asterisks indicate that differences between mean ranks are statistically significant, *** P < 0.0001, Mann-Whitney U test N > 20 cells for each condition. Scale bars, 5 μm.

Thus, in addition to the control exerted on its kinetochore recruitment, other mechanisms likely contribute to ensure that Mps1 activity is tightly regulated throughout mitosis. In line with this, inhibition of Mps1 during late G2 and prophase impaired the formation of pre-mitotic anaphase inhibitory complexes (Maciejowski et al., 2010; Rodriguez-Bravo et al., 2014), indicating that Mps1 activation must precede the assembly of functional kinetochores. This requisite is further exposed in *Drosophila* where Mps1 catalytic activity is required to promote its own recruitment to unattached kinetochores. *In vivo* imaging of 3rd instar neuroblasts revealed that a kinase-dead version of Mps1 (Mps1^{KD}) when expressed in an Mps1-null background (*ald*^{G4422}) failed to localize to prometaphase kinetochores to the same extent as Mps1^{WT} (figure 3.1), confirming previous observations in fly embryos (Althoff et al., 2012). Thus, at least two outstanding questions remain in respect to Mps1 control and consequently SAC regulation: what triggers Mps1 activation and hereby SAC signalling upon mitotic commitment and how Mps1 itself is inactivated upon chromosome biorientation to ensure rapid SAC silencing and timely mitotic exit.

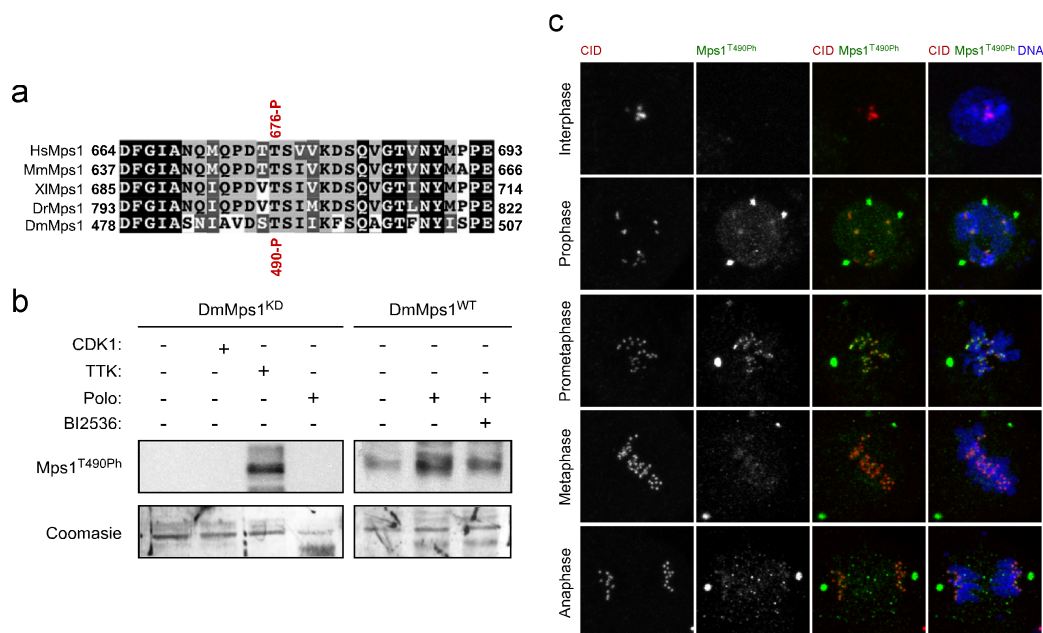


Figure 3.2 – Autophosphorylation of Mps1 T-loop. (a) Conservation among Mps1 activation loop residues. The alignment indicates conservation among Mps1 proteins at positions Thr686 and Tyr689, whereas Ser682 is similar. Residues Thr676 and Ser677 are conserved within metazoan Mps1 proteins. The numbers note specific residues for hMps1 and yeast Mps1, and P indicates a phosphorylated residue. Identical residues are indicated by a black background with white lettering, conserved residues are indicated by gray background with black lettering, and similar residues are indicated by gray background with white lettering (from Mattison et al., 2007). (b) Recombinant versions of *Drosophila* Mps1^{KD} and Mps1^{WT} were incubated with the indicated kinases and BI2536 (100 nM) for

30 min. Mps1 T490 phosphorylation was monitored by western blotting with an antibody specific for T676/T490 phosphorylation. T490 phosphorylation exclusively results from Mps1 autophosphorylation (left panel), which is stimulated by Polo activity (right panel). Note that the antibody does not recognize active Polo. (c) Immunofluorescence analysis of asynchronous S2 cells stained for Mps1T490 phosphorylation (Mps1^{T490Ph}). CID was stained as a kinetochore reference

To address this we examined Mps1 activation in S2 cells resorting to immunofluorescence analysis with an antibody against the conserved autophosphorylation (T490Ph) on the kinase T-loop (Jelluma et al., 2008) (figure 3.2). While no signal could be detected in interphase cells, prophase figures revealed high levels of active Mps1 in the nucleoplasm and on pre-kinetochores (figure 3.3 and figure 3.2 c). Although in flies the Mps1 receptor, Ndc80, associates with the inner-kinetochore only after nuclear envelope breakdown (Venkei et al., 2012), it is possible that unspecific accumulation of the kinase at these sites could drive its activation during prophase.

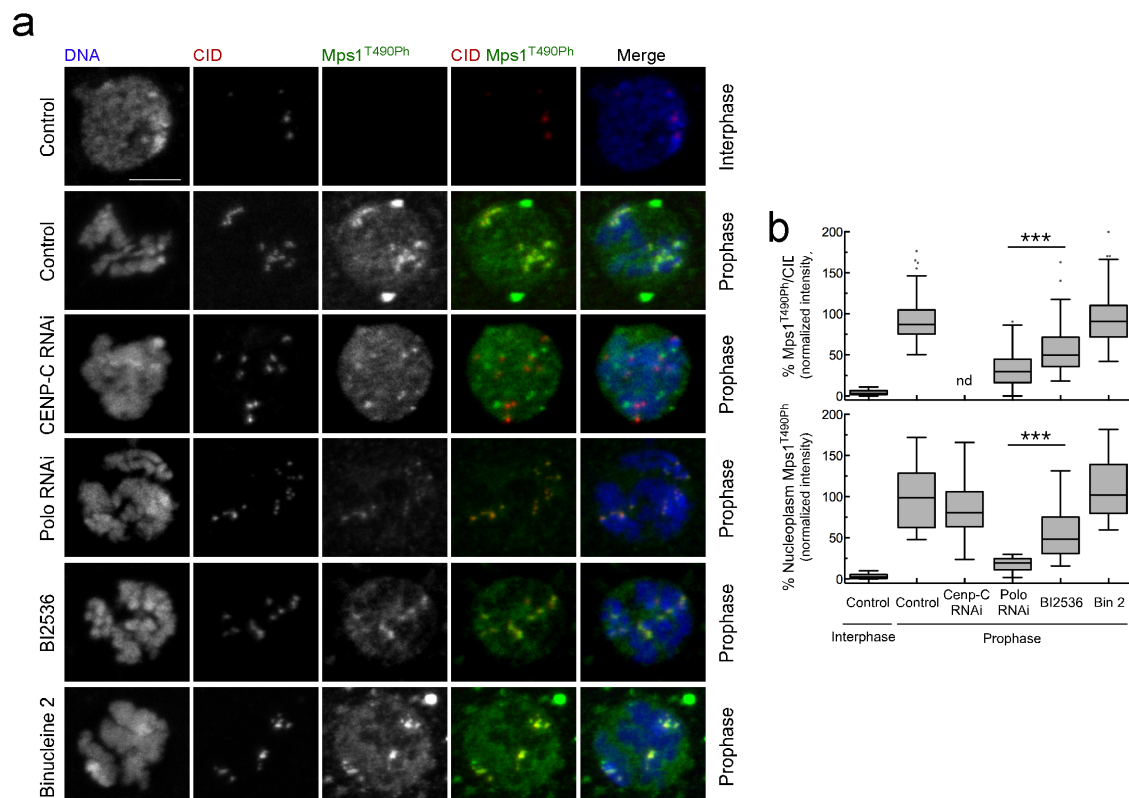


Figure 3.3 – Polo is required to allow Mps1 activation in prophase. (a,b) Representative immunofluorescence images (a) and interquartile representation (b) of Mps1^{T490Ph} levels in the nucleoplasm and pre-kinetochores of S2 cell prophases. Mps1^{T490Ph} fluorescence intensities at pre-kinetochores were determined relative to CID signal. For inhibition of Polo and Aurora B activities, cells

were incubated with BI2536 (100 nM, 6 hours) and Binuclein 2 (40 mM, 2 hours), respectively. Mean values for control cells were set to 100%. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Kruskal-Wallis, Dunn's multiple comparison test. $N > 20$ cells for each condition from 4 different experiments. Scale bars, 5 μm .

To test this, we assessed Mps1 activation in S2 cells lacking pre-kinetochores as a result of CENP-C knockdown. Interestingly, CENP-C depletion did not affect the nucleoplasmic levels of Mps1 T-loop phosphorylation during prophase (figure 3.3), which is consistent with the hypothesis that Mps1 activation takes place before its recruitment to kinetochores. Likewise, treatment with Binucleine 2 to inhibit Aurora B, a kinase required to promote Mps1 kinetochore recruitment and outer kinetochore assembly (Santaguida et al., 2011; Saurin et al., 2011; Conde et al., 2013; Kim and Yu, 2015; Rago et al., 2015), had no effect on Mps1 activation in prophase (figure 3.3).

As Polo activation starts during G2 (Macurek et al., 2008; Seki et al., 2008) and its depletion was shown to impair kinetochore localization of Mps1 during prometaphase (Conde et al., 2013), we analyzed the ability of Mps1 to become active during prophase in the absence of Polo or of its activity. Whereas Polo inhibition with BI2536 led to a 50% reduction of Mps1 T-loop phosphorylation in the nucleoplasm and at inner-kinetochores, RNAi-mediated depletion of Polo virtually prevented Mps1 activation (figure 3.3). This difference in the level of Mps1 activation in prophase conferred different capacities to recruit Mps1 to unattached kinetochores latter during prometaphase. It is important to note, that Polo depletion and inhibition had similar effects on kinetochore levels of Ndc80 and Aurora B activity in prometaphase cells (figure 3.4c-f). The partial activation of Mps1 in BI2536 treated cells seems to be sufficient to allow the recruitment of Mps1 to kinetochores (figure 3.4 a,b).

On the other hand, a more dramatic impairment of Mps1 activation during prophase, as in Polo depleted cells, caused a significant reduction of Mps1 at unattached kinetochores that parallels the observed for the recruitment of catalytically inactive Mps1 (figure 3.4). These results indicate that Polo promotes Mps1 activation during prophase by mechanisms that are both dependent and independent of its kinase function.

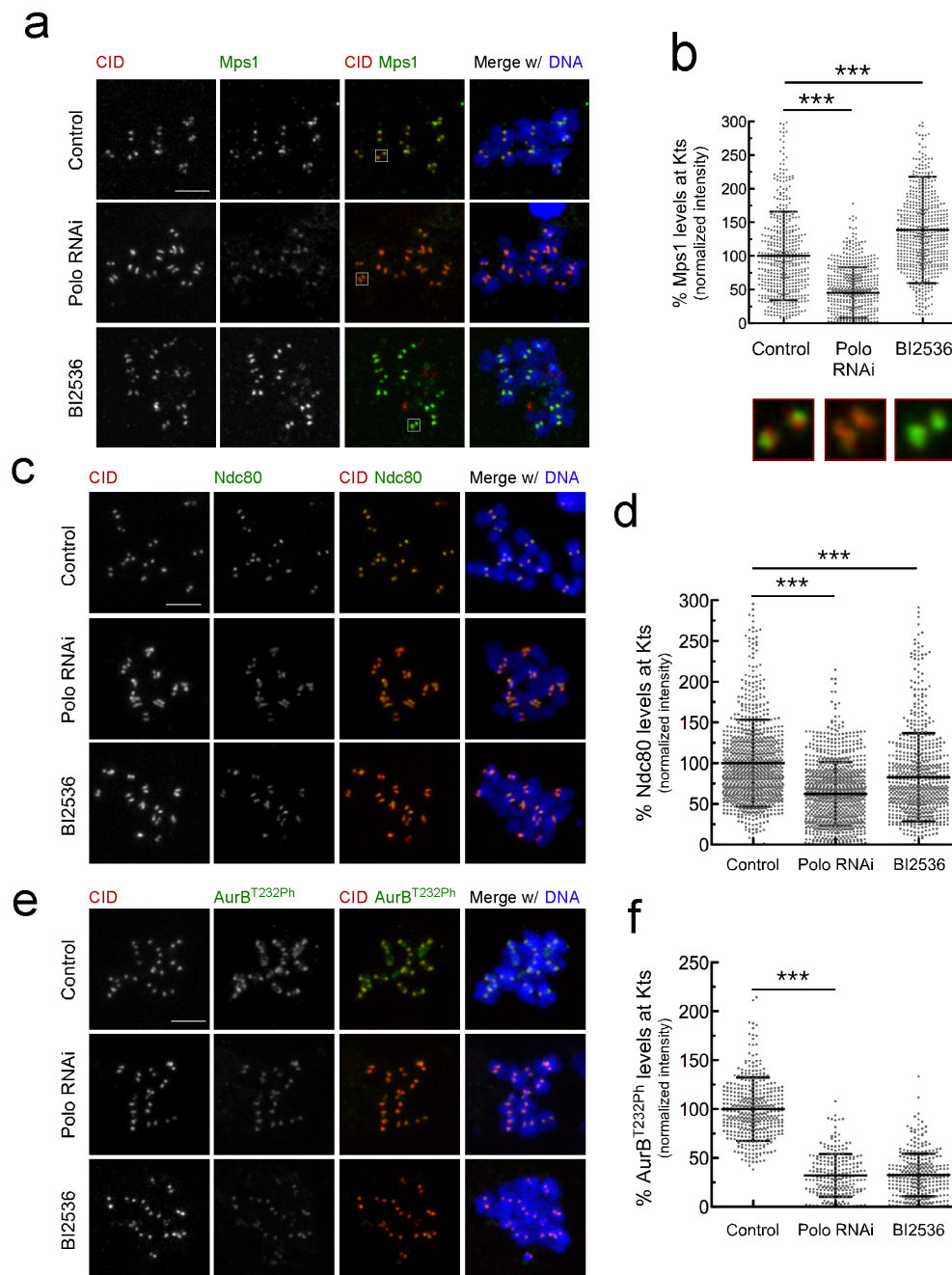


Figure 3.4 – Polo activity is required for proper kinetochore localization of Mps1, Ndc80 and Aurora B activity. (a-f) Representative immunofluorescence images (a,c,e) and quantifications (b,d,f) of Mps1, Ndc80 and Aurora B T232 autophosphorylation at kinetochores/centromeres in S2 cells depleted of Polo or upon inhibition of its kinase activity. Mps1, Ndc80 and Aurora B^{T232Ph} fluorescence intensities were determined relative to CID signal. Cells were incubated with colchicine (25 μ M) for 2 hours. For inhibition of Polo activity, BI2536 (100 nM) was added to the culture 4 hours before microtubule depolymerization with colchicine. Data (b,d,f) represent means $\pm$ s.d. Mean values for control cells were set to 100%. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Mann-Whitney U test. $N > 198$ kinetochores (b), $n > 689$ kinetochores (d) and $n > 234$ kinetochores (f) for each condition from 4 (b) and 2 (b,f) different experiments. Scale bars, 5 μ m.

3.2. Polo phosphorylates Mps1 on its T185 to potentiate its autophosphorylation and SAC activity.

In vitro kinase assays with recombinant *Drosophila* Polo and Mps1 confirmed that Polo activity stimulates Mps1 T-loop autophosphorylation (figure 3.2), as recently observed by others with human orthologues (von Schubert et al., 2015). Surprisingly, although Polo was able to phosphorylate an Mps1 N-terminal fragment (Mps1¹⁵⁰⁻³⁷⁹), we failed to detect phosphorylation of a full-length kinase-dead version of Mps1 (Mps1^{KD}) (figure 3.5a).

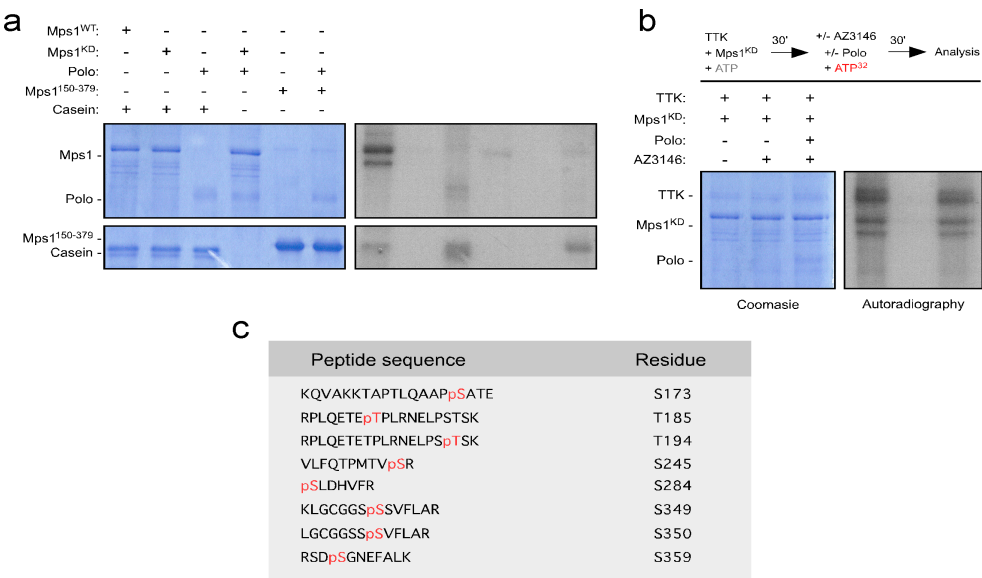


Figure 3.5 – Polo phosphorylates Mps1 N-terminal *in vitro*. (a) *In vitro* kinase assays with the indicated recombinant proteins and peptide in the presence of [γ -³²P]-ATP for 30 min. Phosphorylation of casein was monitored for control. Phosphorylation was detected by autoradiography and levels of proteins and peptide were visualized by comassie blue staining. (b) Two-step *in vitro* kinase assays with the indicated recombinant proteins. *Drosophila* Mps^{KD} was pre-phosphorylated by human TTK with unlabelled ATP (grey ATP) for 30 min. TTK inhibitor AZ3146 (2 μ M), Polo and [γ -³²P]-ATP (red ATP³²) were then added to the reaction as indicated and the reaction stopped after 30 min. Phosphorylation was detected by autoradiography and levels of proteins visualized by comassie blue staining. (c) Recombinant fragments of Mps1¹⁵⁰⁻³⁷⁹ were subjected to mass spectrometry analysis after *in vitro* kinase assay with Polo. Polo phosphorylation sites on Mps1¹⁵⁰⁻³⁷⁹ (red pS/pT) were identified with MaxQuant/Andromeda with a decoy FDR of 0.01 on peptide.

Thus, we hypothesize that catalytically inactive Mps1 assumes an altered structural conformation relative to Mps1^{WT}, which conceals Polo phosphorylation

sites present in its N-terminal region. This suggests that Mps1 must retain its autophosphorylation activity in order to become phosphorylated by Polo. To test this, we pre-incubated *Drosophila* Mps1^{KD} with the human homologue, TTK, which is able to mediate phosphorylation of Mps1^{KD} T-loop (figure 3.2). The reaction occurred in the presence of unlabelled ATP and was stopped by specific inhibition of TTK with AZ3146. This was followed by the addition of ³²P-ATP and Polo, which readily phosphorylated Mps1^{KD} (figure 3.5b). These results strongly suggest that Mps1 autophosphorylation triggers a structural rearrangement that renders the previously cryptic N-terminal now accessible to Polo.

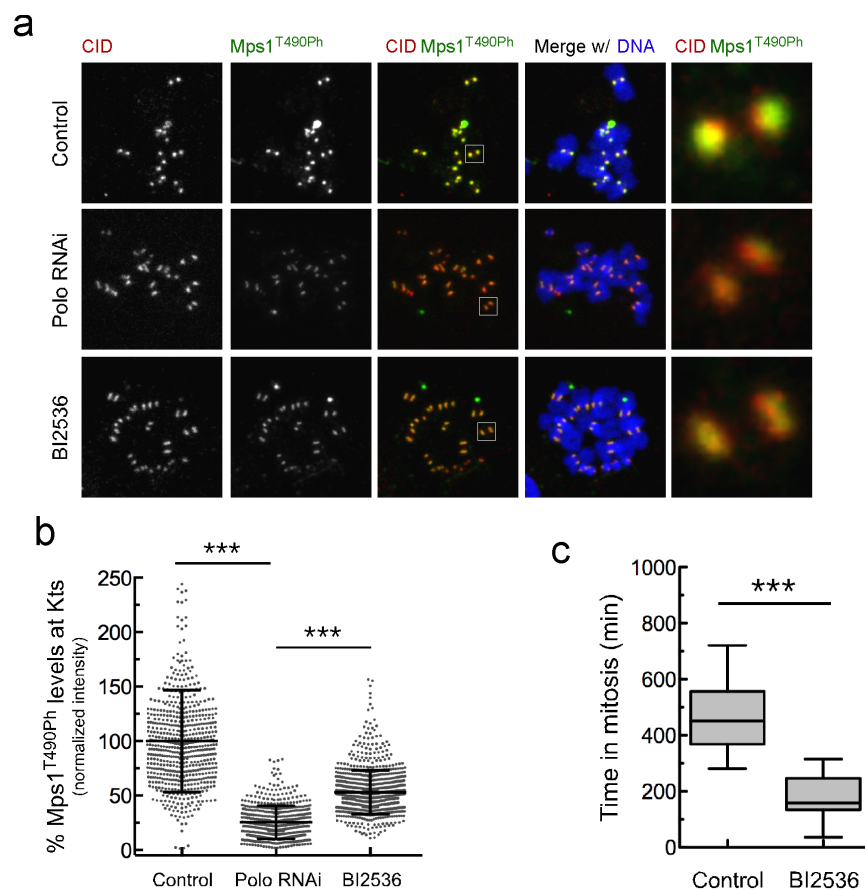


Figure 3.6 – Polo activity is required for proper Mps1 activation and SAC signalling. (a,b) Representative immunofluorescence images (a) and quantification (b) of Mps1^{T490Ph} levels at unattached kinetochores in S2 cells depleted of Polo or upon inhibition of its kinase activity. Mps1^{T490Ph} fluorescence intensities were determined relative to CID signal. Cells were incubated with colchicine (25 μ M) for 2 hours. For inhibition of Polo activity, BI2536 (100 nM) was added to the culture 4 hours before microtubule depolymerization with colchicine. Data (b) represent means $\pm$ s.d. Mean values for control cells were set to 100%. (c) Interquartile representation of the time that S2 cells stably expressing H2B-GFP and mCherry-Tubulin remain in mitosis in the presence of colchicine (25 μ M). Colchicine and

BI2536 (100 nM) were added immediately after nuclear envelope breakdown and mitotic progression monitored by live-cell imaging. Scale bars, 5 μ m.

To evaluate if Polo phosphorylation of Mps1 N-terminal region underlies the observed potentiation of Mps1 auto-activation (figure 3.2b), we mapped Polo phosphorylation sites on Mps1¹⁵⁰⁻³⁷⁹ through mass spectrometry analysis (figure 3.5c) and expressed the respective phosphomutants in S2 cells depleted of endogenous Mps1. Mutation of T185 to alanine (T185A) negatively affected Mps1 T-loop autophosphorylation at unattached kinetochores, whereas mimicking its constitutive phosphorylation (T185D) stimulated Mps1 activation (figure 3.8 and figure 3.7c). Mps1 fast dynamics at unattached kinetochores relies on its kinase activity (Jelluma et al., 2010). In line with the observed reduction of T490 auto-phosphorylation in endogenous Mps1 (figure 3.6a,b) and EGFP-Mps1^{WT} (figure 3.7a,b) at unattached kinetochores, addition of BI2536 caused a 4.5 fold increase of Mps1 half-time at kinetochores (figure 3.8a). In comparison, FRAP analysis of Mps1T185A revealed a 3.5 fold increase in the half-time relative to Mps1WT, while Mps1T185D displayed a faster dynamic exchange at kinetochores, consistent with the notion that phosphorylation of T185 is important to enhance Mps1 activity (figure 3.8a).

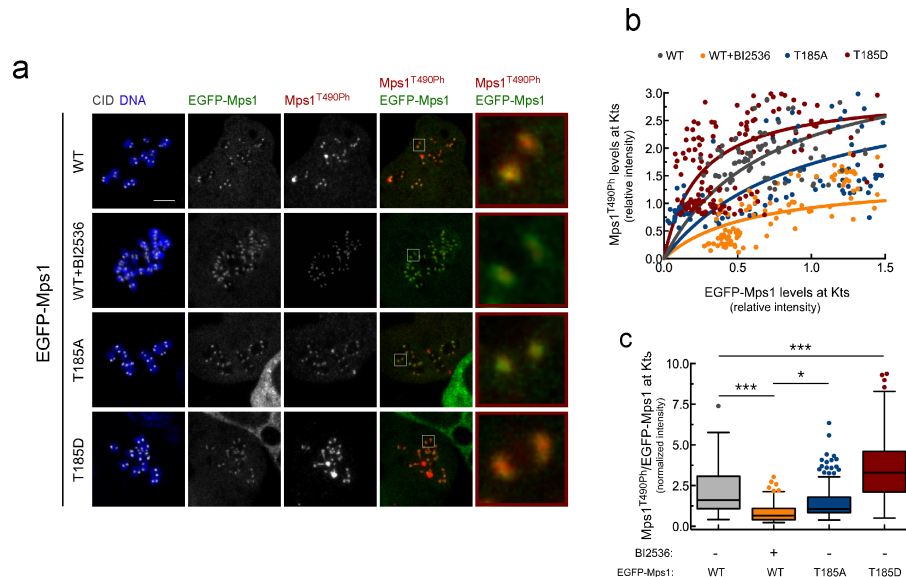


Figure 3.7 – Phosphorylation of Mps1 T185 by Polo promotes Mps1 T-loop autophosphorylation at unattached kinetochores. Representative immunofluorescence images (a) and quantification (b) of EGFP-Mps1 and Mps1^{T490Ph} kinetochore levels in S2 cells depleted of endogenous Mps1 and expressing EGFP-Mps1^{WT} or the indicated phosphomutants. Cells were treated with MG132 (20 μ M) for 1 h followed by a 2 h period of colchicine incubation (30 μ M). When required,

BI2536 (100 nM) was added to the culture 30 min before treatment with colchicine. (b) Mps1^{T490Ph} relative intensities were plotted against EGFP-Mps1 relative intensities at kinetochores. (c) Interquartil representation of Mps1 activation status at kinetochores in S2 cells depleted of endogenous Mps1 and expressing EGFP-Mps1^{WT} or the indicated phosphomutants. Mps1^{T490Ph} fluorescence intensities were determined relative to EGFP-Mps1 signal. Cells were treated with MG132 (20 μ M) for 1 h followed by a 2 h period of colchicine incubation (30 μ M). When required, BI2536 (100 nM) was added to the culture 30 min before treatment with colchicine. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Kruskal-Wallis, Dunn's multiple comparison test. Scale bars, 5 μ m.

To examine its impact on SAC function, we monitored mitotic progression through live-cell imaging. Overexpression of EGFP-Mps1^{WT} in S2 cells results in a prolonged metaphase delay (figure 3.8b) due to persistent SAC signalling (Conde et al., 2013). Overexpression of EGFP-Mps1^{KD} failed to cause an obvious increase in the mitotic timing indicating that the observed arrest requires Mps1 catalytic activity (figure 3.8b). In line with this, Polo inhibition with BI2536 led to a dramatic decrease in the time cells treated with colchicine or overexpressing EGFP-Mps1^{WT} are able to arrest in mitosis (figure 3.6c and figure 3.8b). Consistent with reduced Mps1 activity, overexpression of Mps1^{T185A} resulted in a 3-fold reduction in the mitotic timing relative to cells expressing Mps1^{WT} (figure 3.8b). Conversely, expression of Mps1^{T185D} resulted in a mitotic arrest to the same extent as Mps1^{WT} and partially restored the ability of cells to arrest in mitosis when treated with BI2536 (figure 3.8b). Together, these data indicate that Polo phosphorylates Mps1 on T185 to promote its auto-phosphorylation and consequently its full activation, which is required for sustained SAC function.

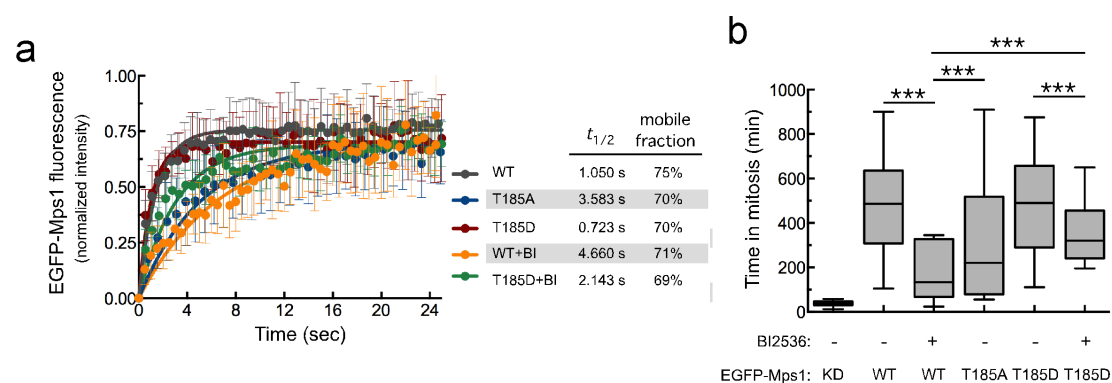


Figure 3.8 – Phosphorylation of Mps1 T185 by Polo is required for Mps1 kinetochore dissociation and robust SAC signalling. (a) FRAP analysis of EGFP-Mps1 in S2 cells depleted of endogenous Mps1 and expressing EGFP-Mps1^{WT} or the indicated phosphomutants in the presence or

absence of BI2536 (100 nM). The graph depicts EGFP fluorescence recovery (means $\pm$ s.d., $n > 11$ cells for each condition) after kinetochore bleaching. Recovery half-life ($t_{1/2}$) and recovery % are depicted. (b) Interquartil representation of the mitotic timing from nuclear envelope breakdown to anaphase onset monitored by live-cell imaging for the indicated conditions. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Kruskal-Wallis, Dunn's multiple comparison test. $N > 12$ cells for each condition from at least 2 different experiments.

Because T185 only becomes accessible to Polo upon Mps1 auto-phosphorylation, its phosphorylation represents a mechanism to potentiate the activation of already active Mps1. The inability of Polo to phosphorylate “inactive” Mps1 and the more severe impairment of Mps1 activation in Polo depleted prophases led us to cogitate additional roles for Polo in Mps1 initial activation, that likely rely on the structural presence of the protein.

3.3. PP1 dephosphorylation of Mps1 T-loop renders the kinase inactive and promotes SAC silencing.

In human cells, KNL1-bound PP1- γ antagonizes Mps1 signalling at kinetochores through dephosphorylation of Mps1 substrates (London et al., 2012; Zhang et al., 2014; Saurin et al., 2015). This allows rapid SAC silencing when kinetochore levels of Mps1 drop upon microtubule attachment (Ji et al., Sacristan et al., 2015). However, expression of active Mps1 in S2 cells caused a prolonged mitotic arrest even when kinetochore assembly was impaired due to CENP-C depletion (Conde et al., 2013). This is in agreement with reports showing that cytosolic Mps1 also contributes for SAC function during mitosis (Maciejowski et al., 2010; Maldonado and Kapoor, 2011; Rodriguez-Bravo et al., 2014) and indicates that dephosphorylation of Mps1 T-loop is required to allow metaphase-anaphase transition even in the absence of kinetochore-mediated SAC signalling. Since Mps1 activation occurs through trans-autophosphorylation, we reasoned that Mps1 itself is targeted by PP1 to inactivate the kinase and ensure timely SAC silencing. To address this, we started by assessing Mps1/TTK auto-activation *in vitro* in the presence of PP1- γ . Simultaneous incubation of PP1- γ with Mps1/TTK led to a concentration-dependent decrease of Mps1/TTK T676 phosphorylation (figure 3.9a). Likewise, PP1- γ was able to dephosphorylate the T-loop of TTK/Mps1 that had been allowed to become active (figure 3.9b). Accordingly, depletion of the PP1-87B orthologue from S2 cells resulted

in a modest, but significant increase of Mps1 T-loop phosphorylation at unattached kinetochores (figure 3.9c,d).

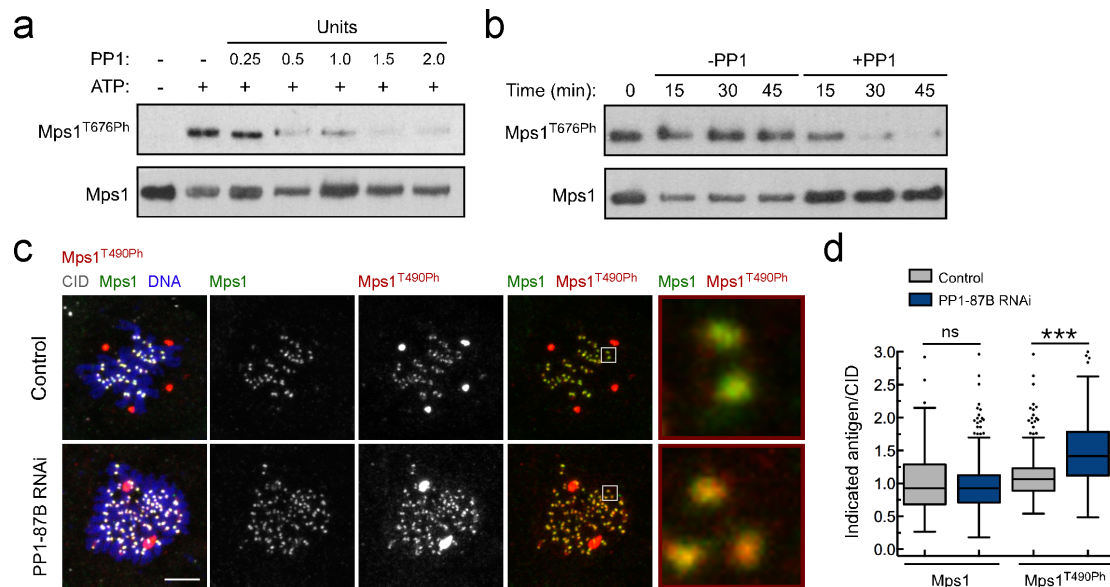


Figure 3.9 – PP1 dephosphorylates Mps1 T-loop *in vitro* and *in vivo*. (a) Recombinant human Mps1 was incubated during 30 min with increasing concentrations of recombinant human PP1- γ or (b) PP1- γ (1.5 U) was added to previously active Mps1 (time 0 min) and phosphorylation of Mps1 T676 monitored over time. Mps1 T676 autophosphorylation and Mps1 loading levels were assessed by western blotting (a,b). (c,d) Representative immunofluorescence images (c) and interquartil representation (d) of Mps1^{T490Ph} and Mps1 relative levels at prometaphase kinetochores of control and PP1-87B depleted cells. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Mann-Whitney U test. $N > 155$ kinetochores (d) for each condition from 2 independent experiments. Scale bars, 5 μ m.

Strikingly, although Mps1 present at kinetochores decreases to residual levels as chromosomes congress to the metaphase plate, it retains higher T-loop phosphorylation in cells depleted of PP1-87B (figure 3.10a,b), which correlates with higher levels of Mad1 at aligned kinetochores and a pronounced metaphase delay (figure 3.10c-f). Together, these data indicate that PP1 antagonizes Mps1 activation through dephosphorylation of its T-loop. This renders the kinase inactive to allow prompt SAC silencing and timely anaphase onset upon chromosome biorientation.

Polo/Plk1-mediated inhibition of PP1 defines a molecular switch to control SAC signalling

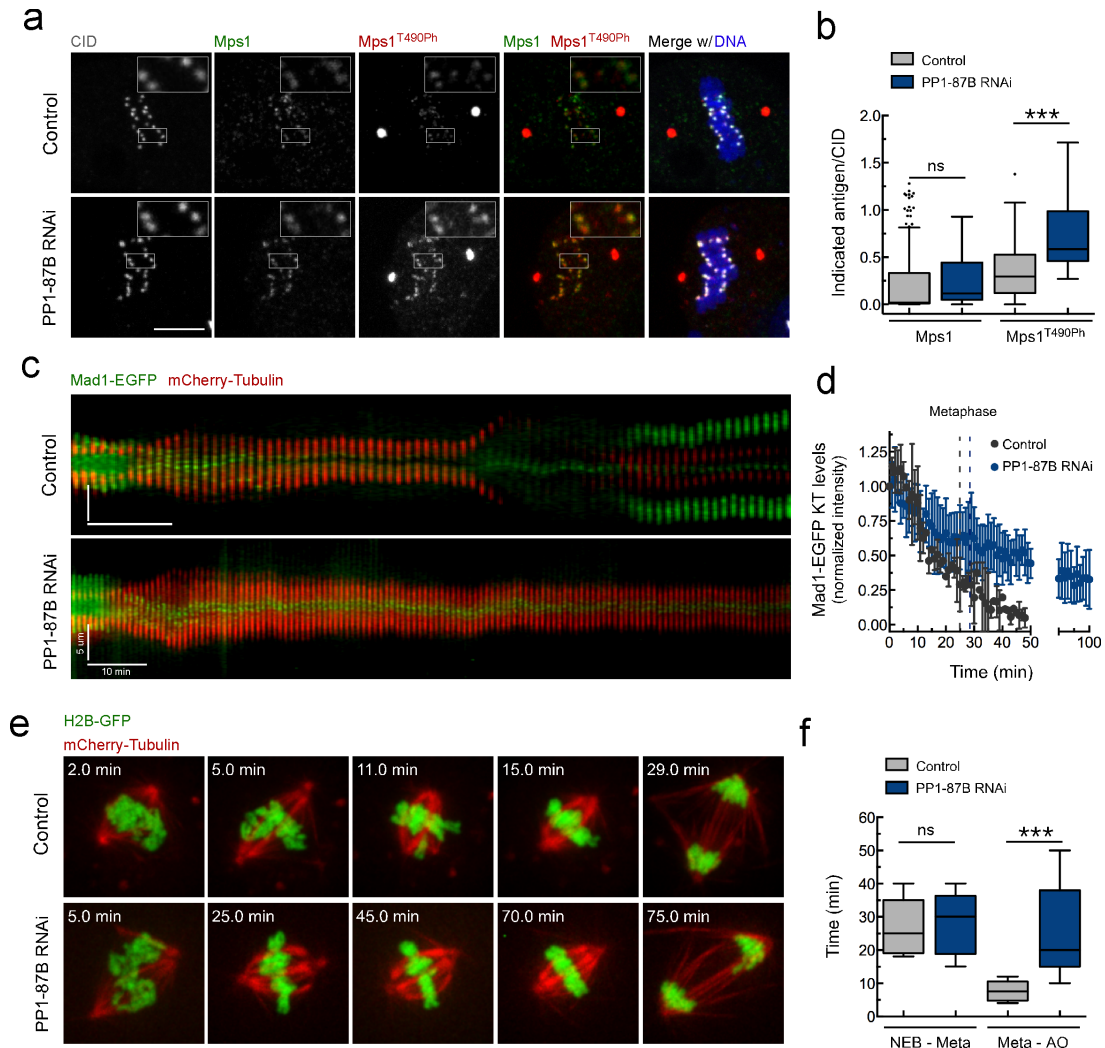


Figure 3.10 – Dephosphorylation of Mps1 T-loop by PP1 is required for prompt SAC silencing. (a,b) Representative immunofluorescence images (a) and interquartil representation (b) of Mps1^{T490Ph} and Mps1 relative levels at metaphase kinetochores of control and PP1-87B depleted cells. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Mann-Whitney U test. $N > 139$ kinetochores (b) for each condition from 2 independent experiments. (c-d) Kymographs analysis of mitotic progression (c) and Mad1-EGFP kinetochore levels (d) of control and PP1-87B depleted S2 cells expressing mCherry-Tubulin and Mad1-EGFP under control of its endogenous promoter. (d) Graphs represent the variation of kinetochore Mad1-EGFP relative levels during mitosis monitored by live-cell imaging. Dashed lines represent the average time from nuclear envelope breakdown to metaphase. Mad1-EGFP fluorescence intensities at kinetochores were corrected for cytosolic signal and normalized to the value determined in the first frame after nuclear envelope breakdown. The insets display magnifications of the outlined regions (a). Horizontal scale bars, 5 μm (a) or 10 min (c). Vertical scale bars, 5 μm (c). (e) Representative stills of mitotic progression of control and PP1-87B depleted S2 cells expressing H2B-GFP and mCherry-Tubulin monitored by time-lapse microscopy. (f) Interquartil representation of the time that cells in (e) spent from nuclear envelope breakdown (NEB) to metaphase and the time from metaphase to anaphase onset (AO). Asterisks indicate that differences between

Polo/Plk1-mediated inhibition of PP1 defines a molecular switch to control SAC signalling

mean ranks are statistically significant, *** $P < 0.0001$, Mann-Whitney U test. $N > 10$ cells for each condition, ns, not significant.

3.4. Polo in its active conformation inhibits PP1 activity independently of its kinase activity.

Thus, PP1 must be tightly regulated to allow Mps1 activation and robust SAC signalling during early stages of mitosis. Phosphorylation of PP1 carboxy terminus (on T320 in humans) by CDK1-Cyclin B has been proposed to repress its activity upon mitotic commitment (Dohadwala et al., 1994; Yamano, et al., 1994).

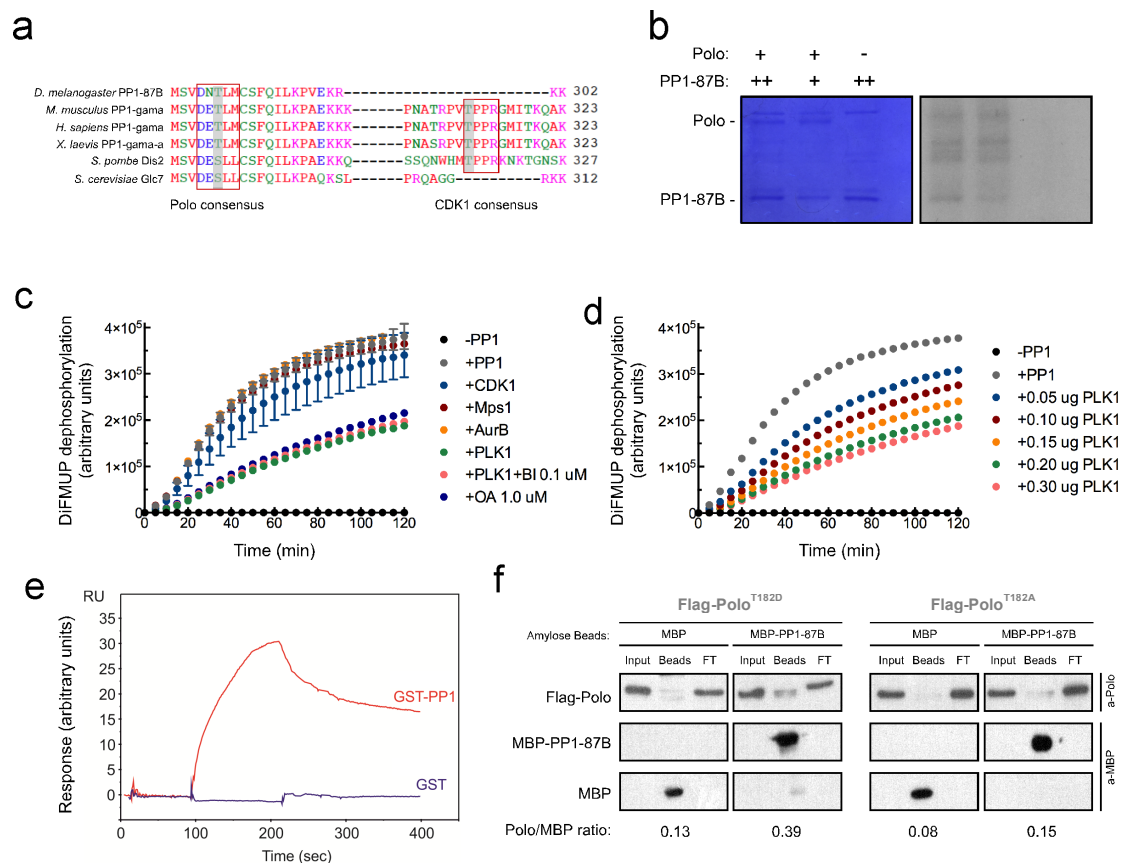


Figure 3.11 – Polo in its active conformation inhibits PP1 activity *in vitro* independently of its kinase activity. (a) Sequence alignment of PP1- γ homologues showing conservation of Polo and Cdk1 consensus sites (red boxes); Cdk1 consensus site is not found in *D. Melanogaster* PP1-87B and *S. Cerevisiae* Glc7. (b) a) *In vitro* kinase assays with the indicated recombinant proteins in the presence of [γ -³²P]-ATP for 30 min. Phosphorylation was detected by autoradiography and levels of proteins were visualized by coomassie blue staining (c,d) EnzChek phosphatase assays of recombinant human PP1- γ . (c) DiFMUP dephosphorylation by γ -PP1 was monitored over time in the presence of 0.5 μ g of the

indicated recombinant human kinases and inhibitors. OA, Okadaic acid; BI, BI2536. (d) DiFMUP dephosphorylation by PP1- γ was monitored over time in the presence of increasing concentrations of recombinant Plk1. PP1- γ was pre-incubated with the indicated kinases and inhibitors for 30 minutes before DiFMUP addition at time 0 min. (e) Interaction between GST-PP1- γ and Plk1 assessed by surface plasmon resonance (SPR). Plk1 was immobilized on the surface of a CM5 sensor chip and both GST-PP1- γ (3.9 μ M) and the control protein GST (3.9 μ M) were used as analytes. Red line corresponds to SPR signal (expressed as resonance units, RU) upon injection of GST-PP1- γ . Blue line corresponds to SPR signal after injection of GST. (f) Pull-downs using immobilized MBP-PP1-87B and total cell lysates from S2 cells expressing Flag-PoloT182D or Flag-PoloT182A and cells treated with colchicine for 2 hours. Immobilized MBP was used as negative control. Input, beads and flow-through (FT) were probed by western blotting for the indicated proteins.

Intriguingly, *in vitro* phosphatase assays with recombinant human PP1- γ that had been previously phosphorylated by CDK1-Cyclin B revealed a minor effect of this phosphorylation on PP1 activity (figure 3.11c), hence failing to recapitulate a more prominent inhibition observed with immunoprecipitated proteins or mitotic extracts from fission yeast and *Xenopus*, respectively (Grallert et al., 2015; Wu et al., 2009). Moreover, *Drosophila* PP1-87B and budding yeast Glc7, which is also required for SAC silencing (Pinsky et al., 2009) lack the CDK1 phosphorylation site (figure 3.11a), indicating that other mechanisms likely contribute to limit PP1 activity during mitosis. We noticed that PP1- γ homologues have a highly conserved consensus signature for Polo phosphorylation (figure 3.11a) and this prompted us to examine whether Polo/Plk1 was able to hinder PP1 activity. Although Polo displayed limited capacity to phosphorylate recombinant PP1-87B (figure 3.11b), Plk1 caused a dramatic reduction of PP1- γ phosphatase activity *in vitro* to the same extent as the presence of Okadaic acid (figure 3.11c,d). Importantly, incubation with BI2536 or lack of ATP did not prevent Polo-mediated inhibition of PP1- γ activity (figure 3.11c), thus indicating that Plk1 structurally inhibits PP1 independently of its kinase activity. Surface plasmon resonance analysis revealed that Plk1 and PP1- γ interact *in vitro* (figure 3.11e).

To examine whether Polo inhibits PP1 activity during mitosis, we assessed the phosphorylation of the Actin-binding protein Moesin on its T559, a well-characterized PP1 substrate (Kunda et al., 2012; Rodrigues et al., 2015). In flies, cortical stability during mitosis relies on phosphorylation of Moesin T559, which is established upon mitotic entry by Sterile20-like kinase (SLIK) (Carreno et al., 2008) and maintained until anaphase due to downregulation of PP1-87B activity (Kunda et al., 2012;

Rodrigues et al., 2015). Consistent with an increment in PP1-87B activity, Polo depletion from S2 cells led to a 2-fold decrease of Moesin^{T559Ph} cortical levels in mitosis (figure 3.12a,b). Co-depletion of Polo and PP1-87B phenocopies the increase of active Moesin observed in PP1-87B depleted cells (figure 3.12a,b), confirming that the reduction of Moesin T559 phosphorylation upon Polo depletion cannot be attributed to decreased SLIK activity. On the other hand, treatment with BI2535 had no noticeable effect on Moesin T559 phosphorylation (figure 3.12a,b), indicating that, similarly to the observed *in vitro*, Polo inhibits PP1 during mitosis independently of its kinase activity.

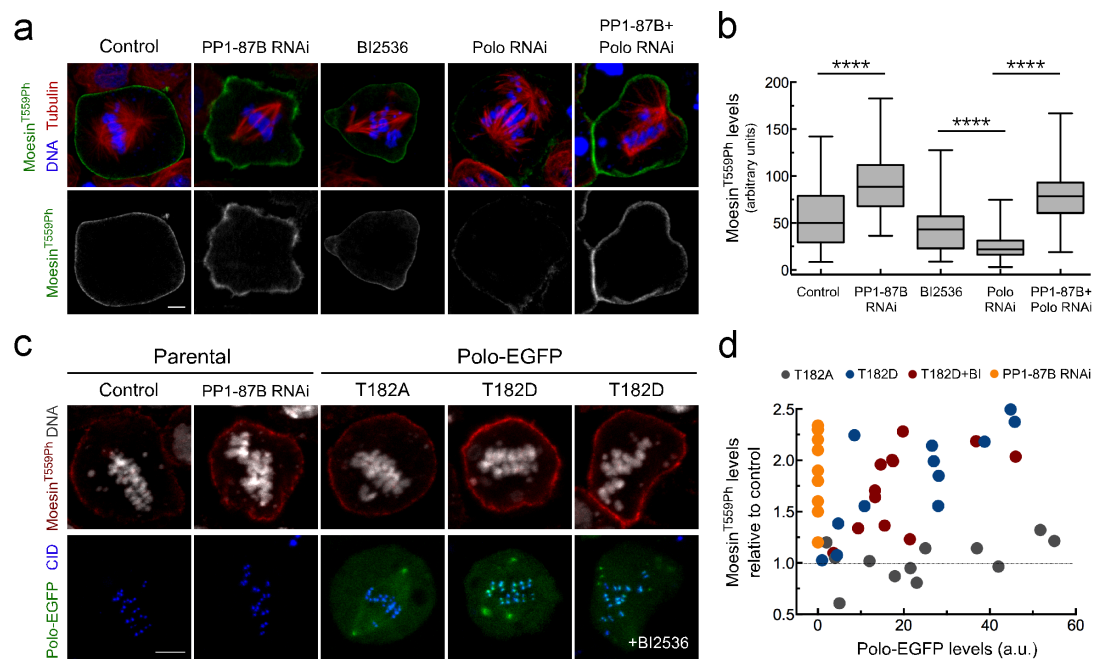


Figure 3.12 – Polo in its active conformation inhibits PP1 activity *in vivo* independently of its kinase activity. (a,b) Representative immunofluorescence images (a) and interquartil representation (b) of prometaphase Moesin^{T559Ph} cortical levels in control S2 cells, cells depleted of the indicate proteins and cells incubated with BI2536 for 3 hours. Asterisks indicate that differences between mean ranks are statistically significant, **** $P < 0.0001$, Kruskal-Wallis, Dunn's multiple comparison test. $N > 10$ cells for each condition from at least 2 different experiments. (c,d) Representative immunofluorescence images (c) and quantifications (d) of Moesin^{T559Ph} cortical levels in control and PP1-87B depleted S2 cells and in S2 cells expressing Polo^{T182A} or Polo^{T182D}. To inhibit Polo activity, cells were incubated with BI2536 (100 nM) for 3 hours. The fold increase of Moesin^{T559Ph} intensities relative to the control mean were plotted against Polo-EGFP cytosolic signal. Scale bars, 5 μ m.

Polo/Plk1 expression is cell cycle regulated. Protein levels start to increase in S and G2, with Polo/Plk1 being detected in the nucleus and cytoplasm (Taniguchi et

al., 2002; Zitouni et al., 2014). However, repression of PP1 activity only takes place at mitotic commitment (Mochida and Hunt, 2011). During interphase, Polo/Plk1 is maintained in an inactive state because the Polo-box domain binds the kinase domain and reduces the flexibility of its hinge region (Xu et al., 2013). Aurora A mediated phosphorylation of the conserved T210 (T182 in *Drosophila*) in the T-loop of Polo/Plk1 relieves this intramolecular auto-inhibition, hence activating the kinase (Zitouni et al., 2014). Polo T-loop phosphorylation starts in G2 and gradually increases until it reaches full activation when cells enter mitosis (Macurek et al., 2009; Seki et al. 2008; Bruinsma et al., 2013). Thus, we reasoned that PP1 inhibition could require Polo in its active conformation, thus restricting the inhibitory mechanism to mitosis.

To test this, we mutated Polo T182 to aspartic acid (T182D) and to alanine (T182A) to render the kinase constitutively active (open) and inactive (closed), respectively. Overexpression of Polo^{T182D} in S2 cells caused an increase of Moesin^{T559Ph} levels similar to the observed in cells depleted of PP1-87B, even when Polo kinase activity was inhibited with BI2536 (figure 3.12c,d). Conversely overexpression of Polo^{T182A} failed to cause an evident increase of active Moesin at the mitotic cortex (figure 3.12c,d), indicating that inhibition of PP1 activity requires Polo T-loop phosphorylation. Moreover, when immobilized PP1-87B fused with MBP was incubated with lysates of S2 treated with colchicine, we were able to detect an interaction with Polo^{T182D} but not with Polo^{T182A} (figure 3.11f). Together, these data indicate that Polo directly inhibits PP1 during mitosis. Although the inhibitory mechanism does not involve Polo catalytic activity, the kinase must assume its active conformation in order to bind PP1 and repress its activity. Because Polo T-loop phosphorylation dramatically decreases upon chromosome biorientation (Conde et al., 2013), this mechanism ensures that inhibition of PP1 occurs in a timely manner coordinated with cell-cycle progression.

3.5. Polo-mediated inhibition of PP1 triggers Mps1 activation in prophase.

To ascertain the importance of PP1 inhibition by Polo/Plk1 in SAC signalling, we started by examining the capacity of Plk1 to avert PP1- γ -mediated inactivation of Mps1/TTK *in vitro*. Addition of Plk1 restored Mps1 T-loop autophosphorylation in the

presence of PP1- γ (figure 3.13a). To exclude the direct contribution of Plk1 activity in promoting Mps1 auto-activation (figure 3.2b), BI2536 was added to the reaction. Under these circumstances, catalytically inhibited Plk1 was able to prevent Mps1/TTK T676 dephosphorylation by PP1- γ (figure 3.13b). These results are in perfect agreement with the effects that Polo inhibition and depletion have on Mps1 activation during prophase.

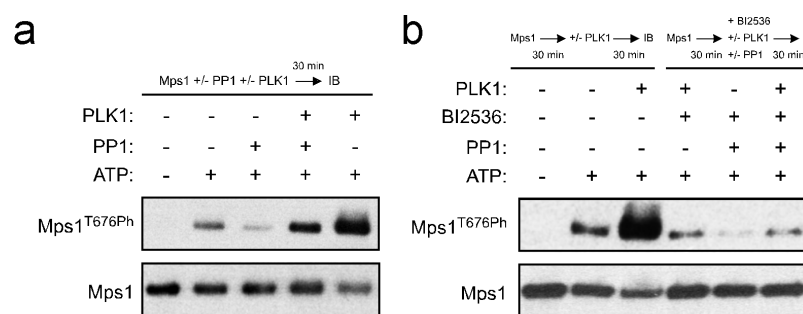


Figure 3.13 – Plk1 prevents dephosphorylation of Mps1 T-loop by PP1 *in vitro*. (a) Recombinant human Mps1 was incubated during 30 min with PP1- γ and Plk1 as indicated and Mps1 T676 phosphorylation and Mps1 loading levels monitored by immunoblotting, IB. (b) PP1- γ , Plk1 and BI2536 (100nM) were added to previously active Mps1 as indicated and Mps1 T676 phosphorylation and Mps1 loading levels monitored by immunoblotting, IB.

Pharmacological inhibition of Polo precludes the phosphorylation of Mps1 T185 and consequently potentiation of Mps1 T-loop autophosphorylation. Nevertheless, Polo retains its active conformation (figure 3.14), which inhibits PP1-87B activity and allows Mps1 to achieve an intermediate level of activation.

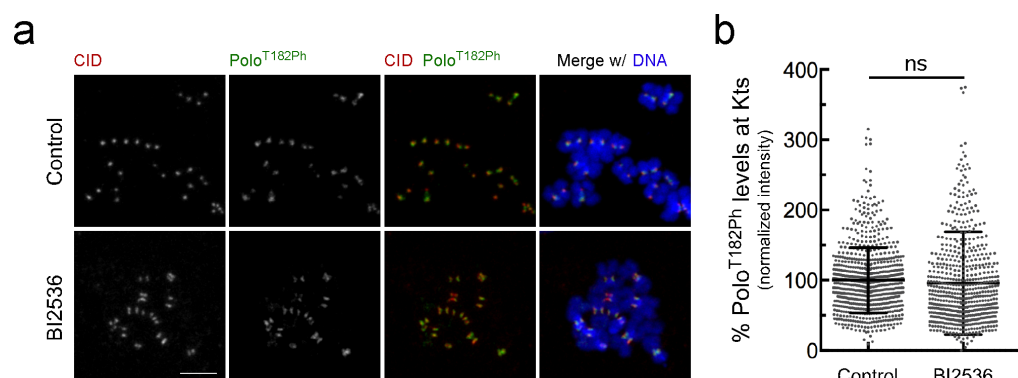


Figure 3.14 – Inhibition of Polo activity has no effect on its T-loop phosphorylation. (a,b) Representative immunofluorescence images (a) and quantification (b) of Polo^{T182Ph} levels at unattached

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kinetochores in control S2 cells or upon inhibition of Polo kinase activity. Polo^{T182Ph} fluorescence intensities were determined relative to CID signal. Cells were incubated with colchicine (25 μ M) for 2 hours. For inhibition of Polo activity, BI2536 (100 nM) was added to the culture 4 hours before microtubule depolymerization with colchicine. Data (b) represent means $\pm$ s.d. Mean values for control cells were set to 100%. Student's *t*-test, unpaired. *N* > 640 kinetochores for each condition, ns, not significant.

Depletion of Polo relieves the restrain on PP1-87B, thus resulting in an increment of phosphatase activity to levels that counteract Mps1 autophosphorylation and prevent its activation. In line with this, co-depletion of Polo and PP1-87B from S2 cells rescued Mps1 T-loop activation in prophase (figure 3.15) to levels that parallel Polo inhibition (figure 3.3). As expected, this restored the ability to recruit Mps1 to unattached kinetochores and led to an increment of Mps1 T-loop phosphorylation in prometaphase (figure 3.16).

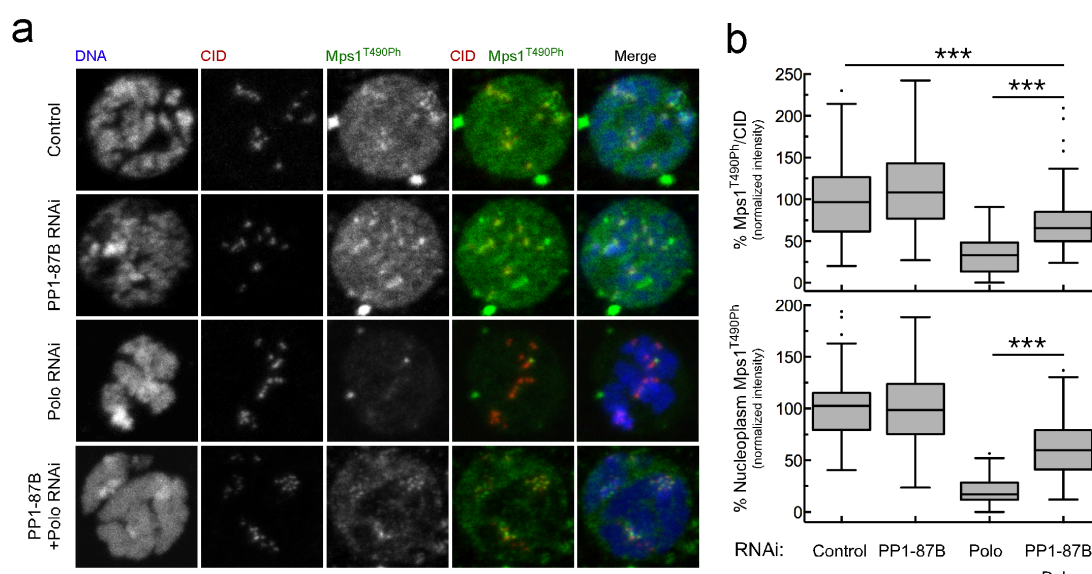


Figure 3.15 – Polo-mediated inhibition of PP1 triggers Mps1 activation in prophase. (a,b) Representative immunofluorescence images (a) and interquartil representation (b) of Mps1^{T490Ph} levels in the nucleoplasm and pre-kinetochores of S2 cell prophases in the indicated conditions. Mps1^{T490Ph} fluorescence intensities at pre-kinetochores were determined relative to CID signal. Mean values in control cells were set to 100%. Asterisks indicate that differences between mean ranks are statistically significant, *** *P* < 0.0001, Kruskal-Wallis, Dunn's multiple comparison test. *N* > 15 cells for each condition from 3 different experiments.

These results indicate that Polo structural inhibition of PP1 activity is required to allow initial activation of Mps1 upon mitotic commitment. To confirm this further, we

assessed the capacity of Polo to drive Mps1 activation during interphase, when PP1 activity is high and Mps1 is naturally inactive. Remarkably, overexpression of Polo^{T182D} led to a prompt increase of Mps1 T-loop phosphorylation levels in the cytoplasm by opposition to the modest effect resulting from the expression of inactive Polo^{T182A} (figure 3.17). Interestingly, inhibition of Polo catalytic activity with BI2536 still led to a significant, albeit lower, activation of Mps1 in interphase cells (figure 3.17). These data confirm the ability of Polo in its active conformation to structurally depress PP1 activity and trigger Mps1 initial activation.

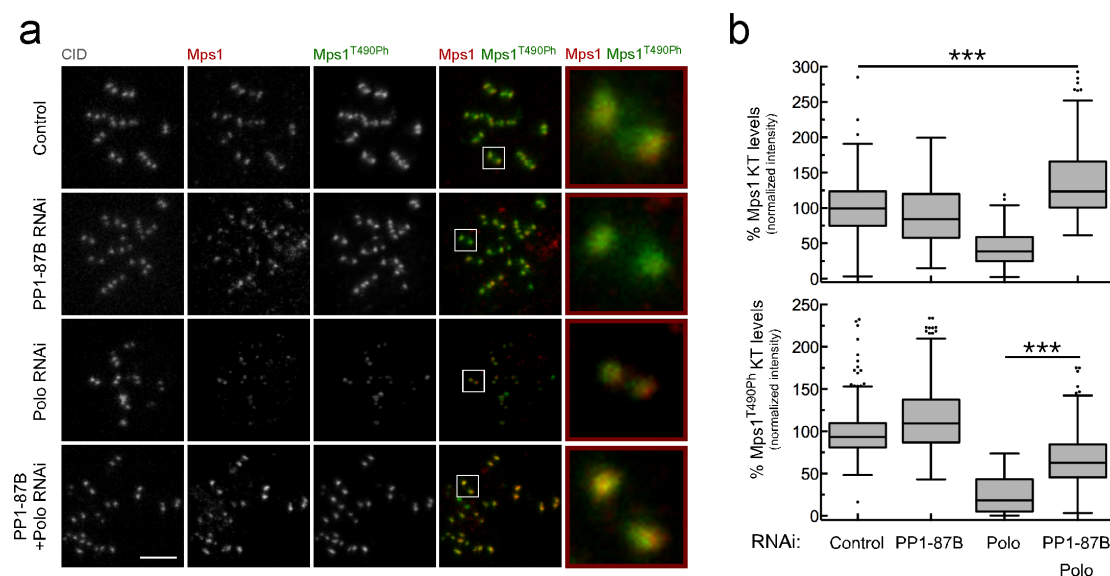


Figure 3.16 – Depletion of PP1-87B rescues Mps1 kinetochore recruitment and activity in the absence of Polo. (a,b) Representative immunofluorescence images (a) and interquartil representation (b) of Mps1^{T490Ph} levels at unattached kinetochores in S2 cells depleted of Polo, PP1-87B or both. Mps1^{T490Ph} fluorescence intensities were determined relative to CID signal. Cells were treated with MG132 (20 μ M) for 1 hour followed by a 2 hours period of colchicine (30 μ M) incubation. Mean values in control cells were set to 100%. Asterisks indicate that differences between mean ranks are statistically significant, *** $P < 0.0001$, Kruskal-Wallis, Dunn's multiple comparison test.

PP1-87B nuclear levels abruptly duplicate within 5 minutes before nuclear envelope breakdown (figure 3.18a,c). This is accompanied by a gradual accumulation of Polo in the nucleus throughout prophase (figure 3.18b,c). Since at this stage Polo is already in its active conformation (Bruinsma et al., 2012), this increment is likely important to ensure timely inhibition of PP1-87B activity and consequently, allow Mps1 to reach an intermediate state of activity as a result of unopposed autophosphorylation. This is then further potentiated by Polo mediated

phosphorylation of Mps1 T185, hence resulting in full Mps1 activation and robust SAC signalling at mitotic entry. During prometaphase, kinetochore accumulation of PP1 is limited by Aurora B-dependent phosphorylation of KNL1/Spc105 SSILK/RVSF motifs (figure 3.18d) (Liu et al., 2010).

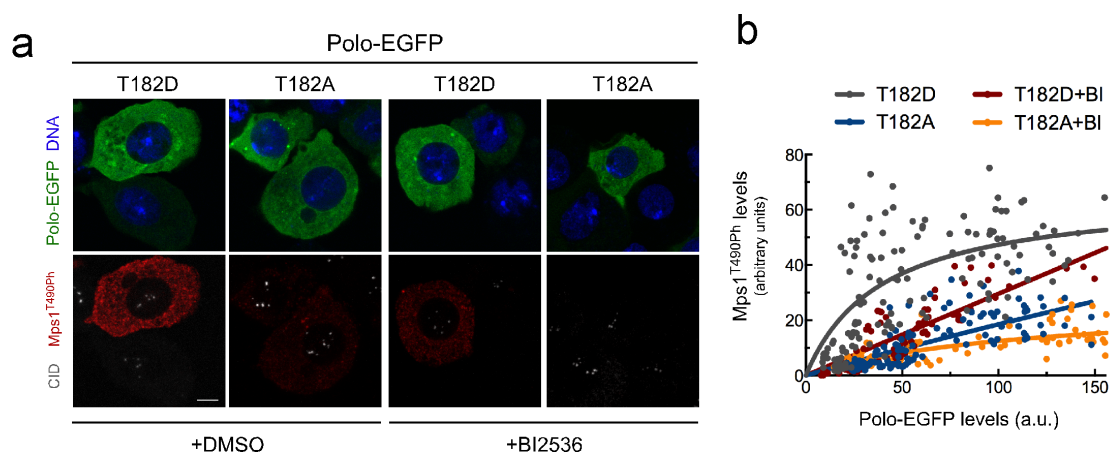


Figure 3.17 – Expression of Polo in its active conformation promotes Mps1 activation in interphase cells. (a,b) Representative immunofluorescence images (a) and quantifications (b) of Mps1^{T490Ph} levels in the cytoplasm of interphase S2 cells expressing Polo^{T182D}-EGFP or Polo^{T182A}-EGFP. To inhibit Polo kinase activity, BI2536 (100 nM) was added to cultured cells before inducing the expression of Polo phosphomutants. (b) Mps1^{T490Ph} cytosolic intensities were plotted against Polo-EGFP cytosolic signal. Scale bars, 5 μ m.

Nevertheless, immunofluorescence analysis revealed that PP1-87B is still able to localize to some extent at kinetochores of colchicine treated S2 cells (figure 3.18e), as previously observed by others in respect to human PP1- γ (Trinkle-Mulcahy et al., 2003; Saurin et al., 2014). Thus, active Polo at kinetochores is continuously required to contain PP1-87B activity at these sites and maintain SAC strength during prometaphase. Upon chromosome biorientation, the levels of active Polo at kinetochores start to decline (Conde et al., 2013), relieving the repression exerted on PP1-87B and ceasing to stimulate Mps1 T-loop autophosphorylation. In addition, decreased phosphorylation of KNL1/Spc105 by Aurora B results in an increment of PP1 levels at kinetochores. Because PP1 dephosphorylates Polo T-loop (Yamashiro, et al., 2008; Zitouni et al., 2014), the raising levels of active PP1 promote further Polo inactivation. This results in a rapid increase of phosphatase activity at kinetochores to levels that ensure Mps1 inactivation and prompt SAC silencing.

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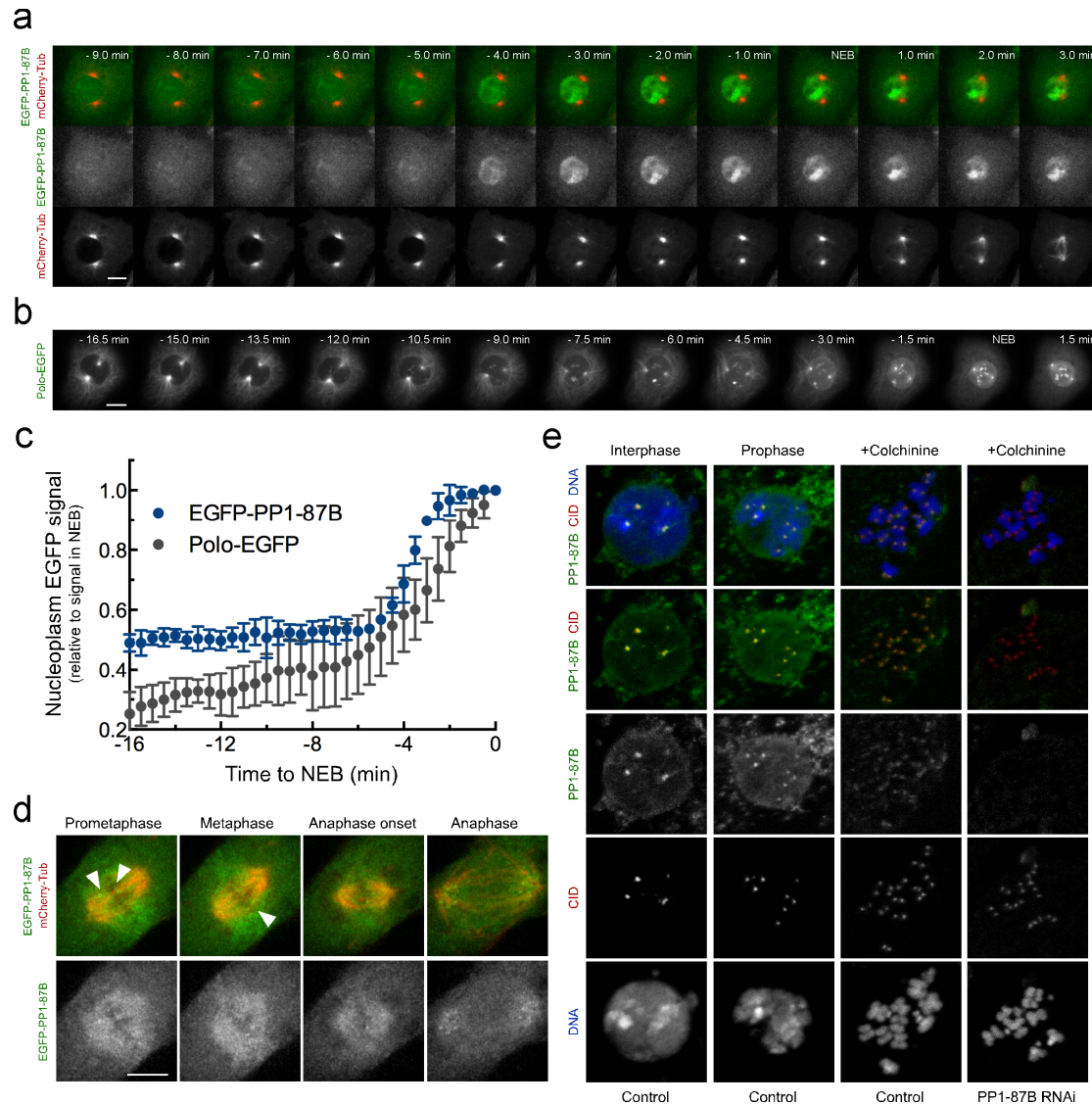


Figure 3.18 – PP1-87B dynamic localization through mitosis. (a-c) Representative prophase progression of S2 cells expressing EGFP-PP1-87B and mCherry-Tubulin (a) or Polo-EGFP (b) monitored by time-lapse microscopy. Selected stills of live-cell imaging are depicted and respective time shown in min. (c) Time-course quantification of EGFP-PP1-87B and Polo-EGFP nuclear levels. Data represent the mean $\pm$ s.d. of at least 8 cells for each experimental condition. EGFP-PP1-87B and Polo-EGFP fluorescence intensities in the nucleus were normalized to the value at nuclear envelope breakdown, NEB. (d) Representative images of S2 cells expressing EGFP-PP1-87B and mCherry-Tubulin in the indicated mitotic stages. Arrows indicate kinetochore localization of EGFP-PP1-87B. (e) Representative immunofluorescence images of S2 cells in the indicated conditions stained with antibodies against PP1-87B and CID, which was used as kinetochore reference. Scale bars, 5 μ m.

3.6. Concluding remarks

This study uncovered a new and likely conserved mechanism underlying regulation of PP1-87B/PP1- γ activity during mitosis. Polo in its active conformation binds and structurally inhibits PP1 activity. On the other hand, Polo activating phosphorylation is antagonistically regulated by PP1. This negative-feedback loop ensures efficient repression of PP1 activity in early mitosis and swift activation upon chromosome biorientation. Here we explored the importance of this regulatory module for SAC signalling. Our data support a model (figure 3.19) in which Polo-mediated inhibition of PP1 during prophase is essential to allow partial Mps1 activation.

We speculate that following Mps1 auto-phosphorylation, the kinase adopts a conformation that favors the phosphorylation of its N-terminal region by Polo. This further stimulates Mps1 autophosphorylation, hence resulting in full kinase activation even before its recruitment to kinetochores. The enrichment of PP1 at kinetochores and the decline of active Polo upon microtubule attachment shift the balance towards phosphatase activity. This promotes fast Mps1 inactivation not only by inactivating its enhancer kinase, but also through direct dephosphorylation of Mps1 T-loop. We propose that this two-pronged regulation of Mps1 by Polo defines a molecular switch that ensures that Mps1 activation occurs in a timely manner and perfectly coordinated with mitotic progression: it triggers robust Mps1 and consequently SAC activation at mitotic commitment and allows prompt Mps1 inactivation and SAC silencing following chromosome biorientation.

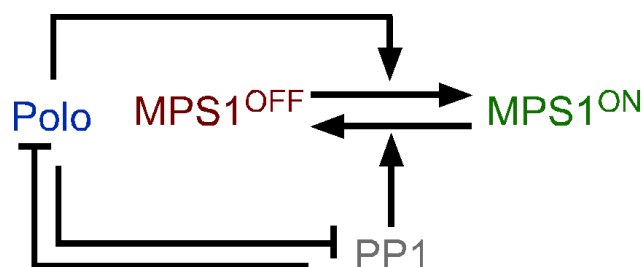


Figure 3.19 – Polo/Plk1-mediated inhibition of PP1 defines a molecular switch to control SAC signalling. Polo/Plk1 in its active conformation structurally inhibits PP1 activity allowing Mps1 activation, which is further potentiated by Polo phosphorylation of Mps1 N-terminal. Because Polo activating phosphorylation is also antagonized by PP1, this two-pronged regulation of Mps1 by Polo defines a molecular switch that ensures robust Mps1 activation on mitotic commitment and swift inactivation following chromosome biorientation.

CHAPTER 4

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