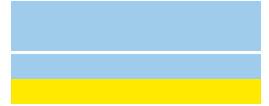


Unravelling molecular cues underlying the accuracy
of kinetochore-microtubule interactions and
chromosome segregation

Margarida Maria de Oliveira Moura

D
2023



Unravelling molecular cues underlying the accuracy
of kinetochore-microtubule interactions
and chromosome segregation

Margarida Maria de Oliveira Mo
ura



Margarida Maria de Oliveira Moura

**Unravelling molecular cues underlying the accuracy of
kinetochore-microtubule interactions and chromosome
segregation**

Tese de Candidatura ao grau de Doutor em
Biologia Molecular e Celular

Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar
da Universidade do Porto)

Orientador – Doutor Carlos Conde

Categoria – Investigador Principal

Afiliação – i3S – Instituto de Investigação e
Inovação em Saúde; IBMC – Instituto de
Biologia Molecular e Celular; ICBAS – Instituto
de Ciências Biomédicas Abel Salazar,
Universidade do Porto

Co-orientador – Professor Doutor Claudio
Sunkel

Categoria – Professor Catedrático

Afiliação – i3S – Instituto de Investigação e
Inovação em Saúde; IBMC – Instituto de
Biologia Molecular e Celular; ICBAS – Instituto
de Ciências Biomédicas Abel Salazar,
Universidade do Porto

PREFACE

The work presented in this thesis was performed at the Cell Division and Genomic Stability (CDGS) group at the Institute for Research and Innovation in Health (i3S/IBMC) at the University of Porto under the supervision of Dr. Carlos Conde and the co-supervision of Prof. Claudio Sunkel.



Margarida Moura is the recipient of a PhD grant (SFRH/BD/123306/2016) and a Research Grant (COVID/BD/152127/2021) from the Portuguese Foundation for Science and Technology (FCT). The research leading to the results presented in this thesis was supported by the following project grants: PTDC7BEX-BCM/1921/2014-PR041602 and IF/01755/2014/CP1241/CT0004, funded by National Funds through FCT; Norte-01-0145-FEDER-000029, funded by Norte Portugal Regional Operational Program (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER).



PUBLICATIONS

This thesis contains data from the following publications:

Moura, M.; Osswald, M.; Leça, N.; Barbosa, J.; Pereira, A.J.; Maiato, H.; Sunkel, C.E.; Conde, C.. "Protein phosphatase 1 inactivates Mps1 to ensure efficient spindle assembly checkpoint silencing". *eLife* 6 (2017) DOI: <http://dx.doi.org/10.7554/eLife.25366.001>

Moura, M.; Conde, C.. "Phosphatases in Mitosis: Roles and regulation". *Biomolecules* 9 2 (2019) DOI: 10.3390/biom9020055

ABSTRACT

Maintenance of genomic stability during cell division relies on the Spindle Assembly Checkpoint (SAC), a conserved signalling pathway that monitors kinetochore-microtubule attachment and delays anaphase onset until all chromosomes are correctly attached to spindle microtubules. Failure in switching off the SAC in a timely manner delays cells in metaphase, which increases the likelihood of merotelic attachments and cohesion fatigue. These errors often result in lagging chromatids and chromatin bridges in anaphase, well-established drivers of aneuploidy and chromosomal instability. Mps1 kinase is a well-established upstream regulator of the SAC. Mps1 becomes active during early mitosis as a result of its T-loop autophosphorylation. However, the mechanism controlling Mps1 inactivation once the SAC is satisfied remains less understood. Here, we demonstrate *in vitro* and in *Drosophila* S2 cells that the serine/threonine phosphatase PP1/PP1 α 87B dephosphorylates Mps1 T-loop to render the kinase inactive. Furthermore, we show that PP1 α 87B-mediated dephosphorylation of Mps1 occurs at kinetochores as well as at the cytoplasm, and the inactivation of both pools of Mps1 is required for timely SAC silencing. Thus, our findings contribute to understand how cells regulate Mps1 and expose a requirement for its kinetochore and cytosolic inactivation to allow prompt SAC silencing.

However, the mechanisms that prevent PP1/PP1 α 87B from prematurely inactivating Mps1 and silencing the SAC had remained ill-defined. Here, we report that Polo phosphorylates PP1 α 87B in its RVxF-binding pocket to limit the phosphatase ability to dephosphorylate SAC substrates in *Drosophila* S2 cells. The phosphorylation occurs at unattached/tensionless kinetochores and decreases as chromosomes congress to the metaphase plate. Replacing endogenous PP1 α 87B with a phospho-mimetic version prevents the inactivation of Mps1 causing a SAC-dependent metaphase delay. Conversely, expression of unphosphorylatable PP1 α 87B mutant weakens the SAC response of S2 cells in the presence of colchicine. Notably, tethering phospho-mimetic PP1 α 87B to kinetochores fails to restore timely SAC inactivation and anaphase onset in asynchronous cultured cells or accelerate mitotic exit when incubated with spindle poisons. These observations suggest that Polo-mediated phosphorylation of PP1 α 87B exerts its inhibitory effect independently of PP1 α 87B kinetochore localization. We also demonstrate that fine-tuning of PP1 α 87B activity by Polo during mitosis assumes critical relevance in proliferative tissues. *In vivo* analysis shows that, unlike for the wild-type phosphatase, expression of phospho-mutants PP1 α 87B transgenes fails to restore the fidelity of chromosome segregation and genome

stability in larval neuroblasts of *pp1 α 87b* mutants. These results uncover Polo-mediated phosphorylation of PP1 α 87B as a new strategy to regulate phosphatase activity during mitosis to ensure a robust and timely SAC and avert aneuploidy.

Altogether, the work presented in this thesis provides fundamental knowledge on the mechanisms controlling SAC signalling, which is instrumental to elucidate how dividing cells maintain genome stability.

SUMÁRIO

A manutenção da estabilidade genómica durante a divisão celular depende em parte do *Spindle Assembly Checkpoint* (SAC), uma via de sinalização conservada que monitoriza a ligação entre cinetocoros e microtúbulos e atrasa a transição para anáfase até que todos os cromossomas estejam corretamente ligados aos microtúbulos do fuso mitótico. Falhas em desligar o SAC atrasam as células em metáfase, o que aumenta a probabilidade de ocorrerem ligações merotélicas entre cinetocoros e microtúbulos e fadiga das coesinas. Estes erros resultam frequentemente em pontes de cromatina e cromossomas atrasados em anáfase, fatores identificados como indutores de aneuploidia e instabilidade cromossómica. A cinase Mps1 é um regulador crítico do SAC. Mps1 é ativado no início de mitose como resultado da auto-fosforilação do seu *T-loop*. No entanto, o mecanismo responsável pela inativação de Mps1 no momento de silenciamento do SAC permanece desconhecido. Neste trabalho, mostramos *in vitro* e em células S2 de *Drosophila*, que a fosfatase de serina/treonina PP1/PP1 α 87B desfosforila o *T-loop* de Mps1 para inativar a cinase. Mostramos ainda que a desfosforilação de Mps1 mediada por PP1 α 87B ocorre tanto nos cinetocoros como no citoplasma, e que a inativação de ambas as populações é necessária para um rápido silenciamento do SAC. Assim, os nossos resultados contribuem para melhor perceber como Mps1 é regulado nas células e sugerem que a inativação de Mps1 citosólico é necessária para permitir um silenciamento atempado do SAC.

Contudo, os mecanismos responsáveis por prevenir o silenciamento prematuro do SAC permanecem indefinidos. Aqui mostramos que Polo fosforila PP1 α 87B no seu local de ligação a RVxF para limitar a capacidade desta desfosforilar os seus substratos mitóticos. A fosforilação é prontamente detetada em cinetocoros desligados/sem tensão de células S2 de *Drosophila* e diminui à medida que os cromossomas efetuam a congressão para a placa metafásica. A Substituição de PP1 α 87B endógena por uma versão fosfo-mimética previne a inativação de Mps1, o que causa um atraso, dependente do SAC, em metáfase. Por outro lado, a expressão do mutante não-fosforilável da PP1 α 87B enfraquece significativamente a resposta do SAC de células S2 tratadas com colchicina. De notar, fundir a versão fosfo-mimética da PP1 α 87B com o cinetocoro não reverte o atraso no silenciamento do SAC e início de anáfase resultantes da depleção de PP1 α 87B em células assíncronas ou na aceleração da saída de mitose em células incubadas com fármacos que afetam o fuso mitótico. Estas observações sugerem que a fosforilação de PP1 α 87B mediada por Polo exerce o seu efeito inibitório independentemente da localização de PP1 α 87B no cinetocoro. Demonstrámos ainda que a regulação da atividade de PP1 α 87B

por Polo em mitose assume uma relevância crucial em tecidos proliferativos. Análises *in vivo* mostram que, ao contrário da fosfatase *wild-type*, a expressão de transgenes de PP1 α 87B mutantes para a fosforilação em estudo não é suficiente para a recuperação da fidelidade da segregação cromossômica e estabilidade genômica em neuroblastos de larvas de mutantes *pp1 α 87b*. Estes resultados permitem descobrir uma nova estratégia de regulação da atividade de PP1 de modo a assegurar uma sinalização atempada do SAC e impedir aneuploidia.

Coletivamente, o trabalho apresentado nesta tese providencia conhecimento fundamental sobre os mecanismos que controlam o SAC, conhecimento esse que é absolutamente necessário para elucidar como é que células em divisão conseguem manter a estabilidade genômica.

TABLE OF CONTENTS

PREFACE	III
PUBLICATIONS	V
ABSTRACT	VII
SUMÁRIO	IX
TABLE OF CONTENTS	XI
FIGURE INDEX.....	XIV
TABLE INDEX	XVIII
ABBREVIATIONS	XIX

GENERAL INTRODUCTION	1
Mitosis and the Spindle Assembly Checkpoint.....	1
CELL CYCLE OVERVIEW.....	3
Cell Cycle Regulation	4
SPINDLE ASSEMBLY CHECKPOINT.....	7
Phospho-regulation of MCC assembly	8
Phosphatases in Mitosis: Roles and Regulation.....	13
INTRODUCTION	15
Serine/Threonine Phosphatases	15
Protein Phosphatase 1	16
Protein Phosphatase 2A	19
Tyrosine Phosphatases	22
Cdc25 Phosphatases.....	23
ROLES OF PROTEIN PHOSPHATASES IN MITOSIS	24
Mitotic Entry: Cdc25 Phosphatases Set Up the Commitment	25
Sister Chromatid Cohesion: PP2A:B56 Holds Them Together	29
To Build a Spindle: A PP1, PP2A, PP4 and PP6 Groundwork.....	32

Kinetochore-Microtubule Attachments: PP1 and PP2A:B56 Keep Them Stable ...	37
Silencing the Spindle Assembly Checkpoint: PP1 and PP2A:B56 Shut It Up.....	44
Mitotic Exit: PP1 and PP2A:B55 Clear the Way.....	49
SPATIOTEMPORAL CONTROL OF PHOSPHATASE ACTIVITIES.....	53
CONCLUDING REMARKS AND FUTURE DIRECTIONS.....	55
Aim of the work.....	57
MATERIALS AND METHODS	61
S2 CELL CULTURES, RNAI AND DRUG TREATMENTS	63
CONSTRUCTS AND S2 CELL TRANSFECTION	64
FLY STOCKS.....	66
RECOMBINANT PROTEINS	67
S2 CELL LYSATES, PULL-DOWNS, IMMUNOPRECIPITATION AND WESTERN BLOTTING	68
<i>IN VITRO</i> KINASE AND PHOSPHATASE ASSAYS	69
IMMUNOFLUORESCENCE ANALYSIS.....	70
LIVE IMAGING.....	72
ANALYSIS OF MICROTUBULE DYNAMICS BY INDUCIBLE FLUORESCENT SPECKLE MICROSCOPY	72
FRAP EXPERIMENTS.....	73
STATISTICAL AND KINETICS ANALYSIS	73
SUPPLEMENTARY INFORMATION	73
EXPERIMENTAL WORK.....	75
CHAPTER I	75
INTRODUCTION	77
RESULTS	78
Depletion of PP1 α 87B results in a metaphase delay with stable kinetochore- microtubules attachments	78
PP1 α 87B antagonizes Mps1 T-loop autophosphorylation at kinetochores.....	82
PP1 α 87B/PP1 γ dephosphorylates Mps1 T-loop	85

PP1-mediated dephosphorylation of Mps1 T-loop inactivates the kinase and promotes SAC silencing	89
Preventing PP1-mediated inactivation of cytosolic Mps1 delays mitotic exit	91
Regulation of PP1 activity during mitosis	93
DISCUSSION.....	94
ANNEXES	99
Figure I.4 – Supplementary figure 1	99
Figure I.7 – Supplementary figure 1	100
Figure I.12 – Supplementary figure 1	100
CHAPTER II	101
INTRODUCTION	103
RESULTS	105
Cdk1 and Aurora B-mediated mechanisms do not fully account for the control of PP1 α 87B activity during early mitosis	105
Polo phosphorylates PP1 α 87B at unattached/tensionless kinetochores	109
Phosphorylation of PP1 α 87B T286 is required for robust SAC signalling.....	112
Mimicking constitutive phosphorylation of PP1 α 87B T286 delays SAC silencing	115
Kinetochores-tethering of PP1 α 87B does not bypass the control exerted by T286 phosphorylation	116
Phospho-regulation of PP1 α 87B on T286 is required for genome stability <i>in vivo</i>	119
DISCUSSION.....	122
ANNEXES	128
Figure II.2 – Supplementary figure 1	128
Figure II.4 – Supplementary figure 1	128
GENERAL DISCUSSION	129
REFERENCES.....	137

FIGURE INDEX

GENERAL INTRODUCTION

Figure 1 – Cell cycle.....	4
Figure 2 – Inhibition of APC/C by the Mitotic Checkpoint Complex prevents mitotic exit ...	8
Figure 3 – MCC formation at unattached kinetochores.....	11
Figure 4 – Structure of PP1 α catalytic subunit and its interaction with the regulatory subunit Repo-Man	17
Figure 5 – Structure of PP2A α holoenzymes.....	20
Figure 6 – Molecular circuitry controlling mitotic entry	27
Figure 7 – PP2A:B56 maintains centromeric cohesion until anaphase onset.....	32
Figure 8 – PP1 prevents premature separation of centrosomes.....	34
Figure 9 – Kinetochores PP2A:B56 and PP1 promote the stability of microtubule attachments and efficient Spindle Assembly Checkpoint silencing in animal cells.....	44
Figure 10 – PP2A:B55 and PP1 activities orchestrate mitotic exit.....	51

EXPERIMENTAL WORK

Chapter I | PP1 inactivates Mps1 to ensure efficient Spindle Assembly Checkpoint silencing

Figure I.1 – Mad1-EGFP localization pattern during mitosis in S2 cells depleted of PP1 α 87B	79
Figure I.2 – Depletion of PP1 α 87B results in a SAC-dependent metaphase delay despite stable kinetochore-microtubule attachments.....	80
Figure I.3 – Analysis of microtubule turnover in control and PP1 α 87B depleted metaphase cells by speckle contrast fade-out	81
Figure I.4 – PP1 α 87B antagonizes Mps1 T-loop autophosphorylation at kinetochores of prometaphase and metaphase cells	82
Figure I.5 – PP1 α 87B antagonizes the T-loop autophosphorylation of Mps1 that remains associated with metaphase kinetochores stably attached to spindle microtubules	83

Figure I.6 – Depletion of Sds22 affects Mps1 T-loop autophosphorylation.....	84
Figure I.7 – PP1 α 87B/PP1 γ dephosphorylates Mps1 T-loop <i>in vitro</i> and <i>in vivo</i>	86
Figure I.8 – Specificity of Mps1 T-loop dephosphorylation <i>in vitro</i>	87
Figure I.9 – Mps1 ^{WT} and Mps1 ^{K231A/F234A} dynamics at kinetochores	88
Figure I.10 – PP1 α 87B-mediated dephosphorylation of Mps1 T-loop renders the kinase inactive and is required for timely SAC silencing	89
Figure I.11 – Stable attachments and Mad1 levels at metaphase kinetochores of S2 cells expressing EGFP-Mps1 ^{WT} and EGFP-Mps1 ^{K231A/F234A}	91
Figure I.12 – PP1-87B-mediated inactivation of cytosolic Mps1 is required for efficient SAC silencing and timely anaphase onset	92
Figure I.13 – Regulation of PP1 α 87B activity during mitosis	93
Figure I.14 – A model for regulation of Mps1 activation/inactivation in mitosis.....	97

Chapter I – Supplementary figures

Figure I.4 – Supplementary figure 1 – Validation of a phospho-specific antibody against <i>Drosophila</i> Mps1 activating T-loop autophosphorylation	99
Figure I.7 – Supplementary figure 1 – Western blotting analysis of S2 cells expressing EGFP-Mps1 ^{WT} and EGFP-Mps1 ^{K231A/F234A}	100
Figure I.12 – Supplementary figure 1 – Western blotting analysis of EGFP-Mps1 ^{WT} , EGFP-Mps1 ^{K231A/F234A} and EGFP-Mps1 ^{K231A/F234A/T490A} expression in CENP C depleted S2 cells..	100

Chapter II | Polo regulates PP1 α 87B to ensure timely Spindle Assembly Checkpoint and genome stability

Figure II. 1 – Forced accumulation of PP1 α 87B at the kinetochore does not promote premature anaphase onset	106
Figure II. 2 – The Spindle Assembly Checkpoint is silenced under high CyclinB:Cdk1 activity.	106
Figure II. 3 – PP1 α 87B-mediated dephosphorylation of Mps1 T-loop in metaphase occurs in the presence of Cyclin B or Cyclin A:Cdk1 activity.....	108

Figure II. 4 – PP1 α 87B-dependent dephosphorylation of Mad1 ^{T726} in metaphase occurs in the presence of Cyclin B or Cyclin A:Cdk1 activity	109
Figure II. 5 – Polo phosphorylates PP1 α 87B at unattached kinetochores.....	110
Figure II. 6 – PP1 α 87B T286 phosphorylation decreases with mitotic progression.....	111
Figure II. 7 – Expression of constitutively active Polo maintains PP1 α 87B T286 phosphorylated at metaphase kinetochores	112
Figure II. 8 – Preventing PP1 α 87B T286 phosphorylation decreases Mps1 T-loop phosphorylation at prometaphase kinetochores	113
Figure II. 9 – Mimicking constitutive phosphorylation of PP1 α 87B T286 extends the metaphase duration	114
Figure II. 10 – Preventing the phosphorylation of PP1 α 87B T286 weakens MPS1 activation and SAC signalling at unattached kinetochores.....	115
Figure II. 11 – Mimicking constitutive phosphorylation of PP1 α 87B T286 impairs Mps1 T-loop dephosphorylation at metaphase kinetochores.....	116
Figure II. 12 – Mimicking constitutive T286 phosphorylation on kinetochore-tethered PP1 α 87B impairs Mps1 T-loop dephosphorylation at metaphase kinetochores.....	117
Figure II. 13 – Mimicking constitutive T286 phosphorylation on kinetochore-tethered PP1 α 87B impairs Mad1 T726 dephosphorylation at metaphase kinetochores	118
Figure II. 14 – Mimicking constitutive T286 phosphorylation on kinetochore-tethered PP1 α 87B delays S2 cells in metaphase	118
Figure II. 15 – Kinetochore-tethered PP1 α 87B harbouring a T286A mutation weakens the SAC	119
Figure II. 16 – Phospho-regulation of PP1 α 87B T286 is required to control Mps1 T-loop phosphorylation in <i>Drosophila</i> neuroblasts	120
Figure II. 17 – Phospho-regulation of PP1 α 87B T286 is required for faithful chromosome segregation and genome stability in <i>Drosophila</i> neuroblasts.....	121
Figure II. 18 – Polo inhibits PP1 to ensure timely Spindle Assembly Checkpoint signalling	126

Chapter II – Supplementary figures

Figure II.2 – Supplementary figure 1 – A Cdk consensus site can be found in the majority of PP1 orthologues but not in *D. melanogaster* PP1 α 87B 128

Figure II.4 – Supplementary figure 1 – Supplementary figure 1 – Mad1^{T726Ph} antibody validation in *D. melanogaster* 128

GENERAL DISCUSSION

Figure 11 – Phospho-regulation of the Spindle Assembly Checkpoint 135

TABLE INDEX

MATERIALS AND METHODS

Table 1 – List of primers used for dsRNA synthesis	63
Table 2 – List of primers used for cloning	65
Table 3 – <i>Drosophila</i> stocks used for immunofluorescence analysis and live imaging.....	66
Table 4 – List of antibodies used for immunofluorescence and western blotting	71

ABBREVIATIONS

AO	Anaphase Onset
APC/C	Anaphase Promoter Complex / Cyclosome
Bub	Budding uninhibited by benzimidazole
BubR1	Bub1-Related
C-Mad2	Closed, active, Mad2
Cdk	Cyclin-dependent kinase
CDS	Coding Sequence
CENP	Centromeric protein
CH	Calponin Homology
CID	Centromere Identifier
DAPI	4,6-diamidino-2-phenylindole
DiFMUP	6,8-Difluoro-4-Methylumbelliferyl Phosphate
DNA	Desoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal Bovine Serum
FRAP	Fluorescence Recovery After Photobleaching
G1	Gap phase 1
G2	Gap phase 2
gDNA	Genomic Desoxyribonucleic Acid
GFP	Green Fluorescent Protein
GST	Glutathione S-transferase
HA	Human influenza hemagglutinin
ISI	Inducible Speckle Imaging

KD	Kinase Dead
KMN	Kn11/Mis12/Ndc80 Complex
Kn11	Kinetochores null 1
KT	Kinetochores
M	Mitosis + Cytokinesis
Mad	Mitotic-arrest deficient
MBP	Maltose-binding Protein
MCC	Mitotic Checkpoint Complex
MELT	Methionine-Glutamate-Leucine-Threonine
Mps1	Monopolar Spindle 1
MT	Microtubule
Ndc80	Nuclear division cycle 80
NDG	Non-degradable
NEB	Nuclear Envelope Breakdown
O-Mad2	Open, inactive, Mad2
PBS	Phosphate Buffered Saline
PBST20	PBS + 0.05% Tween20
RE	Restriction Enzyme
RIPPO	Regulatory Interactor of Protein Phosphatase One
RNAi	RNA interference
Rod	Rough Deal
ROI	Region of Interest
RPE1	Retinal Pigment Epithelial 1 cells
RZZ	Rod/Zw10/Zwisch Complex
S	Synthesis phase
SAC	Spindle Assembly Checkpoint
SD	Standard Deviation
Ska	Spindle- and Kinetochores-Associated

Spc105	Spindle pole body component 105
TTK	Threonine/Tyrosine Kinase
UTR	Untranslated Region
WT	Wild Type
ZW10	Zeste White

GENERAL INTRODUCTION

Mitosis and the Spindle Assembly Checkpoint

CELL CYCLE OVERVIEW

In 1839, Scheiden and Schwann published the *Cell Theory*, which establish the idea that the cell is the fundamental building block of life. Later, in 1855, with the contribution of Rudolf Virchow's work, who is responsible for the famous aphorism *omnis cellula e cellula* [all cells arise from other cells], the idea gained widespread acceptance.

Cell division can be responsible for either reproduction and the formation of new organisms (for unicellular systems) or for tissue proliferation and renewal (in multicellular systems). Additionally, the division of a cell into two new ones can occur symmetrically (formation of two identical cells, i.e. proliferation of a certain cell type) or asymmetrically (formation of a specialized cell and renewal of the mother cell, i.e. differentiation). In either of the cases, the genetic content of the mother cell must be segregated equally into the daughter cells.

The life cycle of a eukaryotic cell comprises 4 phases: G1 and G2, gap phases in which cell growth and protein synthesis take place; S phase, in which DNA replication is achieved; and M phase, that encompasses Mitosis and Cytokinesis, which respectively correspond to the division of the genetic material and the division of the cytoplasm of the cell in two. The first 3 phases are collectively designated as interphase and constitute the larger portion of the cell cycle. In 1879, Walther Flemming observed and described the process of nuclear division and used the term mitosis (from the Greek *mitos*: threads) to name it. Flemming described, in a chronological manner, the behavior of "threads" (chromosomes) during division. Additionally, with his work on epidermal cells from salamander larvae, Flemming also anticipated that chromosomes should divide along their longitudinal axis so that each daughter cell receives an equal copy of the genomic content.

The mitotic process starts with the condensation of the chromatin into highly-packed chromosomes and the separation of the previously duplicated centrosomes to opposite poles where microtubule nucleation and polymerization starts to take place. This phase, named prophase, is ended by the nuclear envelope breakdown (NEB), giving place to the prometaphase in which microtubules start to interact with the chromosomes through proteinaceous structures called kinetochores, present in each sister chromatid, to promote its movement towards the equator of the cell in a process called chromosome congression. Once all chromosomes achieve biorientation (each chromatid bound to microtubules of opposite spindle poles) and position at the cell equator, the cell reaches metaphase. During metaphase, the kinetochore-microtubule interactions are further stabilized, increasing the interkinetochore tension and the cell prepares to segregate the sister chromatids during anaphase. In anaphase, the protein complex Cohesin that glues the sister chromatids together is cleaved promoting their separation and sister chromatids are then pulled apart

towards the spindle poles. Once the chromatids reach the two poles, the nuclear envelope starts to reassemble and the chromatin decondenses, during what is called telophase. The process of nuclear division is accompanied by the division of the cytoplasm, which starts during anaphase with the formation of the contractile ring. The ring contracts along with mitotic progression and the final abscission occurs simultaneously to telophase (Figure 1) (O'Connor, 2008).

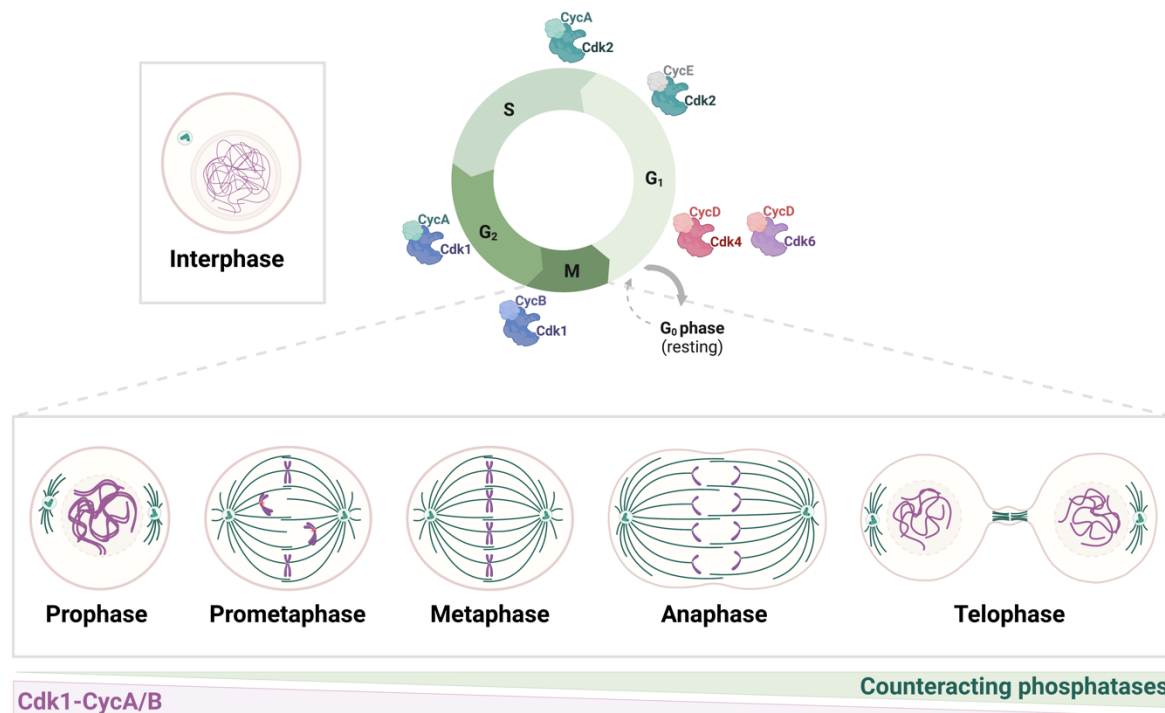


Figure 1 – Cell cycle. The cell division process is composed of four phases: G₁, S, G₂ and M. S is the phase where the DNA replication takes place and M includes Mitosis and Cytokinesis, which correspond to the division of the genetic material and of the cytoplasm of the cell, respectively. G₁ and G₂ are gap phases between the previous two where cell growth and protein synthesis take place. Non-dividing cells can enter a quiescent state named G₀, in which the cell is waiting for appropriate conditions to resume G₁ phase. The Cdk complexes necessary for the regulation of each phase are shown. Mitosis comprises five stages: prophase, prometaphase, metaphase, anaphase and telophase. In prophase, DNA condensation becomes visible and centrosomes begin to separate; after nuclear envelope breakdown, the cell progresses to prometaphase and chromosomes start to interact with spindle microtubules, moving towards the cell equator; when the cell reaches metaphase, all chromosomes are aligned at the metaphase plate and kinetochore-microtubule are correctly attached; only after this configuration is achieved, the cell can progress to anaphase where the sister chromatids are torn apart; 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. Figure created with Biorender.com.

Cell Cycle Regulation

The cell cycle is a unidirectional signalling pathway, meaning that once initiated one phase, a cell cannot rewind to a previous one. The transitions between two phases are accomplished in a switch-like manner as a result of the activation/inactivation of different Cyclin-dependent kinases (Cdk), which activity, as the name indicates, is dependent on the

presence and association to Cyclins (Figure 1). The activation status of these kinases along the cell cycle is tightly controlled by mechanisms of protein-protein interaction, phosphorylation and ubiquitin-dependent proteasome degradation (Poon, 2016).

The progression of the cell cycle is regulated by surveillance mechanisms named checkpoints, to ensure proper cell division so that the genomic integrity is safeguarded. Checkpoints are mechanisms that halt the progression to the next step of a pathway until all conditions for the transition are verified. Cell cycle checkpoints typically entail three components: a sensor that monitors for the presence of defects, a transducer that transmits and amplifies the signal and an effector that blocks the transition (Poon, 2016).

When a cell in G₁ lacks external mitogenic signals to continue to S phase, it will enter the quiescent state G₀. The cell can only pass the Restriction point, stay in the cell cycle and make the transition to S phase, if the growth factors received are sufficient. Once the cell is stimulated to progress the cell cycle, Cyclin D is synthesized, which triggers the formation of active Cdk4/6:Cyclin D complexes (Figure 1). This will in turn allow the phosphorylation of several substrates important for the transcription of genes necessary for the S phase. Cyclin A/E are two of these genes and, in association with Cdk2, replace Cdk4/6:Cyclin D in the phosphorylation of the referred substrates to amplify the signal. (Chen et al, 2009; Henley and Dick, 2012). The G₁ DNA Damage Checkpoint is responsible for monitoring the presence of DNA damage and pause the cell cycle until the DNA can be repaired. DNA damage triggers the activation of the protein kinases ATM and ATR, which initiate a signalling cascade that culminates in the activation of the transcriptional factor p53, which in turn leads to the formation of the Cdk inhibitors p21^{CIP1/WAF1} and p16^{INK4A}. The inhibitors then bind Cdk2:Cyclin A/E and Cdk4/6:Cyclin D, shutting off the above-mentioned signalling pathway. In the absence of DNA damage, a negative feedback loop is responsible for the suppression of p53: one of its transcriptional targets, MDM2, inhibits p53 activity, promotes its exclusion from the nucleus and its ubiquitin-dependent proteasome degradation (Poon, 2016).

In the transition to S phase, Cdk2:Cyclin A/E phosphorylates components from the pre-replication complex (pre-RC), facilitating the activation of the helicase and consequent recruitment of the DNA synthesis machinery (Poon, 2016). Cdk2:Cyclin A/E is also responsible for the coordination, at least in part, of DNA replication initiation and the centrosome cycle (Hinchcliffe and Sluder, 2001). Once the genome has been duplicated, Cyclin E is targeted for proteasome degradation and Cdk2:Cyclin A phosphorylation of specific substrates promotes the dissociation of the origin recognition complexes (ORC) from the chromatin and diminishes the transcription of genes involved in S phase control (Poon, 2016; Dynlacht et. al, 1997; Krek et al, 1995). The Replication Checkpoint monitors

the replication process to ensure the DNA duplication is achieved without errors. Stalled replication forks generate signals that halt cell cycle to provide the cell time to restart or repair the stalled replication fork. This checkpoint relies on the activity of the kinase ATR (Friedel et al, 2009). ATR is recruited to replication forks stopped by insufficient nucleotides supply or lesions and obstacles on DNA. There, it activates a signalling cascade that culminates in the inactivation of Cdk:Cyclin complexes involved in both DNA replication and mitotic entry, thus resulting in cell cycle pause.

For a cell to enter mitosis, the key step is the activation of Cdk1, which despite being present in the cell at constant levels during the cell cycle needs to associate with Cyclins (A and B) and (de)phosphorylation events in order to become active. In mammalian, there are two types of Cyclin A (A1 and A2) and three types of Cyclin B (B1, B2 and B3), which expression pattern is different in somatic and germline cells. Cyclin A is expressed from late G₁ and is degraded during mitosis and Cyclin B starts to be expressed later, in the G₂-M transition and is degraded at mitotic exit. These oscillations are regulated at transcription and degradation levels (Fung and Poon, 2005). The regulation of Cdk1:Cyclin B activity at mitotic entry is dependent on the opposing activities of the kinases MYT1 and WEE1 and the phosphatase Cdc25. The mitotic kinases Plk1 and Aurora A also collaborate to increase Cdk1:Cyclin B activity (see section “Mitotic Entry: Cdc25 Phosphatases Set up the Commitment” in the chapter “Phosphatases in Mitosis: Roles and Regulation”). Similarly to the G₁ DNA Damage Checkpoint, the G₂ DNA Damage Checkpoint also involves the activation of the ATM and ATR kinases. This activation results in the activation of WEE1 and inactivation of Cdc25, leading to Cdk1 inactivation to block mitotic entry (Chen and Poon, 2008).

For a cell to exit mitosis, the key steps are the activation of the Anaphase Promoting Complex or Cyclosome (APC/C), and the degradation of its substrates, including Securin and Cyclin B. During mitosis, Cdc20 binds to the APC/C to activate the complex, which is further stimulated by Cdk1:Cyclin B phosphorylation. Although Cdk1:Cyclin B is promoting its own destruction by phosphorylating the APC/C^{Cdc20}, it is also ensuring that the activation of the complex is only possible after mitotic entry. However, the APC/C^{Cdc20} cannot be activated in the early stages of mitosis, otherwise the irreversible transition to anaphase could occur without proper chromosome alignment and result in segregation errors leading to aneuploidy. To ensure the APC/C is not activated prematurely, the Mitotic Checkpoint or Spindle Assembly Checkpoint (SAC) monitors the status of kinetochore-microtubule attachment and inhibits the transition to anaphase until all chromosomes are correctly attached (Poon, 2016).

SPINDLE ASSEMBLY CHECKPOINT

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 genetic screens in *Saccharomyces cerevisiae* identified most of the key components, namely Mitotic-arrest deficient (Mad) and Budding uninhibited by benzimidazole (Bub) genes (Li and Murray, 1991; Hoyt et al, 1991). These genes are conserved in all eukaryotes (Musacchio and Salmon, 2007), with exception for Mad3. The higher eukaryotes counterpart Bub-Related 1 (BubR1), although it does display homology with Mad3 in the N-terminal sequence, it also contains a C-terminal pseudokinase domain, which suggests an evolutionary relation to Bub1 (Taylor et al, 1998; Suijkerbuijk et al, 2012a). An important hallmark for the comprehension of the SAC mechanism was the discovery that anaphase onset in vertebrate cells can be 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 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”.

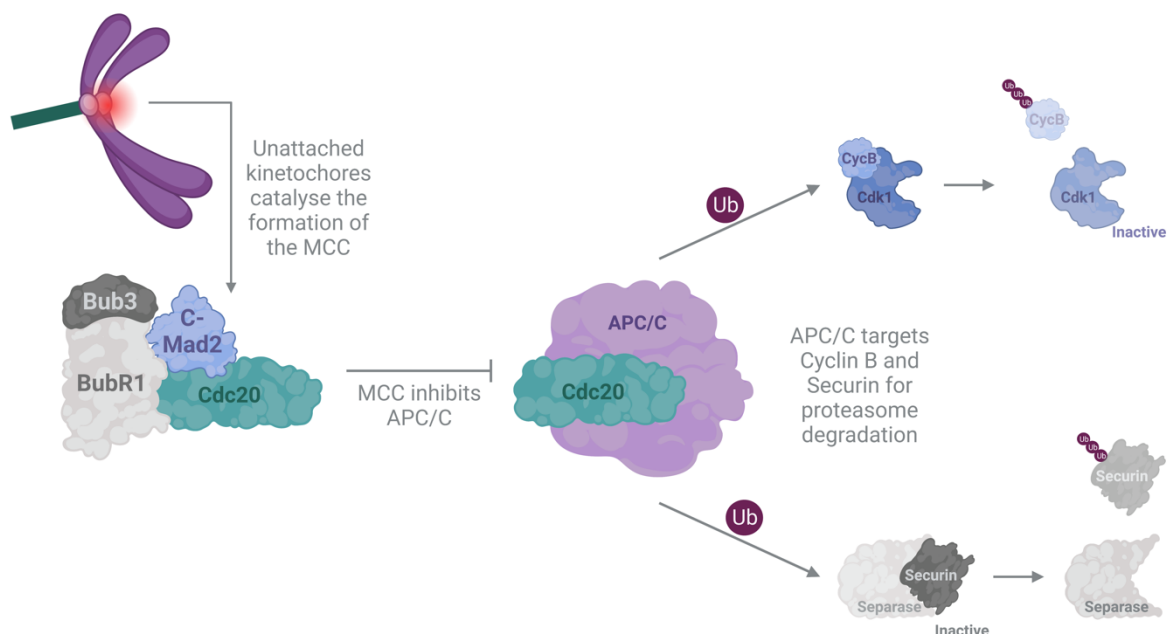


Figure 2 – Inhibition of APC/C by the Mitotic Checkpoint Complex prevents mitotic exit. Unattached kinetochores serve as catalytic platforms for the formation of the Mitotic Checkpoint Complex (MCC), a tetrameric complex composed by Bub3, BubR1, C-Mad2 and Cdc20. MCC binds to and inhibits the APC/C thus restraining its ubiquitin-ligase activity. When APC/C becomes active, it mediates the poly-ubiquitination and proteasome targeting of Cyclin B and Securin, which results, respectively, in the inactivation of Cdk1 and activation of Separase. Both these events are essential for mitotic progression: Cdk1 is responsible for the signalling necessary to maintain cells in mitosis and Separase cleaves the cohesion between sister chromatids. Figure created with Biorender.com.

The ultimate goal of the mitotic checkpoint is the formation of a diffusible inhibitory signal that delays anaphase onset until all kinetochores become attached to spindle microtubules. This inhibitory signal consists of a tetrameric protein complex named Mitotic Checkpoint Complex (MCC) and comprises Bub3, BubR1, Mad2 and Cdc20 (Figure 2). The MCC is catalysed at unattached kinetochores and targets the APC/C inhibiting its E3 ubiquitin ligase activity. SAC silencing terminates MCC assembly, enables Cdc20-dependent activation of the APC/C and, consequently, poly-ubiquitination of Cyclin B and Securin, which targets these proteins for proteasome-mediated degradation. As a result of Cyclin B destruction, Cdk1 is inactivated, while Securin degradation activates the Separase-mediated cleavage of Scc1, a subunit of the Cohesin complex that holds sister chromatids together. Thus, destruction of these mitotic substrates triggers anaphase onset and mitotic exit (Figure 2) (Joglekar, 2016; Musacchio, 2015).

Phospho-regulation of MCC assembly

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 phospho-epitope, present at kinetochores lacking tension (Gorbsky and Ricketts, 1993), led to the initial suggestion that phospho-regulation 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 (Saurin, 2018; Lara-Gonzalez et al, 2021; McAinsh and Kops, 2023).

The serine/threonine kinase Monopolar spindle 1 (Mps1, or TTK in humans) lays on the top of the SAC signalling cascade and acts in several steps along the pathway, namely in the recruitment of Mad1, Mad2, BubR1, Bub1 and Bub3 to unattached kinetochores (Lan and Cleveland, 2010). Mps1 is rapidly activated slightly before nuclear envelope breakdown (Kuijt et al, 2020), which is important for the assembly of pre-mitotic MCC at nuclear pores

(Maciejowski et al, 2010). However, it is right after mitotic entry that Mps1 concentrates on the outer kinetochore and becomes fully activated by *trans*-auto-phosphorylation of its activating T-loop (Howell et al, 2004; Kang et al, 2007; Santaguida et al, 2010; Hewitt et al, 2010). Mps1 binds to the KMN network (Knl1, Mis12 complex and Ndc80 complex) component Ndc80, which is also the main microtubule-binding component of kinetochores, in an 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).

When bound to Ndc80, Mps1 is ideally positioned to phosphorylate Knl1 on its MELT (Methionine-Glutamine-Leucine-Threonine) motifs, which are then able to bind the Bub3:Bub1 complex (Figure 3) (London et al, 2012; Shepperd et al, 2012; Yamagishi et al, 2012). Interestingly, Plk1, another essential mitotic kinase, is also able to phosphorylate, though in a lesser extent, the MELT motifs of Knl1 in *C. elegans* and mammalian cells (von Schubert et al, 2015; Espeut et al, 2015). The number of MELT motifs per Knl1 molecule diverges from one organism to the other, varying from 2 to 27, all distributed across the disordered N-terminal region of the protein (Vleugel et al, 2012). Systematic analyses of the various Knl1 MELT motifs suggest that these may vary in the affinity for Bub1:Bub3 (Moyle et al, 2014; Vleugel et al, 2013; Zhang et al, 2014; Krenn et al, 2014; Roy et al, 2020), possibly allowing to fine-tune the stoichiometry of Bub3:Bub1 complexes per Knl1 molecule in a way responsive to the microtubule attachment status (Etemad et al, 2019). The Bub3:Bub1 dimers recruited to the kinetochore serve, in turn, as a platform for the localization of additional SAC proteins including the Bub3:BubR1 dimers, the Mad1:Mad2 tetramers and Cdc20 (Figure 3) (Joglekar, 2016; Musacchio, 2015; Pachis and Kops, 2018). Interestingly, *Drosophila* Spc105 (Knl1 orthologue) has specific MELT-like motifs that lack the phosphorylatable threonine, hence making Mps1 dispensable for kinetochore recruitment of Bub3:Bub1 and Bub3:BubR1 complexes in flies. Mps1 activity is nevertheless required for BubR1 hyperphosphorylation, which seems to facilitate BubR1:Cdc20 association and consequently efficient MCC formation (Conde et al, 2013).

Mad1 and Mad2 form a hetero-tetrameric complex throughout the cell cycle. The complex localizes at nuclear pore complexes during interphase (Campbell et al, 2001; Lee, S.H. et al, 2008; Lince-Faria et al, 2009), where it may be important for the catalysis of MCC in interphase (Rodriguez-Bravo et al, 2014; Kim et al, 2018). Upon mitotic entry, the Mad1:Mad2 complex is displaced from the nuclear pore and recruited to the kinetochore where it binds Bub1, as a result of Mps1 phosphorylation (Klebig et al, 2009; London and Biggins, 2014a; Zhang, G. et al, 2017; Cunha-Silva et al, 2020; Jackman et al, 2020; Lara-Gonzalez et al, 2021) (Figure 3). The recruitment of the Mad1:Mad2 complex is one of the most important events for SAC signalling, as it catalyses the assembly of the MCC at

kinetochores. In fact, tethering Mad1 to the kinetochore is sufficient to trigger a constitutive SAC (Maldonado and Kapoor, 2011; Ballister et al, 2014; Henrich et al, 2014; Kruse et al, 2014). The Mad1:Mad2 complex serve as a platform for the recruitment and activation of cytosolic Mad2, which allows the formation of MCC. Mad2 can adopt two different conformations: open (O, inactive) and closed (C, active), being the first found free in the cytosol and the second bound to its interactors Mad1 or Cdc20 (Yu, 2006; Mapelli and Musacchio, 2007). According to the “template model” for SAC activation, the C-Mad2 conformer constitutively bound to Mad1, rather than Mad1 itself, is the kinetochore receptor for the cytosolic O-Mad2. In this model, C-Mad2 serves as a template for the conversion of O- into C-Mad2 (De Antoni et al, 2005). Cdc20, which binding to Bub1 puts in the right position, directly assists Mad2 conversion (Piano et al, 2021; Lara-Gonzalez, 2021; Fischer et al, 2022). C-Mad2 gets entrapped in the process. This further stabilizes the interaction between Cdc20 and BubR1 leading to a repositioning of Cdc20 from Bub3:Bub1 to Bub3:BubR1, which completes MCC formation. Mps1 also controls this step through phosphorylation of Mad1, which enables the unfurling of Cdc20 N-terminal to promote interaction with Mad2 (Ji et al, 2017; Faesen et al, 2017) (Figure 3). This conformational change process is highly energy-consuming, making this the rate-limiting step for MCC formation (Simonetta et al, 2009).

It is worth to mention that in higher eukaryotes, Mad1:Mad2 also localizes to the fibrous corona, an expanded structural mesh composed of several outer kinetochore proteins that forms in the absence of microtubule attachment and contributes both to SAC signalling and kinetochore-microtubule interactions. Mad1:Mad2 recruitment to the corona occurs via the RZZ complex and requires Cyclin B (Allan et al, 2020). Although corona-anchored Mad1 was shown to be important for a robust SAC signalling (Allan et al, 2020; Conde and Gassmann, 2020), the C-terminal Mps1-dependent Mad1 phosphorylation, which is necessary for MCC catalysis, is restricted to the kinetochore, suggesting that only the Bub1-bound Mad1 is phosphorylated at the C-terminal. Electron microscopy data shed light into this matter: the elongated shape of Mad1 may enable its anchoring in the corona through its N-terminal region while the C-terminal region interacts with Bub1 at the outer kinetochore, which supports an integrated view of SAC signalling, in which the Bub1 and corona pools of Mad1 converge on the same site of MCC production (Allan et al, 2020; Conde and Gassmann, 2020).

Once all chromosomes become attached to spindle microtubules, the SAC is silenced, to allow mitotic exit. Upon microtubule attachment, a significant amount of Mps1 is removed from the kinetochore, as a result of the competition between microtubules and Mps1 for Ndc80 binding, since microtubules and Mps1 interact with Ndc80 through partially

overlapping domains. This suggests a direct regulation between kinetochore-microtubule attachment and Mps1 removal to silence the SAC (Ji et al, 2015; Hiruma et al, 2015). Mps1 removal from the kinetochore prevents the continuous phosphorylation of MELT motifs and other substrates in the SAC signalling cascade. A non-mutually exclusive alternative model argues that, as microtubules bind, the kinetochore is stretched along the longitudinal kinetochore-microtubule attachment axis. This results in a physical separation of Ndc80 and Knl1, further preventing any remnant Mps1 from phosphorylating MELT repeats (Aravamudhan et al, 2015). Importantly, the recruitment of opposing phosphatases is also required to antagonize the phosphorylation of SAC substrates (for further detail see next section “Phosphatases in Mitosis: Roles and Regulation”). In higher eukaryotes, the removal of SAC proteins from kinetochores is facilitated by the minus-end motor protein Dynein, which transports SAC proteins along the microtubules following attachment, in particular the Mad1-Mad2 complex, the main catalyst for MCC assembly at kinetochores (Musacchio, 2015).

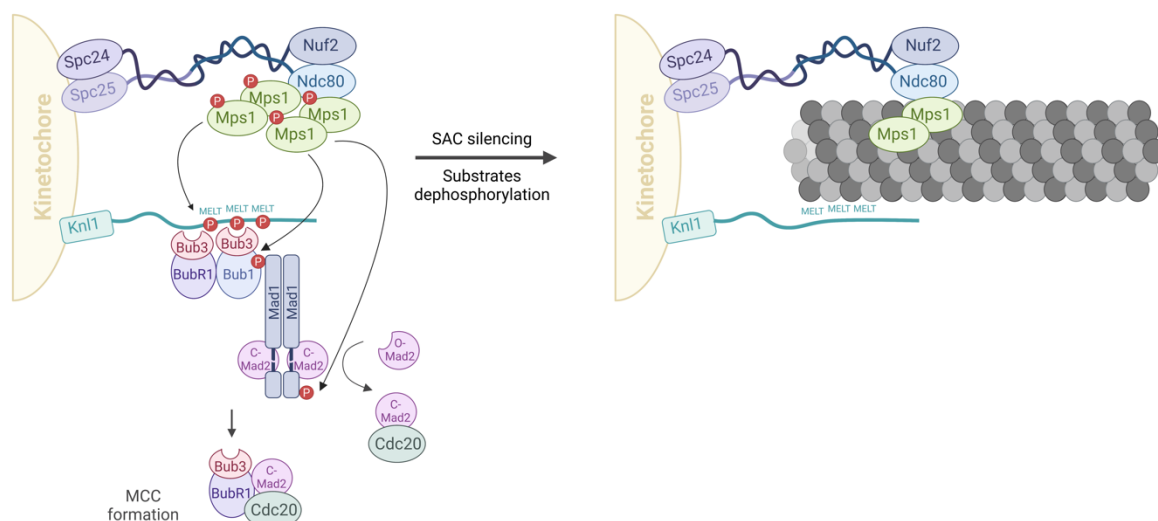


Figure 3 – MCC formation at unattached kinetochores. Mps1 is recruited to the Ndc80 complex at unattached kinetochores. The local concentration increase allows Mps1 to dimerize and *trans*-autophosphorylate, becoming active. Mps1 is then responsible for the phosphorylation of Knl1 MELT motifs that serve as a scaffold for the recruitment of Bub3:Bub1 and subsequently Bub3:BubR1. Mps1 phosphorylation of Bub1 promotes the interaction with the tetramer Mad1-C-Mad2, which after Mps1 mediated phosphorylation of Mad1 will serve as a catalyst for the conversion of O-Mad2 into C-Mad2. The conformation alteration from O- to C-Mad2 increases the affinity of the protein to Cdc20, facilitating the formation of the MCC (Bub3, BubR1, Mad and Cdc20 complex). Figure created with Biorender.com.

GENERAL INTRODUCTION

Phosphatases in Mitosis: Roles and Regulation

This chapter is based on the following published paper: Moura M, Conde C (2019) Phosphatases in Mitosis: Roles and Regulation. *Biomolecules* 9(2):55. DOI: 10.3390/biom9020055

INTRODUCTION

Protein phosphorylation is the most profuse post-translational modification in eukaryotes. It plays a critical role in controlling protein function, activity, and molecular interactions. The phosphorylation state of a given protein is established by the balance of activities between protein kinases and protein phosphatases. Kinases catalyse the transfer of a phosphate group to tyrosine (Tyr), threonine (Thr), or serine (Ser) residues, while protein phosphatases are responsible for the counteracting hydrolytic removal of the phosphate moiety. Phospho-proteomic analyses revealed that protein phosphorylation occurs primarily at Ser and Thr residues (83% and 15% of sites, respectively), with Tyr phosphorylation accounting for only up to 2% of the total phospho-proteome (Olsen et al, 2006). The importance and regulatory role of protein kinases is well established, whereas protein phosphatases were long regarded as passive housekeeping enzymes. However, it is now evident that phosphatases are also subjected to stringent control and assume equally critical roles in the regulation of protein function and signalling responses. The human genome contains 189 known and predicted protein phosphatase genes (Chen et al, 2017). Their evolutionary history can be traced back to a core catalytic domain that evolved through a series of gene duplication events and by adopting the use of regulatory subunits and/or fusion with novel functional modules or domains (Chen et al, 2017). Thus, contrasting with kinases, whose catalytic domains are commonly from a single protein structural fold (Manning et al, 2002), protein phosphatase activity is associated with numerous different protein folds and catalytic mechanisms (Andersen et al, 2001; Shi, 2009). Based on substrate specificity, protein phosphatases are typically classified as serine/threonine phosphatases, tyrosine phosphatases, or as dual-specificity phosphatases (Brautigan and Shenolikar, 2018).

Serine/Threonine Phosphatases

Serine/threonine phosphatases are distributed through three broad families in accordance to their primary structure and catalytic mechanism: the phospho-protein phosphatase (PPP), the protein phosphatase metal-dependent (PPM), and the haloacid dehydrogenases (HAD) families (Shi, 2009). The most abundant serine/threonine phosphatases in eukaryotic cells are protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Brautigan and Shenolikar, 2018). These phosphatases are members of the PPP family, which also includes PP2B, PP4, PP5, PP6, and PP7. All PPPs are phospho-monoesterases (EC3.1.3) and share a similar core catalytic domain, with two metal ions at the active site. This bimetallic centre, typically a Zn::Fe in native enzymes or a Mn::Mn pair in recombinant PPPs, is bridged by a water molecule that acts as a nucleophile in the hydrolysis of

phosphorylated Ser (pSer) and pThr substrates (Mitic et al, 2014; Heroes et al, 2015; Verbinnen et al, 2017; Brautigan and Shenolikar, 2018). The core catalytic domains of all PPPs exhibit modest substrate specificity when assayed *in vitro*. Unlike most kinases, PPPs fail to recognize a consensus sequence surrounding or adjacent to the phosphorylated residue (Imaoka et al, 1983; Mumby et al, 1987; McNall and Fischer, 1988; Agostinis et al, 1990; Agostinis et al, 1992). Tight substrate selectivity is, however, achieved in a cellular context through association with specific regulatory subunits, which also ensure the control of the phosphatase subcellular localization and modulate overall activity (Andersen et al, 2001; Brautigan and Shenolikar, 2018). Despite overall similarities, PP1 and PP2A catalytic subunits show some, albeit limited, intrinsic substrate specificity *in vitro* due to structural variations in their catalytic sites. Protein phosphatase 2A, for instance, is inhibited by subnanomolar concentrations of okadaic acid, due to hydrophobic residues in its catalytic cleft that bind tightly to okadaic acid, whereas PP1 inhibition requires 100-fold higher concentrations (Xing et al, 2006; Barr et al, 2011). Furthermore, isolated catalytic subunits of PP1 and PP2A show distinct preferences for different subunits of phosphorylase b kinase (Ingebritsen and Cohen, 1983). However, there is compelling evidence that PP1 and PP2A do not exist as free catalytic subunits in cells but operate as multimeric holoenzyme complexes composed of the catalytic subunit and by one or more regulatory and scaffolding subunits that control the phosphatase specificity and activity under physiological settings.

Protein Phosphatase 1

A PP1 holoenzyme contains one of four isoforms of the PP1 catalytic subunit – PP1 α , PP1 β , PP1 γ 1, or PP1 γ 2 (alternative splicing variant of PP1 only expressed in testis) and one or two of the numerous regulatory subunits (Okano et al, 1997; Sinha et al, 2012; Boens et al, 2013; Sinha et al, 2013). Catalytic subunits of PP1 show extreme phylogenetic and functional conservation. This is illustrated by the ability of any human PP1 isoform to rescue PP1/Glc7 loss in budding yeast (Gibbons et al, 2007). Biochemically, PP1 α , PP1 β , PP1 γ 1, or PP1 γ 2 are identical enzymes (Alessi et al, 1993; Shi, 2009; Brautigan and Shenolikar, 2018) with extensive functional overlap, although some specific functions have been attributed to PP1 γ 2 in testis and to PP1 β in some tissues (Sinha et al, 2012; Sinha et al, 2013; Liu et al, 2015). Protein phosphatase 1 isoforms adopt a compact α/β fold, with a β sandwich wedged between two α -helical domains (Egloff et al, 1995; Goldberg et al, 1995). The catalytic site resides at the intersection of three potential substrate/adaptor binding regions: the hydrophobic, acidic, and C-terminal grooves. These are shallow and converge at the catalytic centre, forming a Y-shaped surface feature (Figure 4 A) (Shi, 2009).

More than 200 regulatory subunits of PP1 have been described. These are generically designated as PP1-interacting proteins (PIPs) and its diversity provides the cell with a wide array of different PP1 holoenzymes to meet specificity and regulatory requirements (Bollen et al, 2010; Heroes et al, 2013). PIPs are mostly structurally unrelated proteins whose interaction with PP1 is frequently mediated by short linear motifs (SLiMs) that bind to the surface grooves of PP1 (Figure 4 B) (Roy and Cyert, 2009; Marsh et al, 2010; Davey et al, 2012; Davey et al, 2015). Several distinct PP1-binding motifs have now been characterized, enabling a huge combinatorial potential to generate unique PP1 holoenzymes with specific properties (Bollen et al, 2010; Heroes et al, 2013; Brautigan and Shenolikar, 2018). The most frequent and best characterized PP1-binding SLiM is the RVxF-motif. This motif conforms to the consensus sequence $[K/R]x(0-1)[V/I]x[F/W]$ and is present in nearly 70% of identified PIPs (Wakula et al, 2003; Meiselbach et al, 2006). Structural insights revealed that the RVxF-motif docks into a hydrophobic pocket on the PP1 surface through contacts that are primarily mediated by the hydrophobic side-chains of valine (Val) and phenylalanine (Phe) (Egloff et al, 1997). Additional examples of PP1-binding SLiMs include the SILK, MyPhoNE (Myosin Phosphatase N-terminal Element), and SpiDoC (Spinophilin Docking site for the C-terminal groove) motifs (Bollen et al, 2010; Heroes et al, 2013). The binding of PIPs to PP1 can also occur through highly structured segments, such as leucine-rich repeats or ankyrin repeats, respectively present on SDS22 and MYPT1 regulatory subunits (Hirano et al, 1997; Ceulemans et al, 2002; Terrak et al, 2004). Different PP1-binding motifs associate with different regions of the phosphatase surface. Therefore, a given PP1:PIP interaction might be mediated by several PP1-binding motifs to provide a higher affinity and enhance the complex stability. Importantly, although the same PP1 surface can establish contact with different PIPs, the interaction occurs in a highly specific manner due to the unique combination of PP1-binding motifs in each PIP.

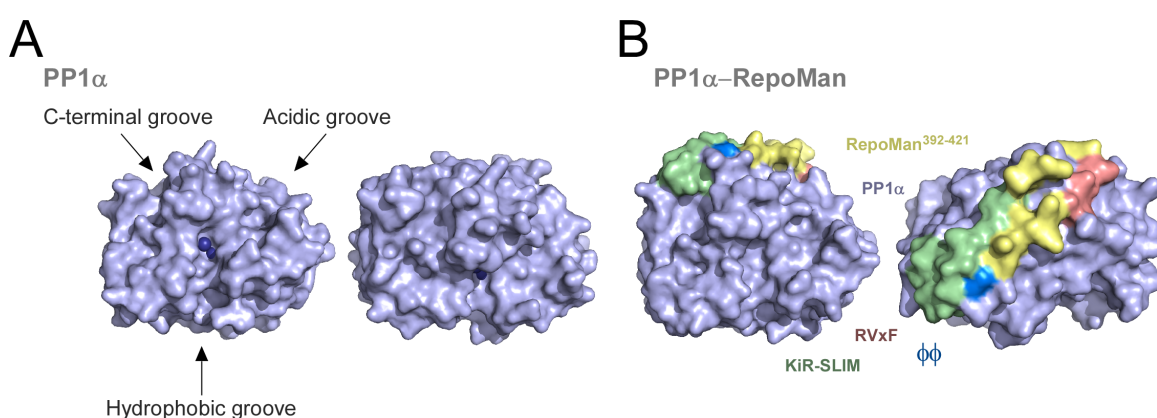


Figure 4 – Structure of PP1 α catalytic subunit and its interaction with the regulatory subunit Repo-Man. (A) Surface representation of the three-dimensional (3D) structure of PP1 α catalytic subunit. Manganese atoms in the active site are represented as dark blue spheres and lie at the Y-shaped intersection of the three substrate-binding grooves. Protein Data Bank (PDB) accession identification (ID) for the structure: 4MOV. (B) Surface

representation of the three-dimensional structure of PP1 α catalytic subunit in complex with Repo-Man³⁹²⁻⁴²¹. The PP1 α catalytic subunit is represented in light blue and the Repo-Man³⁹²⁻⁴²¹ peptide is represented in yellow. The interaction is mediated by RVxF (red), $\Phi\Phi$ (blue) and KiR-SLiM (green) motifs on Repo-Man³⁹²⁻⁴²¹ that bind to specific pockets on PP1 α surface. PDB accession ID: 5IOH.

Crystallography studies show that binding to PIPs does not impose a discernible conformational change on the PP1 catalytic domain. Instead, most PIPs limit PP1 activity towards specific substrates through a combination of functional domains involved in PP1-anchoring, PP1-inhibition, substrate-recruitment, and/or subcellular-targeting (Bollen et al, 2010; Heroes et al, 2013; Brautigan and Shenolikar, 2018). Therefore, controlled dephosphorylation can be achieved by a multitude of mechanisms. Some PIPs, such as spinophilin or protein phosphatase 1 nuclear-targeting subunit (PNUTS), bind to the PP1 C-terminal groove and sterically hinder the access to substrates recruited through this region (Ragusa et al, 2010; Choy et al, 2014). Nuclear inhibitor of protein phosphatase 1 (NIPP1), on the other hand, defines PP1 substrate selectivity through a polybasic stretch in its PP1-anchoring domain, which dramatically changes the electrostatic charge distribution of the NIPP1:PP1 surface, and by a N-terminus forkhead-associated (FHA) domain that mediates the recruitment of specific substrates (O'Connell et al, 2012). Other PIPs target PP1 to specific subcellular compartments through domains that may also be involved in substrate recruitment. This raises the local concentration of PP1 and enables the dephosphorylation of resident substrates. The regulatory subunit inhibitor-2 (I-2) binds to the acidic and hydrophobic grooves of PP1. This positions the IDoHA (Inhibitor-2 Docking site for the Hydrophobic and Acidic grooves) motif in contact with the catalytic centre, obstructing its access to substrates and causing the release of metals required for catalysis (Hurley et al, 2007). Other PIPs, such as inhibitor-1 (I-1), CPI-17, and MYPT1, associate with PP1 in a phospho-dependent manner and may inhibit its activity by posing as pseudosubstrates or act as anchoring subunits (Goldberg et al, 1995; Barford et al, 1998; Eto et al, 2007; Dumitru et al, 2017). Several heterotrimeric PP1 holoenzymes have been identified where PP1 associates with one targeting- or substrate-specifying PIP and one inhibitory PIP. The I-1, I-2, inhibitor-3 (I-3), and CPI-17 are examples of inhibitory PIPs that are able to interact with various PP1-PIPs dimers. The crystallographic structure of PP1 bound to spinophilin, and to I-2 has revealed major structural rearrangements in both regulatory subunits to assemble the heterotrimeric complex (Terry-Lorenzo et al, 2002; Dancheck et al, 2011). The RVxF motif of spinophilin has a higher affinity for the hydrophobic pocket on PP1 and displaces the RVxF motif of I-2, which remains, however, anchored to PP1 through a SILK motif. The IDoHA motif of I-2 dislodges the β -helix of spinophilin. These rearrangements enhance the flexibility of the holoenzyme, which is

thought to increase its ability to bind substrates or provide new protein interaction sites that were previously concealed and thereby mediate access to additional targets. In summary, amongst the vast repertoire of identified PIPs, there are subcellular/substrate-specifiers, inhibitors, and substrates that also act as inhibitory regulators of PP1. Their combinatorial association with each of the PP1 catalytic subunits provides the cell with a multitude of PP1 holoenzymes with unique structural and biochemical properties to enable the spatiotemporally controlled dephosphorylation of specific targets under physiological conditions.

Protein Phosphatase 2A

The PP2A catalytic subunit has two isoforms (PP2A α and PP2A β) that share 97% of their sequence identity with each other (Arino et al, 1988; Janssens and Goris, 2001). Like other PPPs, PP2A catalytic subunits operate as members of discrete holoenzyme complexes to achieve substrate specificity and controlled activity in cells. PP2A holoenzymes are predominantly heterotrimers consisting of a catalytic subunit (C) bound to a scaffold subunit (A), which in turn recruits a variety of mutually exclusive regulatory subunits (B) with distinct substrate and subcellular specificities. The human genome encodes two scaffolding subunits that share 87% of their sequence identity with each other (PP2A α and PP2A β isoforms), and 16 regulatory subunits grouped into four families according to their primary sequence and structure: B55 (PR55/B), B56 (PR61/B'), B72 (PR72/B''), and Striatin (PR93/B''') (Seshacharyulu et al, 2013; Nasa and Kettenbach, 2018).

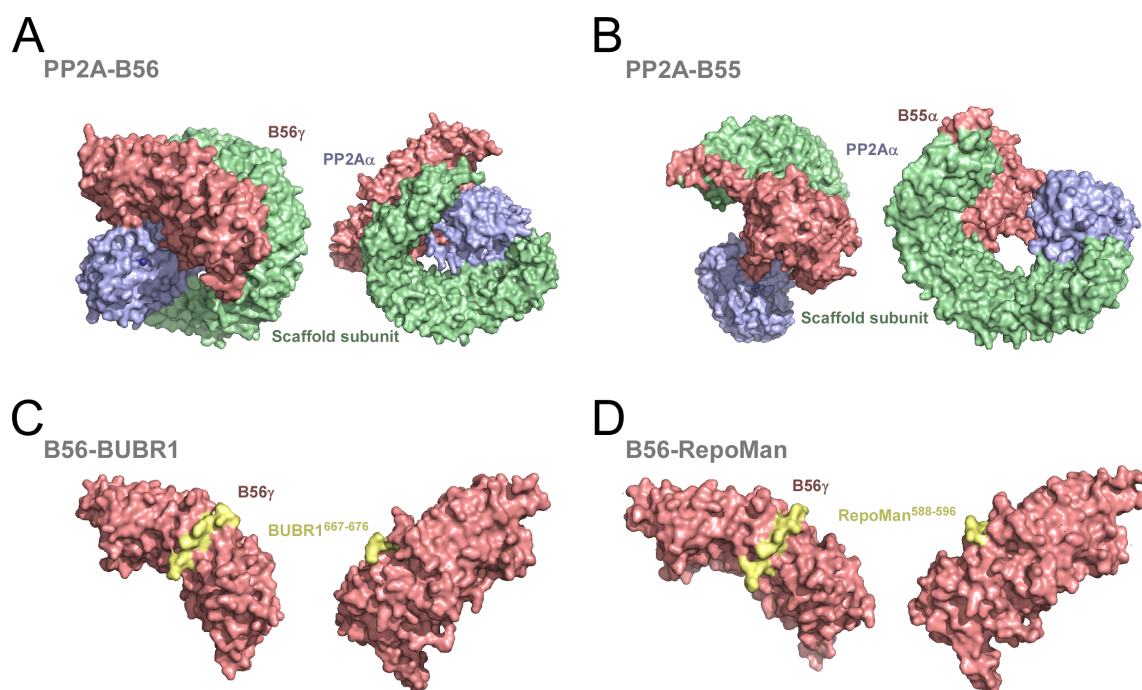


Figure 5 – Structure of PP2A α holoenzymes. (A,B) Surface representation of the three-dimensional (3D) structure of the PP2A α catalytic subunit (light blue) in complex with the scaffold subunit and the regulatory subunits, B56 γ (A) or B55 α (B). The PP2A catalytic subunit is represented in light blue with manganese atoms in the active site represented as dark blue spheres. The scaffold subunit is represented in green and the regulatory subunits are depicted in light red. Protein Data Bank (PDB) accession ID: 2NPP (A) and 3DW8 (B). (C,D) Surface representation of the three-dimensional structure of B56 γ (light red) interacting with BubR1⁶⁶⁷⁻⁶⁷⁶ and Repo-Man⁵⁸⁸⁻⁵⁹⁶ peptides (yellow). Both peptides encompass a LxxIxE motif that binds to a conserved basic pocket at the concave surface of B56 γ . PDB accession ID: 5K6S (C) and 5SW9 (D).

The PP2A scaffold subunit contains 15 tandem helical HEAT (huntingtin-elongation-A subunit-TOR, target of rapamycin) repeats assembled into an elongated, horseshoe-shaped structure (Groves et al, 1999) (Figure 5 A). PP2A(C) specifically binds to the conserved rim of HEAT repeats 11–15 to form the heterodimeric core enzyme (Xing et al, 2006). Although the PP2A catalytic subunit shares extensive sequence similarity with other PPPs, the majority of the latter fail to associate with PP2A α or PP2A β because the interaction is mediated by specific amino acids that have been replaced by non-conserved residues in PP1, PP2B, PP5, and PP7 (Shi, 2009). Roughly one-third of PP2A catalytic subunits are present in mammalian cells in the form of heterodimers with a scaffolding subunit (Kremmer et al, 1997). Whether this PP2A heterodimeric complex is physiologically relevant or merely represents a readily available intermediate towards holoenzyme assembly remains unclear.

The association of a regulatory B subunit to the core enzyme provides substrate specificity as well as spatially and temporally controlled functions (Slupe et al, 2011). Structural analysis revealed that the B56 subunit extensively interacts with both the scaffold and the catalytic subunit (Figure 5 A). The B56 subunit contains eight HEAT-like repeats and is structurally similar to the scaffold subunit. The convex surface of B56 binds to HEAT repeats 2–8 of the scaffold subunit, whereas the concave surface is tilted toward the active site pocket of the catalytic subunit to define the substrate-recognizing surface (Figure 5 A) (Cho and Xu, 2006; Xu et al, 2006). The B55 subunit on the other hand adopts a WD40 fold. It comprises a seven-bladed propeller and a protruding β -hairpin handle that respectively bind to the HEAT repeats 3–7 and 1–2 of the scaffold subunit (Xu et al, 2008). Contrasting with the B56 subunit, B55 makes few contacts with the catalytic subunit and the interaction with substrates is mediated by the acidic central groove of the propeller (Figure 5 B). Thus, notwithstanding evident structural differences and their specific interactions with the scaffold subunit, both B55 and B56 generate PP2A holoenzymes in which the substrate-binding site is located on the top face of the regulatory subunit at a position that is proximal to the active site of the catalytic domain (Shi, 2009) (Figure 5 A,B). Different B subunits generate different substrate-recognition interfaces. The PP2A core enzyme is only able to

efficiently dephosphorylate the microtubule-binding protein, Tau, when in complex with the B55 subunit (Xu et al, 2008). Two lysine-rich sequences in Tau are predicted to bind to the acidic groove on the top face of the propeller. The residues on B55 required for the interaction have been mapped, but further studies will be required to establish the precise Tau sequences that contact with B55 (Xu et al, 2008). Additional critical insight into the B55 substrate recognition mechanism was recently provided through a combination of phosphoproteome screens and kinetic modelling. The strategy allowed the global identification of B55 mitotic substrates, most of which conform to the cyclin-dependent kinase 1 (Cdk1) phosphorylation signature (Cundell et al, 2016). This specificity of B55 is provided by a bipartite positively charged polybasic motif located upstream and downstream of the Cdk1 site that acts as a binding determinant for the acidic surface of B55 (Cundell et al, 2016). Notably, substrates that are dephosphorylated more rapidly by PP2A:B55 are generally more basic than substrates that are dephosphorylated at slower rates (Cundell et al, 2016). This strongly suggests that the cooperative electrostatic interactions between the negatively charged residues in B55 and the positively charged amino acids on the polybasic motif control the dephosphorylation kinetics (Cundell et al, 2016; Pereira and Schiebel, 2016). Moreover, PP2A:B55 exhibits a preference towards pThr over pSer residues that is not determined by electrostatic interactions with B55, as it has also been observed in other PP2A holoenzymes (Pinna et al, 1976; Deana et al, 1982; Deana and Pinna, 1988; Godfrey et al, 2017). This preference seems to reflect instead an inherent property of the PP2A catalytic subunit. It is possible that the additional methyl side group of threonine could affect the binding affinity or even the catalytic reaction (Hein et al, 2017).

Recent studies shed new light on the mechanisms by which PP2A:B56 holoenzymes engage with some of its substrates. The B56 subfamily in humans comprises five isoforms (α , β , γ , δ and ϵ) with no discernible differences in their substrate binding pockets (Nasa and Kettenbach, 2018). These subunits recognize SLiMs conforming to the [LMFI]xx[ILV]xE sequence, henceforward referred to as the LxxlxE motif (Hertz et al, 2016; Wang, J. et al, 2016; Wang, X. et al, 2016; Wu et al, 2017). Structural analysis of B56 interaction with the kinetochore protein, BubR1, or with the nuclear scaffolding protein, Repo-Man, revealed that the LxxlxE motif binds to a conserved basic pocket between the third and the fourth HEAT repeats on the concave surface of B56 (Wang, J. et al, 2016; Wang, X. et al, 2016) (Figure 5 C,D). Peptide phage display of intrinsically disordered regions of the human proteome identified 126 ligands of B56 and confirmed substantial specificity overlap between PP2A:B56 α and PP2A:B56 γ holoenzymes as expected from the highly conserved LxxlxE binding surface in all B56 isoforms (Wu et al, 2017). Nevertheless, B56 isoforms were recently shown to bind differentially to LxxlxE motifs on mitotic proteins (Vallardi et al,

2019). This apparent selectivity is proposed to allow B56 α and B56 γ to localize at discrete subcellular compartments and control separate mitotic processes. Thus, although functional redundancy has been attributed to B56 isoforms, some sub-functionalization appears to occur under certain circumstances. The molecular features underlying B56 isoform-specific interactions during mitosis are discussed in the section “Kinetochore-Microtubule Attachments: PP1 and PP2A:B56 Keep Them Stable”.

Tyrosine Phosphatases

Although recurrently underrepresented in global phospho-proteomes, tyrosine phosphorylation plays a critical role in numerous signal transduction pathways that underlie a broad spectrum of fundamental physiological processes. Protein tyrosine phosphatases (PTPs) are defined by the active-site signature motif HCX5R, in which the cysteine residue acts as a nucleophile essential for catalysis (Tonks, 2006; Tiganis and Bennett, 2007). Protein tyrosine phosphatases are categorized as classical tyrosine-specific phosphatases (CTSPs) or as dual-specificity phosphatases (DUSPs) (Andersen et al, 2001; Alonso et al, 2004; Andersen et al, 2004). Contrasting with PPP catalytic subunits, PTPs are selective enzymes that exhibit striking substrate specificity in a cellular context. Hence, instead of occurring *in vivo* in complex with a multitude of regulatory subunits, PTPs comprise a range of structurally distinct catalytic units that are encoded by nearly 100 genes in the human genome (Tonks, 2006; Kin and Ryu, 2012; Sacco et al, 2012). The use of alternative promoters, alternative splicing, and post-translational modifications introduce further structural diversity (Tonks, 2006). The catalytic domain of classical PTPs (CTSPs) comprises approximately 280 residues and is defined by the HCX5R motif that functions as a phosphate-binding loop at the active site and by a mobile general acid/base loop (WPD) (Andersen et al, 2001). Despite relatively large sequence variations in the X5 segment, the P-loop conformation is strictly conserved and ensures that the cysteine and arginine residues remain in close proximity to efficiently remove the phosphate group from the substrate tyrosine. The sulphur atom of the cysteine thiolate serves as a nucleophile and attacks the phosphorus of the phospho-tyrosyl target, whereas arginine contributes to substrate binding and stabilizes the cysteine-phosphate intermediate (Barford et al, 1994; Jia et al, 1995). This intermediate is resolved by an activated water molecule aided by an aspartate in the WPD loop (Barford et al, 1994; Jia et al, 1995). Dual-specificity phosphatases are the largest sub-group of PTPs with approximately 70 members (Tonks, 2006). These phosphatases also encompass a typical HCX5R signature motif in their active site, but have smaller catalytic domains and are more structurally diverse than the CTSPs. Notably, the active site pocket of DUSPs is wider and shallower, which allows them to

accommodate pSer and pThr residues as well as pTyr residues (Yuvaniyama et al, 1996). Dual-specificity phosphatases share the same catalytic mechanism as the CTSPs (Yuvaniyama et al, 1996). Although DUSPs do not possess a WPD loop, a conserved aspartate residue in the corresponding position is proposed to act as a general acid/base during catalysis (Kim and Ryu, 2012). With the notable exception of the inhibitory phosphorylation of Cdk1 on Tyr15, reversible tyrosine phosphorylation was long thought to play a minor role in mitosis. However, SFKs, ABL1, EGFR, and PKM2 are examples of PTPs whose inhibition was shown to compromise mitotic accuracy (Hitosugi et al, 2009; Matsumura et al, 2012; Jiang et al, 2014; Wee et al, 2015). Furthermore, the master mitotic regulator, polo-like kinase 1 (Plk1), was recently shown to be phosphorylated on Tyr217 during mitosis (Caron et al, 2016). This phosphorylation occurs within the kinase activation segment and is proposed to repress the activity of selected pools of Plk1 during cell division (Caron et al, 2016). Hence, available new evidence suggests that mitotic pTyr signalling may be more prevalent than previously appreciated. Nevertheless, a comprehensive and mechanistic knowledge of mitotic events controlled by tyrosine phosphorylation remains limited and the only PTPs with well-established essential roles in mitosis are the dual-specificity phosphatases, cell division cycle 14 (Cdc14) and cell division cycle 25 (Cdc25) (Trinkle-Mulcahy and Lamond, 2006; Queralt and Uhlmann, 2008; Wurzenberger and Gerlich, 2011). Phosphatases of the Cdc25 family have evolutionarily conserved functions in controlling key transitions between cell cycle phases. Cdc14, on the other hand, is a key mitotic exit phosphatase in budding yeast, but its relevance for mitotic exit in metazoans remains ill-defined. Instead, a growing body of evidence indicates that mitotic exit in animal cells relies primarily on regulatory networks orchestrated by PP1 and PP2A phosphatases (Wurzenberger and Gerlich, 2011).

Cdc25 Phosphatases

The human genome encodes three Cdc25 phosphatases: Cdc25A, Cdc25B, and Cdc25C (Sadhu et al, 1990; Galaktionov and Beach, 1991; Nagata et al, 1991). The three isoforms are ~60% identical in their C-terminal catalytic domain, but diverge considerably in their N-terminal regulatory region (Kristjánsdóttir and Rudolph, 2004; Rudolf, 2007). All three Cdc25 phosphatases act at various points of the cell cycle to control the activity of specific Cdk:Cyclin subpopulations (Boutros et al, 2007; Aressy and Ducommun, 2008; Lavecchia et al, 2012). Not surprisingly, Cdc25 phosphatases are often overexpressed in cancer cells (Kristjánsdóttir and Rudolph, 2004). Structural characterization of Cdc25A and Cdc25B catalytic domains revealed a near identical folding that is clearly distinct from that of any PTP (Fauman et al, 1998; Reynolds et al, 1999). The small catalytic domains adopt a

rhodanese-like structure with a central five-stranded parallel β -sheet sandwiched by three α -helices from below and two α -helices from above. The active sites containing the HCX5R signature are flat and shallow and lack auxiliary loops or obvious features for mediating substrate recognition. Rather, this seems to rely on three hotspot residues (R488, R492, and Y497 on Cdc25B) that form a substrate docking site remotely located from the catalytic cysteine (Sohn et al, 2004; Rudolph, 2007; Sohn et al, 2007). In line with that, the primary sequence surrounding the site of dephosphorylation does not seem to contribute significantly for substrate recognition (Rudolph et al, 2001). For instance, Cdc25A and Cdc25B phosphatases exhibit a clear preference towards phosphorylated Cdk2 (on Thr14 and Tyr15) in complex with Cyclin A (Cdk2-pThr14pTyr15:CyclinA) over artificial substrates or phosphorylated peptides that harbour the same target sequence (Rudolph, 2007). Both catalytic domain constructs and respective full-length phosphatases exhibit the same affinity for Cdk2-pThr14pTyr15:CyclinA, indicating that all the elements required for substrate recognition are in the catalytic domain. Interestingly, Cdc25C is significantly less active towards Cdk2-pThr14pTyr15:CyclinA, but highly efficient in dephosphorylating Cdk1-pThr14pTyr15:CyclinB (Wilborn et al, 2001). Such differential reactivity is attributed to differences in the C-terminal tail of each Cdc25 phosphatase. The C-terminus corresponds to the least conserved region of the catalytic domain and remains undefined in crystal structures, presumably due to its intrinsic flexibility or disordered nature. Nevertheless, two arginine residues present in the C-terminal tail of Cdc25A and Cdc25B, but absent from Cdc25C were shown to dramatically enhance the affinity for Cdk2-pThr14pTyr15:CyclinA (Rudolph, 2007). The catalytic mechanism of Cdc25 phosphatases is very similar to the well-established mechanism of PTPs (Denu and Dixon, 1995; Denu et al, 1996; Rudolph, 2007). The cysteine in the active site forms a transient covalent phospho-cysteine. Departure of the leaving group and formation of the intermediate are facilitated by protonation of the oxyanion by aspartate acting as the catalytic acid. In a second chemical step, water serves as a nucleophile in the hydrolysis of the phospho-enzyme intermediate assisted by the aspartate, which now acts as a base to activate the water molecule. Extensive experimental evidence confirmed this general mechanism for Cdc25 phosphatases, but the identity of the catalytic acid remains to be unambiguously established (Chen et al, 2000; Rudolph, 2002; McCain et al, 2002; Rudolph, 2007).

ROLES OF PROTEIN PHOSPHATASES IN MITOSIS

The unidirectional transitions between the phases of the cell cycle are governed by reversible phosphorylation and proteolysis. The highest occupancy of phosphorylation sites

is observed in mitosis. Over 32,000 protein phosphorylation/dephosphorylation events occur as cells go into, progress through, and exit from mitosis (Sharma et al, 2014; Kuilman et al, 2015; McCloy et al, 2015; Burgess et al, 2017). Such an extensive network of dynamic phosphorylations underlies the physical events that distribute one copy of the genome to each daughter cell and their subsequent resumption to an interphase state. Mitotic entry in animal cells involves dramatic cellular and structural reorganization, including cell rounding, disassembly of the nuclear envelope, condensation of chromatin into tightly packed chromosomes and formation of the microtubule-based mitotic spindle. These events occur in concert with regulatory and surveillance pathways to enable the attachment of chromosomes to microtubules from opposite spindle poles and are primarily driven by elevated activity of the Cdk1:Cyclin B complex. This attachment configuration allows sister chromatids to be pulled towards opposite poles during mitotic exit, hence providing the same genetic content to progeny cells. To safeguard the accuracy of chromatid partitioning, cells only commit to irreversibly exit mitosis when sister kinetochores of each chromosome attain proper amphitelic attachments. Chromosome segregation and decondensation, cytokinesis, and reassembly of interphase cell structures are the major events defining mitotic exit, all of which require inactivation of Cdk1 as well as spatially and temporally confined removal of mitotic phosphorylations to occur. These are respectively driven by the degradation of Cyclin B and controlled activity of protein phosphatases.

Mitotic Entry: Cdc25 Phosphatases Set Up the Commitment

Activation of Cdk:Cyclin B has long been established as the trigger that drives the transition into mitosis. The activity of Cdk1 throughout the cell cycle is regulated by the quantity and subcellular localization of Cyclin B and by the phosphorylation status of Cdk1 itself. Expression of Cyclin B increases during the G2 phase of the cell cycle, thus allowing formation of the Cdk1:Cyclin B complex before mitotic entry (Pines and Hunter, 1989). The activity of Cdk1:Cyclin B is however repressed by inhibitory phosphorylations on Cdk1 Thr14 and Tyr15 catalysed by WEE1 and MYT1 kinases (Parker and Piwnicka-Worms, 1992; Morgan, 1995). These residues are in the Cdk1 catalytic domain and their phosphorylation induces conformational changes in the ATP-binding loop that render the kinase inactive. Dephosphorylation of pThr14 and pTyr15 catalysed by Cdc25 dual-specificity phosphatases is considered the rate-limiting step in the transition from G2 into mitosis (Figure 6 A). In human cells, all three Cdc25 isoforms contribute for mitotic entry. The Cdc25B isoform is responsible for the initial activation of Cdk1:Cyclin B at centrosomes during late G2 (Gabrielli et al, 1996; De Souza et al, 2000; Lindqvist et al, 2005). The kinase is subsequently translocated into the nucleus, where it becomes completely activated by

Cdc25C at the onset of mitosis (Gabrielli et al, 1997). The Cdc25A phosphatase is proposed to activate Cdk1:Cyclin B complexes that trigger chromosome condensation (Molinari et al, 2000; Mailand et al, 2002; Lindqvist et al, 2005; Boutros et al, 2006). Partially active Cdk1:Cyclin B directly stimulates Cdc25B and Cdc25C activities and promotes the inhibition of WEE1 and MYT1 kinases, hence establishing both a positive and a double-negative feedback loop that drives swift and sustained Cdk1 activation and the irreversible transition to mitosis (Figure 6 A) (Booher et al, 1997; O'Farrell, 2001; Nakajima et al, 2003; Watanabe et al, 2004; Lindqvist et al, 2009).

In addition to Cdk1:Cyclin B, multiple other mechanisms contribute to regulate Cdc25 phosphatases (Figure 6 B). These include changes in Cdc25 protein levels and subcellular localization, inhibitory phosphorylations, and binding to regulatory proteins. The Cdc25A isoform is predominantly expressed in G1 and subjected to rapid turnover during interphase as a result of SCF β TRCP ubiquitin-mediated degradation (Donzelli et al, 2002). The phosphatase becomes, however, stabilized at the G2/M transition upon Cdk1:Cyclin B-mediated phosphorylation on Ser17 and Ser115 (Jinno et al, 1994; Mailand et al, 2002), thus contributing to the phosphatase pool required to fully activate Cdk1:Cyclin B. The levels of Cdc25A decrease rapidly at the end of mitosis due to APC/C:Cdh1-mediated targeting for proteasomal degradation (Donzelli et al, 2002). The levels of Cdc25B begin to accumulate during mid S-phase, peak at mitotic entry, and decrease as a result of SCF β TRCP-dependent degradation at the metaphase-anaphase transition (Gabrielli et al, 1996; Baldin et al, 1997; Garner-Hamrick and Fisher, 1998; Kanemori et al, 2005; Kieffer et al, 2007). Contrasting with the other isoforms, Cdc25C protein levels remain constant throughout the cell cycle (Boutros et al, 2007). However, the phosphatase is kept inactive during interphase due to Ser216 phosphorylation. This phosphorylation can be catalysed by a number of kinases, including Chk1, Chk2, C-Tak, CaMKII, and PKA (Furnari et al, 1997; Kumagai et al, 1998; Peng et al, 1998; Zeng et al, 1998; Furnari et al, 1999; Duckworth et al, 2002), and generates a binding site for the 14-3-3 family of proteins (Peng et al, 1997; Sanchez et al, 1997; Kumagai, Yakowec and Dunphy, 1998). The interaction of Cdc25C with 14-3-3 masks a nuclear localization signal, resulting in cytoplasmic sequestration of the phosphatase and consequently in the inhibition of mitotic entry (Boutros et al, 2006; Hermeking and Benzinger, 2006; Boutros et al, 2007). Moreover, studies with *Xenopus* oocytes indicate that pSer216 might also directly suppress Cdc25C catalytic activity independently of 14-3-3 binding (Margolis et al, 2006). Similarly, phosphorylation of Cdc25B on the equivalent residue (Ser323) by p38 MAPK or pEG3 promotes binding to 14-3-3 and thereby cytoplasmic retention of the phosphatase during interphase. Interestingly, 14-3-3 also binds to Cdc25A following phosphorylation of the latter on Thr505 by Chk1.

This, however, does not affect the phosphatase nuclear localization but rather impairs its interaction with Cdk1:Cyclin B to control proper timing of mitotic onset (Chen et al, 2003).

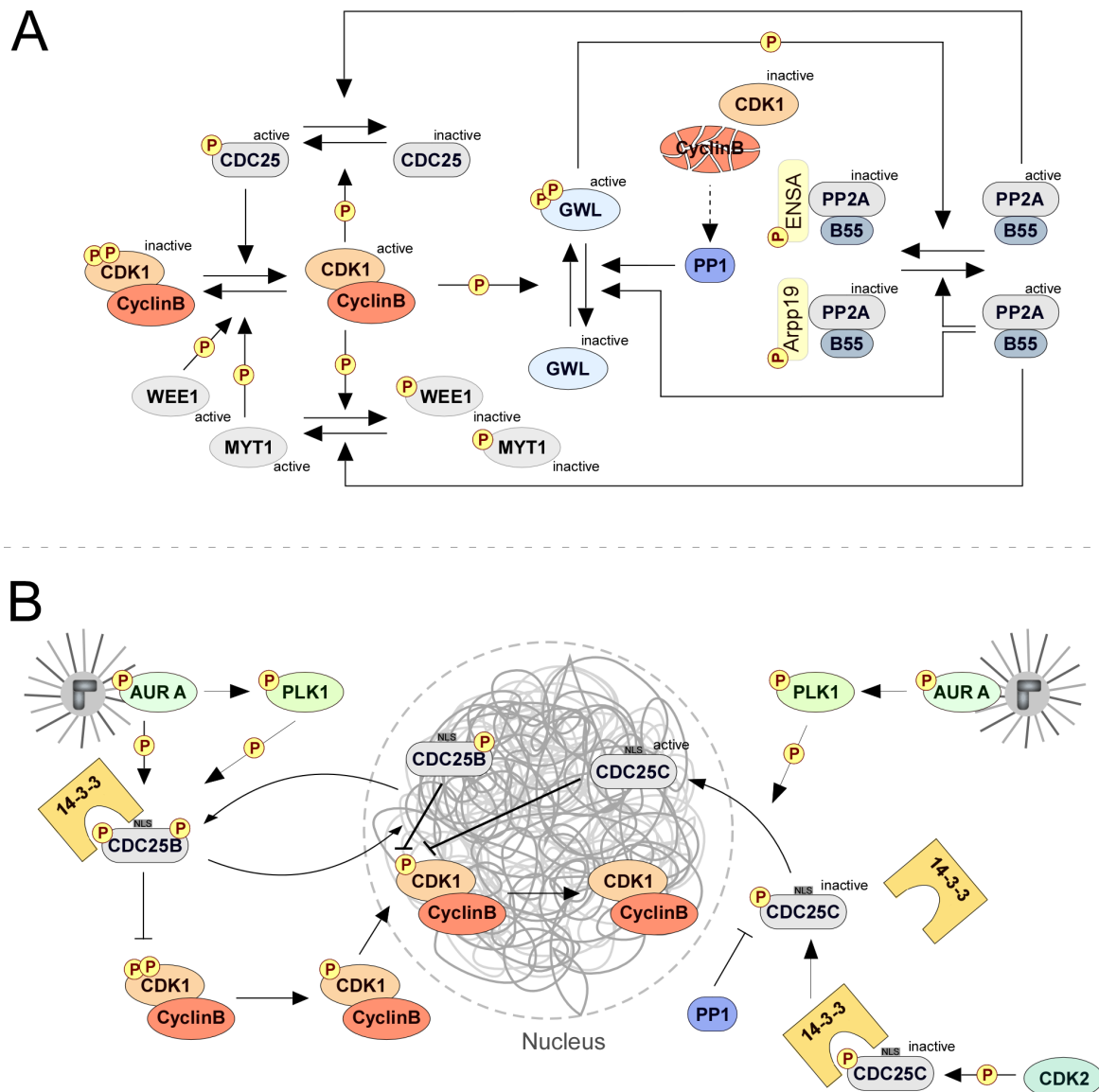


Figure 6 – Molecular circuitry controlling mitotic entry. (A) Cdc25 phosphatases dephosphorylate and activate Cdk1:Cyclin B to promote mitotic entry. Cdc25 activity antagonizes the inhibitory phosphorylations on Cdk1 (Thr14 and Tyr15) catalysed by MYT1 and WEE1. Active Cdk1:Cyclin B respectively inhibits and promotes MYT1/WEE1 and Cdc25 activities, establishing both a double negative feedback loop and a positive feedback loop. This regulatory circuitry defines a bistable trigger that ensures irreversible transition into mitosis. Cdk1:Cyclin B further promotes the activation of G2L kinase, which subsequently phosphorylates ENSA and Arpp19. The phosphorylated forms of these endosulfines bind to PP2A:B55 and inhibit the phosphatase activity towards Cdk1:Cyclin B substrates, including MYT1, WEE1, and Cdc25. (B) The Cdc25B and Cdc25C isoforms become active during late G2 to drive Cdk1:Cyclin B activation. During interphase, Cdc25B and Cdc25C are kept inactive by phosphorylation and are retained in the cytoplasm due to binding to 14-3-3 proteins. The activity of Cdc25B is proposed to be responsible for the initial activation of Cdk1:Cyclin B at centrosomes. Activation of Cdc25B is triggered by Aurora A- and Plk1-dependent phosphorylations on the phosphatase N-terminus, which also promote Cdc25B shuttling between the cytoplasm and the nucleus. Following activation, the centrosomal pool of Cdk1:Cyclin B is translocated to the nucleus, where it enhances Cdc25C activity, initiating an amplification loop that drives the cell into mitosis. The inhibitory phosphorylation on Cdc25C is removed by PP1 phosphatase upon dissociation of the 14-3-3 protein. This concurs with Plk1-mediated phosphorylation of the Cdc25C N-terminus to promote the phosphatase nuclear import and directly stimulate the phosphatase catalytic activity.

Mitotic kinases have also been shown to operate as active regulators of Cdc25 phosphatases (Figure 6 B). Centrosome-localized Aurora A phosphorylates Cdc25B on Ser353 during prophase. Although the molecular underpinnings remain elusive, this phosphorylation correlates with the re-localization of Cyclin B to the nucleus and Cdk1 activation during mitotic entry (Dutertre et al, 2004; Cazales et al, 2005). Aurora A is also implicated in promoting timely activation of Cdc25C, though indirectly through Plk1 (Seki et al, 2008). While the requirement of Plk1 activity for mitotic entry upon the G2 DNA-damage checkpoint is well established, its relevance during unperturbed cell cycles has long remained controversial (Kumagai and Dunphy, 1996; Qian et al, 1998; Karaïskou et al, 1999; Roshak et al, 2000; van Vugt et al, 2004; Lobjois et al, 2011). Recent studies have now demonstrated that complete inhibition of Plk1 delays mitotic entry even in the absence of DNA-damage events. Plk1 is rapidly activated in late G2 shortly before Cdk1:Cyclin B and is dependent on Aurora A and Cdk1:Cyclin A activities (Gheghiani et al, 2017; Qi et al, 2018; Vigneron et al, 2018). Polo-like kinase 1 subsequently phosphorylates the Cdc25C N-terminal region on multiple sites, promoting its nuclear import (Toyoshima-Morimoto et al, 2002; Lobjois et al, 2009) and possibly directly stimulating the phosphatase catalytic activity (Kumagai and Dunphy, 1996; Roshak et al, 2000). Therefore, Aurora A- and Plk1-mediated control of Cdc25 phosphatases represent critical events in the molecular circuitry that ensures robust Cdk1:Cyclin B activation and mitotic commitment (Figure 6 B).

Data from *Xenopus* oocytes revealed that prompt Cdc25C activation further requires the activity of PP1 phosphatase (Figure 6 B). During mitotic entry, Cdk2 activity promotes the dissociation of 14-3-3 protein from Cdc25C, exposing the inhibitory phosphorylation on Ser287 (corresponding to Ser216 in the human Cdc25C orthologue) for PP1-mediated removal (Margolis et al, 2003). This enables the initial activation of Cdc25C and a consequent increment in Cdk1:Cyclin B activity. Cdk1:Cyclin B subsequently phosphorylates Cdc25C on Ser285 and enhances the recruitment of PP1 to Cdc25C, hence accelerating S287 dephosphorylation and thereby warranting prompt and sustained phosphatase activation (Margolis et al, 2006).

Along with increased Cdk1:Cyclin B activity, the inhibition of PP2A:B55 phosphatase is essential for mitotic compliance (Figure 6 A). B55-type regulatory subunits confer specificity of PP2A towards Cdk1 phospho-sites, which suggests that PP2A:B55 holoenzymes might represent a critical opposing activity to Cdk1:Cyclin B (Xu et al, 2008). Accordingly, PP2A:B55 mediated removal of numerous Cdk1-generated phosphorylations was shown to be critical for mitotic exit (Clarke et al, 1993; Mayer-Jaekel et al, 1994; Mochida et al, 2007; Castilho et al, 2009; Mochida et al, 2009; Schmitz et al, 2010; Cundell et al, 2013; Cundell et al, 2016; Godfrey et al, 2017). In order to prevent premature dephosphorylation of

Cdk1:Cyclin B substrates and ensure mitotic commitment, PP2A:B55 activity is repressed by the Ser/Thr kinase Greatwall (GWL) at the G2/M transition (Figure 6 A). Two key studies using *Xenopus* egg extracts uncovered the underlying biochemical pathway. GWL phosphorylates Arpp19 and ENSA, two small and unstructured proteins of the Endosulfine family. Phosphorylation occurs on specific serine residues (Ser62 in Arpp19 and Ser67 in ENSA) within a highly conserved FDSpGDY motif and allows Arpp19 and Ensa to bind PP2A:B55 in a highly specific manner, causing its near-complete inhibition (Gharbi-Ayachi et al, 2010; Mochida et al, 2010). This mechanism was also shown to be essential for mitotic entry in flies, starfish oocytes, and in human cultured cells (Yu et al, 2004; Archambault et al, 2007; Burgess et al, 2010; Lorca et al, 2010; Voets and Wolthuis, 2010; Hara et al, 2012). A notable exception was observed in mouse embryonic fibroblasts, where the control of PP2A:B55 by GWL seems to be dispensable for the G2/M transition, but nevertheless required after nuclear envelope breakdown to prevent mitotic collapse (Álvarez-Fernández et al, 2013; Diril et al, 2016). As expected, and by opposition to PP2A:B55, GWL activity is undetectable throughout interphase, abruptly increases at the G2/M transition, and remains elevated until anaphase onset (Yu et al, 2006; Voets and Wolthuis, 2010; Hara et al, 2012). Activation of GWL is triggered by Cdk1:Cyclin B-mediated phosphorylation of two conserved residues located in the kinase presumptive activation loop. This primes GWL to intramolecularly autophosphorylate its C-terminal tail and stabilize the kinase in its active conformation (Yu et al, 2006; Vigneron et al, 2011; Blake-Hodek et al, 2012). In frame of the G2/M signalling circuitry, the inhibition of PP2A–B55 orchestrated by GWL has been proposed to increase the levels of activating phosphorylation events on Cdc25 phosphatases and inhibitory phosphorylation events on WEE1 and MYT1 (Zhao et al, 2008; Wurzenberger and Gerlich, 2011) (Figure 6 A).

Sister Chromatid Cohesion: PP2A:B56 Holds Them Together

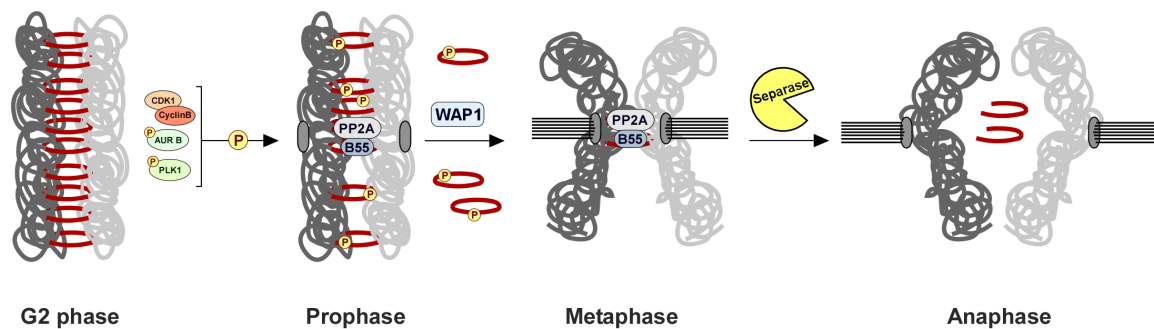
Mitotic entry is accompanied by the condensation of DNA into chromosomes, each comprised of two sister-chromatids held together from DNA replication until anaphase onset by proteinaceous ring-like cohesin complexes (Peters et al, 2008; Nasmyth et al, 2009) (Figure 7 A). These are composed by heterodimers of Smc1 and Smc3 bound to the α -Kleisin subunit, Scc1/Mcd1/Rad21, forming a closed tripartite structure that encircles the replicated DNA (Haering et al, 2002). The Kleisin subsequently binds to Scc3/SA, which when associated with the heterodimer, Wapl-Pds5, forms a subcomplex that regulates the maintenance of cohesion on chromatin (Gandhi et al, 2006; Kueng et al, 2006) (Figure 7 B). During prophase, cohesin is displaced from chromosome arms by Wapl, possibly through disruption of the Smc3-Scc1 interface (Figure 7 A,B) (Peters et al, 2008; Buheitel

and Stemmann, 2012; Chan et al, 2012; Chatterjee et al, 2013; Eichinger et al, 2013; Ouyang et al, 2013). Prior to mitotic entry, however, cohesin rings are insensitive to Wapl as a result of Smc3 acetylation by Eco1/2 acetyltransferases (Ben-Shahar et al, 2008; Rowland et al, 2009; Sutani et al, 2009; Ünal et al, 2008; Zhang et al, 2008). Acetylation of Smc3 during DNA replication promotes the recruitment of Sororin, which antagonizes Wapl by impairing its binding to Pds5 (Lafont et al, 2010; Nishiyama et al, 2010). Notably, loss of protection against Wapl during early mitosis does not involve Smc3 deacetylation. It is instead elicited through extensive phosphorylation of Sororin and Scc3/SA2 by Aurora B, Cdk1, and Plk1 (Losada et al, 2002; Sumara et al, 2002; Giménez-Abián et al, 2004; Hauf et al, 2005; Dreier and Bekier, 2011; Zhang et al, 2011; Liu et al, 2013; Nishiyama et al, 2013). Aurora B- and Cdk1-dependent phosphorylation of Sororin causes its dissociation from Pds5, hence consenting Wapl binding to Pds5 (Figure 7 A,B) (Liu et al, 2013; Nishiyama et al, 2013). Moreover, Wapl-mediated removal of cohesin further requires phosphorylation of Scc3/SA2 by Plk1. The underlying mechanism remains, however, unknown. Loss of cohesion from chromosome arms in prophase seems to occur exclusively in metazoan cells, where is thought to facilitate sister DNA decatenation and allow timely cohesin reloading in the subsequent cell cycle (Wang et al, 2010; Deardorff et al, 2012; Haarhuis et al, 2013; Tedeschi et al, 2013). Cohesion must, however, persist at centromeres until the kinetochores of all chromosomes are correctly attached to microtubules of opposite spindle poles. Centromeric cohesin facilitates this process by promoting the back-to-back arrangement of sister kinetochores and grants resistance to microtubules pulling forces so that separation can only occur upon mitotic checkpoint silencing (Nicklas, 1997; Tanaka et al, 2000; Haarhuis et al, 2014; Morales and Losada, 2018).

Centromeres are protected against Wapl-dependent cohesin removal by the activity of Sgo1-associated PP2A:B56 (McGuinness et al, 2005; Kitakima et al, 2006; Riedel et al, 2006; Tang et al, 2006; Liu et al, 2013). Recruitment of PP2A:B56 to centromeres of mammalian cells is mediated both by Sgo1 and Sgo2 (Rivera and Losada, 2009; Xu et al, 2009; Tanno et al, 2010). Although Sgo2 is required for cohesion protection during meiosis, it seems to be dispensable for this function in mitotic cells, where instead Sgo1 assumes primary relevance (Kitajima et al, 2005; McGuinness et al, 2005; Kitajima et al, 2006; Tang et al, 2006; Huang et al, 2007; Lee, J. et al, 2008; Llano et al, 2008; Rivera and Losada, 2009; Tanno et al, 2010; Gutiérrez-Caballero et al, 2012). Structural analysis of an N-terminal fragment of human Sgo1 bound to PP2A:B56 γ reveals a bipartite interaction between Sgo1 and the regulatory and catalytic subunits of the PP2A:B56 γ holoenzyme (Xu et al, 2009). Sgo1 N-terminus forms a parallel coiled-coil homodimer whose N- and C-

terminal ends bind to PP2A and B56 γ , respectively. Each binding interface contains a hydrophobic core as well as peripheral hydrogen bonds and salt bridges. Binding of Sgo1 to PP2A:B56 γ does not seem to impose significant alterations in PP2A structure or catalytic activity. In a cellular context, this interaction likely places PP2A:B56 γ in close proximity to Sororin and Scc3/SA2 to counteract Aurora B, Cdk1, and Plk1 destabilizing phosphorylations and thereby prevent the dissociation of centromeric cohesin complexes (Figure 7 B). Several lines of evidence concur in support of this model: binding of PP2A:B56 γ to Sgo1 is required for protection of centromeric cohesion (Tang et al, 2006; Xu et al, 2009), Sgo1 physically interacts with cohesin complexes during mitosis through Scc1:Scs3/SA2 heterodimers, and an unphosphorylatable version of Sororin prevents chromatid separation that would normally follow Sgo1 depletion (Liu et al, 2013).

A



B

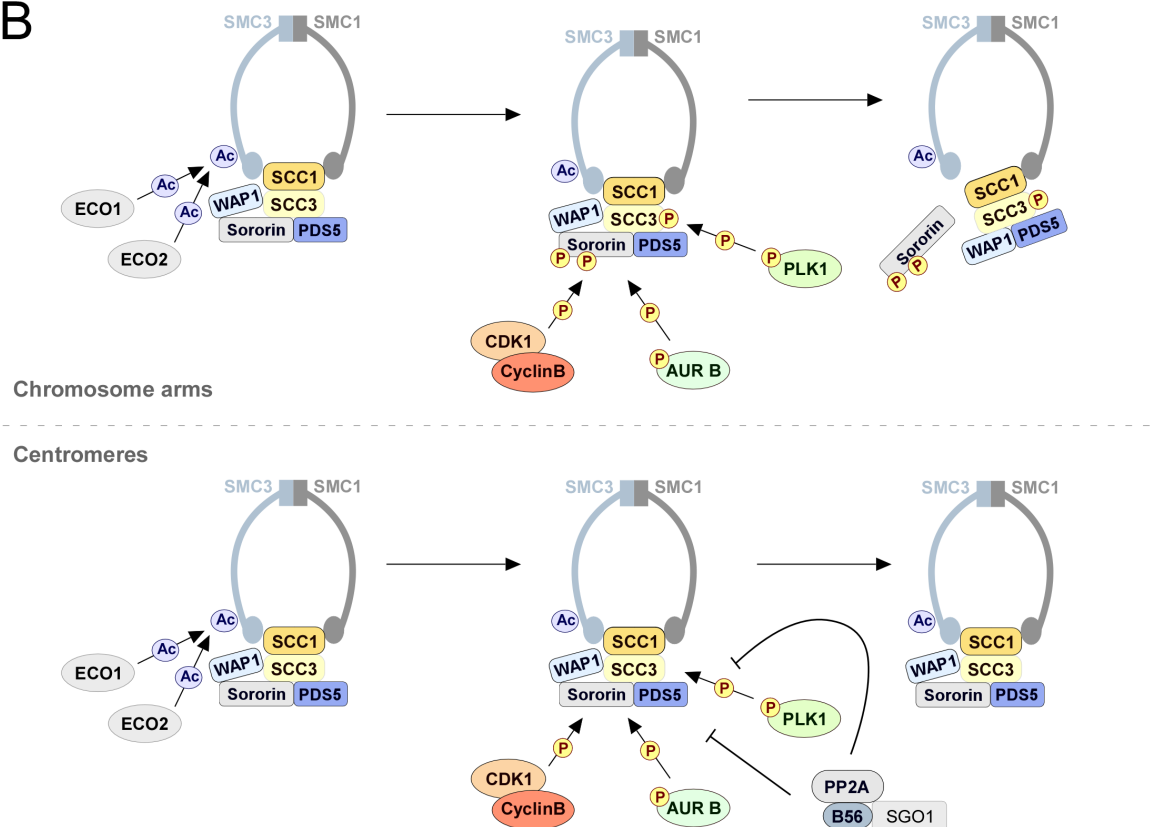


Figure 7 – PP2A:B56 maintains centromeric cohesion until anaphase onset. (A) Following DNA replication, sister chromatids are held together by cohesin-ring complexes. During prophase, Cdk1, Plk1, and Aurora B trigger Wapl-mediated removal of cohesin from chromosome arms. Centromere cohesion is protected from Wapl by the activity of PP2A:B56 bound to Sgo1. Centromeric PP2A:B56 antagonizes the phosphorylations catalysed by the mitotic kinases, hence maintaining cohesion at centromeres until kinetochores of all chromosomes are correctly attached to microtubules of opposite spindle poles. At this stage, Separase catalyses the proteolytic degradation of cohesin complexes, abolishing cohesion and enabling sister chromatid separation in anaphase. (B) During G2, cohesin rings are insensitive to Wapl by Sororin recruited to acetylated Smc3. The presence of Sororin prevents Wapl binding to Pds5. Following mitotic entry, Aurora B- and Cdk1-dependent phosphorylation of Sororin causes its dissociation, thus consenting Wapl binding to Pds5. Wapl-mediated removal of cohesin also requires phosphorylation of Scc3 by Plk1. At centromeres, PP2A:B56 in complex with Sgo1 counteracts Aurora B, Cdk1, and Plk1 destabilizing phosphorylations and thereby prevents the dissociation of cohesin complexes by Wapl.

To Build a Spindle: A PP1, PP2A, PP4 and PP6 Groundwork

Accurate segregation of chromosomes relies on the formation of a bipolar spindle structure (Wittmann et al, 2001). This requires the maturation and separation of duplicated centrosomes to originate two independent microtubule-organizing centres correctly positioned at opposite poles of the cell. Centrosome maturation occurs when cells prepare to enter mitosis and results from a dramatic expansion in the organized matrix of pericentriolar material (PCM) surrounding mother centrioles and a concomitant increase in its microtubule nucleation capacity (Mennella et al, 2014; Woodruff et al, 2014; Conduit et al, 2015). Expanded PCM serves as a catalytic scaffold for γ -tubulin ring complexes (γ -TuRC) to mediate microtubule nucleation (Stearns and Kirschner, 1994; Sunkel et al, 1995) and its assembly involves the recruitment of numerous PCM proteins orchestrated by Plk1 and Aurora A kinases (Conduit et al, 2015; Lattao et al, 2017). The Ser/Thr phosphatase PP4 is predominantly enriched at mitotic centrosomes of metazoan cells and its activity was shown to be required for γ -tubulin accumulation and microtubule nucleation in *Drosophila* and *Caenorhabditis elegans* embryos, as well as in human cultured cells. The mechanism by which PP4 promotes the maturation of centrosomes remains unknown, but the phosphatase appears to be necessary for proper centrosomal localization of Plk1 (Sumiyoshi et al, 2002) and activation of the Aurora A-CEP192 complex (Martin-Granados et al, 2008). Conversely, disruption of PP4 in MEFs has no impact on γ -tubulin levels, but leads to unstable contacts between PCM and microtubules minus-ends (Toyo-oka et al, 2008).

Regulation of microtubule dynamics at the centrosomes and spindle poles relies in part on the activity of the microtubule-severing Katanin complex (McNally and Vale, 1993; McNally, 2000), whose centrosomal localization is mediated by the interaction of its p60 subunit with the Dynein-motor regulator Ndel1 (Toyo-oka et al, 2005). Binding of p60 Katanin to Ndel1 increases during mitotic entry as a result of Ndel1 phosphorylation at Ser198, Thr219, and

Ser231 by Cdk1:Cyclin B (Toyo-oka et al, 2005). These phospho-sites are directly antagonized by PP4 phosphatase, which limits the accumulation of Katanin activity at centrosomes and thereby promotes the stabilization of microtubule connections (Toyo-oka et al, 2008). This is important to enable organized outgrowing of microtubules into a functional mitotic spindle, a process that additionally requires discrete activities of PP2A and PP6 phosphatases. Interestingly, in *C. elegans*, PP4 directly dephosphorylates the Katanin catalytic subunit homologue MEI-1 to promote microtubule-severing activity in meiosis (Han et al, 2009; Gomes et al, 2013).

Plus-end growth of microtubules emanating from centrosomes relies on the activity of the microtubule-polymerase ch-TOG/XMAP215/MSPS (Srayko et al, 2005), whose mechanism was recently uncovered in *Xenopus* egg extracts (Thawani et al, 2018). The C-terminus of XMAP215 directly interacts with γ -tubulin whereas the N-terminus TOG domains bind to soluble α/β -tubulin dimers to endorse their assembly onto γ -TuRCs (Thawani et al, 2018). The number of microtubules that grow out from centrosomes is limited by the activity of the microtubule depolymerizing kinesin MCAK/XKCM1/KLP-7 (Srayko et al, 2005; Hinoshita et al, 2005). In *C. elegans* embryos, the regulatory subunit, PR72/RSA-1, targets PP2A to centrosomes through a direct interaction with the PCM-associated RSA-2 protein. Centrosomal PP2A-PR72/RSA-1 promotes microtubule outgrowth and spindle stability by controlling KLP-7 and the spindle assembly factor, TPX2/TPXL-1. The PP2A-PR72/RSA-1 complex restricts the levels of KLP-7 through an unknown mechanism and directly mediates the accumulation of TPX2/TPXL-1 at centrosomes, thus increasing the rate of microtubules' nucleation as well as the length of centrosomal microtubules (Schlitz et al, 2007). No obvious RSA-2 orthologues have been identified so far and the requirement of PP2A-PR72 for spindle formation in other metazoans remains to be demonstrated. In fact, TPX2 displays limited centrosomal localization in vertebrate cells, accumulating preferentially on spindle microtubules (Kufer et al, 2002; Ozlü et al, 2005), where it plays a critical role in recruiting and promoting Aurora A activity required for chromatin-driven spindle assembly (Wittmann et al, 2000; Meunier and Vernos, 2016). Following nuclear envelope breakdown, the RanGTP that is produced by the chromatin-associated GEF RCC1 relieves TPX2 from inhibitory interactions with Importin- α/β (Gruss et al, 2001; Kalab and Heald, 2008; Cavazza and Vernos, 2016). This licenses TPX2 to bind and allosterically activate Aurora A (Kufer et al, 2002; Bayliss et al, 2003; Eysers et al, 2003; Tsai et al, 2003). The TPX2-Aurora A complex oligomerizes and binds to the RHAMM- γ -TuRC complex, which becomes competent for microtubule nucleation after Aurora A-mediated phosphorylation of the γ -TuRC adaptor subunit, Nedd1 (Groen et al, 2004; Pinoyl et al, 2013; Scrofani et al, 2015). Interestingly, microtubule-associated TPX2-Aurora A is more active in the purlieu of spindle

poles. This is owed to the spatially controlled activity of PP6 phosphatase and is critical for the stability of the assembling spindle (Zeng et al, 2010). In addition to allosteric regulation by TPX2 binding, dimerization-mediated T-loop autophosphorylation (Thr288) further enhances Aurora A activation (Zorba et al, 2014; Cyphers et al, 2017; Ruff et al, 2018). Moreover, the interaction with TPX2 protects the activating phosphorylation from the antagonizing action of several phosphatases, including PP1 and PP2A (Andrésson and Ruderman, 1998; Walter et al, 2000; Bayliss et al, 2003; Eysers et al, 2003; Tsai et al, 2003). The Ser/Thr phosphatase, PP6, is, however, able to recognize and dephosphorylate the T-loop of the TPX2-complexed form of Aurora A and its depletion was shown to impair proper spindle assembly (Zeng et al, 2010; Rusin et al, 2015). Although PP6 is uniformly distributed through the cytoplasm of mitotic cells, its activity towards TPX2-Aurora A is inhibited in the vicinity of centrosomes by action of Plk1 (Kettenbach et al, 2018). Following Cdk1-dependent priming, Plk1 binds to and phosphorylates the PP6-regulatory subunit, PP6R2, at multiple sites (Kettenbach et al, 2018). These phosphorylations repress PP6 activity possibly by preventing its interaction with substrates. Because Plk1 is particularly enriched at centrosomes, PP6-mediated dephosphorylation of TPX2-Aurora A complexes located on proximal microtubules is repressed, thus ensuring maximal Aurora A activation at spindle poles and moderate activation on distal spindle microtubules (Dodson and Bayliss, 2012; Joukov and Nicolo, 2018; Ruff et al, 2018). This enhances Aurora A-catalysed phosphorylation of transforming acidic coiled-coil (TACC) proteins at spindle poles, which enables TACC-ch-TOG complexes to efficiently bind to and stabilize the minus-ends of nascent microtubules and, in this way, promote growth of microtubules from the centrosome (Tournebize et al, 2000; Lee et al, 2001; Gergely, 2002; Giet et al, 2002; Gergely et al, 2003; Barros et al, 2005; Kinoshita et al, 2005; Peset et al, 2005; LeRoy et al, 2007; Burgess et al, 2018; Peset and Vernos, 2008). Hence, coordinated activities of Plk1, PP6, and Aurora A favour the centrosomes as dominant sites of spindle assembly in mitosis.

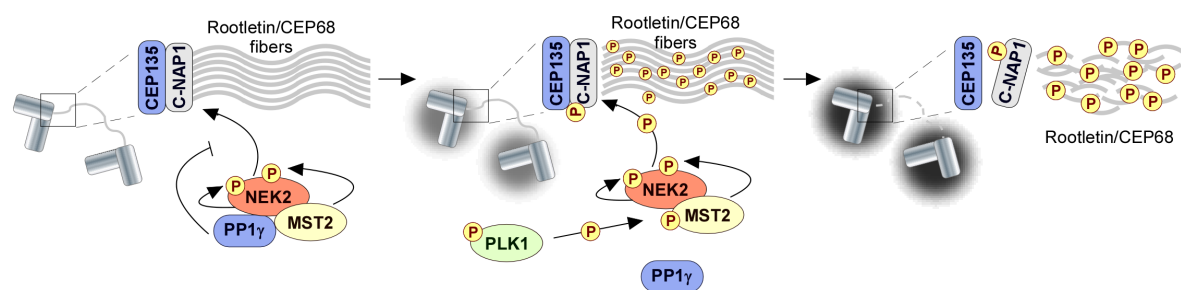


Figure 8 – PP1 prevents premature separation of centrosomes. Centrosome splitting during late G2 involves the dissolution of the Rootletin/CEP68 linker that holds duplicated centrosomes together. This process is orchestrated by Nek2A-mediated phosphorylation of C-Nap1 and of linker proteins. Phosphorylation of C-Nap1 abrogates its interaction with CEP135 docked on the proximal ends of parental centrioles, thus causing C-Nap1 displacement from centrosomes. Phosphorylation of Rootletin and of associated proteins disrupts the linker structure and enables centrosome disjunction. PP1 γ suppresses Nek2A activity up until late G2, thereby preventing premature resolution of centrosomes. During interphase, PP1 γ bound to Nek2A in complex with Mst2

is able to efficiently antagonize phosphorylations catalysed by the kinase. Increasing activity of Plk1 in late G2 results in Mst2 phosphorylation and consequential dissociation of PP1 γ from the complex. This renders Nek2A phosphorylations unopposed, hence allowing pre-mitotic resolution of centrosomes.

Setting two independent microtubule-organizing centres at opposite poles of a mitotic cell requires the separation of duplicated centrosomes, which typically occurs before nuclear envelope breakdown in organisms that undergo an open mitosis (Tanenbaum and Medema, 2010). Centrosome splitting during late G2 involves the dissolution of a fibrous proteinaceous linker that holds the duplicated centrosomes together, a process elicited by an increase in Nek2A kinase activity and controlled by PP1 phosphatase (Ghosh et al, 1998; Meraldi and Nigg, 2001; Agircan et al, 2014; Wang et al, 2014) (Figure 8). The primary target of Nek2A is C-Nap1, which is docked to the proximal ends of parental centrioles through the centriolar protein, CEP135, and serves as a platform for the assembly of Rootletin/CEP68 fibres (Fry et al, 1998; Yang et al, 2002; Bahe et al, 2005; Yang and Adamian, 2006; Graser et al, 2007). Data from STED microscopy and 3D-STORM analysis suggest that Rootletin/CEP68 filaments coming from each centriole are entwined into a web-like structure that probably underpins the flexible nature of the centrosomal linker (Vlijm et al, 2018). Multisite phosphorylation of C-Nap1 by Nek2A abrogates C-Nap1 interaction with CEP135, hence causing its displacement from centrosomes (Hardy et al, 2014). This concurs with Nek2A-mediated phosphorylation of Rootletin and of associated proteins to ultimately disrupt the linker structure and enable centrosome disjunction (Figure 8) (Fry et al, 1998; Faragher and Fry, 2003; Bahmanyar et al, 2008; He et al, 2013; Hardy et al, 2014). The kinase activity of Nek2A is tightly regulated to prevent premature centrosome separation, whose occurrence was shown to result in aberrant nuclear positioning and incorrect spindle orientation (Zhang, Y. et al, 2012; Nam and Deursen, 2014; Decarreau et al, 2017). Activation of Nek2A occurs through homodimerization-induced autophosphorylation of the activation loop (Fry et al, 1999; Rellos et al, 2007; Croasdale et al, 2011). The kinase is also phosphorylated in its non-catalytic domain by sterile-20 like kinase Mst2, which significantly enhances its localization at centrosomes (Mardin et al, 2010). However, Nek2A activity towards C-Nap1, and possibly other substrates, is kept at residual levels up until late G2 by action of PP1 γ , which binds to the Nek2A-Mst2-hSAV1 complex through an RVxF motif on the Nek2A C-terminus (Helps et al, 2000; Eto et al, 2002; Mardin et al, 2011). Within the complex, PP1 γ antagonizes Nek2A phosphorylations on C-Nap1, thus maintaining the linker integrity and, consequently, the centrosomes tethered (Mardin et al, 2011). Whether PP1 γ or PP1 α dephosphorylates the Nek2A T-loop to directly inactivate the kinase remains unclear (Eto et al, 2002; Mi et al, 2007). Loss of centrosome cohesion is triggered by the activation of Plk1, which subsequently

phosphorylates Mst2 on Ser15, Ser18, and Ser516, and disrupts PP1 γ from the complex (Figure 8) (Mardin et al, 2010). The mechanism by which these phosphorylations abolish PP1 γ interaction with Nek2A is currently unknown. The absence of PP1 γ leaves Nek2A-mediated phosphorylations of linker proteins unopposed, thereby allowing pre-mitotic resolution of centrosomes and consequently minimizing the rate of erroneous interactions that the mitotic spindle might establish with chromosomes (Hames et al, 2001; Magidson et al, 2011; Kaseda et al, 2012; Silkworth et al, 2012).

Phosphatases not only control the assembly of the mitotic spindle, but also its orientation. The position that the spindle assumes in mitosis defines the axis of cell division and plays a key role in cell fate determination in tissues. Depletion of PP4 from progenitor cells of the mouse neocortex causes premature differentiation into neurons and severe defects in cortical layering and brain cytoarchitecture (Xie et al, 2013). Biochemical and functional studies demonstrated that PP4-mediated dephosphorylation of Ndel1 at Cdk1 sites promotes its binding to Lis1-Dynein to efficiently generate microtubule pulling forces that orient the spindle in parallel to the neuroepithelial surface (Xie et al, 2013). This mechanism was shown to be critical at the onset of neurogenesis to ensure extensive symmetric cell division required for the expansion of the progenitor pool (Xie et al, 2013). The orientation of the mitotic spindle is also regulated upstream by the distribution of the conserved G α i-LGN-NuMA module at the cell cortex (Siller and Doe, 2009; di Petro et al, 2016; Bergstralh et al, 2017; Seldin and Macara, 2017). Cortical NuMA recruits Dynein complexes that interact with the plus ends of astral microtubules and generate pulling forces to orient the spindle apparatus (Nguyen-Ngoc et al, 2007; Kotak et al, 2012; Laan et al, 2012). Hence, the localization of NuMA must be tightly controlled, as both insufficient and excessive levels at the cortex impair accurate spindle orientation (Du and Macara, 2004; Kotak et al, 2012). The association of NuMA with the cell cortex is dynamically modulated by concerted action of Cdk1:Cyclin B, Aurora A, Plk1, and several phosphatases. Prior to anaphase onset, phosphorylation of NuMA by Aurora A directs it from the spindle poles to the cortex (Gallini et al, 2016; Kotak et al, 2016), whereas phosphorylation by Plk1 and Cdk1:Cyclin B displaces NuMA from the cortex (Kotak et al, 2013; Sana et al, 2018). Importantly, phosphorylation of Thr2055 by Cdk1 is continuously antagonized by PP2A activity. This results in moderate levels of cortical NuMA during metaphase to ensure accurate spindle positioning and contributes to the enhancement of its cortical recruitment following Cdk1 inactivation, which is required to sustain robust spindle elongation in anaphase (Kotak et al, 2013; Zheng et al, 2014). Protein phosphatase 1- α in complex with its regulatory subunit Repo-Man also promotes cortical accumulation of NuMA in anaphase. This occurs, however, through a separate pathway that is kept in check during metaphase by

p37/UBXN2B, a cofactor of the p97 AAA ATPase (Lee et al, 2018). Although exactly how p37/UBXN2B limits PP1 α -Repo-Man activity towards NuMA is unknown, this balance is critical to prevent excessive Dynein-dependent forces and concomitant spindle orientation defects. Future studies are also expected to cast new light on the mechanism by which PP1 α -Repo-Man controls NuMA cortical localization. Moreover, at least in *C. elegans*, PP6 was also shown to positively regulate pulling forces during anaphase by promoting the cortical localization of LGN and NuMA homologues (Afshar et al, 2010; Kotak et al, 2016). However, the underlying mechanism remains elusive.

Kinetochores-Microtubule Attachments: PP1 and PP2A:B56 Keep Them Stable

To ensure faithful genome partitioning, sister kinetochores must become attached to microtubules emanating from opposite spindle poles. This attachment configuration is termed amphitelic or bioriented and enables the dividing cell to segregate sister chromatids towards opposite sides during anaphase and in this way distribute one copy of each chromosome per daughter nucleus. Amphitelic attachments assume an end-on arrangement with the plus-end of microtubules embedded within the kinetochore. This is believed to provide sufficient stability to sustain the force released by the depolymerisation of kinetochore-associated microtubules during anaphase (Musacchio and Desai, 2017). However, the initial contacts occurring between kinetochores and the spindle microtubules are asynchronous and stochastic, which implies that amphitelic attachments are often preceded by immature or erroneous microtubule interactions. Following nuclear envelope breakdown, kinetochores might establish lateral (kinetochores interact with the side of microtubules), syntelic (both kinetochores bind microtubules from the same spindle pole), or merotelic attachments (one kinetochore binds microtubules that originate from both spindle poles) (Cimini et al, 2001; Tanaka, et al, 2005; Kapoor et al, 2006; Magidson et al, 2011; Thompson and Comptom, 2011; Foley and Kapoor, 2013; Barisic et al, 2014). Consequently, accurate chromosome segregation requires the destabilization of the inadequate kinetochore-microtubule interactions and the formation of robust amphitelic end-on attachments, events that are orchestrated by the coordinated activities of molecular motors, kinases, and phosphatases (Bakhoum, Genovese et al, 2009; Bakhoum, Thompson et al, 2009; Zaytsev and Grishchuk, 2015).

A central player in the formation of stable end-on attachments is the Ndc80 complex at the outer-kinetochore, which directly binds microtubules through the CH domain and the N-terminal tail of Ndc80/Hec1 (Cheeseman et al, 2006; Wei et al, 2006; Ciferri et al, 2008). The CH domain encompasses several positively charged residues that contact both α and

β Tubulin at the intra- and inter-tubulin interfaces (Wei et al, 2006; Ciferri et al, 2008; Alushin et al, 2011). The highly basic nature of the structurally disordered N-terminal tail further increases the affinity for microtubules by directly interacting with negatively charged E-hooks of tubulin subunits (Alushin et al, 2011). This binding arrangement and the oligomerization of the Ndc80 complex confers the kinetochore the capacity to maintain a processive association with dynamic microtubules and in this way couple chromosome movement to microtubule depolymerization/polymerization (Alushin et al, 2011; Foley and Kapoor, 2013). The interaction between Ndc80 and tubulin is, however, destabilized by Aurora B-mediated phosphorylation of multiple residues on the Ndc80 N-terminal tail. The negatively charged phosphates neutralize the intrinsic positive charge of the Ndc80 N-terminus, significantly reducing its binding affinity for microtubules (Cheeseman et al, 2006; DeLuca et al, 2006; Wei et al, 2006; Ciferri et al, 2008; Guimarães et al, 2008; Miller et al, 2008; Alushin et al, 2011; Zaytsev et al, 2014; Zaytsev et al, 2015). Notably, this destabilization mechanism acts preferentially on incorrect kinetochore-microtubules attachments. Although the basis for this selectivity is not fully understood, the prevailing model is that the level of tension across centromeres and/or kinetochores plays an important role in controlling the capacity of Aurora B to phosphorylate Ndc80 (Tanaka et al, 2002; Liu et al, 2009; Lampson and Cheeseman, 2011; Krenn and Musacchio, 2015). Aurora B is the catalytic subunit of the chromosome passenger complex (CPC) that also comprises INCENP, Survivin, and Borealin as scaffold subunits. Survivin and Borealin mediate CPC recruitment to centromeres, which subsequently promotes Aurora B T-loop activation through *trans*-autophosphorylation (Bishop and Schumacher, 2002; Honda et al, 2003; Yasui et al, 2004; Sessa et al, 2005; Kelly et al, 2007; Wang et al, 2011; Zaytsev et al, 2016; Ruppert et al, 2018). Microtubules responsible for amphitelic attachments impose tension or alterations on the kinetochore architecture so that the distance between centromeric active Aurora B and the outer kinetochore increases enough to preclude Ndc80 phosphorylation (Figure 9 A-D) (Maresca and Salmon, 2009; Uchida et al, 2009; Wan et al, 2009; Maresca and Salmon, 2010). On the other hand, erroneous attachments fail to produce sufficient tension, thus leaving Ndc80 within reach of Aurora B activity to ultimately cause microtubule detachment (Lampson et al, 2004; Pinsky et al, 2006; Liu et al, 2009; Welburn et al, 2010). Because unattached kinetochores are tensionless, this error correction mechanism might seemingly represent a problem for the formation of initial end-on microtubule attachments (Figure 9 A). In that respect, it is important to consider that the early contacts that kinetochores establish with microtubules are predominantly lateral and that PP2A:B56 at outer-kinetochores was shown to play a critical role in antagonizing Aurora B dependent phosphorylations (Figure 9 B). The interaction of kinetochores with the side of microtubules is mediated by the molecular motors Dynein and CENP E and are

therefore impervious to the phosphorylation state of Ndc80 (Tanaka et al, 2005; Kapoor et al, 2006; Magidson et al, 2011; Foley and Kapoor, 2013; Barisic et al, 2014; Shrestha et al, 2017). Dynein minus-end motor activity transports chromosomes towards the spindle pole, exposing the kinetochores to a higher density of microtubules that might favor direct end-on capture (Li et al, 2007; Yang et al, 2007; Vorozhko et al, 2008; Monda and Cheeseman, 2018). As spindle microtubules become more stable and detyrosinated, CENP E motility increases to promote the movement of the persisting laterally attached chromosomes towards the equator (Kapoor et al, 2006; Cai et al, 2009; Barisic, 2015; Iemura and Tanaka, 2015; Barisic and Maiato, 2016; Itoh et al, 2018). Once it reaches microtubule ends, CENP E converts from a lateral transporter into a microtubule tip-tracker that tethers kinetochores to the dynamic microtubule tips, thus directly contributing for the formation and stability of end-on attachments (Lombillo et al, 1995; Gudimchuk et al, 2013; Shrestha and Draviam, 2013). Importantly, lateral attachments exert intermediate levels of tension that increase the distance between Aurora B and the outer-kinetochore. This concurs with the dephosphorylation of the Ndc80 N-terminal tail by PP2A:B56 γ to allow the formation of stable end-on attachments (Suijkerbuijk et al, 2012b; Kruse et al, 2013; Xu et al, 2013; Shrestha et al, 2017) (Figure 9 B-D). The recruitment of PP2A:B56 γ to the outer-kinetochore is directly mediated by a LxxIxE motif within the KARD domain of BubR1 (Suijkerbuijk et al, 2012b; Kruse et al, 2013; Xu et al, 2013; Wang, J. et al, 2016; Vallardi et al, 2018). A conserved pocket in the B56 γ regulatory subunit recognizes and binds to the LxxIxE motif (Hertz et al, 2016; Wang, J. et al, 2016; Wang, X. et al, 2016; Wu et al, 2017) (Figure 5 C). Phosphorylations within and around the LxxIxE motif, respectively catalysed by Cdk1 and Plk1, significantly strengthen the interaction (Elowe et al, 2007; Huang et al, 2008; Suijkerbuijk et al, 2012b; Kruse et al, 2013; Wang, J. et al, 2016; Wang, X. et al, 2016). Notably, although the binding pocket is conserved in all B56 isoforms, BubR1 binds preferentially to B56 γ and possibly to B56 δ and presents limited association with B56 α and B56 ϵ , which instead display preference for centromeric Sgo2 (Rivera et al, 2012; Nijenhuis et al, 2014; Meppelink et al, 2015; Vallardi et al, 2018). Similarly, several other mitotic proteins harboring LxxIxE motifs were shown to bind preferentially to B56 γ over B56 α (Vallardi et al, 2019). A possible explanation for B56 differential binding, and therefore localization, was recently attributed to specific differences in a small loop at the C-terminus of each B56 isoform. The identity of four critical amino acids within the loop seems to modulate its capacity to repress or allow binding to LxxIxE motifs (Vallardi et al, 2018). The residues (Glu405, Pro409, Val412, and Ala413) define the EPVA loop of B56 α and B56 ϵ subunits, which not only hampers binding to LxxIxE motifs, but is also critical to enable B56 α and B56 ϵ interaction with Sgo2. Remarkably, replacing the EPVA signature amino

acids by the corresponding residues of the B56 γ isoform (Thr405, Lys409, His412, and Gly413) enhanced B56 α ability to bind BubR1 and other LxxIxE-containing proteins (Vallardi et al, 2019). Although structural details on how the C-terminal loop controls B56 specificity remain unknown, these observations clearly demonstrate sub-functionalization between the B56 isoforms. Whether such specialization allows PP2A activity to be differently regulated in specific subcellular compartments represents an alluring possibility that could be addressed in future studies.

Because BubR1 associates with Knl1 in the absence of microtubules, the binding of PP2A:B56 γ to BubR1 places the phosphatase in the close proximity of Ndc80 to efficiently counteract Aurora B destabilizing activity and enable initial end-on microtubule interactions (Foley et al, 2011; Suijkerbuijk et al, 2012b; Kruse et al, 2013; Xu et al, 2013) (Figure 9 A,B). As microtubule occupancy increases, the outer-kinetochore is further pulled away from Aurora B zone of influence and attachments stability concomitantly increases. At this point, PP1 activity at kinetochores increases to silence the mitotic checkpoint (as discussed in the following section) and ensure that mature end-on microtubule attachments remain stabilized (Liu et al, 2010; Shrestha et al, 2017; Vallardi et al, 2017; Saurin, 2018) (Figure 9 C,D). Recruitment of PP1 to kinetochores is mediated in part by SILK and RVSF motifs on Knl1 N-terminus (Liu et al, 2010). At tensionless kinetochores, both PP1-docking motifs are phosphorylated by Aurora B, which significantly represses their ability to interact with PP1 (Liu et al, 2010) (Figure 9 A). As end-on attachments mature, Knl1 N-terminus is no longer accessible to Aurora B and PP2A:B56 γ dephosphorylates SILK and RVSF to allow PP1 binding (Figure 9 B-D).

The PP1 γ -Knl1 complex further suppresses kinetochore Aurora B activity, hence enhancing the stability of amphitelic attachments (Posch et al, 2010; Wurzenberger et al, 2012). Disrupting the PP1-Knl1 interaction destabilizes kinetochore-microtubule attachments on metaphase-aligned chromosomes while failing to cause a discernible impact on chromosome congression (Liu et al, 2010; Shrestha et al, 2017). Thus, although PP1 γ :Knl1 activity seems to be dispensable for initial microtubule binding, it does contribute to preserve attachment stability following biorientation. In that respect, PP1 γ :Knl1 antagonizes the activating T-loop autophosphorylation of Aurora B (Murnion et al, 2001; Rosasco-Nitcher et al, 2008; Posch et al, 2010) and enables the recruitment of the Ska complex to the KMN network (Cheerambathur et al, 2017; Janczyk et al, 2017) (Figure 9 C,D). The Ska complex at the kinetochore-microtubule interface is proposed to enhance the microtubule-binding affinity and end-tracking ability of the Ndc80 complex (Schmidt et al, 2012). This increases the stability of attached microtubule bundles and maintains the kinetochore associated with dynamic microtubules, thus enhancing the ability of end-on attachments to sustain load-

bearing force and couple chromosome movement to microtubules plus-end dynamics (Hanisch et al, 2006; Gaitanos et al, 2009; Raaijmakers et al, 2009; Welburn et al, 2009; Jeyaprakash et al, 2012; Schmidt et al, 2012; Auckland et al, 2017; Cheerambathur et al, 2017; Monda et al, 2017; Helgeson et al, 2018). Aurora B-mediated phosphorylation of the Ska complex reduces its binding affinity for Ndc80, which might provide a safety strategy to minimize premature stabilization of erroneous attachments during early prometaphase (Chan et al, 2012; Jeyaprakash et al, 2012; Schmidt et al, 2012; Abad et al, 2014; Cheerambathur et al, 2017). As chromosomes congress and PP1 γ -Knl1 activity increases, Ska recruitment is triggered and the complex becomes maximally enriched at amphitelically attached kinetochores (Raaijmakers et al, 2009; Chan et al, 2012; Jeyaprakash et al, 2012; Redli et al, 2016). In *C. elegans*, timely association of the Ska complex with bioriented kinetochores is facilitated by dephosphorylation of the Ndc80 N-terminal tail (Cheerambathur et al, 2017). Whether PP1 γ :Knl1 cooperates with PP2A:B56 γ to dephosphorylate Aurora B phospho-sites on the Ndc80 tail remains unclear, as well as PP1 γ :Knl1 aptitude to directly target Aurora B-mediated phosphorylations of the Ska complex. Interestingly, the Ska complex directly binds PP1 γ through the C-terminal domain (CTD) of its Ska1 subunit and independently of Knl1, thus representing an additional pathway to increment PP1 γ at kinetochores of bioriented chromosomes (Sivakumar et al, 2016). Although PP1 γ :Ska significantly contributes to timely inactivation of the mitotic checkpoint, its role in kinetochore-microtubule attachments is unclear (Sivakumar et al, 2016). The accumulation of PP1 on bioriented kinetochores can be further endorsed by spindle motors. The budding yeast Kinesin 5, CIN8, associates with PP1/GL7 through a canonical RVxF motif (Suzuki et al, 2018). Kinetochore localization of CIN8 dramatically increases during metaphase in a microtubule-dependent manner and is mediated by a direct interaction with the Dam1 complex, a functional analogue of the Ska complex in vertebrates (Gaitanos et al, 2009; Welburn et al, 2009; Suzuki et al, 2018). Binding to Dam1 places the PP1/GL7:CIN8 complex near the N-terminus of Ndc80, likely resulting in dephosphorylation of its N-terminal tail. This increases the affinity of Ndc80 for microtubules, and consequently, the stability and tension required for robust attachments (Suzuki et al, 2018). Human cells engage in a similar strategy, but differ in the choice of motor, as the human Kinesin-5 orthologue does not localize at kinetochores. Instead, PP1 γ binds to RVxF motifs on the plus-end directed motors, Kif18A (De Wever et al, 2014; Hafner et al, 2014) and CENP E (Kim et al, 2010). Pools of PP1 γ :Kif18A and PP1 γ :CENP E accumulate at the ends of kinetochore-attached microtubules as cells progress from prometaphase to metaphase, thus providing a localized delivery of PP1 to oppose Ndc80 phosphorylation and promote attachment stability (Kim et al, 2010; Kim and Stumpff, 2018).

Importantly, Aurora A-mediated phosphorylation of CENP E on its PP1-docking motif averts premature binding of PP1. This phosphorylation is maximal on polar chromosomes and is required for CENP E-powered congression along kinetochore-microtubule bundles (Kim et al, 2010). As chromosomes move away from Aurora A, dephosphorylation of CENP E takes place, enabling PP1 recruitment. Although it is presently uncertain which phosphatase pool dephosphorylates the PP1-docking motif on CENP E, this was shown to be essential for the formation of stable end-on attachments and biorientation of chromosomes that congressed from the spindle poles (Kim et al, 2010).

Several other PIPs have been shown to act as important kinetochore-targeting factors of PP1, but their precise impact on microtubule attachment has been difficult to discern. For instance, PP1 bound to MYPT1 was shown to antagonize the phosphorylated T-loop of Plk1, which consequently renders the kinase inactive (Totsukawa et al, 1999; Yamashiro et al, 2008). Contrasting with the previously described PP1-PIP complexes, MYPT1-PP1 associates with kinetochores preferentially during early mitosis and depends on Cdk1:Cyclin A activity (Dumitru et al, 2017). Cdk1:Cyclin A-mediated phosphorylation of MYPT1 promotes its interaction with Plk1, hence dampening Plk1 activity and therefore the stability of kinetochore-microtubule attachments (Dumitru et al, 2017). This is thought to generate a temporally-defined microtubule-destabilizing environment to facilitate the correction of erroneous attachments typically produced during early prometaphase (Kabeche and Compton, 2013). The observation that Plk1 activity is suppressed prior to chromosome biorientation seems somehow difficult to reconcile with its requirement for initial end-on attachments. However, it is becoming increasingly clear that Plk1 modulates the stability of kinetochore-microtubule interactions through multiple kinetochore/centromere pools and possibly responding to different inputs (Nishino et al, 2006; Elowe et al, 2007; Lénárt et al, 2007; Li et al, 2010; Bader et al, 2011; Hood et al, 2012; Liu et al, 2012; Maia et al, 2012; Moutinho-Santos et al, 2012; Suijkerbuijk et al, 2012b; Amin et al, 2014; Kakeno et al, 2014; Shao et al, 2015; Lera et al, 2016; Ehlen et al, 2020). The conserved regulatory subunit, Sds22/PPP1R7, has been reported to be essential for the activity of multiple PP1 isoforms at kinetochores. Depletion of Sds22/PPP1R7 in human cells perturbs kinetochore-microtubule attachments and results in chromosome alignment defects (Posh et al, 2010; Wurzenberger et al, 2012). Paradoxically, Sds22/PPP1R7 does not seem to localize at kinetochores and PP1 fails to dephosphorylate its kinetochore substrates while bound to Sds22/PPP1R7 (Eiteneuer et al, 2014). Critical insight into this apparent discrepancy came from the observation that Sds22 and I3 transiently act as chaperones during PP1 biogenesis (Weith et al, 2018). Binding of Sds22 and I3 stabilizes newly-synthesized PP1 in an inactive state until it is under control

of substrate specifier PIPs (Lesage et al, 2007; Eiteneuer et al, 2014; Cheng and Chen, 2015; Weith et. a., 2018). The p97 AAA-ATPase complex subsequently catalyses the disassembly of the heterotrimeric complex, which enables PP1 to associate with one of its numerous adaptor subunits and form a functional holoenzyme (Weith et al, 2018).

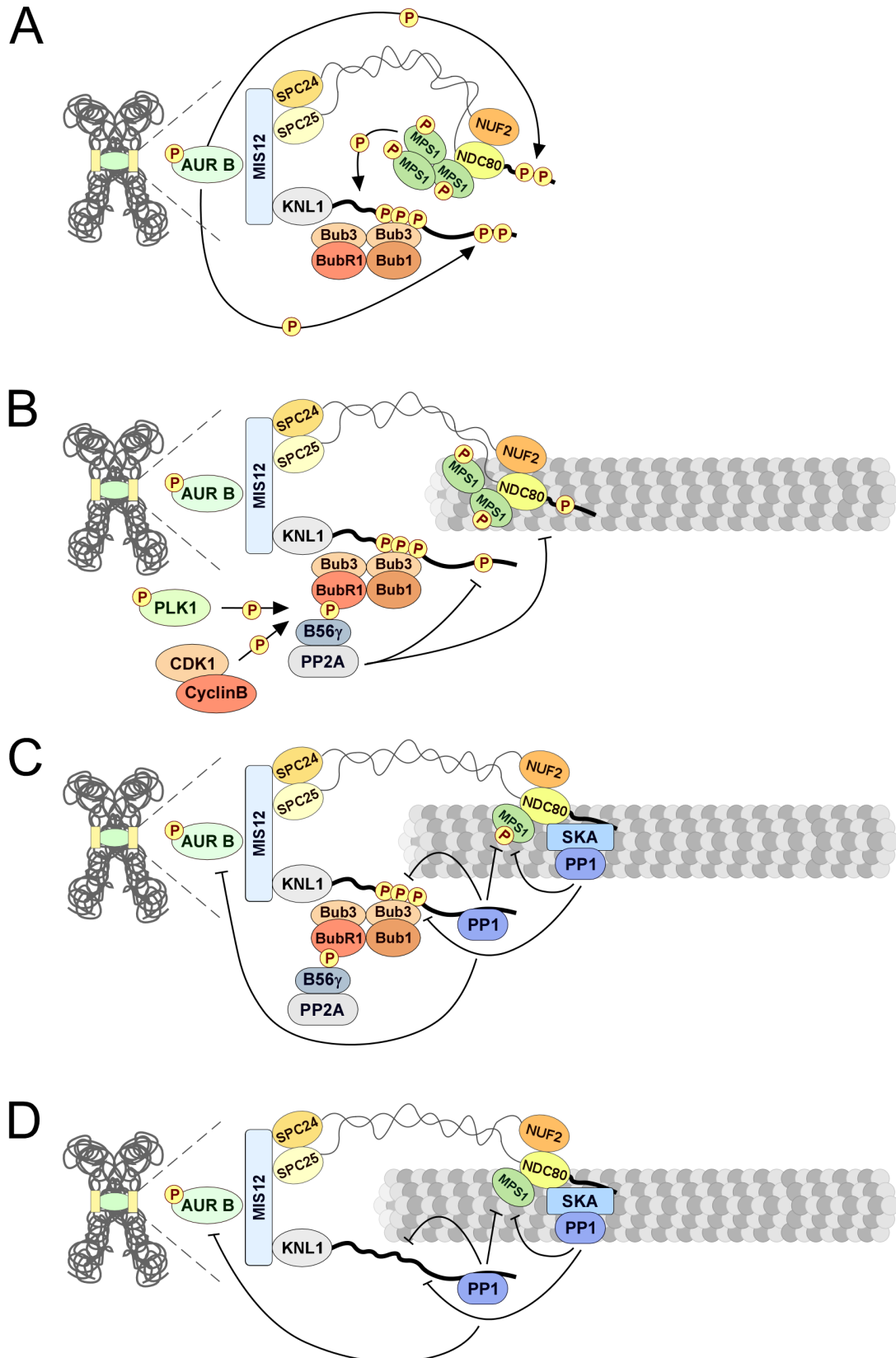


Figure 9 – Kinetochore PP2A:B56 and PP1 promote the stability of microtubule attachments and efficient Spindle Assembly Checkpoint silencing in animal cells. (A,B) In the absence of microtubules, Mps1 kinase efficiently accumulates at kinetochores through direct binding to the Ndc80 complex. This enables Mps1 trans-autoactivation and instates SAC signalling. Mps1 phosphorylates several MELT motifs on Knl1, generating docking sites for the hierarchical recruitment of SAC proteins required for MCC assembly. This also enables the recruitment of PP2A:B56 γ to kinetochores, following Plk1-mediated phosphorylation of BubR1 KARD motif. PP2A:B56 γ bound to BubR1 has critical roles in allowing the initial binding of microtubules to Ndc80 and in establishing SAC responsiveness. The activity of PP2A:B56 γ :BubR1 antagonizes microtubule-destabilizing phosphorylations on the Ndc80 N-terminal tail. These phosphorylations are catalysed by centromeric Aurora B and predominate at tensionless kinetochores to enable the correction of erroneous attachments. Dephosphorylation of the Ndc80 N-terminal tail by PP2A:B56 γ :BubR1 provides Ndc80 an opportunity to bind microtubules. (C,D) As microtubules occupancy increases, kinetochore tension or alterations on the kinetochore architecture place outer-kinetochore proteins further apart from the Aurora B zone of influence. This renders PP2A:B56 γ :BubR1 activity largely unopposed, which ultimately results in complete dephosphorylation of Ndc80 and in the formation of stable end-on attachments. At this point, several pools of PP1 γ accumulate at the kinetochore to preserve the stability of bioriented attachments and promote prompt SAC silencing. The recruitment of PP1 is mediated in part by its interaction with PP1-binding motifs on the Knl1 N-terminus. PP1 γ :Knl1 dephosphorylates phospho-MELTs, causing the dissociation of SAC proteins from kinetochores and consequently ceasing MCC assembly. Binding of PP1 γ to Knl1 is limited at unattached or tensionless kinetochores due to Aurora B-mediated phosphorylation of both PP1-docking motifs, which significantly represses their ability to interact with the phosphatase. These phosphorylations are counteracted by the activity of PP2A:B56 γ bound to BubR1. Thus, as end-on attachments are established, the Knl1 N-terminus becomes inaccessible to Aurora B and PP2A:B56 γ ensures efficient recruitment of PP1 γ . Increasing levels of PP1 γ :Knl1 further suppress Aurora B activity at kinetochores, hence enhancing the stability of microtubule attachments. PP1 γ :Knl1 is also proposed to directly antagonize the activating T-loop autophosphorylation of Aurora B, which likely favours the recruitment of the Ska complex to dephosphorylated Ndc80. The Ska complex directly binds PP1 γ through its Ska1 subunit and independently of Knl1, thus representing an additional pathway to deliver PP1 γ to kinetochores of bioriented chromosomes. Importantly, because Mps1 and microtubules have partially overlapping binding surfaces on the Ndc80 complex, the formation of stable end-on attachments imposes a substantial reduction in the amount of Mps1 at kinetochores. Residual Mps1 molecules remaining on bioriented kinetochores are dephosphorylated and inactivated by PP1. These mechanisms concur to efficiently avert SAC function at the top of the signalling cascade.

Silencing the Spindle Assembly Checkpoint: PP1 and PP2A:B56 Shut It Up

To ensure that each daughter nucleus receives one copy of the genome, chromosome segregation can only occur after all sister-kinetochore have established stable amphitelic attachments. Timely control of anaphase onset is achieved through the spindle assembly checkpoint (SAC), a conserved signalling pathway primarily instated by unattached/tensionless kinetochores that inhibits the ubiquitin-dependent proteolysis of Securin and Cyclin B. Degradation of Securin triggers the cleavage of centromeric cohesion by the protease Separase to enable sister chromatid separation, whereas reduced levels of Cyclin B lead to inactivation of Cdk1, which consequently reverts the cell into an interphase state. According to the prevailing mechanistic model of SAC signalling, unattached/tensionless kinetochores catalyse the assembly of a diffusible protein tetramer known as mitotic checkpoint complex (MCC) that binds to the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) and thereby prevents it from targeting Securin and Cyclin B for degradation (London and Biggins, 2014b; Musacchio, 2015; Joglekar, 2016;

Corbett, 2017). A weakened SAC signalling was shown to allow anaphase onset in the presence of one or few unattached chromosomes, which resulted in varied levels of aneuploidy (Michel et al, 2001; Dai et al, 2004; Iwanaga et al, 2007; Schwartzman et al, 2010). On the other hand, failing to switch off the SAC in a timely manner delays the cells in metaphase, thus increasing the likelihood of merotelic attachments and cohesion fatigue, errors that often lead to lagging chromosomes and chromatin bridges (Schwartzman et al, 2010; Gorbsky, 2013). Hence, to safeguard against genome imbalances, the SAC must be robust enough so that a single unattached kinetochore is sufficient to halt the transition to anaphase, but also highly responsive to allow swift mitotic exit upon formation of stable microtubule attachments (Rieder et al, 1995; Dick and Gerlich, 2013; Saurin, 2018). Activation and strength of SAC signalling is driven by the activity of multiple mitotic kinases, with monopolar spindle 1 (Mps1) assuming the leading role (Liu and Winey, 2012; Pachis and Kops, 2018). Unsurprisingly, counteracting phosphatases play a critical role in extinguishing the checkpoint signal and ensure timely cell cycle progression.

Mps1 associates with unattached kinetochores and phosphorylates Knl1 on its MELT repeats, creating docking sites for the hierarchical recruitment of additional SAC proteins necessary for MCC assembly (London et al, 2012; Shepperd et al, 2012; Yamagishi et al, 2012; Primorac et al, 2013; Vleugel et al, 2015) (Figure 9 A). Heterodimers of Bub1-Bub3 bind to phosphorylated MELTs and recruit BubR1:Bub3 through hetero-dimerization (Overlack et al, 2015). Mps1 phosphorylates Knl1-associated Bub1 to promote the binding of Mad1-Mad2 complexes (London and Biggins, 2014a; Mora-Santos et al, 2016; Faesen et al, 2017; Ji et al, 2017; Qian et al, 2017; Zhang, G et al, 2017). Subsequently, Mps1-mediated phosphorylation of Mad1 dramatically accelerates the structural conversion of Mad2 into a conformer that binds to Cdc20, the rate-limiting step for MCC assembly (Faesen et al, 2017; Ji et al, 2017). These multi-target phosphorylations along the SAC signalling cascade render the checkpoint highly responsive to variations in Mps1 activity (Ciliberto and Hauf, 2017). Activation of Mps1 is triggered during early mitosis by T-loop *trans*-autophosphorylation. The CH domains of Ndc80 and Nuf2 directly mediate the recruitment of Mps1 to unattached kinetochores (Hiruma et al, 2015; Ji et al, 2015), which likely favors the kinase trans-autoactivation (Kang et al, 2007; Mattison et al, 2007; Jelluma et al, 2008a; Tyler et al, 2009; Wang et al, 2009; Hewitt et al, 2010). Kinetochore tethering of Mps1 and its activity are enhanced by Aurora B, Plk1, and Cdk1, but the underlying mechanisms remain largely unknown (Santaguida et al, 2011; Saurin et al, 2011; Morin et al, 2012; Conde et al, 2013; Nijenhuis et al, 2013; von Schubert et al, 2015; O'Connor et al, 2016; Ikeda and Tanaka, 2017). On the other hand, Mps1 promotes centromeric accumulation of Aurora B via the Bub1:H2A:Sgo1 axis and was shown to directly foster

Aurora B activity, thus setting a positive feedback loop that ensures prompt and robust activation of both kinases at the onset of mitosis (Jelluma et al, 2008b; van der Waal et al, 2012).

Once all kinetochores attach to microtubules and become stably bioriented, the SAC must be silenced to permit APC/C activation and initiate anaphase. This primarily entails mechanisms that prevent and revert the phosphorylations catalysed by Mps1. The region of the Ndc80 CH domain that interacts with Mps1 partially overlaps with the microtubule binding interface (Hiruma et al, 2015), which significantly restrains Mps1 ability to associate with kinetochores at a high microtubule occupancy. This biochemical competition imposes a severe reduction on the amount of Mps1 present at kinetochores once stable end-on attachments are established (Hiruma et al, 2015; Ji et al, 2015) (Figure 9 A-D). This concurs with increased kinetochore localization of PP1, which in *Drosophila* cells was shown to dephosphorylate the T-loop of Mps1 to ensure the inactivation of residual Mps1 molecules remaining at kinetochores and thereby efficiently avert the phosphorylation of SAC substrates during metaphase (Moura et al, 2017) (Figure 9 C,D). PP2A:B55 was also shown to dephosphorylate Mps1 in MEFs (Diril et al, 2016), but the functional relevance of the targeted residue remains unclear (Tyler et al, 2009; Morin et al, 2012).

Suppressing Mps1 activity at bioriented kinetochores is not sufficient to promptly silence the SAC, as the phosphorylations carried out to instate the checkpoint still need to be reversed. This task has been almost consensually attributed to PP1 (Bloecher and Tatchell, 1999; Sassoone et al, 1999; Pinsky et al, 2009; Vanoosthuyse and Hardwick, 2009; Meadows et al, 2011; Rosenberg et al, 2011; Espeut et al, 2012; London et al, 2012; Nijenhuis et al, 2014). Several PP1-PIP complexes were shown to contribute for SAC silencing. PP1 γ bound to Knl1 is particularly well positioned, as it lies adjacent to the site of MCC production. Impairing PP1 γ interaction with Knl1 prevents the dephosphorylation of MELT motifs and delays exit from mitosis in different organisms (Meadows et al, 2011; Rosenberg et al, 2011; Espeut et al, 2012; London et al, 2012; Nijenhuis et al, 2014; Zhang et al, 2014). Human cells lacking the PP1 γ Knl1 complex and incubated with microtubule depolymerizing drugs were able to remain in mitosis even after Mps1 inhibition, suggesting that PP1 directly targets the phospho-MELTs to cease the recruitment of SAC proteins and consequently silence the SAC (Nijenhuis et al, 2014). Efficient binding of PP1 γ to Knl1 relies on the dephosphorylation of SILK and RVxF motifs by PP2A:B56 γ in complex with BubR1, which is itself indirectly recruited to Knl1 upon MELT phosphorylation (Liu et al, 2010; London et al, 2012; Shepperd et al, 2012; Yamagishi et al, 2012; Nijenhuis et al, 2014) (Figure 9 A-C). This negative feedback loop enables the checkpoint to prime its own inhibition, thus rendering signalling quenching highly responsive to microtubule binding

(Meppelink et al, 2015). Prompt SAC silencing following end-on attachment is further ensured by pools of PP1 γ delivered to kinetochores through microtubules (Meadows et al, 2011; Tang and Toda, 2015; Sivakumar et al, 2016) (Figure 9 C,D). In fission yeast, Dis2/PP1 bound to the kinesin-8 motor Klp5:Klp6 heterodimer cooperates with Spc7/Knl1-associated Dis2/PP1 in switching off the SAC (Meadows et al, 2011). However, the kinetics of checkpoint silencing mediated by each pathway differs considerably, with Dis2:Spc7 acting firstly under low microtubule occupancy, while Dis2:Klp5:Klp6 appears to operate during later time points as attachments stabilize (Meadows et al, 2011; Tang and Toda, 2015). Preventing PP1 γ binding to the KIF18A orthologue in human cells delays the transition to anaphase, but whether this is caused by inefficient SAC silencing is unclear as the activity of PP1 γ :Kif18A is required for the stability of kinetochore-microtubule attachments (Hafner et al, 2014; Kim and Stumpff, 2018). Likewise, disruption of the interaction between PP1 γ and Ska1 through deletion of Ska1 CTD results in a prolonged metaphase delay (Sivakumar et al, 2016). Notably, replacing the CTD by a PP1-binding motif or by a direct fusion to PP1 γ largely restores timely anaphase onset, suggesting that the recruitment of PP1 γ mediated by the Ska complex is important to efficiently silence the SAC at bioriented kinetochores (Sivakumar et al, 2016). However, Ska1 CTD has been shown to be required for the stability of kinetochore-microtubule attachments (Schmidt et al, 2012; Abad et al, 2014), which calls for some caution in ascribing a direct role for PP1 γ :Ska1 in switching off the SAC. It is possible that PP1 γ :Ska1 might instead counteract destabilizing phosphorylations on Ndc80 and thereby indirectly promote SAC silencing by increasing the stability of end-on attachments (Saurin, 2018). In line with that, the Ska complex was shown to directly interact with multiple regions of Ndc80 (Zhang, G. et al, 2012; Cheerambathur et al, 2017; Janczyk et al, 2017; Zhang, Q. et al, 2017; Helgeson et al, 2018; Zhang et al, 2018). On the other hand, this also means that PP1 γ is favourably positioned to dephosphorylate and inactivate Mps1 when robust microtubule attachments are established (Saurin, 2018). It is therefore important to determine which specific phosphorylations are targeted by each pool of PP1 γ present at kinetochores. This will be critical to better understand their role and how the activities of PP1 γ :Knl1 and PP1 γ complexes recruited through microtubules are coordinated to ensure efficient SAC silencing. In that respect, recent structural data have shown that PP1-docking motifs in Knl1 overlap extensively with domains that also mediate binding to microtubules, and, at least *in vitro*, these are mutually exclusive (Bajaj et al, 2018). This hints that in mammalian cells, as described for fission yeast (Meadows et al, 2011; Tang and Toda, 2015), PP1 γ :Knl1 operates mainly at a low microtubule occupancy earlier in mitosis and sets up the recruitment of microtubule-dependent pools of PP1 γ to bioriented kinetochores. This would

be consistent with the observation that Knl1 mutants defective in PP1 γ binding fail to switch off the SAC in nocodazole (Nijenhuis et al, 2014), while exhibiting only a modest metaphase delay under unperturbed conditions (Saurin et al, 2011), whereas loss of the PP1 γ :Ska1 complex results in a prominent SAC-dependent metaphase arrest, but only marginally affects mitotic exit when Mps1 is inhibited in the absence of microtubules (Sivakumar et al, 2016; Saurin, 2018).

Interestingly, PP2A:B56 γ bound to BubR1 might also directly contribute to dephosphorylate phospho-MELT motifs (Espert et al, 2014) and was recently shown to antagonize Mps1-mediated phosphorylation of Bub1 Thr461 (Qian et al, 2017). This phosphorylation stimulates Mad1 binding (Ji et al, 2017; Qian et al, 2017; Zhang, G. et al, 2017) and appears to be only transiently required for SAC initiation immediately upon nuclear envelope breakdown (Qian et al, 2017). Dephosphorylation of Bub1 Thr461 is triggered during early prometaphase concomitantly with the recruitment of PP2A:B56 γ to BubR1. Phosphorylation of Bub1 Thr461 is virtually undetectable in metaphase or after a drug-elicited prometaphase arrest, suggesting that Bub1 dephosphorylation by PP2A:B56 γ is impervious to kinetochore attachment status (Qian et al, 2017). Thus, Mps1 and PP2A:B56 γ :BubR1 establish an incoherent feedforward loop that restricts Bub1 Thr461 phosphorylation to the period of time required to assemble PP2A:B56 γ :BubR1 at kinetochores (Qian et al, 2017). Because this transient pulse in Bub1 Thr461 phosphorylation is not affected by microtubule occupancy, it was proposed to function as a biochemical timer defining the minimum length of time the cell spends in mitosis (Qian et al, 2017). Bub1 further promotes APC/C inhibition through an MCC-independent mechanism. Kinetochore Bub1 acts as a scaffold for Plk1-mediated phosphorylation of the APC/C co-activator, Cdc20. Although the mechanistic underpinnings remain elusive, this phosphorylation was shown to inhibit Cdc20 ability to bind and activate the APC/C (Jia et al, 2016). The inhibitory phosphorylation was proposed to be removed by PP2A:B56 γ :BubR1 (Jia et al, 2016). However, Cdc20 phosphorylation remained more stable in cells incubated with okadaic acid than in cells depleted of all B56 isoforms, suggesting that PP1 or additional phosphatases might contribute to dephosphorylate Cdc20 and enable APC/C activation (Labit et al, 2012; Craney et al, 2016). Further inhibition of Cdc20 is promoted by Cdk1:Cyclin B, which paradoxically also phosphorylates the APC/C to ensure its proper ubiquitination activity (Labit et al, 2012; Craney et al, 2016; Hein et al, 2017; Kim et al, 2017; Lee et al, 2017). PP2A:B55 has been implicated in opposing the inhibiting and activating Cdk1 phosphorylations on Cdc20 and APC/C, respectively (Hein et al, 2017). The intrinsic preference of PP2A:B55 towards pThr over pSer is proposed to provide a short period of time in which dephosphorylated Cdc20 is able to interact with phosphorylated and functional APC/C (Hein et al, 2017). However, the APC/C-Cdc20 is

known to be active prior to reactivation of PP2A:B55 (Nilsson, 2018), which in fact requires APC/C-Cddc20 dependent repression of Gwl activity. Hence, removal of Cdk1 inhibitory phosphorylations on Cdc20 likely relies on the activity of other phosphatases, an undertaking recently attributed to PP1 γ -Kn1 in *C. elegans* (Kim et al, 2017).

Mitotic Exit: PP1 and PP2A:B55 Clear the Way

Once the SAC is switched off and APC/C:Cdc20-dependent degradation of Securin and Cyclin B occurs, the cell becomes irreversibly committed to exit mitosis. Resumption of an interphase state requires remodeling of the spindle architecture, reassembly of the functional nuclear envelope, and decondensation of chromatin (Wurzenberger and Gerlich, 2011). This extensive structural reorganization relies not only on the inactivation of Cdk1, but also on the removal of mitotic phosphorylations, an assignment that in animal cells is primarily led by PP2A:B55 and PP1 phosphatases (Wurzenberger and Gerlich, 2011; Cundell et al, 2016). A key event during anaphase is the reorganization of spindle microtubules into a structure referred to as the central spindle. This is assembled between the segregating chromosomes to define the position of the cleavage furrow, thus ensuring spatial and temporal coordination of cytokinesis with genome partitioning (Neef et al, 2007; Petronczki et al, 2007; Santamaria, et al, 2007; Wolfe et al, 2009; Wurzenberger and Gerlich, 2011). Formation of the central spindle requires PRC1, a microtubule-bundling protein that stabilizes and organizes microtubules in an antiparallel overlapped fashion and promotes anaphase spindle elongation (Walczak and Shaw, 2010). Phosphorylation of PRC1 by Cdk1:Cyclin B prevents its microtubules' bundling activity before anaphase (Jiang et al, 1998; Mollinari et al, 2002; Mishima et al, 2004; Zhu et al, 2006). Timely PRC1 dephosphorylation is catalysed by PP2A:B55 (Cundell et al, 2013) to enable PRC1 interaction with the kinesin motor Kif4 and its consequent translocation to the plus ends of antiparallel interdigitating microtubules (Zhu et al, 2005; Walczak and Shaw, 2010) (Figure 10 A). Furthermore, PP2A:B55-mediated dephosphorylation promotes PRC1 homodimerization that is required for its microtubule bundling activity and thereby for the assembly of the central spindle (Zhu et al, 2006; Bieling et al, 2010; Subramanian et al, 2010; Cundell et al, 2013) (Figure 10 A).

Reformation of the nuclear envelope and associated nuclear pore complexes around chromatin occurs at the end of mitosis to establish functional interphase nuclei (de Castro et al, 2016; Schellhaus et al, 2016; LaJoie and Ullman, 2017). Disassembly of the nuclear envelope and of nuclear pore complexes at mitotic entry is triggered by a myriad of phosphorylations catalysed by several mitotic kinases (Figure 10 B). Phosphorylation of

Lamins by Cdk1 promotes depolarization of the nuclear lamina, the filamentous protein meshwork underlying the nuclear membranes (Heald and McKeon, 1990; Peter et al, 1990). Cdk1 further cooperates with Plk1 in the phosphorylation of several nucleoporins to elicit disintegration of nuclear pore complexes (Macaulay et al, 1995; Favreau et al, 1996; Mansfeld et al, 2006; Glavy et al, 2007; Laurell et al, 2011; de Castro et al, 2017; Linder et al, 2017; Martino et al, 2017). Moreover, phosphorylation of the chromatin binding protein BAF by VRK reduces its affinity for chromatin and towards the LEM family of inner nuclear membrane proteins interacting with the nuclear lamina (Nichols et al, 2006; Gorjánácz et al, 2007; Margalit et al, 2007). Loss of the BAF-mediated link between chromatin and the inner nuclear membrane at mitotic entry is proposed to contribute to nuclear envelope disassembly. Phosphorylations on BAF are however removed during mitotic exit by action of PP2A:B55 (Figure 10 B). This restores BAF interaction with chromatin and with components of the nuclear periphery, which was shown to be required for timely nuclear envelope reformation in worms, flies, and human cells (Asencio et al, 2012; Mehsen et al, 2018). Furthermore, recent findings in *Drosophila* cells suggest that PP2A:B55 might also target Lamin and Nup107 to direct their assembly into the nuclear envelope during mitotic exit (Mehsen et al, 2018). Further evidence supporting PP2A:B55 involvement in nuclear pore reformation come from the observation that the phosphatase dephosphorylates Nup153 (as well as Nup107) to promote its recruitment to chromatin in human cells (Cundell et al, 2016) (Figure 10 B).

Several findings indicate that PP1 is equally important for reassembly of functional nuclear envelopes. Binding to AKAP149 localizes PP1 on the assembling nuclear envelope, where it dephosphorylates Lamin B to promote its polymerization and accelerates nuclear lamina assembly (Thompson et al, 1997; Steen et al, 2000; Steen et al, 2003) (Figure 10 B). The regulatory subunit, Repo-Man, targets PP1 γ to the periphery of late anaphase chromosomes, where the initial reassembly events take place (Güttinger et al, 2009; Vagnarelli et al, 2011; LaJoie and Ullman, 2017; Ungricht and Kutay, 2017). There, the N-terminus of Repo-Man directly mediates the recruitment of Importin- β to control early nuclear envelope formation and nucleoporin deposition (Zhang et al, 2002; Harel et al, 2003; Walther et al, 2003; Clarke and Zhang, 2008; Rotem et al, 2009; Vagnarelli et al, 2011) (Figure 10 B). Binding of Importin- β to Repo-Man does not require PP1 γ catalytic activity and is repressed during mitosis by Cdk1 phosphorylations on the Repo-Man N-terminus (Vagnarelli et al, 2011). Localized activity of PP1 seems, however, to be required for later steps of nuclear pore complex formation. One subset of the nucleoporins that is controlled by Importin- β is the Nup107–Nup160 complex (Hoelz et al, 2011), which is recruited to chromatin by the nuclear pore complex assembly factor, ELYS/MEL-28

(Fernandez and Piano, 2006; Galy et al, 2006; Rasala et al, 2006; Franz et al, 2007; Gillespie et al, 2007). This requires RanGTP-dependent relief of Nup107:Nup160 nucleoporins from an inhibitory association with importin- β , most likely docked at the vicinity of ELYS/MEL-28 by Repo-Man (Güttinger et al, 2009; Vagnarelli et al, 2011; Ungricht and Kutay, 2017). This results in the formation of chromatin-bound pre-pores onto which additional nucleoporins are subsequently assembled, a process that in *C. elegans* was recently shown to require the contribution of PP1c/GSP-2 bound to ELYS/MEL-28 (Hattersley et al, 2016). However, direct evidence for dephosphorylation events catalysed by PP1c/GSP-2 and a mechanistic understanding of its role in the reassembly of nuclear pore complexes are still missing.

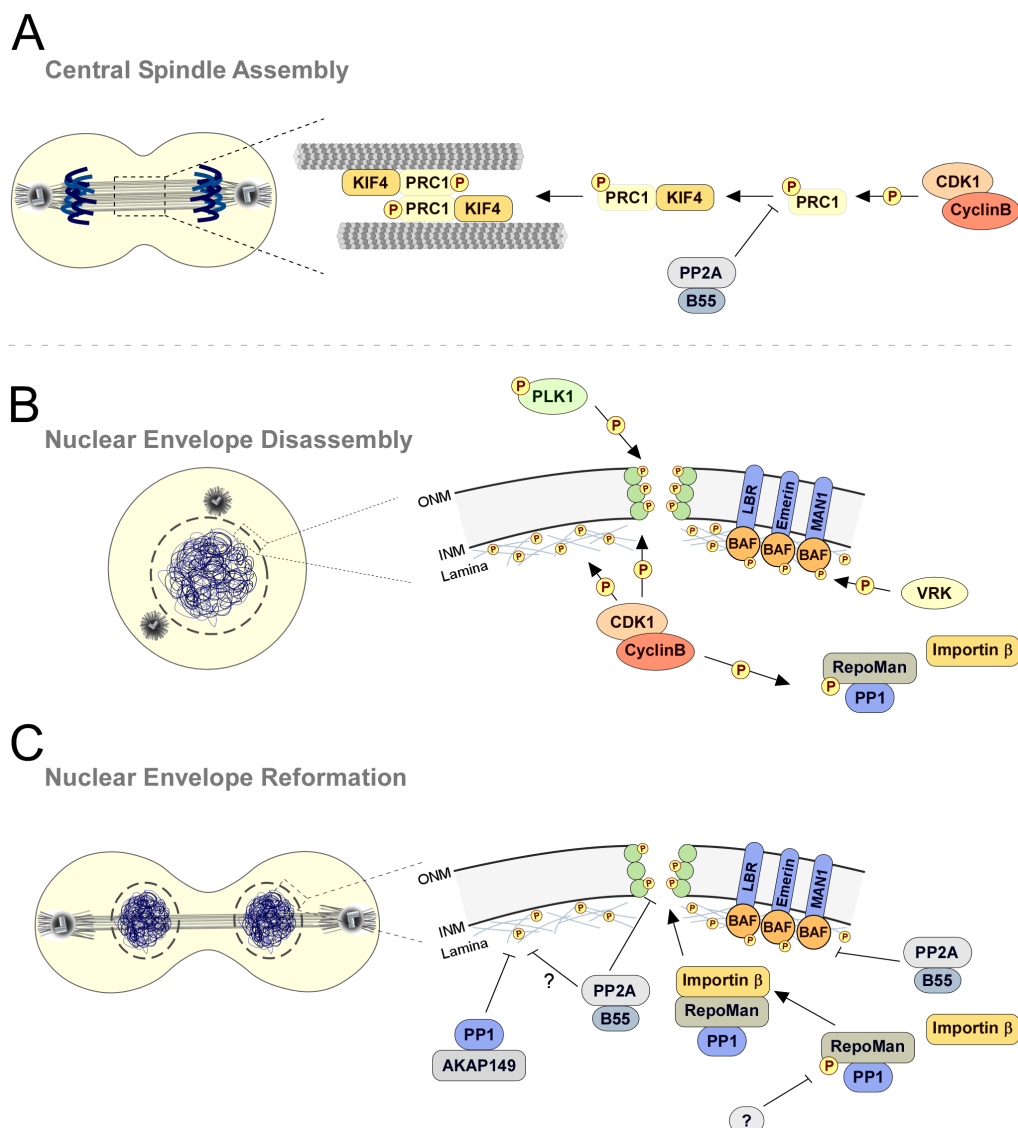


Figure 10 – PP2A:B55 and PP1 activities orchestrate mitotic exit. (A) Formation of the central spindle during anaphase relies on the activity of PRC1. Phosphorylation of PRC1 by Cdk1:Cyclin B precludes its microtubule bundling activity. Dephosphorylation of PRC1 is catalysed by PP2A:B55 during anaphase and enables PRC1 interaction with the kinesin motor Kif4, its consequent translocation to the plus ends of antiparallel interdigitating microtubules, and the protein homodimerization required for its microtubule bundling activity and thereby for the assembly of the central spindle. (B) Reformation of the nuclear envelope and associated nuclear pore

complexes around chromatin occurs at the end of mitosis. This requires removal of the multiple phosphorylations that were catalysed during mitotic entry to promote the disassembly of the nuclear envelope and of nuclear pore complexes. During late anaphase/telophase, PP2A:B55-mediated dephosphorylation of BAF restores its interaction with chromatin and with components of the inner nuclear membrane (INM). PP2A:B55 might also target Lamin and several nucleoporins to direct their assembly into the nuclear envelope. Multiple pools of PP1 cooperate with PP2A:B55 to promote nuclear envelope reformation. AKAP149 localizes PP1 on the assembling nuclear envelope, where it dephosphorylates Lamin B to promote its polymerization and accelerate nuclear lamina assembly. Repo-Man targets PP1 γ to the periphery of late anaphase chromosomes. The N-terminus of Repo-Man directly mediates the recruitment of Importin- β to control early nuclear envelope formation and nucleoporin deposition.

As the nuclear envelope reassembles, the segregated chromatin decondenses to re-establish an interphase structure compatible with DNA replication and transcription (Antonin and Neumann, 2016). This relies on the activity of PP1 γ , which, prior to its accumulation at the periphery of late anaphase chromosomes, is homogeneously distributed over chromatin during early stages of anaphase. This initial recruitment of PP1 γ to chromatin is also mediated by the regulatory subunit, Repo-Man, although through a separate C-terminal module that is dispensable for the peripheral localization (Trinkle-Mulcahy et al, 2006; Vagnarelli et al, 2006; Vagnarelli et al, 2011). Analogously to inhibitory phosphorylations on the Repo-Man N-terminal domain, phosphorylation of the C-terminus chromatin-binding module by Cdk1 and Aurora B prevents Repo-Man binding to histones before anaphase onset (Vagnarelli et al, 2011; Qian et al, 2013). PP1 γ :Repo-Man antagonizes the mitotic phosphorylation of Thr3 and Ser10 on histone H3, respectively catalysed by Haspin and Aurora B (Qian et al, 2011; Vagnarelli et al, 2011; Qian et al, 2015). Dephosphorylation of Thr3 allows the translocation of the CPC from chromosomes to the central spindle, whereas dephosphorylation of Ser10 triggers decondensation and enables the re-association of HP1 to H3K9me3 after mitosis (Fischle et al, 2005; Qian et al, 2011). Intriguingly, depletion of Repo-Man fails to cause a dramatic impairment of chromatin decondensation (Vagnarelli et al, 2011). Because the recruitment of PP1 γ to anaphase chromatin is also mediated by Ki-67 (Booth et al, 2014; Kumar et al, 2016), it is possible that the pool of PP1 γ :Ki-67 compensates for the loss of Repo-Man (Antonin and Neumann, 2016). However, the exact contribution of PP1 γ :Ki-67 holoenzyme for chromatin decondensation remains to be demonstrated. Another chromatin targeting factor for PP1 γ is the regulatory subunit PNUTS. Binding of PP1 γ to PNUTS through a canonical RVxF motif was shown to enhance chromatin decondensation *in vitro* (Landsverk et al, 2010). However, the association of PP1 γ :PNUTS with chromatin is only detected during telophase, after H3 Ser10 dephosphorylation and nuclear envelope reformation (Landsverk et al, 2010). Therefore, the precise targets of PP1 γ :PNUTS and its relevance for chromatin decondensation are still elusive.

SPATIOTEMPORAL CONTROL OF PHOSPHATASE ACTIVITIES

The accuracy of mitosis requires tight spatiotemporal control of phosphatase activities. Although PP1 does contribute to mitotic exit progression, the bulk of Cdk1-mediated phosphorylations is removed by PP2A when in complex with B55 (Cundell et al, 2016). Thus, PP2A:B55 must be kept inhibited from nuclear envelope breakdown to anaphase onset for the sake of mitotic compliance, becoming then reactivated to orchestrate the major events underlying anaphase and mitotic exit. As mentioned before, repression of PP2A:B55 is triggered by Cdk1-mediated activation of Gwl, which subsequently phosphorylates Ensa and Arpp19 to promote their inhibitory binding to PP2A:B55 (Gharbi-Ayachi et al, 2010; Mochida et al, 2010; Blake-Hodek et al, 2012; Vigneron et al, 2011) (Figure 6 A). Both inhibitors are high affinity substrates of PP2A:B55, but because their dephosphorylation rate is orders of magnitude lower than Cdk1-phosphorylated targets, this efficiently restrains PP2A:B55 activity in what is known as “unfair competition” (Williams et al, 2014). Degradation of Cyclin B upon transition into anaphase initiates the cascade that activates PP2A:B55. Decreasing Cdk1:Cyclin B activity is proposed to render PP1 active to dephosphorylate and partially inactivate Gwl (Heim et al, 2015; Ma et al, 2016; Rogers et al, 2016; Ren et al, 2017) (Figure 6 A). This means that the phosphorylations that are ultimately removed from Ensa and Arpp19 by PP2A:B55 now fail to be efficiently replaced, hence allowing PP2A:B55 to target Cdk1-phosphorylated substrates (Williams et al, 2014). In that respect, PP2A:B55 further dephosphorylates Gwl to ensure its complete inactivation and thereby maintain elevated PP2A:B55 activity to promote mitotic exit (Heim et al, 2015; Ma et al, 2016; Rogers et al, 2016; Ren et al, 2017) (Figure 6 A).

Importantly, active PP2A:B55 seems to follow a timely defined dephosphorylation program so that events in anaphase, telophase, and cytokinesis are carefully coordinated and regulated to ensure a successful cell division (Cundell et al, 2013; Cundell et al, 2016). It is absolutely critical that dephosphorylation of PRC1 and of other spindle proteins occurs before the dephosphorylation of Lamins and of nucleoporins, so that the nuclear envelope and nuclear pores can only assemble and import proteins once chromosomes are successfully segregated (Cundell et al, 2016; Pereira and Schiebel, 2016). The timing of substrate dephosphorylation during mitotic exit is determined by the net basic charge of the two polybasic regions upstream and downstream of the Cdk1-phosphorylated site. Spindle proteins have pronounced polybasic regions and are therefore readily selected and dephosphorylated by PP2A:B55 early in anaphase, whereas nuclear envelope proteins comprise an inferior number of basic residues and are therefore dephosphorylated at lower rates. This differential catalytic efficiency enables PP2A:B55 to achieve temporal dephosphorylation of Cdk1 sites during mitotic exit and, thereby, coordinate the segregation

of chromosomes with nuclear envelope reformation (Cundell et al, 2016). Moreover, the intrinsic preference of the PP2A catalytic subunit towards pThr over pSer residues provides an additional regulatory element to ensure ordered dephosphorylation of substrates during mitotic exit (Godfrey et al, 2017; Hein et al, 2017). Early anaphase events seem to be predominantly driven by pThr dephosphorylation, whereas pSer-bearing substrates are dephosphorylated with slower dynamics, thus contributing for sequential execution of the mitotic exit program (Hein et al, 2017).

The mechanism controlling PP1-mediated inactivation of Gwl remains, however, somewhat contentious. It has been proposed that PP1 activity is globally repressed in mitosis by a Cdk1 inhibitory phosphorylation on its C-terminus (Dohadwala et al, 1994; Kwon et al, 1997; Grallert et al, 2015) and by binding to I-1, an inhibitory PIP whose interaction with PP1 is promoted by a PKA-dependent phosphorylation residue (Wu et al, 2009). Notably, both phosphorylations are targeted by PP1 itself. Thus, declining Cdk1:Cyclin B activity following SAC silencing is thought to be sufficient to allow PP1 autodephosphorylation and I-1 dephosphorylation, thereby resulting in increased activation of PP1 to levels that drive mitotic exit (Wu et al, 2009; Heim et al, 2015; Ma et al, 2016; Rogers et al, 2016; Ren et al, 2017). This sequential activation of PP1 and PP2A:B55 occurring in animal cells resembles a phosphatase relay reported in fission yeast, which also operates as a timer for the orderly dephosphorylation of mitotic substrates at the end of division (Grallert et al, 2015). Interestingly, not all PP1 molecules are inhibited by Cdk1 during mitosis (Olsen et al, 2010) and, therefore, are likely to be involved in the phosphatase relay. In fact, the activity of multiple PP1 holoenzymes is known to be required for the stability of kinetochore-microtubule attachments and efficient SAC silencing, events that occur before APC/C-Cdc20-triggered degradation of Cyclin B. It would be interesting to identify which specific pools of PP1 are under control of Cdk1:Cyclin B to understand the mechanism that selectively renders some PP1 holoenzymes impervious to this inhibition. The activity of PP1-PIP complexes operating in mitosis is, however, locally regulated, frequently by controlling PP1 interaction with regulatory subunits or substrates. This generally entails the phosphorylation of RVxF PP1-binding motifs by Aurora B, which abrogates the PIP ability to bind PP1 (Nasa et al, 2018). This regulatory strategy enables the cell to tightly control the levels of PP1 at centromeres and at kinetochores in response to microtubule attachment status (Hengeveld et al, 2017; Nasa et al, 2018) (Figure 9 A-D). The inhibitory phosphorylations on the Knl1 PP1-docking motif are removed by BubR1:PP2A:B56, which allows PP1 to associate with Knl1. This comprises another phosphatase relay, now at kinetochores and required for efficient SAC silencing and timely anaphase onset.

The activity of PP2A:B56 at kinetochores is modulated to avert deleterious dephosphorylation during early mitosis, when correction of erroneous attachments is often required. This is accomplished by Bod1, a small protein that shares sequence similarity with Ensa and Arpp19, and that specifically binds to PP2A:B56 when phosphorylated by Cdk1 (Porter et al, 2013). Bod1-mediated inhibition of PP2A:B56 is maximal at early prometaphase, possibly to prevent premature dephosphorylation of the Ndc80 N-terminus and of Knl1 PP1-docking motifs (Schleicher et al, 2017). As the cell progresses through mitosis, relief of PP2A:B56 from the inhibitory interaction with Bod1 is essential for the formation and stabilization of end-on attachments. The mechanism responsible for Bod1 dephosphorylation remains, however, undetermined.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In the past decades, we have witnessed the emergence of protein phosphatases as critical regulators of mitosis. In recent years, the list of identified substrates and regulators has increased considerably, as has our understanding of phosphatases' roles in mitotic progression.

Structural and biochemical determinants of phosphatase holoenzyme assembly and specificity have been unveiled. Research has made important advances in the functional characterization of protein phosphatases and has generated critical molecular knowledge to better understand their spatiotemporal control. Importantly, these achievements have provided new and renovated perspectives on the regulation of mitotic events, whose accurate execution is absolutely critical for genomic stability.

Nonetheless, enduring unanswered questions deserve further investigation. It remains relevant to dissect the regulatory network underlying the activity of Cdc25 isoforms. The field will also benefit from a comprehensive identification of the precise substrates that are targeted by specific Ser/Thr phosphatase holoenzymes and of the upstream signalling inputs controlling the subcellular localization and activity of the different holoenzymes. These have been hampered by the inability to routinely tackle specific interactions of phosphatases with substrates or regulators. Further structural insights into SLiM-mediated interactions might reveal critical mechanisms to enable the disruption of specific phosphatase complexes in a way that is compatible with systematic studies. Furthermore, the development of specific inhibitors targeting phosphatase-SLiM contacts should also fuel their potential use as therapeutic strategies in disease. This might represent a valuable alternative, or complement, to protein kinase inhibitors that are often associated with on-target resistance.

GENERAL INTRODUCTION

Aim of the work

To efficiently guard against chromosome mis-segregation the SAC must be robust but also highly responsive to microtubule attachments. A defective SAC or a failure to switch it off in a timely manner has been shown to cause aneuploidy, a hallmark of cancer. Hence, the main aim of this PhD thesis was to unravel how cells establish robust and timely SAC signalling. This requires a comprehensive understanding of how the activities of Mps1 and PP1, master SAC orchestrators, are regulated during mitosis. In that respect, the work presented here addressed the following specific questions:

- (1) How is Mps1 inactivated to ensure timely SAC silencing and mitotic exit?
- (2) How is PP1 controlled during mitosis to avoid premature SAC silencing and anaphase onset?

Mps1 recruitment to kinetochores is thought to cease upon microtubule attachment. However, this appears to be insufficient to completely silence the checkpoint as a population of Mps1 is still present in attached kinetochores and cytosolic Mps1 may also contribute for mitotic arrest. Given the spatiotemporal profile of PP1 activity, and its prominent role in SAC silencing, we hypothesized that PP1 could dephosphorylate Mps1 T-loop at metaphase kinetochores and contribute to ensure efficient Mps1 inactivation and thereby prompt SAC silencing. Experimental results testing this hypothesis are described and discussed in Chapter I.

PP1 phosphatase activity towards SAC substrates plays a critical role in switching off the SAC in metaphase. Thus, PP1 activity must be tightly regulated throughout mitosis to allow robust SAC activation at prometaphase kinetochores but also timely SAC silencing following amphitelic attachments. However, exactly how PP1 is regulated so that SAC signalling is coordinated with kinetochore-microtubule attachment status remains elusive. Chapter II delves into this matter and sheds new light on our understanding of PP1 mitotic regulation, which is instrumental to elucidate how timely SAC signalling is established.

These issues were addressed using *Drosophila melanogaster* as a model organism. Both *Drosophila* S2 cultured cells and tissue cells from 3rd instar larvae were used in the experiments presented in this work. Recombinant proteins were used in *in vitro* assays. Collectively, the findings reported in this thesis represent fundamental knowledge on the mechanisms controlling SAC signalling, thus advancing our understanding of how dividing cells prevent aneuploidy.

MATERIALS AND METHODS

This part describes the materials and methods used to perform the experiments presented in the section Experimental Work

(Chapters I and II)

S2 CELL CULTURES, RNAi AND DRUG TREATMENTS

Drosophila S2 cells were cultured at 25°C in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS). The *Drosophila* S2 – DGRC cell line (stock#006A9) was acquired from the *Drosophila* Genomics Resource Center, Indiana University, and was not independently authenticated. The cell lines were routinely tested negative for mycoplasma contamination.

For each depletion, 10^6 S2 cells/mL of Schneider's Insect Medium (Sigma-Aldrich) were plated in twelve-well plates (0.5 mL) and 30 µg of the respective dsRNAi were added. After one hour at 25°C, cells were supplemented with 1 mL of Schneider's Insect Medium (Sigma-Aldrich) + 10% FBS. The incubation period was 120 hours. At selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy or immunoblotting. When required, cells were subjected to several drug treatments before being collected and processed for the desired analysis. In order to promote microtubule depolymerisation, cells were incubated with 30 mM Colchicine (Sigma-Aldrich) for 2-12 h. To inhibit Aurora B activity, selected concentrations of Binucleine 2 (Sigma-Aldrich) were added to cultured cells for two hours. To inhibit Cdk1:Cyclin B activity, 10 mM RO-3306 (Sigma-Aldrich) were added to cultured cells for one hour. To inhibit Polo activity, 100 nM BI2536 (Boehringer Ingelheim) were added to cultured cells for two hours. When required, 20mM MG132 (Calbiochem) were added to cultured cells to inhibit the proteasome.

Expression of EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A}, EGFP-PP1α87B^{WT}, EGFP-PP1α87B^{T286A} and EGFP-PP1α87B^{T286D} transgenes was induced by treatment of the S2 transfected cells with 100 µM CuSO₄ at least eight hours before analysis. To induce expression of Polo^{WT}-EGFP, Polo^{T182D}-EGFP, Mis12-EGFP-PP1α87B^{WT}, Mis12-EGFP-PP1α87B^{T286A} and Mis12-EGFP-PP1α87B^{T286D}, under regulation of Hsp70 promoter, cells were incubated for 30 minutes at 37°C at least eight hours prior to cell processing.

Table 1 – List of primers used for dsRNA synthesis

Target	Primer sequence
BubR1	5' – <u>TAATACGACTCACTATAGGG</u> GAGCTTTTAAATCGACACAGGG – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GAGTAGATGCTTAGTCCGACGC – 3'
CENP C	5' – <u>TAATACGACTCACTATAGGG</u> GTGTAGTTTTAGCGAAATAAG – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GCTGCAGCGGATTTTC – 3'

Mps1 (CDS)	5' – <u>TAATACGACTCACTATAGGG</u> TCTTCCAAACACCTATGACCG – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> CGTTTAGATATCCCTGCACCA – 3'
Mps1 (UTRs)	5' – <u>TAATACGACTCACTATAGGG</u> GGTCACACTTGAATA – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GCTTGCAACCCGATTT – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GCACGAAAGCTCA – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> AAGGATTTATGATGT – 3'
Ndc80	5' – <u>TAATACGACTCACTATAGGG</u> TTTGATCCGGATGTTTTGTTGTAG – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> TTTTTTAATTTGAAAACAAAGAATACTCTT – 3'
PP1 α 87B (UTRs)	5' – <u>TAATACGACTCACTATAGGG</u> GAGACGGCAGTGTGGCAACATCAGCTA – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GAGAGTTTGCGTGCGAAAGTGTGGATCTG – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GAGATATCACAACTGCAGCACCAC – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> GAGAGTTTCATGCATCATTTGATGTGTTG – 3'
Polo	5' – <u>TAATACGACTCACTATAGGG</u> TCAGATCACATGAAGCTCATCC – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> CTATAGCGCACTACGAACGC – 3'
Sds22	5' – <u>TAATACGACTCACTATAGGG</u> CCAATCTGGAAAAGCTGGAA – 3'
	5' – <u>TAATACGACTCACTATAGGG</u> CAACGCCGTGGATTAGTCTT – 3'

Note: The underlined sequence corresponds to the T7 RNA polymerase promoter site.

CONSTRUCTS AND S2 CELL TRANSFECTION

The pCaSpeR4 harbouring EGFP-Mps1^{WT} under control of Mps1 cis-regulatory region was a gift from Christian Lehner (Althoff et al, 2012). Mad1-EGFP construct under regulation of Mad1 native promoter cloned in the pMT backbone (Invitrogen) was a gift from Thomas Maresca (University of Massachusetts, Amherst). pMT-Cyclin B^{NDG} construct was a gift from Hélder Maiato (i3S, Porto). The constructs pMT-EGFP-Mps1^{WT}, pAC-H2B-GFP, pAC-mCherry- α -Tubulin, pMT-EGFP-Cyclin B^{WT}, pHWG-Polo^{WT} and pHWG-Polo^{182D} were already available in the lab and have been previously described (Conde et al, 2013).

All the other constructs were cloned according to Table 2. PCR reactions were performed with Phusion polymerase (New England Biolabs). For cloning with restriction enzymes (RE), PCR products were digested with the appropriate RE (New England Biolabs) for at least two and a half hours. PCR product and destination vector were purified (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel) and roughly 75 ng of the insert were mixed with 25

ng 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 DH5 α bacteria and selected for positives. For site directed mutagenesis and Fast Cloning (Li et al, 2011), PCR products were digested with DpnI restriction enzyme (New England Biolabs), used to transform competent DH5 α bacteria and selected for positives. To produce the pH-Mis12-GW-PP1 α 87B construct, pENTR-PP1 α 87B was recombined with pH-Mis12-GW using Gateway LR Clonase II (Invitrogen), according to the manufacturer's instructions.

Stable cell lines expressing the indicated constructs were obtained by co-transfection with pCo-Blast or pAC-mCherry- α -Tubulin[Blast] and selection in medium with 25 ug/mL Blasticidin. Transfections of recombinant plasmids and pCoBlast or pAC-mCherry- α -Tubulin[Blast] into *Drosophila* S2 cells were performed using Effectene Transfection Reagent (Qiagen), according to the manufacturer's instructions. The S2 cell line expressing CID-mCherry and GFP- α -Tubulin was a gift from Hélder Maiato.

Table 2 – List of primers used for cloning

Construct	Template	Primer sequence
RE Cloning		
pMT-EGFP-PP1 α 87B	gDNA	5' - GAC <u>GGTACCA</u> TGGGCGACGTGATGAATATCGAC - 3'
		5' - GAT <u>CCCGGGT</u> TACTTTTTACGCTTGTCGGCTGG - 3'
Fast Cloning		
1) pH-Mis12-GW	Mis12 cDNA	5' – GAGAAGCCCGCCACCATGGACTTCAATAGCCTAGC – 3'
		5' – GCCCTTGCTCACCATAAGCTTGAGCTCGAGATCAGTCTCCTTCT – 3'
	pHGW (Gateway)	5' – ATGGTGAGCAAGGGCGA – 3'
		5' – GGTGGCGGGCTTCTCTAG – 3'
pENTR-PP1 α 87B	pMT-EGFP-PP1 α 87B	5' – GCTGCTCCATTTACAATGGGCGACGTGATGAATA – 3'
		5' - AGCGCGTCCACCTTTTTACTTTTTACGCTTGTC – 3'
	pENTR (Gateway)	5' – TGTAAATGGAGCAGCCGCGGAGC – 3'
		5' – AAAGGTGGACGCGCTGACCCAGCTTTCTT – 3'
2) pAC-mCherry- α -Tubulin [Blast]	pAC-mCherry- α -Tubulin	5'- GAGACCCCGGATCGGATGGTGAGCAAGGGCGA-3'
		5'- CTTACCTTCGAACCGCTTAGTACTCCTCAGCGCCCTC-3'
	pAC[Blast]	5'- CCGATCCGGGGTCTCTGGATTAGAC- 3'
		5'- GCGGTTCGAAGGTAAGCCTATCCC- 3'

Site directed Mutagenesis		
3)		5' - ATTTCCGTGCGCAG GC AGTGCTC G CCCAAACAC - 3'
Mps1 ^{K231A/K234A}	Mps1 ^{WT}	5' - CTGCGCACGGAAATCATT C GACACCGC - 3'
3)		5' - GT C GA C TCG G CGAGCATCATCAAATTCTC - 3'
Mps1 ^{K231A/K234A} /T490A	Mps1 ^{K231A/K234A}	5' - CGAGTC G ACAGCGATATTGCTGGCTATGC - 3'
4)		5' - ATGTCCGTGGACGAT G CGCTGATGTGC - 3'
PP1 α 87B ^{T286A}	PP1 α 87B ^{WT}	5' - GTCCACGGACATCATTGCGCCGGCATT - 3'
4)		5' - ATGTCCGTGGACGAT G ATCTGATGTGCTCG - 3'
PP1 α 87B ^{T286D}	PP1 α 87B ^{WT}	5' - GTCCACGGACATCATTGCGCCGGCATT - 3'

Notes: The RE recognition sites are underlined and the point mutations introduced in the primers are shown in bold. 1) this construct was made by Mariana Osswald; 2) this construct was made by Sofia Cunha-Silva; 3) these primers were used for pMT-EGFP-Mps1 and pCaSpeR-EGFP-Mps1 constructs; 4) these primers were used for pMT-EGFP-PP1 α 87B and pENTR-PP1 α 87B constructs.

FLY STOCKS

All fly stocks used in this work are described on the table below. To produce the pUASg-PP1 α 87B-HA-attB (WT, T286A or T286D) constructs, pENTR-PP1 α 87B (WT, T286A or T286D) were recombined with pUASg-HA-attB using Gateway LR Clonase II (Invitrogen), according to the manufacturer's instructions. The constructs were sent to BestGene to inject embryos and create stocks carrying the transgenes. The stock used for injection was #9748.

Table 3 – *Drosophila* stocks used for immunofluorescence analysis and live imaging

Fly stock	Origin	Reference
<i>w1118</i>		
<i>ald</i> ^{G4422}	Bloomington Stock Center	#30079
<i>inscutable</i> Gal4	Bloomington Stock Center	#8751
UAS-PP1 α 87B ^{RNAi}	Bloomington Stock Center	#32414
gEGFP-Mps1 ^{WT}	Gift from Christian Lehner	
gEGFP-Mps1 ^{KD} (D478A)	Gift from Christian Lehner	
UAS-GFP- α -Tubulin	Bloomington Stock Center	#7374
His2Av-mRFP	Bloomington Stock Center	#23651
PP1 α 87B ^{87Bg3}	Bloomington Stock Center	#23696

PP1 α 87B ¹	Bloomington Stock Center	#6207
UAS-PP1 α 87B ^{WT} -HA	BestGene	26205-2-M3-M
UAS-PP1 α 87B ^{T286A} -HA	BestGene	26205-3-M2-M
UAS-PP1 α 87B ^{T286D} -HA	BestGene	26205-4-M4-M

RECOMBINANT PROTEINS

To generate pFastBac-Mps^{WT/KD} for expression of *Drosophila* Mps1^{WT} (Wild type) and Mps1^{KD} (Kinase-dead, D478A) in the baculovirus/Sf21 system, pMT-EGFP-Mps1^{WT/KD} (Conde et al, 2013) and pFastBac HT A were digested with EcoRI and XbaI restriction enzymes (New England Biolabs). A 1414 bp fragment harboring Mps1^{WT/KD} coding region was cloned into the destination vector. The obtained recombinant pFastBac-Mps^{WT/KD} was used to transform DH10EMBacY competent cells in order to generate recombinant bacmid His6-Mps1^{WT/KD}. Positive clones were identified by blue/white screening and baculoviral DNA was isolated with QIAprep Spin Miniprep Kit (QIAGEN) and used to transfect 10⁶ Sf21 cells in 6-well plate. Forty-eight hours after transfection, the supernatant was collected (V₀) and used to infect Sf21 cells. Cell cultures in shaker flasks were split every twenty-four h until proliferation arrest. Protein expression was monitored by YFP fluorescence and confirmed by SDS-PAGE. Cells were harvested forty-eight h post infection and lysed in 50 mM Tris-HCl pH 7.5, 500 mM NaCl and 10 mM Imidazole. Lysates were sonicated and clarified by centrifugation at 4°C. Recombinant His6-Mps1^{WT/KD} were purified by affinity chromatography in a His-TrapTM-HP nickel-column (Amersham). The protein was eluted with 250 mM Imidazole and dialysed against 20 mM Hepes pH 7.5, 150mM NaCl, 10mM MgCl₂ and 1mM DTT. [The production of recombinant proteins in the baculovirus/Sf21 system was performed by Carlos Conde and Mariana Osswald].

To generate the MBP-PP1 α 87B construct for expression in bacteria, PCR products harbouring PP1 α 87B coding sequence were cloned into StuI/XbaI sites of pMal-c2 (New England Biolabs) vector. The recombinant construct was used to transform BL21-star competent cells and protein expression induced with 0.1 mM IPTG at 15°C, overnight. Lysates were sonicated and clarified by centrifugation at 4°C. Recombinant MBP-PP1 α 87B^{WT} was purified with amylose magnetic beads (New England Biolabs) and eluted with 10 mM Maltose, 200 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA and 1 mM DTT. To generate 6xHis-N-Mps1^{WT} and 6xHis-N-Mps1^{K231A/F234A} constructs for expression in bacteria, PCR products with the coding sequence corresponding to N-terminus 104-330 amino acids from Mps1^{WT} and Mps1^{K231A/F234A} were cloned into KpnI/XhoI sites of pET30a

(+) vector (Novagen). Recombinant constructs were used to transform BL21-star competent cells and protein expression induced with 0.05 mM IPTG at 15°C, overnight. Lysates were sonicated and clarified by centrifugation at 4°C. Recombinant 6xHis-N-Mps1^{WT} and 6xHis-N-Mps1^{K231A/F234A} were purified with Novex Dynabeads (Invitrogen) and eluted with 50 mM Imidazole, 200 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA and 1 mM DTT. Recombinant human Mps1/TTK, PP1- γ and λ -Phosphatase were purchased from SignalChem, MRC-PPU Reagents (University of Dundee) and New England Biolabs, respectively.

S2 CELL LYSATES, PULL-DOWNS, IMMUNOPRECIPITATION AND WESTERN BLOTTING

For preparation of S2 cell lysates used in pull-down and immunoprecipitation experiments, S2 cultured cells overexpressing EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} were treated with Colchicine for twelve hours. Overexpression of EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} was induced with 0.1 mM CuSO₄ during twelve hours. Cells were harvested through centrifugation at 5000 rpm for 10 min at 4°C and afterwards washed with 2 mL PBS supplemented with protease inhibitors cocktail (Roche). Cell pellet was resuspended in lysis buffer (150 mM KCl, 75 mM HEPES, pH 7.5, 1.5 mM EGTA, 1.5 mM MgCl₂, 15% glycerol, 0.1% NP-40, 1x protease inhibitors cocktail (Roche) and 1x phosphatase inhibitors cocktail 3 (Sigma-Aldrich)) before disruption through freezing in liquid nitrogen. Cell lysates were then clarified through centrifugation at 8000 rpm for 10 min at 4°C and quantified by Bradford protein assay (Bio-Rad). EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} were immunoprecipitated from 0.5 mg of total cell lysates using the GFP-Trap_MA system (Chromotec GmbH) according to the manufacturer's instructions. For pull-down assays using S2 cell protein extracts, 0.5 mg of total cell lysates were diluted in a final volume of 500 μ L of column buffer (250 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 0.05% Tween-20, 1x protease inhibitors cocktail (Roche) and 1x phosphatase inhibitors cocktail 3 (Sigma-Aldrich)) and incubated with 5 μ L of MBP or MBP-PP1 α 87B bound to amylose magnetic beads (New England Biolabs) for 90 min at room temperature with rotation. The magnetic beads and bound protein fraction were collected and washed 3 times with 750 μ L of column buffer. For pull-down assays with purified recombinant proteins, 5 μ L of 6xHis-N-Mps1^{WT} or 6xHis-N-Mps1^{K231A/F234A} were incubated with 3 μ L of MBP or MBP-PP1 α 87B bound to amylose magnetic beads (New England Biolabs) in a final volume of 30 μ L of column buffer (250 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT) for 60 min at room temperature with rotation. The magnetic beads and bound protein fraction

were collected and washed 3 times with 100 mL of column buffer. Magnetic beads and bound protein were resuspended in Laemmli sample 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. For western blot analysis, 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). The membrane was blocked for one hour at room temperature with 5% powder milk in PBS-Tween20 0.05%. All the primary and secondary antibodies were diluted in PBST20 containing 5% powder milk (exception for anti-pMps1 which was diluted in PBST20 containing 1% powder milk) and the membranes were incubated overnight at 4°C under agitation, then washed three times 10 min with PBST20 and immediately incubated with secondary antibodies for one hour at room temperature under agitation. Detailed information about the primary antibodies used can be found in table 4. Secondary antibodies conjugated to Horseradish peroxidase (Jackson ImmunoResearch) were used at 1:5000. Blots were developed with Clarity Western ECL Substrate (BioRad) according to manufacturer's protocol and detected on X-ray film (Fuji Medical X-Ray Film).

IN VITRO KINASE AND PHOSPHATASE ASSAYS

For *in vitro* kinase assays, 6xHis-DmMps1^{WT}, 6xHis-DmMps1^{KD}, HsMps1/TTK (0.25 mg) (SignalChem) or immunoprecipitated EGFP-Mps1 were incubated for 30 min in a total volume of 15-30 mL kinase reaction buffer (5 mM MOPS, pH 7.2, 2.5 mM β -glycerol-phosphate, 5 mM MgCl₂, 1 mM EGTA, 0.4 mM EDTA, 0.25 mM DTT and 100 μ M ATP). Reactions were carried out at 25°C for His6-DmMps1^{WT}, His6-DmMps1^{KD} and immunoprecipitated EGFP-Mps1 and at 30°C when using HsMps1/TTK. For inhibition of HsMps1/TTK activity, AZ3146 (Tocris) was added to the reaction at a final concentration of 10 mM. For phosphatase assays, recombinant human PP1- γ was added either to the kinase reaction at t0 or after Mps1/TTK inhibition. Reactions were carried out at 30°C for 30 min in PMP buffer (New England Biolabs) containing 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 and 1 mM MnCl₂. Reactions were stopped by addition of Laemmli sample buffer (4% SDS, 10% β -mercaptoethanol, 0.125 M Tris-HCl, 20% glycerol, 0.004% bromophenol blue) and heated for 5 min at 95°C. Peptides were resolved in 8% SDS-PAGE. The activating autophosphorylation on T676 or T490 (for human Mps1/TTK and *Drosophila* Mps1, respectively) was detected by western blotting with a phospho-specific antibody provided by Geert Kops (Jelluma et al, 2008a).

IMMUNOFLUORESCENCE ANALYSIS

For immunofluorescence analysis of S2 cells, 10^5 cells were centrifuged onto slides for 5 min, at 1500 rpm (Cytospin 2, Shandon), and simultaneously fixed and extracted in 3.7% formaldehyde (Sigma-Aldrich), 0.5% Triton X-100 in PBS for 10 min followed by three washing steps in PBS-Tween20 0.05% (PBST20) for 5 min each. PBST20 was replaced by PBS for the immunofluorescence with pMps1 and pMad1 antibodies presented in Chapter II. For immunofluorescence analysis of *Drosophila* neuroblasts, third-instar larval brains were dissected in PBS and incubated with 50 μ M Colchicine for one and a half hours at 25°C. The larval brains were transferred to coverslips (1 brain per coverslip) and fixed for 5 min in 1.8% formaldehyde + 45% acetic acid. After fixation, a slide was placed over the coverslip and the brain was squashed in the middle. After the squash, slides were freeze in liquid nitrogen, the coverslips were removed and the slides with the squashed brains were incubated in cold ethanol (100%) for 10 min and washed for 10 min in PBS with 0.1% Triton X-100.

Fixed cells were blocked for one hour in PBST20 supplemented with 10% FBS 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-labelled secondary antibodies diluted in PBST20 + 10% FBS for one hour at room temperature. Lastly, 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). Detailed information about the primary antibodies used can be found in table 4. Secondary antibodies Alexa 488 and 568 (Invitrogen) were used at 1:2000 and Alexa 647 (Invitrogen) was used at 1:1000.

Images presented on Chapter I were collected in Leica TCS SP5 II laser scanning confocal microscope (Leica Microsystems). Images presented on Chapter II were collected in Leica SP8 laser scanning confocal microscope (Leica Microsystems). Data stacks were analysed and projected using ImageJ software (<http://rsb.info.nih.gov/ij/>). For immunofluorescence quantification, the mean pixel intensity was obtained from maximum projected raw images acquired with fixed exposure acquisition settings. For kinetochore proteins, the mean fluorescence intensity was quantified for individual kinetochores, selected manually by CID or Spc105 staining. The size of the region of interest (ROI) was predefined so that each single kinetochore could fit into it. After subtraction of background intensities, estimated from regions of the cell with no kinetochores, the intensity was determined relative to CID or Spc105 reference and averaged over multiple kinetochores.

Table 4 – List of antibodies used for immunofluorescence and western blotting

Antibody name (target and code)		Species	Dilutions used		Origin	Reference
			IF	WB		
BubR1	Rb666	Rabbit	1:3000	1:3500	In house	AB_2566989
CENP C	Rb1	Rabbit	1:5000	1:8000	In house	
CID	Rat4	Rat	1:250		In house	
GST	B-14	Mouse	1:2000		Santa Cruz Biotechnology	
Mad1	Rb1	Rabbit	1:2500		In house	
MBP		Mouse	1:5000		New England Biolabs	AB_1559738
Mps1	Gp15	Guinea Pig	1:250	1:5000	Gift from Scott Hawley	AB_2567774
Mps1	NT clone 3-472-1	Mouse		1:5000	Milipore	AB_309903
Ndc80	Rb272	Rabbit	1:5000		Gift from Michael Goldberg	
Phosphorylated Thr232-Aurora B		Rabbit	1:1000		Rockland,	
Phosphorylated ser10-Histone H3	p-H3	Rabbit	1:5000		Milipore,	AB_565299
Phosphorylated Thr716-Mad1		Rabbit	1:1000		Gift from Adrien Saurin	
Phosphorylated Thr676-Mps1	T676	Rabbit	1:10000	1:2000	Gift from Geert Kops	
Phosphorylated Thr288-PP1 γ		Rabbit	1:500		Eurogentec	
PP1 α 87B		Sheep	1:5000		MRC-PPU Reagents	

Spc105		Rat	1:250	In house	
α -Tubulin	B512	Mouse	1:4000	Sigma-Aldrich	AB_86546
α -Tubulin	DM1A	Mouse	1:10000	Sigma-Aldrich	AB_477593

LIVE IMAGING

For live imaging of S2 cells, cells were plated onto dishes with coverslip bottoms (MatTek Corporation) previously treated with 0.2 mg/mL concanavalin A (Sigma-Aldrich). For time-lapse recording of *Drosophila* neuroblasts, brains from third-instar larvae were dissected in PBS and placed on a drop of PBS in a coverslip. The preparation was gently squashed using a smaller coverslip and excess media was removed. The sample was sealed with Halocarbon oil 700 (Sigma-Aldrich). 4D 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 561nm) were used for near-simultaneous excitation of EGFP and mCherry or mRFP. The system was driven by iQ software (Andor). Time-lapse imaging of z stacks with 0.8 nm steps covering the entire volume of the cell were collected and image sequence analysis and video assembly done with ImageJ and iQ software. For quantification of EGFP-Mad1 fluorescence at kinetochores, the mean intensity was calculated within the area corresponding to kinetochores and corrected for cytosolic signal. The changes in fluorescence intensity with time were plotted as normalized signal relative to the signal measured at NEB. For quantification of EGFP-Cyclin B^{WT}, EGFP-Cyclin B^{NDG} and Mad1-mCherry fluorescence at kinetochores, the mean intensity was calculated within the area corresponding to kinetochores and corrected for cytosolic signal and plotted against the time in mitosis.

ANALYSIS OF MICROTUBULE DYNAMICS BY INDUCIBLE FLUORESCENT SPECKLE MICROSCOPY

Inducible speckle imaging (ISI) was used to measure spindle microtubule turnover in *Drosophila* S2 cells as previously described (Pereira et al, 2016). Speckle patterns were imprinted using ~200- to 500-ms-long pulses with a bleaching strength (γ) between 1.5 and 2, a regime which maximizes speckle fluorescence amplitude. A rectangular ROI was defined enclosing a significant portion of the spindle area. Intensity contrast was then determined at each time point of the acquired sequence after the subtraction of a mean

dark reference level from each frame. The microtubule turnover half-time was calculated by fitting a double-exponential curve ($y_0 + A_1 \cdot \exp[-(t - t_0)/\tau_1] + A_2 \cdot \exp[-(t - t_0)/\tau_2]$) to the mean intensity squared-contrast from independent cells for each time point. Before averaging, contrast curves were normalized to a 0-to-1 contrast change defining the pre- and post-ISI transition.

FRAP EXPERIMENTS

FRAP experiments were performed in S2 cells expressing EGFP-Mps1^{WT}, EGFP-Mps1^{KD} or EGFP-Mps1^{K231A/F234A}. When required, Colchicine was added to cultures at least 2 h prior FRAP experiment. Datasets were collected at 25°C with a Leica TCS SP5II scanning confocal microscope (Leica Microsystems). The EGFP tag 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 EGFP fluorescence signal had become stable. Fluorescence intensity in the bleached area was acquired every 537 ms, 555 ms or 777 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 phase exponential association curve fitting to normalized data using GraphPad Prism software.

STATISTICAL AND KINETICS ANALYSIS

All statistical and enzyme kinetics analysis was performed with GraphPad Prism V6.0f (GraphPad Software, Inc.).

SUPPLEMENTARY INFORMATION

The phospho-antibody that recognizes human Mps1 T676 autophosphorylation can be used to detect the conserved activating T-loop autophosphorylation on *Drosophila* Mps1 (T490). The phospho-antibody generated against human Mps1 T676 autophosphorylation readily detected in immunoblots the autophosphorylation of a recombinant wild type version

of *Drosophila* Mps1 (His6-Mps1^{WT}) contrasting with the absence of evident phosphorylation on a catalytically inactive version of the kinase (His6-Mps1^{KD}) (Figure I.4 – supplementary figure 1 B). Moreover, immunofluorescence analyses of 3rd instar larval neuroblasts revealed a significant decrease in Mps1 T490 phosphorylation at kinetochores of *ald*^{G4422} strong hypomorphic Mps1 mutants (Figure I.4 – supplementary figure 1 C, D). Importantly, a similar reduction was observed following the expression of kinase dead EGFP-Mps1 in *ald*^{G4422} background when compared to mutants expressing the Mps1 wild-type transgene (Figure I.4 – supplementary figure 1 E,F). Taken together, these data validate the phospho-antibody as a read-out for the activation status of *Drosophila* Mps1. The phospho-antibody that recognizes human Mps1 phosphorylation on Mad 1 T716 can be used to detect the conserved residue on *Drosophila* Mad1 (T726). Immunofluorescence analyses of S2 cells revealed a significant decrease in Mad1 T726 phosphorylation at prometaphase kinetochores of Mps1 depleted cells (Figure II.4 – supplementary figure 1), indicating that the phospho-antibody can be used as a read-out for *Drosophila* Mps1 activity.

EXPERIMENTAL WORK

CHAPTER I

PP1 inactivates Mps1 to ensure efficient Spindle Assembly Checkpoint silencing

This chapter is based on the following published paper: Moura M, Osswald M, Leça N, Barbosa J, Pereira AJ, Maiato H, Sunkel C and Conde C* (2017) Protein Phosphatase 1 inactivates Mps1 to ensure efficient Spindle Assembly Checkpoint silencing. *eLife* 6: e25366. DOI: <http://dx.doi.org/10.7554/eLife.25366.001>

INTRODUCTION

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 attached to microtubules emanating from opposite spindle poles (Musacchio and Salmon, 2007; Foley and Kapoor, 2013). Mps1 kinase is a master regulator of SAC function (Liu and Winey, 2012). Mps1 phosphorylates MELT motifs on Spc105/Knl1 creating docking sites for the hierarchical recruitment of additional SAC proteins required for the assembly of the Mitotic Checkpoint Complex (MCC), a tetrameric protein complex that inhibits the Anaphase Promoting Complex (APC/C) and thereby prevents anaphase onset (Shepherd et al, 2012; Primorac et al, 2013; Vleugel et al, 2015; London et al, 2012; Yamagishi et al, 2012). Moreover, Mps1-mediated phosphorylation of Mad1 dramatically accelerates the structural conversion of Mad2 into its closed conformer, the rate-limiting step for MCC assembly and thus, of SAC activation (Faesen et al, 2017; Ji et al, 2017). The recruitment of Mps1 to unattached kinetochores is directly mediated by the Ndc80 complex and promoted by Aurora B kinase (Santaguida et al, 2011; Saurin et al, 2011; Conde et al, 2013; Nijenhuis et al, 2013). This localized accumulation of Mps1 is thought to potentiate its trans-autophosphorylation on the T-loop to ensure full kinase activity and robust SAC signalling (Kang et al, 2007; Sacristan and Kops, 2015).

The events that underlie SAC inactivation are less well understood. The phosphatases PP1 γ and PP2A:B56 appear to play an important role in this process by dephosphorylating Knl1-MELT motifs (London et al, 2012; Espert et al, 2014; Nijenhuis et al, 2014), which contributes to the removal of SAC proteins from kinetochores following microtubule attachments (Etemad and Kops, 2016). Furthermore, elegant *in vitro* studies demonstrated that binding of Mps1 to the calponin homology (CH) domains of Ndc80 and Nuf2 is inhibited by microtubules (Hiruma et al, 2015; Ji et al, 2015). Although this competition mechanism precludes the recruitment of Mps1 to bioriented kinetochores, it is however insufficient to remove all Mps1 from kinetochores even after the formation of stable end-on attachments (Aravamudhan et al, 2015; Hiruma et al, 2015; Ji et al, 2015). Work in budding yeast proposes that end-on attachments physically separate residual Mps1 from the Spc105/Knl1 phospho-domain, hence disrupting MELT phosphorylation at metaphase kinetochores (Aravamudhan et al, 2015). While this may contribute to silence the SAC in yeast, this model does not account for the highly dynamic behaviour of active Mps1 that has been observed in human cells (Jelluma et al, 2010). The association of Mps1 with the Ndc80 complex is very transient and the kinase diffuses rapidly into the cytoplasm, which is likely to allow Mps1 to overcome the increased spatial separation between its kinetochore receptor and

Spc105/Knl1 MELT motifs. Moreover, kinetochore recruitment of Mad1:Mad2 in metazoa is also mediated through a Spc105/Knl1-independent pathway (Schittenhelm et al, 2009; Caldas et al, 2015; Silió et al, 2015). This is known to rely on the recruitment of the Rod:ZW10:Zwilch (RZZ) complex to unattached kinetochores, an event that is also controlled by Mps1 kinase (Santaguida et al, 2010). Importantly, in addition to its role at unattached kinetochores, Mps1 also contributes to SAC signalling in the cytoplasm. Soluble Mps1 is required for the assembly of pre-mitotic MCC that determines the peak time of anaphase onset in the absence of kinetochore–microtubule attachment problems (Rodriguez-Bravo et al, 2014). Moreover, Mps1 lacking its kinetochore-binding domain is sufficient to delay mitotic exit in mouse embryonic fibroblasts and RPE1 cells challenged with spindle poisons (Fojer et al, 2014; Maciejowski et al, 2010) and cytosolic Mps1 was shown to be required to support SAC arrest caused by kinetochore-tethered Mad1 (Maldonado and Kapoor, 2011). Therefore, in addition to the control exerted on Mps1 kinetochore recruitment and to alterations on kinetochore architecture, other mechanisms must contribute to disrupt SAC signalling upon stable end-on microtubule attachments.

RESULTS

Depletion of PP1 α 87B results in a metaphase delay with stable kinetochore-microtubules attachments

In yeast and human cells, Knl1-bound PP1 γ antagonizes Mps1 signalling at kinetochores through dephosphorylation of MELT motifs, which leads to a reduction in Bub and Mad proteins at metaphase kinetochores (London et al, 2012; Zhang et al, 2014; Nijenhuis et al, 2014). Since *Drosophila* Spc105/Knl1 inherently lacks phospho-regulatable MELTs (Schittenhelm et al, 2009; Conde et al, 2013) but PP1 is still required for timely mitotic exit in flies (Chen et al, 2007), we resorted to this model organism to uncover novel SAC-silencing mechanisms that might have been overlooked in other systems. Live cell-imaging analysis showed that depletion of PP1 α 87B orthologue from *Drosophila* S2 cells results in an abnormal accumulation of Mad1 at aligned kinetochores and a pronounced extension of the metaphase duration (Figure I.1; Figure I.2 A).

Co-depletion of Mps1 prevented the anaphase onset delay, indicating it is due to active SAC signalling (Figure I.1; Figure I.2 A). Aurora B phosphorylates KMN proteins to destabilize erroneous kinetochore-microtubules interactions (Liu et al, 2009; Welburn et al, 2010; Lampson and Cheeseman, 2011). Since in human cells PP1 was shown to modulate Aurora B activity by antagonizing its activating T232 autophosphorylation (Liu et al, 2010; Posch et al, 2010; Wurzenberger et al, 2012), the metaphase delay observed in PP1 α 87B

depleted cells could be caused by labile kinetochore-microtubule attachments continuously engaging the SAC. We found that depletion of PP1 α 87B from *Drosophila* S2 cells increased Aurora B activation at centromeres/kinetochores of prometaphase and metaphase chromosomes, as expected (Figure I.2 C,D). However, the inter-kinetochore distances on aligned chromosomes were similar to those measured in control cells (Figure I.2 E), indicating that kinetochores are under mechanical tension compatible with stably attached microtubules (Li and Nicklas, 1995; Akiyoshi et al, 2010; Khodjakov and Pines, 2010; Ye et al, 2016). In agreement, PP1 α 87B depleted cells revealed the presence of cold stable kinetochore-fibers, as observed in control cells (Figure I.2 F,G).

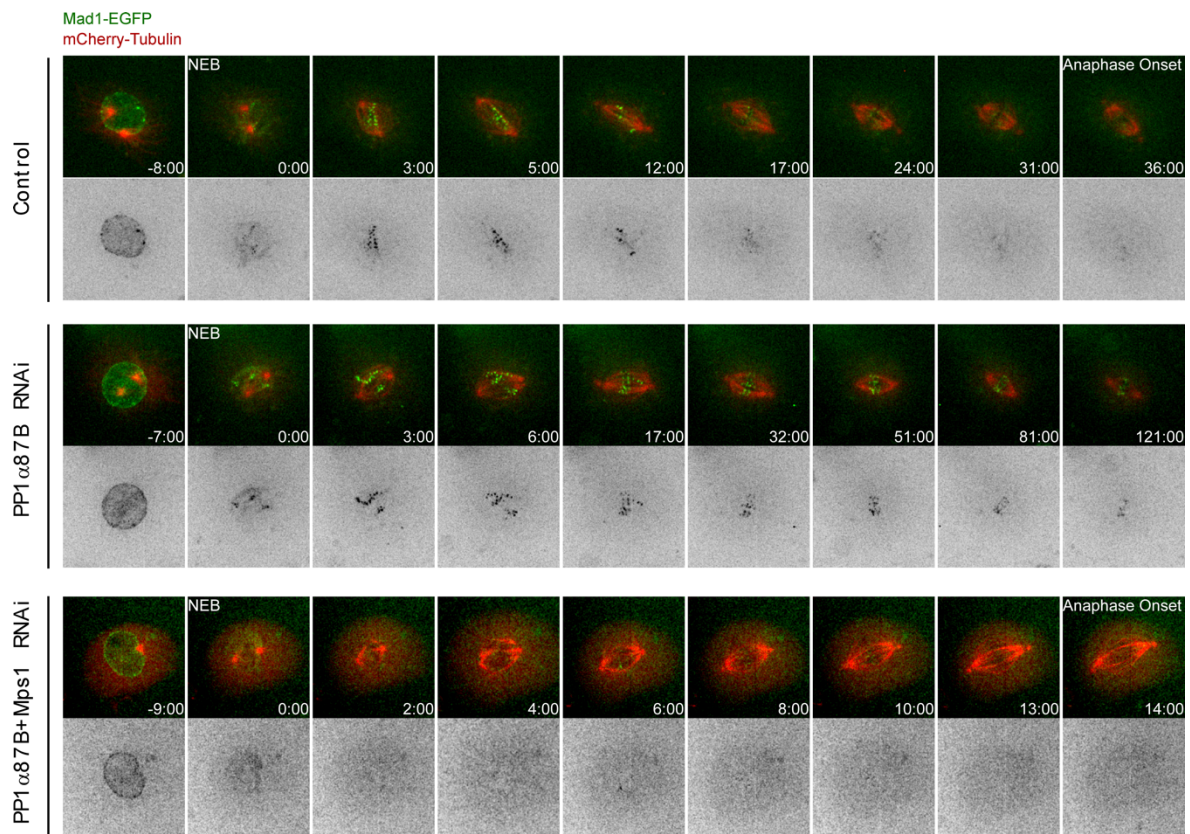


Figure I.1 – Mad1-EGFP localization pattern during mitosis in S2 cells depleted of PP1 α 87B. Representative mitotic progression of control, PP1 α 87B depleted or Mps1 and PP1 α 87B co-depleted *Drosophila* S2 cells co-expressing Mad1-EGFP and mCherry- α Tubulin. Mitotic progression was monitored by time-lapse microscopy. (NEB) nuclear envelope breakdown. Time is in min:sec.

Furthermore, we quantified the stability of kinetochore-microtubule attachments by measuring microtubule turnover rates with inducible speckle imaging (ISI) (Pereira et al, 2016). Briefly, a speckled laser beam imprints a bleaching pattern on GFP- α Tubulin that dissipates (by fade-out of the bright spots and fade-in of the dark spots) in a timescale indicative of microtubule lifetime. In contrast to conventional photobleaching experiments, ISI imprinting is 3D and immune to aberrations.

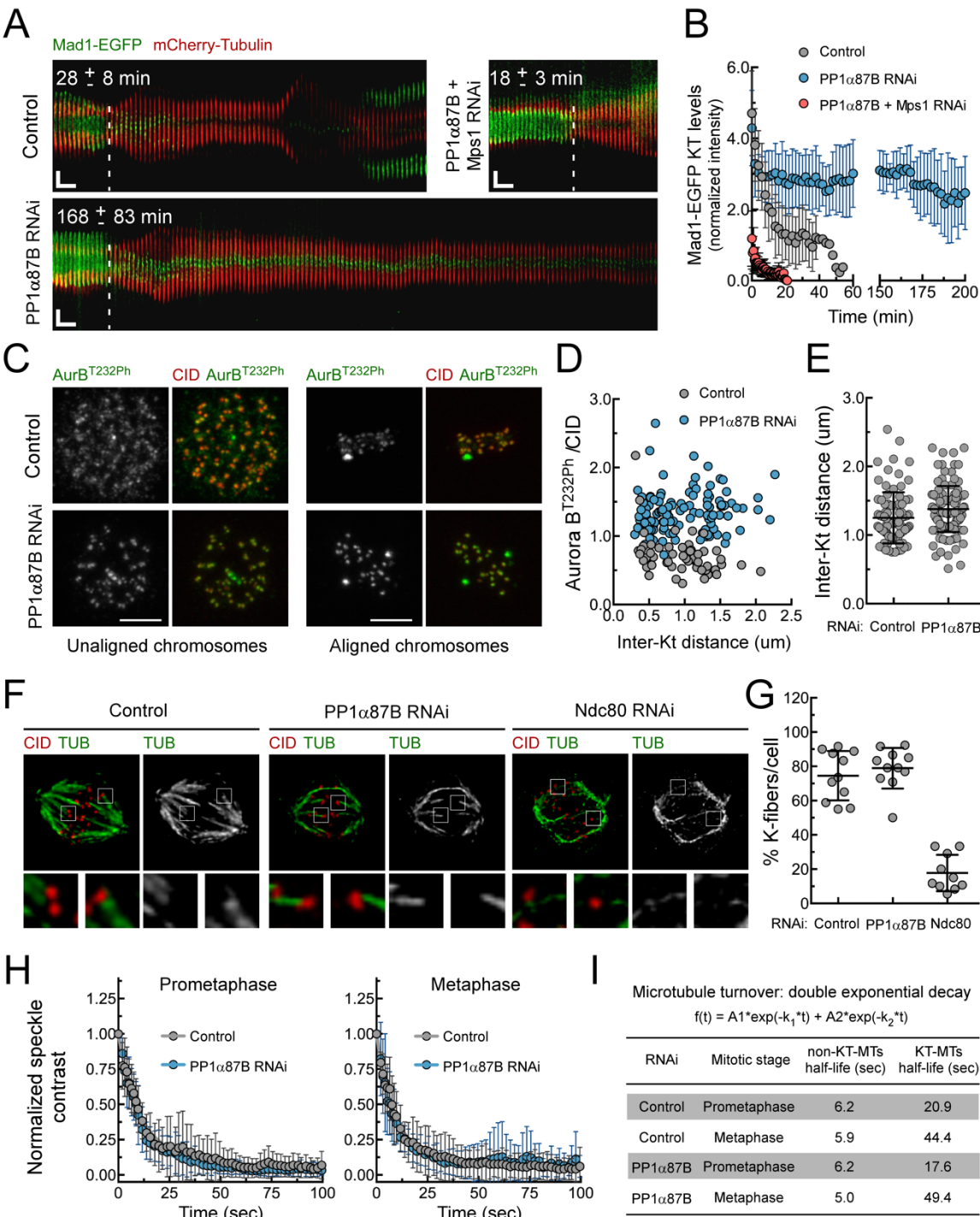


Figure I.2 – Depletion of PP1α87B results in a SAC-dependent metaphase delay despite stable kinetochore-microtubule attachments. (A,B) Kymograph analysis of mitotic progression monitored by live-cell imaging (A) and corresponding quantification of Mad1-EGFP kinetochore levels (B) of control, PP1α87B depleted or Mps1 and PP1α87B co-depleted *Drosophila* S2 cells expressing mCherry-αTubulin and Mad1-EGFP under control of Mad1 promoter. The mean time from nuclear envelope breakdown to anaphase onset for each condition is displayed in the corresponding kymograph. Mad1-EGFP fluorescence intensities at kinetochores were corrected for cytosolic signal ($n \geq 9$ cells for each condition). Vertical dashed line indicates the frame corresponding to nuclear envelope breakdown. Horizontal scale bar: 5 min. Vertical scale bar: 5 μm. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of inter-kinetochore distances and relative levels of Aurora B T232 phosphorylation (AurB^{T232Ph}) at unaligned and aligned chromosomes in control and PP1α87B depleted S2 cells. AurB^{T232Ph} relative levels were plotted over the inter-kinetochore distance measured as the distance between centroids of CID pairs. AurB^{T232Ph}

fluorescence intensities were determined relative to CID signal ($n \geq 58$ kinetochore pairs for each condition). Scale bar: 5 μm . (E) Quantification of inter-kinetochore distances measured in chromosomes aligned at the metaphase plate of control and PP1 α 87B depleted S2 cells. Inter-kinetochore distances were measured as the distance between centroids of identified CID pairs ($n \geq 86$ kinetochores for each condition). (F,G) Representative immunofluorescence images (F) and corresponding quantification (G) of cold-stable kinetochore fibres in control, PP1 α 87B and Ndc80 depleted S2 cells. CID immunolocalization was used as kinetochore reference. The insets display magnifications of the outlined regions. The graph represents the % of kinetochores attached to cold-stable microtubules per cell ($n \geq 10$ cells for each condition). (H,I) Analysis of microtubules turnover rates by speckle contrast fadeout of GFP- α Tubulin. (H) Contrast fadeout–time curves of GFP- α Tubulin fluorescent speckles (lines) and their time point means (dots) measured in rectangular areas enclosing the spindle of control and PP1 α 87B-depleted S2 cells in prometaphase and metaphase. The rate of speckle contrast fadeout was calculated to obtain microtubule turnover rates. (I) Table showing microtubules half-lives of non-kinetochore- (non-KT-MTs) and kinetochore-microtubules (KT-MTs) of control and PP1 α 87B-depleted S2 cells determined by inducible speckle imaging in prometaphase and metaphase. The average speckle intensity squared-contrast at each time point was fit to a double-exponential curve $A1 \cdot \exp(-k1 \cdot t) + A2 \cdot \exp(-k2 \cdot t)$, in which t is time, $A1$ represent the less stable population (non-KT-MTs) and $A2$ the more stable population (KT-MTs) with decay rates of $k1$ and $k2$, respectively. The turnover half-life for each population was calculated as $\ln 2/k$ ($n \geq 7$ metaphase cells for each condition). Data information: in (A), (B), (E), (G) and (H) data are presented as mean $\pm$ SD.

Depletion of PP1 α 87B fails to cause significant changes in the stability of kinetochore-microtubule attachments in metaphase cells and there are no significant differences in the fractions of microtubules in the stable (KT-MT) versus unstable (non-KT-MT) populations when compared to control cells (Figure I.2 H,I; Figure I.3). Taken together, these results clearly show that Mad1 accumulation at metaphase-aligned kinetochores and the delayed anaphase onset observed in PP1 α 87B-depleted cells are not caused by unattached kinetochores transiently generated as a result of increased Aurora B activity.

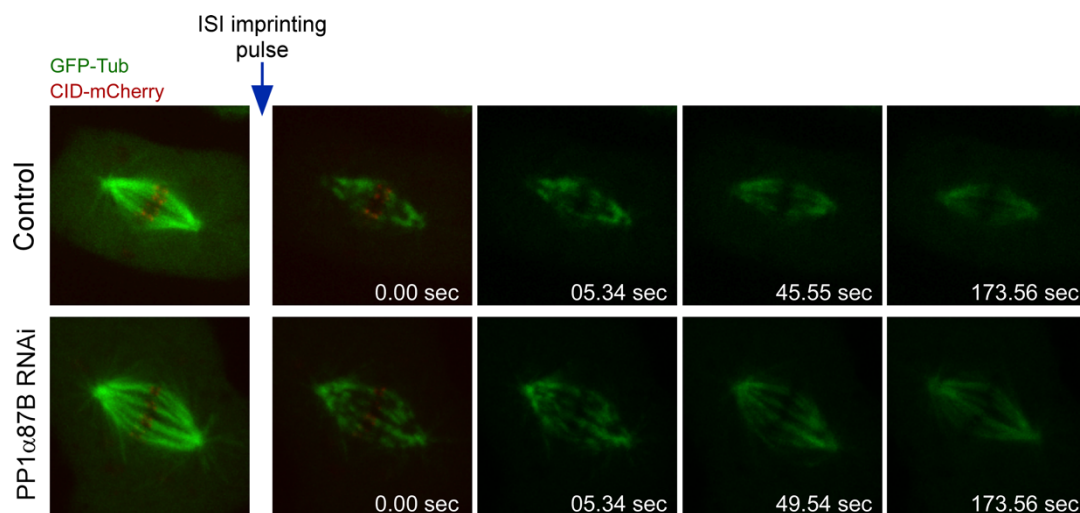


Figure I.3 – Analysis of microtubule turnover in control and PP1 α 87B depleted metaphase cells by speckle contrast fade-out. Representative inducible speckle imaging (ISI) imprinting and progressive contrast fade-out of GFP- α Tubulin in control and PP1 α 87B depleted metaphase S2 cells. The observed speckle contrast fadeout is due to microtubule turnover. Time is sec.

PP1α87B antagonizes Mps1 T-loop autophosphorylation at kinetochores

The results described in the previous section suggest that the metaphase delay evoked by PP1α87B depletion results from problems in SAC silencing. Although Mps1 is dispensable for kinetochore localization of Bub1 and BubR1 in flies (Schittenhelm et al, 2009; Conde et al, 2013), its activity is nonetheless required for Mad1 and Mad2 recruitment and therefore for SAC signalling (Althoff et al, 2012; Conde et al, 2013). In accordance, co-depletion of Mps1 with PP1α87B abolished Mad1 kinetochore recruitment and led to a premature anaphase onset, demonstrating that Mps1 is required for the SAC-dependent metaphase delay observed in PP1α87B RNAi (Figure I.2 A,B).

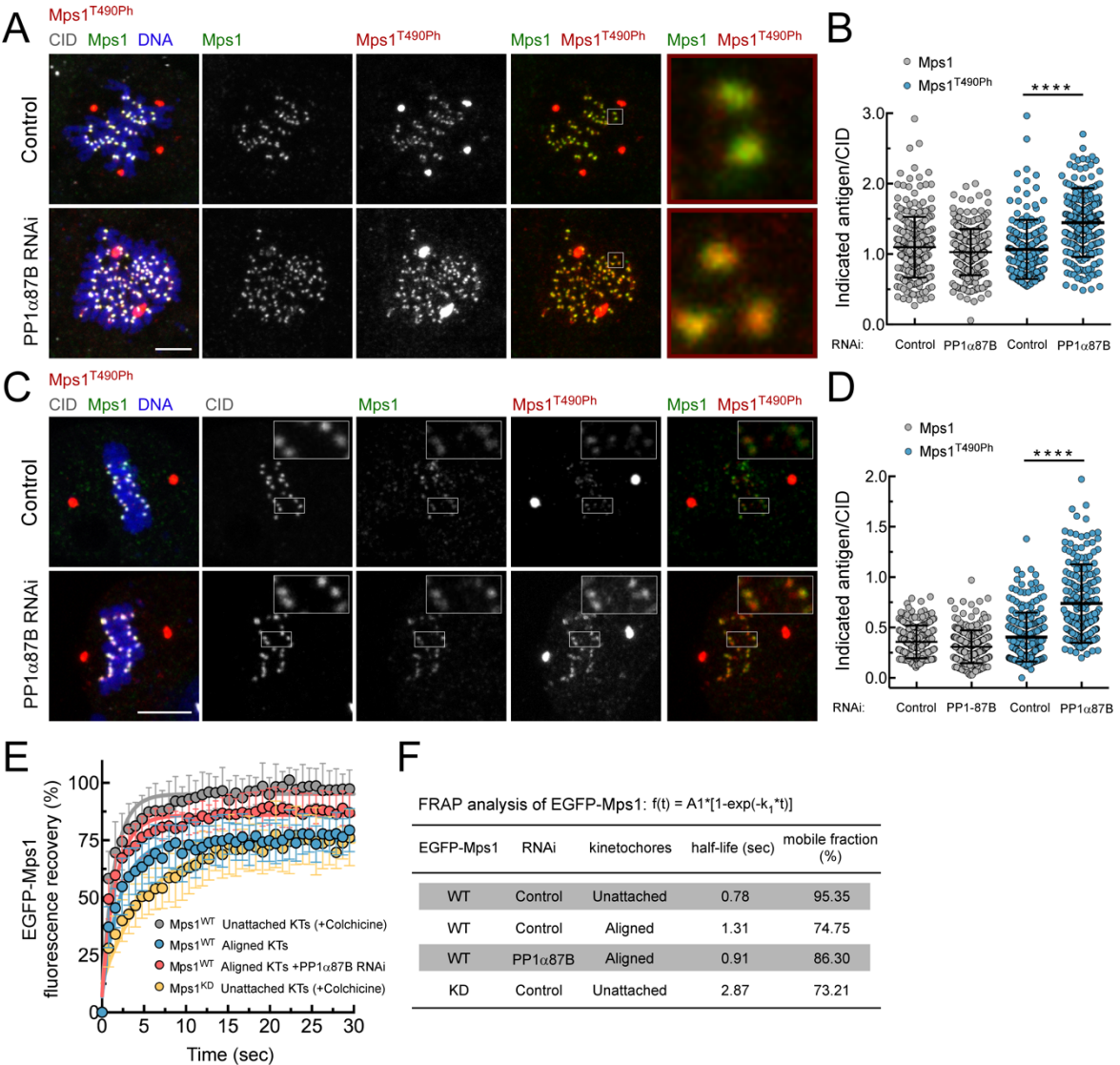


Figure I.4 – PP1α87B antagonizes Mps1 T-loop autophosphorylation at kinetochores of prometaphase and metaphase cells. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) and Mps1 relative levels at prometaphase kinetochores of control and PP1α87B depleted *Drosophila* S2 cells. The insets display magnifications of the outlined regions. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal ($n \geq 252$ kinetochores from at least 10 cells for each condition). Scale bar: 5 μ m. (C,D) Representative immunofluorescence images

(C) and corresponding quantifications (D) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) and Mps1 relative levels at metaphase kinetochores of control and PP1 α 87B depleted S2 cells. The insets display magnifications of the outlined regions. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal ($n \geq 217$ kinetochores from at least 15 cells for each condition). Scale bar: 5 μ m. (E,F) FRAP analysis of EGFP-Mps1^{WT} (wild type) and EGFP-Mps1^{KD} (kinase dead) at unattached or metaphase-aligned kinetochores of control and PP1 α 87B depleted S2 cells. To generate unattached kinetochores, S2 cells were treated with colchicine (30 μ M) for 2h prior to FRAP experiments (E) Graph displays recovery-time curves of EGFP-Mps1 fluorescence (lines) and their time point means (dots) after bleaching of individual kinetochores in the indicated conditions. (F) Table showing the recovery half-lives and the % of mobile EGFP-Mps1 population obtained after fitting the average fluorescence intensity at each time point to a single-exponential one-phase association curve ($n \geq 9$ cells for each condition). Data information: in (B), (D) and (E) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Mann-Whitney U test).

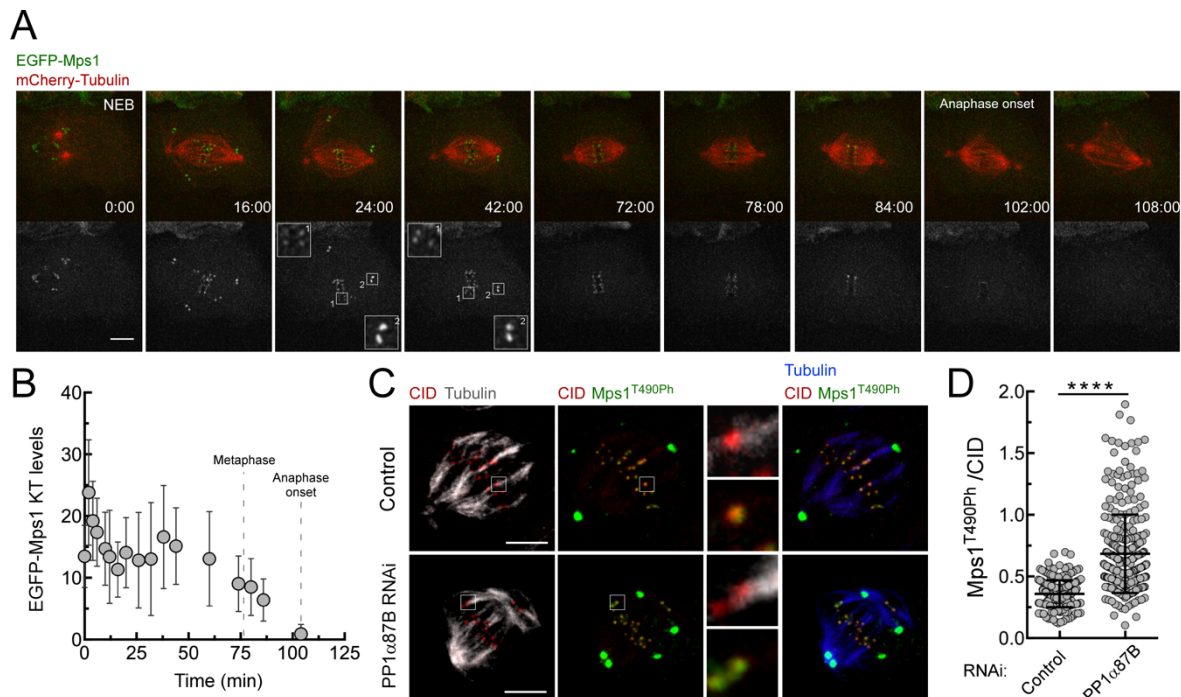


Figure I.5 – PP1 α 87B antagonizes the T-loop autophosphorylation of Mps1 that remains associated with metaphase kinetochores stably attached to spindle microtubules. (A) Detailed analysis of mitotic progression of *Drosophila* S2 cells expressing mCherry- α Tubulin and EGFP-Mps1. Mitotic progression was monitored by time-lapse microscopy. The insets display magnifications of the outlined regions. Time is in min:sec. (B) Quantification of EGFP-Mps1 kinetochores levels from (A). The average fluorescence intensity at kinetochores was corrected for the average EGFP-Mps1 signal in the cytoplasm. Time 0 min corresponds to nuclear envelope breakdown (NEB). Metaphase duration was defined by the length of time between the first still in which all kinetochore pairs were perfectly aligned at the cell equator (Metaphase) and anaphase onset. Dashed lines indicate the time from NEB to metaphase and to anaphase onset. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) levels at metaphase kinetochores attached to cold-resistant microtubules bundles of control and PP1 α 87B depleted *Drosophila* S2 cells. The insets display magnifications of the outlined regions. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal ($n \geq 270$ kinetochores from at least 12 cells for each condition). Scale bar: 5 μ m. Data information: in (B) and (D) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Mann-Whitney U test).

Therefore, we sought to examine Mps1 kinetochore recruitment and activation in S2 cells depleted of PP1 α 87B. Immunofluorescence analysis showed that depletion of PP1 α 87B has no significant impact on Mps1 kinetochore localization. Mps1 accumulates at prometaphase kinetochores and its levels decrease significantly as microtubules attach, with a fraction of Mps1 persisting at kinetochores of metaphase chromosomes (Figure I.4 A-D). This residual pool of Mps1 is retained at bioriented kinetochores until anaphase onset, even after establishment of inter-kinetochore tension, as revealed by live imaging of S2 cells expressing EGFP-Mps1 (Figure I.5 A,B). To evaluate Mps1 activation status we used a phospho-specific antibody that recognizes the conserved activating autophosphorylation (T490Ph) on Mps1 T-loop (Jelluma et al, 2008a). Interestingly, we found that depletion of PP1 α 87B resulted in a substantial increase of Mps1 T-loop autophosphorylation during prometaphase and on metaphases kinetochores stably attached to microtubule bundles (Figure I.4 A–D; Figure I.5; Figure I.4 – Supplementary figure 1).

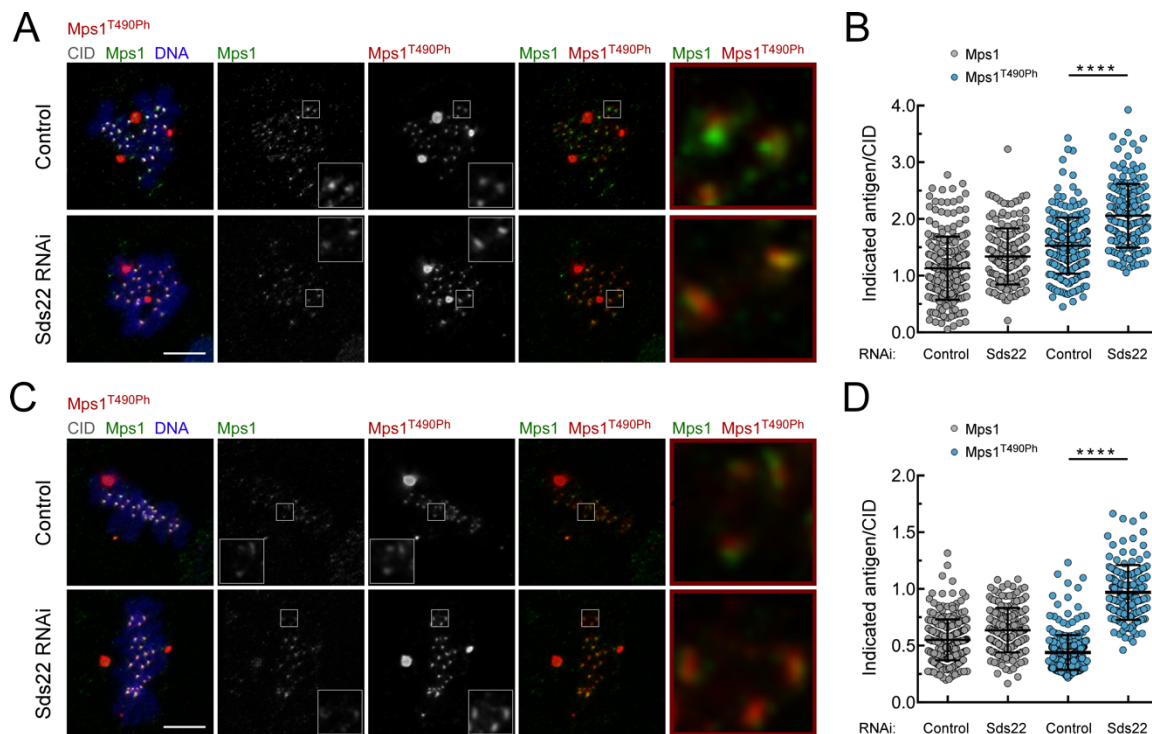


Figure I.6 – Depletion of Sds22 affects Mps1 T-loop autophosphorylation. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) and Mps1 relative levels at prometaphase kinetochores of control and Sds22 depleted S2 cells. The insets display magnifications of the outlined regions. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal ($n \geq 177$ kinetochores from at least 8 cells for each condition). Scale bar: 5 μ m. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) and Mps1 relative levels at metaphase kinetochores of control and Sds22 depleted S2 cells. The insets display magnifications of the outlined regions. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal ($n \geq 186$ kinetochores from at least 8 cells for each condition). Scale bar: 5 μ m. Data information: in (B) and (D) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, ****p<0.0001 (Mann-Whitney U test).

Moreover, depletion of PP1 noncatalytic subunit Sds22/PPP1R7, which positively regulates the phosphatase activity at kinetochores (Posch et al, 2010; Wurzenberger et al, 2012; Heroes et al, 2013), mimicked PP1 α 87B RNAi by increasing Mps1 T490 phosphorylation both at unattached and aligned kinetochores (Figure I.6). Mps1 association with prometaphase kinetochores in human cells is highly transient (Jelluma et al, 2010). Likewise, *Drosophila* Mps1 exhibits a fast turnover rate at unattached kinetochores that relies in part on its kinase activity, as revealed by FRAP measurements of wild type (WT) and kinase-dead (KD) versions of EGFP-Mps1 expressed in S2 cells (Figure I.4 E,F). In line with the observed reduction in Mps1 T490 autophosphorylation, both the half-life and stable population of Mps1 increase at metaphase kinetochores (Figure I.4 E,F). However, in the absence of PP1 α 87B, Mps1 displays a faster dynamic exchange at aligned kinetochores, as expected from enhanced kinase activity (Figure I.4 E,F). Collectively, these results demonstrate that PP1 α 87B activity is required to represses the activating autophosphorylation of Mps1 that remains associated with metaphase kinetochores.

PP1 α 87B/PP1 γ dephosphorylates Mps1 T-loop

To evaluate whether PP1 directly antagonizes Mps1 T-loop activating autophosphorylation, we started by assessing *in vitro* the capacity of PP1 γ to dephosphorylate the kinase T-loop. Simultaneous incubation of PP1 γ with recombinant versions of human (Mps1/TTK) and *Drosophila* Mps1 orthologues led to a concentration-dependent decrease of T676 (T676Ph) and T490 (T490Ph) autophosphorylation, respectively (Figure I.7 A,C). Moreover, PP1 γ was able to dephosphorylate the T-loop of Mps1/TTK that had been previously activated by autophosphorylation (Figure I.7 B). In contrast, λ -phosphatase failed to antagonize to the same extent the autophosphorylation of human Mps1/TTK T676 and of *Drosophila* Mps1 T490 (Figure I.7 C; Figure I.8), indicating a higher specificity of PP1 γ towards Mps1 T-loop *in vitro*.

We then sought to examine whether PP1 directly dephosphorylates Mps1 T-loop in a cellular context and determine its implications for SAC silencing. Because PP1 has numerous mitotic substrates acting on a multitude of pathways, its depletion results in pleiotropic phenotypes that preclude a clear and direct analysis of individual events. To circumvent this, we devised a strategy to specifically prevent Mps1 dephosphorylation without impacting the remaining PP1 α 87B cellular activity. The association of PP1 with the majority of its interactors is mediated by short motifs that bind to the phosphatase hydrophobic groove (Egloff et al, 1997; Wakula et al, 2003). *Drosophila* Mps1 N-terminus contains a clear match for a PP1-docking motif of the [R/K]VxF type (Figure I.7 D). To

assess the relevance of Mps1 KVL²³¹⁻²³⁴ for the interaction with PP1 α 87B, we converted K231 and F234 to alanine and expressed both the wild type (EGFP-Mps1^{WT}) and mutant versions (EGFP-Mps1^{K231A/F234A}) of Mps1 in S2 cells.

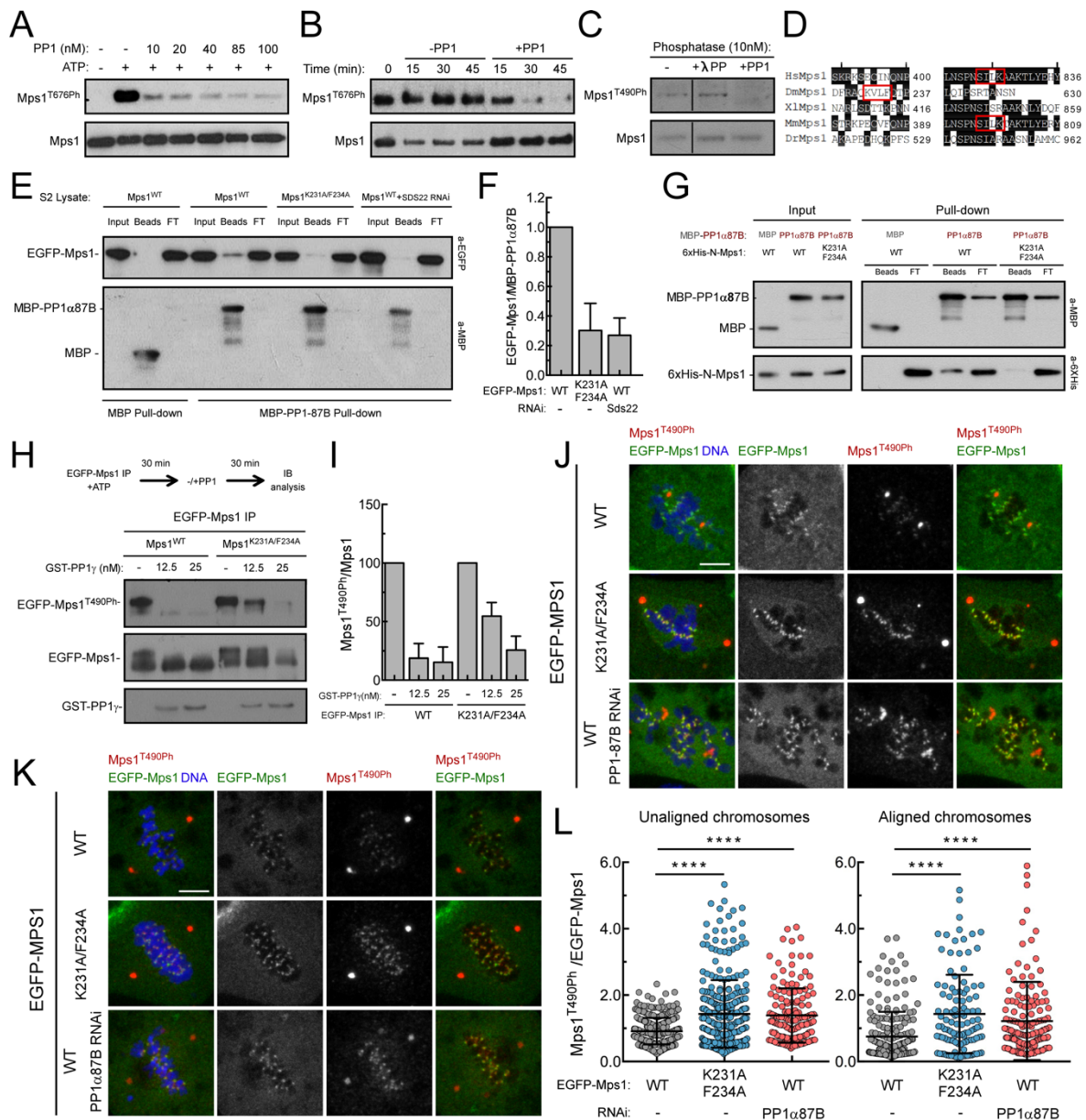


Figure I.7 – PP1 α 87B/PP1 γ dephosphorylates Mps1 T-loop *in vitro* and *in vivo*. (A,B) Western blot analysis of Mps1/TTK T676 autophosphorylation. Recombinant human Mps1 was incubated during 30 min with increasing concentrations of recombinant human PP1 γ (A) or PP1 γ (42.7 nM) was added to previously active Mps1 and phosphorylation of Mps1 T676 (Mps1^{T676Ph}) monitored over time (B). (C) Western blot analysis of *Drosophila* Mps1 T490 autophosphorylation. Recombinant *Drosophila* Mps1 was incubated during 30 min with 10 nM of recombinant human PP1 γ or λ -PP and phosphorylation of Mps1 T490 (Mps1^{T490Ph}) monitored. (D) Clustal W alignments of amino acid residues of indicated Mps1 orthologues. Red boxes highlight putative PP1-docking motifs identified in silico by the Eukaryotic Linear Motif (ELM) resource. (E) MBP-PP1 α 87B pull-downs from total cell lysates of S2 cells expressing EGFP-Mps1^{WT} or EGFP-Mps1^{K231A/F234A} or EGFP-Mps1^{WT} upon depletion of Sds22. Immobilized MBP was used as negative control. Input, beads and flow-through (FT) were probed by western blotting for the indicated proteins. (F) Quantification of Mps1 binding to PP1 α 87B from the pull-downs in (E). The chemiluminescence signal intensity of EGFP-Mps1 was determined relative to the signal of MBP-PP1 α 87B beads. The graph represents the quantification of relative levels of EGFP-Mps1 in MBP-

PP1 α 87B pull-downs from at least two independent experiments. The values obtained for EGFP-Mps1^{WT} from control cells were set to 1. (G) MBP-PP1 α 87B pull-downs of purified recombinant Mps1 N-terminus region (104–330 amino acids) harbouring the wild-type (6xHis-N-Mps1^{WT}) or mutated PP1-docking motif (6xHis-N-Mps1^{K231A/F234A}). Immobilized MBP was used as negative control. Input, beads and flow-through (FT) were probed by western blotting for the indicated proteins. (H) Western blot analysis of EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} T-loop dephosphorylation by PP1 γ . EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} were immunoprecipitated from mitotic S2 lysates and incubated with ATP for 30 min to allow Mps1 T-loop autophosphorylation. Increasing concentrations of recombinant GST-PP1 γ were subsequently added to the reaction mixture and Mps1 T490 phosphorylation (Mps1^{T490Ph}) assessed after 30 min. Immunoprecipitates were probed by immunoblotting (IB) for the indicated proteins. (I) Quantification of Mps1^{T490Ph} levels from the dephosphorylation assay in (H). The chemiluminescence signal intensity of Mps1^{T490Ph} was determined relative to the corresponding signal of EGFP-Mps1 immunoprecipitates. The values obtained for each control reaction were set to 100%. The graph represents the quantification of Mps1^{T490Ph} relative levels from two independent experiments. (J–L) Representative immunofluorescence images (J,K) and corresponding quantifications (L) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) at kinetochores of unaligned (J) and aligned (K) chromosomes from S2 cells expressing EGFP-Mps1^{WT} or EGFP-Mps1^{K231A/F234A} and from PP1 α 87B depleted S2 cells expressing EGFP-Mps1^{WT}. Mps1^{T490Ph} fluorescence intensities were determined relative to EGFP-Mps1 signal ($n \geq 150$ unaligned kinetochores from at least 8 cells for each condition and $N \geq 111$ aligned kinetochores from at least 7 cells for each condition). Scale bar: 5 μ m. Data information: in (F), (I) and (L) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

The interaction between Mps1 and PP1 α 87B was confirmed in pull-down assays in which EGFP-Mps1^{WT} from S2 cell lysates bound efficiently to recombinant MBP-PP1 α 87B but not to the MBP used as negative control (Figure I.7 E). Conversely, mutating Mps1 KVLF²³¹⁻²³⁴ to AVLA²³¹⁻²³⁴ markedly decreased the ability of EGFP-Mps1 to interact with MBP-PP1 α 87B, hence validating KVLF as a PP1-binding motif (Figure I.7 E,F). To demonstrate that PP1 α 87B directly interacts with Mps1 via the KVLF motif, we performed pull-down assays with MBP-PP1 α 87B and bacterially purified fragments of Mps1 N-terminus (104–330 aa) harboring the wild-type (6xHis-N-Mps1^{WT}) or mutated PP1-docking motif (6xHis-N-Mps1^{K231A/F234A}). While 6xHis-Mps1^{WT} bound efficiently to MBP-PP1 α 87B, the fragment 6xHis-N-Mps1^{K231A/F234A} did not (Figure I.7 G), thus confirming that the KVLF motif mediates a direct interaction between Mps1 N-terminus and PP1 α 87B. In accordance, *in vitro* assays with EGFP-Mps1 immunoprecipitated from mitotic S2 cell lysates showed that recombinant PP1 γ antagonized with discernible less efficiency the T-loop autophosphorylation of EGFP-Mps1^{K231A/F234A} in comparison to EGFP-Mps1^{WT} (Figure I.7 H,I).

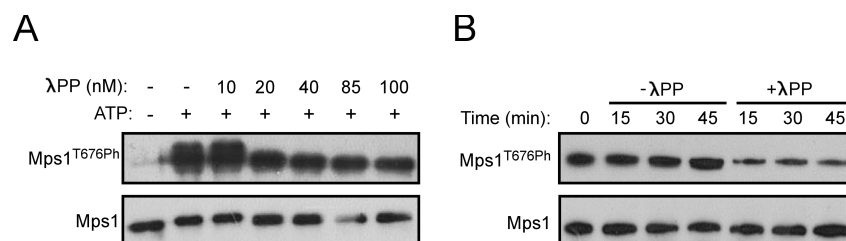


Figure I.8 – Specificity of Mps1 T-loop dephosphorylation *in vitro*. (A,B) Western blot analysis of Mps1T676 autophosphorylation. Recombinant human Mps1 was incubated during 30 min with increasing concentrations

of recombinant human λ -phosphatase (λ PP) (A) or λ -PP (42.7 nM) was added to previously active Mps1 and phosphorylation of Mps1 T676 (Mps1^{T676Ph}) monitored over time (B).

Collectively, these results indicate that Mps1 T-loop is a direct substrate of PP1 α 87B/PP1 γ . However, we found that MBP-PP1 α 87B failed to pull-down EGFP-Mps1^{WT} from S2 cells depleted of Sds22/PPP1R7 (Figure I.7 E,F), which suggests that in addition to the KVLF motif on the kinase N-terminus, the Sds22/PPP1R7 regulatory subunit is also required for competent binding of full-length Mps1 to PP1 α 87B in cell extracts. To analyse the impact of diminished PP1 α 87B binding on cellular Mps1 activation and SAC signalling, we monitored Mps1 T-loop phosphorylation and mitotic progression in S2 cells expressing EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} (Figure I.7 – Supplementary figure 1). Phosphorylation of T490 at unaligned kinetochores was significantly higher for EGFP-Mps1^{K231A/F234A} relative to EGFP-Mps1^{WT} (Figure I.7 J,L). A similar increment in the T-loop activation status was observed for EGFP-Mps1^{WT} of prometaphase cells depleted of PP1 α 87B (Figure I.7 J,L). Notably, the mutation KVLF²³¹⁻²³⁴ prevented the dephosphorylation of Mps1 T-loop also at bioriented kinetochores, mimicking the result obtained in metaphase cells expressing EGFP-Mps1^{WT} in the absence of PP1 α 87B (Figure I.7 K,L). As expected from increased T490 autophosphorylation, EGFP-Mps1^{K231A/F234A} exhibits faster dynamics than EGFP-Mps1^{WT} at aligned kinetochores (Figure I.9). From these results, we conclude that PP1 α 87B directly controls Mps1 T-loop dephosphorylation at kinetochores during prometaphase and metaphase.

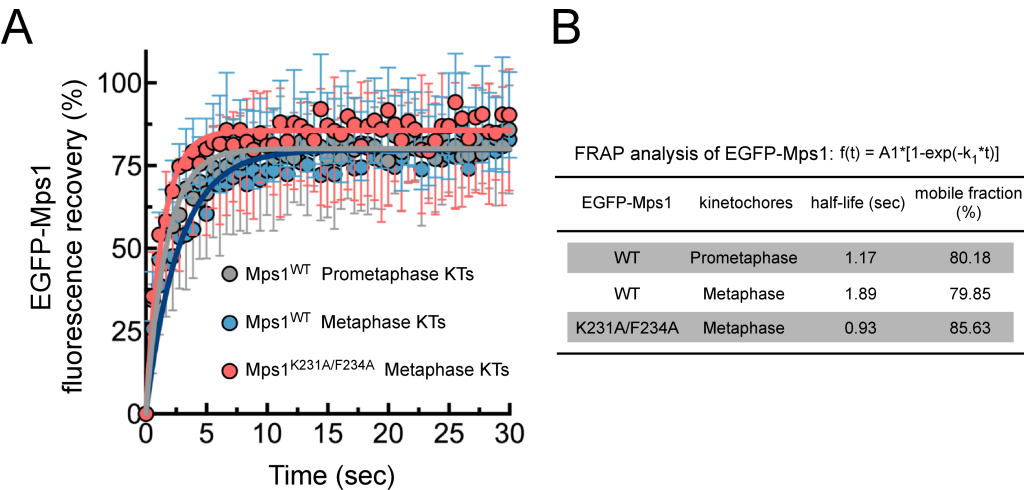


Figure I.9 – Mps1^{WT} and Mps1^{K231A/F234A} dynamics at kinetochores. (A,B) FRAP analysis of EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} at prometaphase and metaphase kinetochores. (A) Graph displays recovery-time curves of EGFP-Mps1 fluorescence (lines) and their time point means (dots) after bleaching of individual kinetochores in the indicated conditions. (B) Table showing the recovery half-lives and the % of mobile EGFP-Mps1 populations obtained after fitting the average fluorescence intensity at each time point to a single-exponential one-phase association curve ($n \geq 10$ cells for each condition).

PP1-mediated dephosphorylation of Mps1 T-loop inactivates the kinase and promotes SAC silencing

Live-cell imaging revealed that expression of EGFP-Mps1^{K231A/F234A} caused a pronounced metaphase delay when compared to EGFP-Mps1^{WT} expressing cells (Figure I.10 A,B). Depletion of BubR1 prevented this delay showing it is SAC-dependent (Figure I.10 A,B).

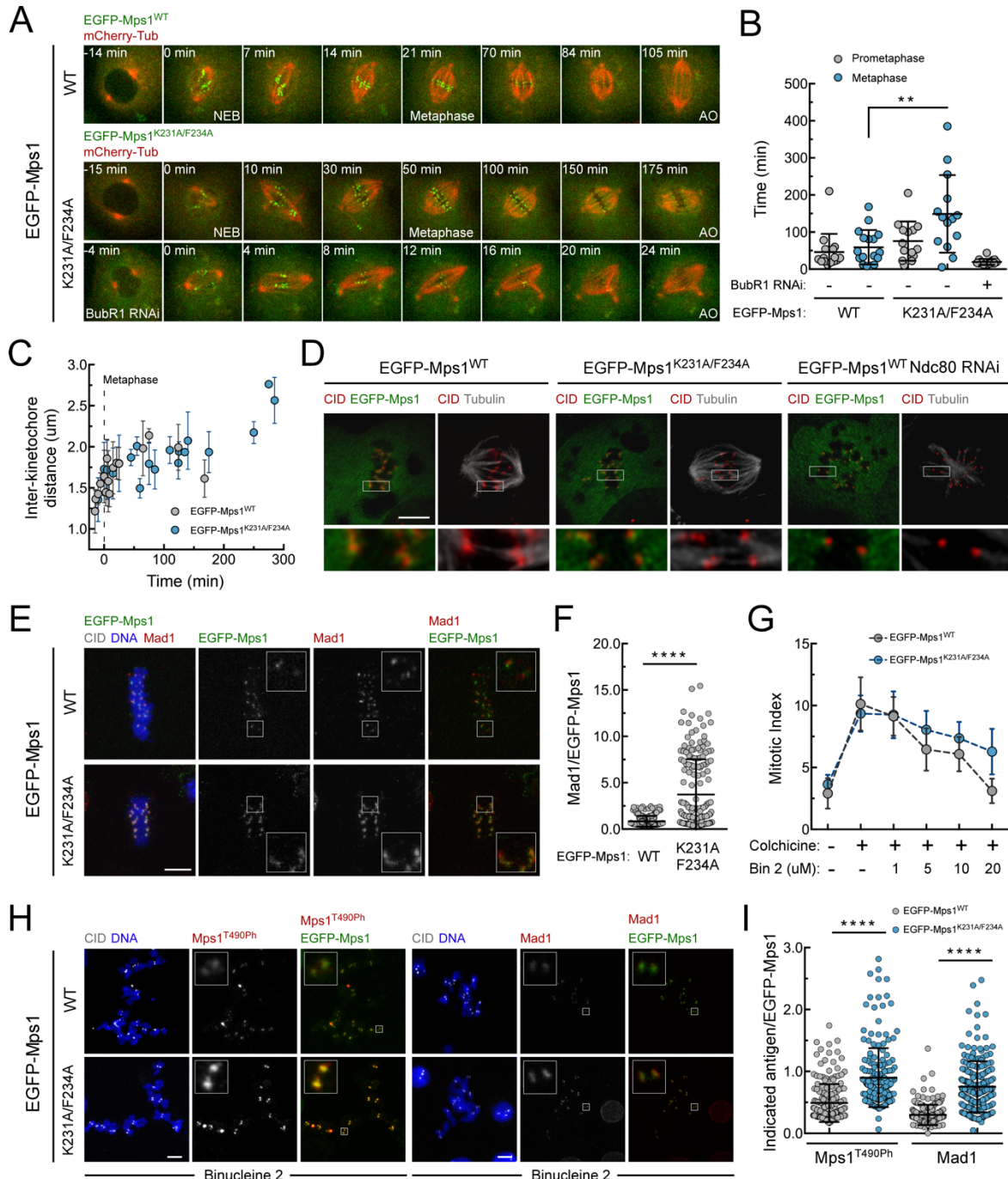


Figure I.10 – PP1 α 87B-mediated dephosphorylation of Mps1 T-loop renders the kinase inactive and is required for timely SAC silencing. (A,B) Mitotic progression (A) and prometaphase and metaphase duration (B) of S2 cells expressing mCherry- α Tubulin and EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} or EGFP-Mps1^{K231A/F234A} in a BubR1-depleted background. Mitotic progression was monitored by time-lapse microscopy. The metaphase duration was defined by the length of time measured between the first still in which all

kinetochore pairs were perfectly aligned at the metaphase plate (Metaphase) and anaphase onset (AO) ($n \geq 14$ cells for each condition, from at least two independent experiments). (C) Quantification of inter-kinetochore distances throughout mitosis in S2 cells expressing EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A}. Inter-kinetochore distances were measured as the distance between centroids of identified EGFP-Mps1 pairs at selected prometaphase and metaphase time frames. Time 0 min corresponds to the first metaphase frame. (D) Representative immunofluorescence images of cold-stable kinetochore fibers in S2 cells expressing EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} or EGFP-Mps1^{WT} upon Ndc80 depletion. CID immunolocalization was used as kinetochore reference. The insets display magnifications of the outlined regions. Scale bar: 5 μ m. (E,F) Representative immunofluorescence images (E) and corresponding quantifications (F) of Mad1 levels at metaphase kinetochores of S2 cells expressing EGFP-Mps1^{WT} or EGFP-Mps1^{K231A/F234A}. Mad1 fluorescence intensities were determined relative to EGFP-Mps1 signal ($n \geq 162$ kinetochores from at least 10 cells for each condition). Scale bar: 5 μ m. (G–I) Mitotic index (G), representative immunofluorescence images (H) and quantification of Mps1^{T490Ph} and Mad1 levels at unattached kinetochores (I) of colchicine treated S2 cells expressing EGFP-Mps1^{WT} or EGFP-Mps1^{K231A/F234A} in the presence of Aurora B inhibitor, binucleine 2 (Bin2) for 2h. Mitotic index in (G) was determined through p-H3 staining of cultured cells incubated in the absence or presence of colchicine (30 μ M) for 12h. Increasing concentrations of Bin2 were added to cultures 10h after colchicine and mitotic index determined 2h later. In (H and I) Mps1^{T490Ph} and Mad1 fluorescence intensities were determined relative to EGFP-Mps1 signal in cells treated with Bin2 (20 μ M) and colchicine as in (G). Proteasome inhibitor MG132 (20 μ M) was added to cultured cells 1h prior to Bin2 incubation to prevent mitotic exit ($n \geq 185$ kinetochores from at least 9 cells for each condition). Scale bar: 5 μ m. Data information: in (B), (C), (F), (G) and (I) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, ** $p < 0.05$, **** $p < 0.0001$ (Mann-Whitney U test).

However, the delay is not caused by unstable kinetochore-microtubule interactions, since the inter-kinetochore distances monitored throughout metaphase and the attachments of bioriented kinetochores to cold-resistant microtubule bundles in EGFP-Mps1^{K231A/F234A} cells were similar to those observed in metaphase cells expressing the wild-type kinase (Figure I.10 C,D; Figure I.11 A). Nevertheless, cells expressing EGFP-Mps1^{K231A/F234A} retained higher levels of Mad1 at aligned kinetochores (Figure I.10 E,F; Figure I.11 B,C), which is consistent with lasting Mps1 activation and SAC signalling in metaphase. These results strongly suggest that impairing PP1-mediated dephosphorylation of the residual pool of Mps1 that remains associated with kinetochores of bioriented chromosomes is sufficient to delay SAC silencing and prevent anaphase onset despite normal chromosome attachment and high inter-kinetochore tension. To further confirm the increment in Mps1 activity in EGFP-Mps1^{K231A/F234A} and its impact on SAC signalling, we treated cells with colchicine to generate unattached kinetochores and monitored their capacity to arrest in mitosis. As expected for SAC competent cells, this led to an evident increase in the mitotic index of both EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} expressing cells (Figure I.10 G).

Consistent with the role of Aurora B in potentiating Mps1 activation and SAC signalling, inhibition of the former with increasing concentrations of Binucleine 2 (Smurnyy et al, 2010) weakened the SAC in EGFP-Mps1^{WT} cells, as revealed by the gradual reduction in the mitotic index of colchicine incubated cultures (Figure I.10 G). In accordance, Aurora B inhibition negatively affected Mps1 T-loop autophosphorylation and Mad1 accumulation at

unattached kinetochores. Strikingly, expression of EGFP-Mps1^{K231A/F234A} partially rescued Mps1 T490 phosphorylation, Mad1 kinetochore recruitment and consistently attenuated the decline of SAC function following Aurora B inhibition (Figure I.10 G-I). Collectively, these results demonstrate that compromising Mps1 T-loop dephosphorylation is sufficient to delay SAC silencing and mitotic exit. Thus, we conclude that PP1-mediated dephosphorylation of Mps1 T-loop is required for efficient Mps1 inactivation and consequently for prompt SAC silencing.

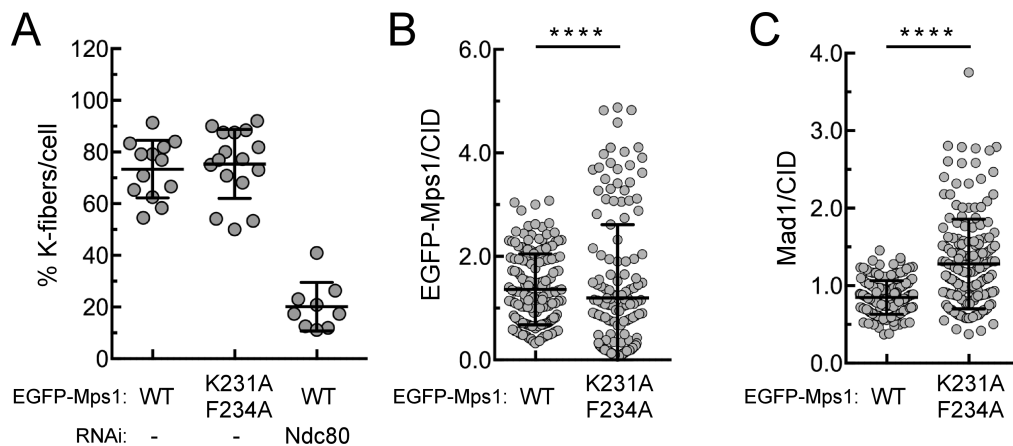


Figure I.11 – Stable attachments and Mad1 levels at metaphase kinetochores of S2 cells expressing EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A}. (A) Quantification of cold-stable kinetochore fibers in S2 cells expressing EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} and EGFP-Mps1^{WT} upon Ndc80 depletion. The graph represents the % of kinetochores attached to cold-stable microtubules per cell ($n \geq 9$ cells for each condition). (B,C) Quantifications of EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} (B) and of Mad1 (C) levels at metaphase kinetochores of S2 cells expressing EGFP-Mps1^{WT} or EGFP-Mps1^{K231A/F234A}. EGFP-Mps1 and Mad1 fluorescence intensities were determined relative to CID signal ($n \geq 162$ kinetochores from at least 10 cells for each condition). Scale bar: 5 μ m. Data information: in (A–C) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Mann-Whitney U test).

Preventing PP1-mediated inactivation of cytosolic Mps1 delays mitotic exit

Mps1 T-loop activation is first detected in the nucleoplasm and on pre-kinetochores during prophase (Figure I.12 A). Interestingly, kinetochore-null S2 cells engendered through CENP C knockdown retain high levels of nucleoplasmic Mps1 T490 phosphorylation (Figure I.12 A,B), indicating that Mps1 activation at mitotic entry can occur through kinetochore-independent mechanisms. Since previous studies have shown that cytosolic Mps1 also contributes for SAC function (Maciejowski et al, 2010; Maldonado and Kapoor, 2011; Rodriguez-Bravo et al, 2014), we reasoned that the activation status of soluble Mps1 must be tightly controlled during mitosis to allow timely anaphase onset. This prompted us to examine whether PP1 α 87B also represses cytoplasmic Mps1 activation and whether this is relevant for SAC signalling. To assess the impact of PP1-mediated dephosphorylation specifically on cytosolic Mps1 and completely avert contributions from kinetochore-

generated MCC, we monitored mitotic progression in CENP C depleted cells (Figure I.12 C; Figure I.12 – Supplementary figure 1).

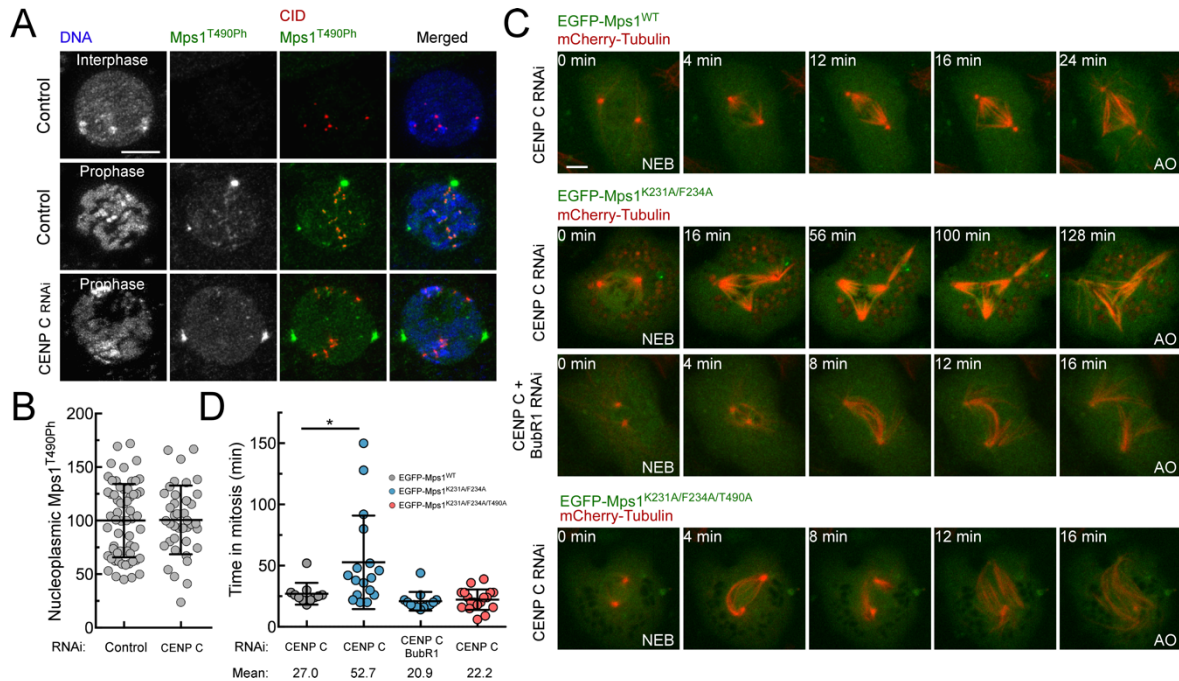


Figure I.12 – PP1-87B-mediated inactivation of cytosolic Mps1 is required for efficient SAC silencing and timely anaphase onset. (A,B) Representative immunofluorescence images (A) and corresponding quantification (B) of Mps1^{T490Ph} levels in the nucleoplasm of control and CENP C depleted cells in prophase. Mean values for control cells were set to 100% ($n \geq 40$ cells for each condition from four independent experiments). Scale bar: 5 μ m. (C,D) mitotic progression (C) and mitotic timing (D) of CENP C depleted S2 cells expressing mCherry- α Tubulin and EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} or EGFP-Mps1^{K231A/F234A/T490A}. Mitotic progression was monitored through time-lapse microscopy and the mitotic timing was defined by the length of time between nuclear envelope breakdown (NEB) and anaphase onset (AO) ($n \geq 11$ cells for each condition from at least two independent experiments). Scale bar: 5 μ m. Data information: in (B) and (D) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, $*p < 0.05$ (Mann-Whitney U test).

Live-cell imaging revealed that in the absence of kinetochores, cells expressing EGFP-Mps1^{WT} rapidly progressed through mitosis, whereas expression of EGFP-Mps1^{K231A/F234A} markedly delayed anaphase onset (Figure I.12 C,D). Importantly, preventing MCC assembly through BubR1 co-depletion or expressing Mps1^{K231A/F234A} harbouring unphosphorylatable T490 (EGFP-Mps1^{K231A/F234A/T490A}), failed to cause an obvious increase in the mitotic timing indicating that the delay in mitosis observed in kinetochore-null Mps1^{K231A/F234A} cells is SAC-dependent and requires Mps1 activating T-loop autophosphorylation (Figure I.12 C,D). These results show that impairing T-loop dephosphorylation of cytosolic Mps1 prolongs mitosis in a SAC-dependent manner even in the absence of a kinetochore-generated ‘wait anaphase’ signal. Therefore, timely transition to anaphase requires that SAC extinction at stably attached kinetochores coincides with the inactivation of cytosolic Mps1. These results allow us to conclude that PP1-mediated

dephosphorylation of non-kinetochore Mps1 is a critical mechanism to efficiently silence the SAC and ensure prompt anaphase onset.

Regulation of PP1 activity during mitosis

The activity of PP1 must be tightly regulated to allow robust Mps1 activation during early mitosis and to ensure its inactivation and SAC silencing when all kinetochores become stably attached to spindle microtubules. Immunofluorescence analysis of S2 cells revealed that PP1 α 87B preferentially accumulates on kinetochores when chromosomes are bioriented at the metaphase plate (Figure I.13 A,B).

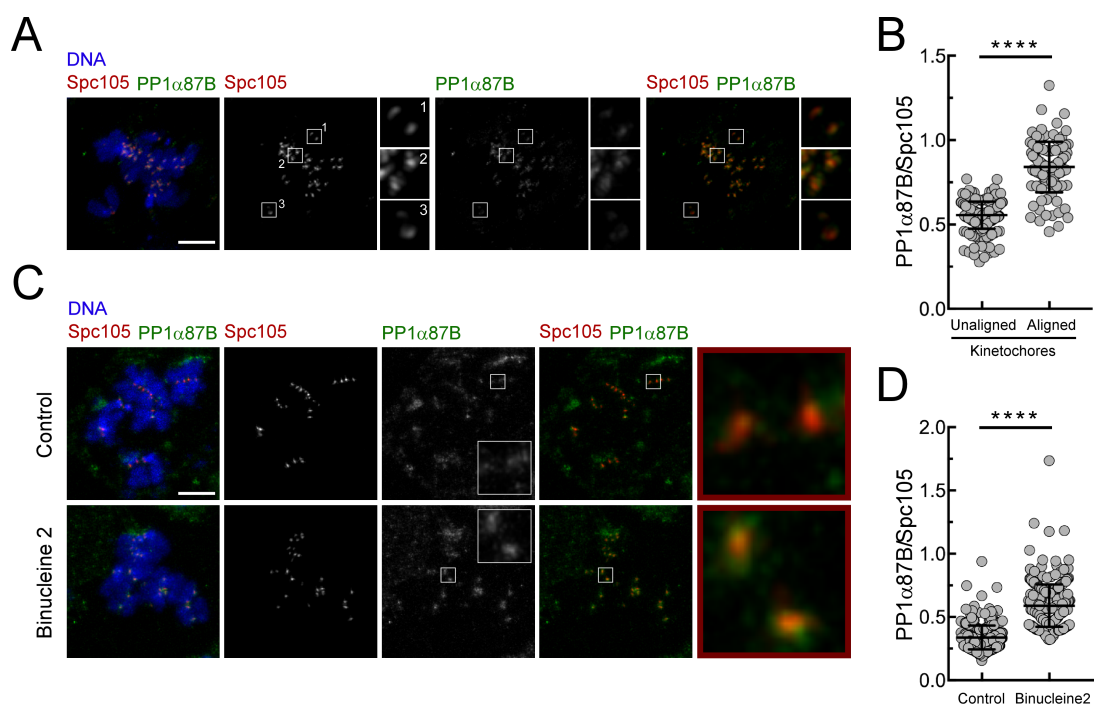


Figure I.13 – Regulation of PP1 α 87B activity during mitosis. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of PP1 α 87B levels at kinetochores of unaligned and aligned chromosomes in S2 cells. PP1 α 87B fluorescence intensities were determined relative to Spc105 signal ($n = 241$ unaligned kinetochores and $N = 110$ aligned kinetochores from at least 6 cells). Scale bar: 5 μ m. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of PP1 α 87B levels at unattached kinetochores of control and Binucleine 2-treated S2 cells. Cells were treated with MG132 (20 μ M) for 1h followed by a 2h incubation with colchicine (30 μ M) to generate unattached kinetochores. Binucleine 2 (20 μ M) was added to cultures 30 min prior to colchicine treatment. PP1 α 87B fluorescence intensities were determined relative to Spc105 signal ($n \geq 282$ kinetochores from at least 12 cells for each condition). Scale bar: 5 μ m. Data information: in (B) and (D) data are presented as mean $\pm$ SD. Asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Mann-Whitney U test).

Kinetochore recruitment of PP1 in human cells is mediated in part by SILK and RVSF motifs present on Knl1 N-terminus. Aurora B-dependent phosphorylation of both motifs prevents the binding of PP1 during prometaphase (Liu et al, 2010). As *Drosophila* Spc105/Knl1 also

contains a RVSF motif in its N-terminus, we examined whether a similar mechanism operates in S2 cells to control PP1 α 87B kinetochore localization. Cultured cells were incubated with Aurora B inhibitor Binucleine 2 in the presence of colchicine and of the proteasome inhibitor MG132, which respectively generate unattached kinetochores and prevent mitotic exit resulting from SAC abrogation. Inhibition of Aurora B led to an increase of PP1 α 87B levels at unattached kinetochores (Figure I.13 C,D), suggesting that similarly to the described for human cells, Aurora B activity limits the association of PP1 α 87B with unattached/ unaligned kinetochores in flies. In addition to the control exerted by Aurora B on PP1 kinetochore localization, Cdk1 phosphorylation of PP1 C-terminus was shown to repress the phosphatase activity during early mitosis (Dohadwala et al, 1994; Yamano et al, 1994; Wu et al, 2009; Grallert et al, 2015).

DISCUSSION

How the SAC is rapidly extinguished allowing anaphase to proceed when the last chromosome becomes bioriented has remained a key unanswered question. The present study shows that efficient SAC silencing requires the inactivation of its most upstream regulator, Mps1 kinase. We demonstrate *in vitro* and *in vivo* that PP1 directly antagonizes Mps1 T-loop autophosphorylation rendering the kinase inactive at metaphase kinetochores and in the cytoplasm. In *Drosophila* this relies on a [R/K]VxF motif that is present on Mps1 N-terminus and requires the PP1 regulatory subunit Sds22. Importantly, Mps1 orthologues encompass several other PP1-binding motifs within different regions of the kinase, such as SILK signatures in the C-terminus of human and mouse Mps1. This suggests that despite the diversification in type and localization of PP1-docking motifs among Mps1 orthologues, the dephosphorylation of the kinase T-loop by PP1 is likely to be conserved.

The calponin homology (CH) domains of Ndc80 and Nuf2 directly mediate Mps1 accumulation at unattached kinetochores, which is proposed to promote the kinase activation required for efficient SAC signalling (Kemmler et al, 2009; Santaguida et al, 2011; Saurin et al, 2011; Nijenhuis et al, 2013). Because microtubules bind to the same surface of the CH domains that interacts with Mps1, microtubules forming end-on attachments outcompete Mps1 for Ndc80 binding (Hiruma et al, 2015; Ji et al, 2015). This competition mechanism is consistent with the reduction of Mps1 from metaphase kinetochores but is, however, insufficient to dislodge all Mps1 molecules. As in human and budding yeast (Vázquez-Novelle and Petronczki, 2010; Aravamudhan et al, 2015), *Drosophila* kinetochores retain a fraction of Mps1 even after the formation of end-on attachments that exert proper kinetochore tension. Intriguingly, although low levels of Mps1 were shown to

be sufficient to delay anaphase onset (Herriott et al, 2012; Foijer et al, 2014), the residual pool of Mps1 at metaphase kinetochores does not prevent SAC silencing. Work in budding yeast proposes that this is due to changes on kinetochore architecture imposed by end-on attachments. Stably bound microtubules exert mechanical tension on kinetochores, which increases the distance between the CH domains of the Ndc80 complex and the MELT motifs on Spc105/Knl1, thus preventing phosphorylation of the latter by Mps1 and allowing SAC silencing (Aravamudhan et al, 2015). However, since in higher eukaryotes kinetochore-localized Mps1 dynamically exchanges with the cytoplasmic pool (Jelluma et al, 2010), free diffusion of Mps1 is expected to overcome the physical separation between the kinase receptor and its substrates at metaphase kinetochores. Moreover, the recruitment of Mad1:Mad2 to budding yeast kinetochores occurs exclusively through a linear pathway in which Mps1-phosphorylated MELTs function as docking sites for the binding of Bub1:Bub3 complexes, which in turn act as a loading platform for Mad1:Mad2 localization (London and Biggins, 2014a). In *Drosophila* and in human cells, however, a Spc105/Knl1- and Bub1-independent pathway also recruits Mad1:Mad2 to unattached kinetochores (Schittenhelm et al, 2009; Caldas et al, 2015; Silió et al, 2015). This relies on the localization of the RZZ complex at kinetochores, which is itself regulated by Mps1 activity (Santaguida et al, 2010) and is sufficient to activate the SAC in response to unattached kinetochores, even in the absence of Knl1 (Schittenhelm et al, 2009; Silió et al, 2015). Hence, in addition to the physical separation between the Ndc80 complex and Spc105/Knl1 phospho-domain, other mechanisms must operate in metazoans to limit the activity of kinetochore-associated Mps1 and extinguish SAC signalling in metaphase. Here we demonstrate that this is accomplished by PP1-mediated dephosphorylation of Mps1 T-loop. We found that depletion of PP1 α 87B or preventing its interaction with Mps1 increases the activation status of the residual fraction of Mps1 that persists at kinetochores of bioriented chromosomes. This correlates with higher levels of Mad1 retained on kinetochores and a prolonged metaphase delay that is caused by chronic SAC engagement despite stable end-on attachments and establishment of kinetochore tension. Thus, we reason that intra-kinetochore tension or alterations on kinetochore architecture, which also correlate with SAC satisfaction in *Drosophila* and human cells (Maresca and Salmon, 2009; Uchida et al, 2009), are important not only to disrupt the interaction of Mps1 with its kinetochore substrates but also to increase the recruitment of PP1 to metaphase chromosomes. Similarly to human cells (Liu et al, 2010), kinetochore levels of PP1 α 87B in *Drosophila* S2 cells are significantly higher at bioriented chromosomes. The association of PP1 γ with human kinetochores is directly mediated by SILK and [R/K]VxF motifs on Spc105/Knl1 N-terminus and by the Spindle- and Kinetochore-Associated (Ska) complex

(Sivakumar et al, 2016). At tensionless kinetochores, the recruitment of PP1 γ is limited due to the phosphorylation of Spc105/Knl1 N-terminus and of the Ska complex by Aurora B, which respectively inhibits the binding of PP1 γ to Spc105/Knl1 and Ska (Liu et al, 2010; Chan et al, 2012). As microtubules bind to kinetochores and tension is established, the spatial repositioning of the KMN network decreases Aurora-B mediated phosphorylation of the Ska complex and of Spc105/Knl1 N-terminus (Welburn et al, 2010), hence allowing bulk accumulation of PP1 γ , which then catalyses the dephosphorylation of Mps1 T-loop. This provides a mechanism to ensure the complete inactivation of any vestigial Mps1 residing at microtubule-attached kinetochores during late stages of mitosis.

In addition to the MCC generated at unattached kinetochores in prometaphase, APC/C inhibitory complexes are also assembled at the nuclear pore and in the cytoplasm during late interphase and prophase (Sudakin et al, 2001; Lopes et al, 2005; Lince-Faria et al, 2009; Maciejowski et al, 2010; Schweizer et al, 2013; Rodriguez-Bravo et al, 2014). This pre-mitotic MCC defines the minimum length of time a cell will spend in mitosis as it ensures APC/C inhibition until newly assembled kinetochores are able to generate an efficient SAC response. Interestingly, inhibition of Mps1 causes a substantial decrease in pre-mitotic MCC levels and a consequent reduction in Cyclin B levels already in G2 and prophase (Maciejowski et al, 2010; Rodriguez-Bravo et al, 2014). Moreover, a truncated form of Mps1 unable to localize at kinetochores failed to promote the recruitment of Bub1 to unattached kinetochores but was nevertheless sufficient to sustain the assembly of mitotic APC/C inhibitory complexes and delay anaphase onset (Maciejowski et al, 2010). In addition, cytosolic Mps1 activity was shown to be critical to maintain the metaphase arrest caused by Mad1 constitutively tethered to stably attached bioriented kinetochores that were stripped from Mps1 (Maldonado and Kapoor, 2011). These observations indicate that the activity of soluble Mps1 is required to generate the MCC and/or prevent its disassembly during mitosis, further strengthening the idea that removing the kinase from kinetochores is not sufficient for efficient SAC silencing. Therefore, determining how cytosolic Mps1 is inactivated after the SAC signal is extinguished at stably attached kinetochores becomes essential to understand the mechanism that allows timely mitotic exit. Here we show that PP1 directly controls Mps1 activation, not only at kinetochores but also in the cytoplasm. We demonstrate that preventing PP1-mediated dephosphorylation of soluble Mps1 maintains the kinase active in the cytosol and that this is sufficient to significantly delay mitotic exit even in the absence of kinetochore-generated MCC. Although kinetochore recruitment of Mps1 substantially increases the cell capacity to sustain a prolonged mitotic arrest when challenged with spindle poisons (Nijenhuis et al, 2013), during an unperturbed mitosis in which MCC production at kinetochores is minimal (Collin et al, 2013; Dick and

Gerlich, 2013), controlled activity of soluble Mps1 is expected to be particularly relevant. We envisage that the inactivation of cytosolic Mps1 is critical to ensure SAC responsiveness at kinetochores during late mitosis so that transition to anaphase occurs without delay after the last unattached kinetochore forms stable attachments.

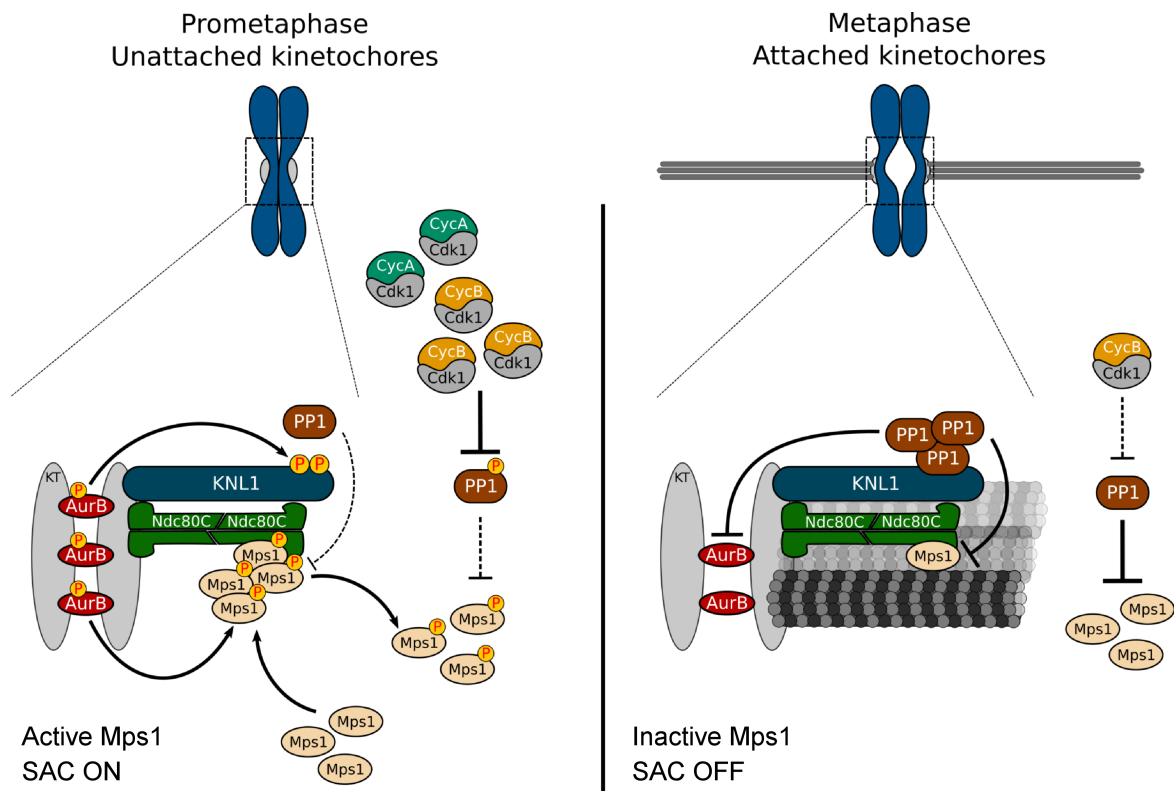


Figure I.14 – A model for regulation of Mps1 activation/inactivation in mitosis. Proposed model for regulation of soluble- and kinetochore-Mps1 activation in mitosis. During prometaphase, Aurora B potentiates the recruitment of Mps1 to unattached kinetochores and phosphorylates Knl1/Spc105 to limit PP1 kinetochore association. This allows the accumulation of active Mps1 at unattached kinetochores to instate efficient MCC assembly. In parallel, high levels of active Cdk1 repress PP1 activity in the cytoplasm, which prevents the dephosphorylation and consequently inactivation of soluble Mps1. Active Mps1 in the cytoplasm promotes the assembly and/or prevents the disassembly of APC/C inhibitory complexes through mechanisms that are yet to be described. Cytoplasmic- and kinetochore generated-MCC orchestrated by Mps1 cooperate to ensure efficient APC/C inhibition during unperturbed early mitosis. End-on attachment of microtubules mediated by the Ndc80 complex and Knl1 prevent the recruitment of Mps1 and exert tension across kinetochores and/or impose alterations on kinetochore architecture. Under these conditions, phosphorylation of Knl1 by Aurora B is minimal, and PP1 becomes enriched at bioriented kinetochores, where it dephosphorylates and inactivates the remaining residual pool of Mps1. Declining levels of active Cdk1 allow PP1 to auto-activate and repress Mps1 activity in the cytoplasm, hence ensuring efficient SAC silencing and prompt anaphase onset.

Taking our results together with previously reported work (Dohadwala et al, 1994; Yamano et al, 1994; Liu et al, 2010; Mochida and Hunt, 2012; Grallert et al, 2015; Qian et al, 2015; Rogers et al, 2016), we propose a model that controls Mps1 activation in a timely manner and perfectly coordinated with mitotic progression. Elevated Cdk1 and Aurora B activities during prometaphase, respectively repress PP1 activity and avert the phosphatase localization at unattached kinetochores. This ensures high levels of active kinetochore- and

cytosolic Mps1 and, therefore, robust SAC signalling in early mitosis (Figure I.14). As kinetochores become stably attached to spindle microtubules, Mps1 levels drastically decrease whereas PP1 accumulation increases. This occurs concomitantly with a drop in Cyclin B (and Cyclin A) that allows prompt PP1-mediated inactivation of Mps1 at kinetochores and in the cytoplasm (Figure I.14). We propose that this coincides with the dephosphorylation of Mps1 substrates, with the removal of other SAC proteins and with modifications on kinetochore organization. As Mps1 promotes MCC assembly through multi-target phosphorylations along the SAC signalling cascade, the checkpoint is highly responsive to changes in Mps1 activity (Faesen et al, 2017; Ji et al, 2017). Inactivation of Mps1 by PP1 prevents the kinase from counteracting the dephosphorylation of its substrates, thus avoiding futile cycles and ensuring efficient and rapid SAC silencing. This guarantees a swift anaphase onset following chromosome biorientation.

ANNEXES

Figure I.4 – Supplementary figure 1

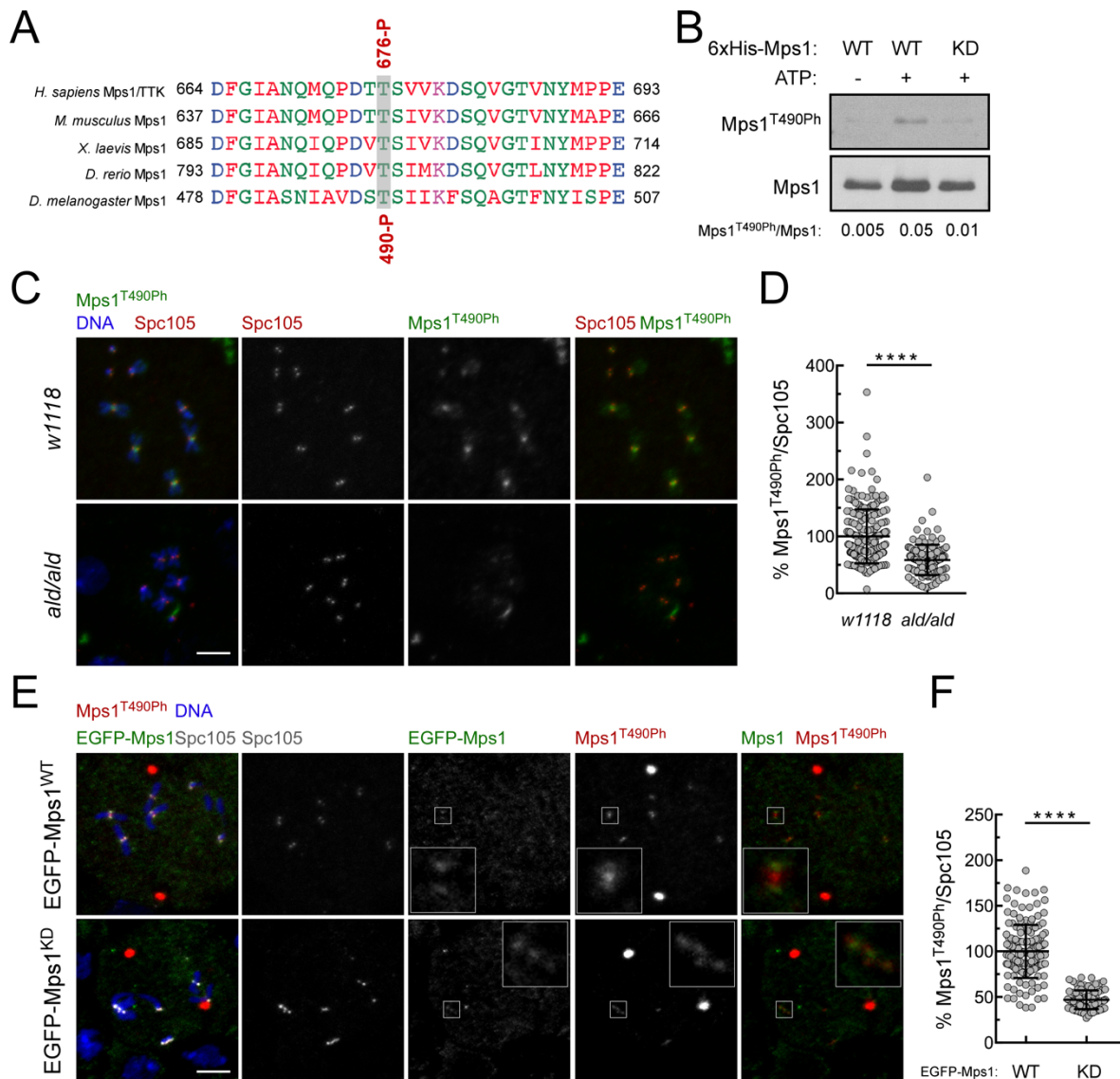


Figure I.4 – Supplementary figure 1 – Validation of a phospho-specific antibody against *Drosophila* Mps1 activating T-loop autophosphorylation. (A) Conservation among Mps1 T-loop residues. Clustal W alignments of T-loop residues of indicated Mps1 orthologues. The conserved Mps1 activating autophosphorylation threonine is highlighted in grey shading. The red numbers note specific residues for human Mps1/TTK (Thr676) and *Drosophila* Mps1 (Thr490), and P indicates phosphorylation. (B) *In vitro* autophosphorylation of *Drosophila* Mps1 detected by western blotting. Recombinant wild type (WT) and kinase dead (KD) versions of recombinant *Drosophila* Mps1 were incubated with ATP for 30 min and phosphorylation of T490 (Mps1^{T490Ph}) probed with a phospho-specific antibody. The chemiluminescence signal intensity of Mps1^{T490Ph} was determined relative to the signal of total Mps1. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) at prometaphase kinetochores of third instar larval neuroblasts from w1118 and ald^{G4422} homozygous (ald/ald). Mps1^{T490Ph} fluorescence intensities were determined relative to Spc105 signal and the mean values for control w1118 neuroblasts were set to 100% (n ≥ 135 kinetochores from at least 12 neuroblasts for each condition from two independent experiments). Scale bar: 5 μm. (E,F) Representative immunofluorescence images (E) and corresponding quantifications (F) of Mps1 T490 phosphorylation (Mps1^{T490Ph}) at prometaphase kinetochores of third instar larval neuroblasts from ald^{G4422} homozygous expressing EGFP-Mps1^{WT} (wild type) or EGFP-Mps1^{KD} (kinase dead). The insets display magnifications of the outlined regions. Mps1^{T490Ph} fluorescence intensities were determined relative to Spc105

signal and the mean values for neuroblasts expressing EGFP-Mps1^{WT} were set to 100% (n ≥ 87 kinetochores from at least 10 neuroblasts for each condition from two independent experiments). Scale bar: 5 μm. Data information: in (D) and (F) data are presented as mean ± SD. Asterisks indicate that differences between mean ranks are statistically significant, ****p<0.0001 (Mann-Whitney U test).

Figure I.7 – Supplementary figure 1

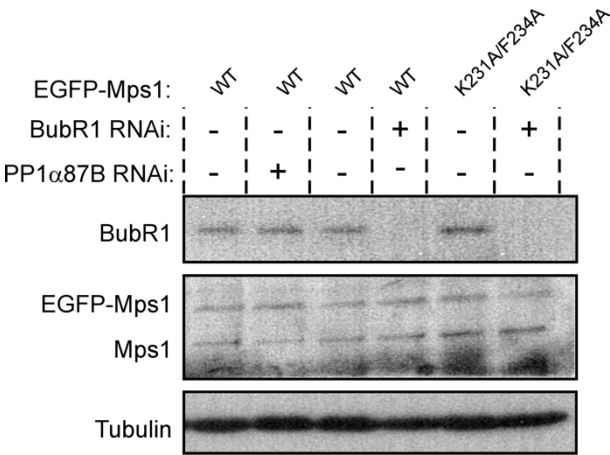


Figure I.7 – Supplementary figure 1 – Western blotting analysis of S2 cells expressing EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A}. Western blot of analysis of BubR1 depletion and of EGFP-Mps1^{WT} and EGFP-Mps1^{K231A/F234A} expression in *Drosophila* S2 cells used in the studies described in Figure I.7 J-L. αTubulin was used as loading control.

Figure I.12 – Supplementary figure 1

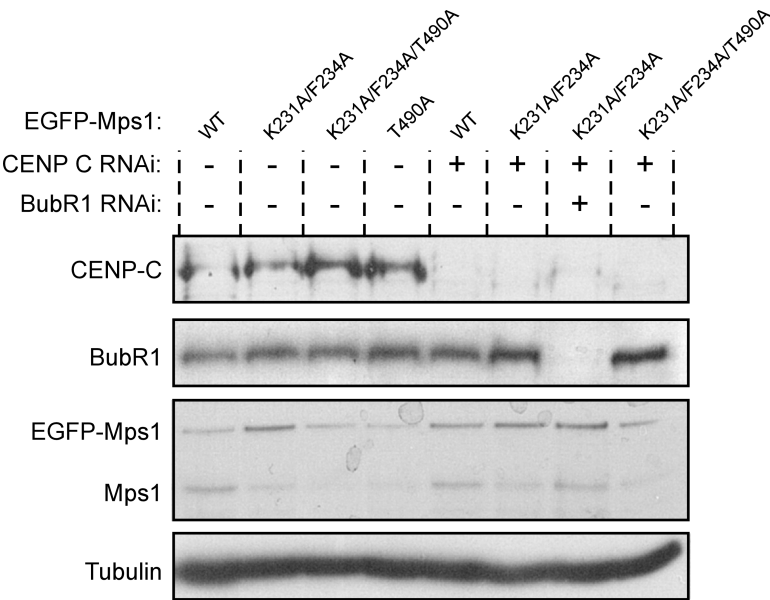


Figure I.12 – Supplementary figure 1 – Western blotting analysis of EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} and EGFP-Mps1^{K231A/F234A/T490A} expression in CENP C depleted S2 cells. Western blot of analysis of CENP C and BubR1 depletion and of EGFP-Mps1^{WT}, EGFP-Mps1^{K231A/F234A} and EGFP-Mps1^{K231A/F234A/T490A} expression in S2 cells used in the studies described in Figure I.12 C. αTubulin was used as loading control. Expression of EGFP-Mps1 transgenes was under control of Mps1 cis-regulatory region.

EXPERIMENTAL WORK

CHAPTER II

Polo regulates PP1 α 87B to ensure timely
Spindle Assembly Checkpoint and
genome stability

INTRODUCTION

To ensure accurate genome partitioning during cell division, eukaryotic cells evolved a surveillance mechanism known as the Spindle Assembly Checkpoint (SAC) that halts anaphase onset until all the kinetochores are correctly bound to spindle microtubules (Musacchio and Salmon, 2007; Foley and Kapoor, 2013; Lara-Gonzalez et al, 2021). The downstream target of the SAC is the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase that targets Securin and Cyclin B for proteasome-mediated proteolysis. Degradation of Securin and Cyclin B cause sister chromatid separation and Cyclin-dependent kinase 1 (Cdk1) inactivation, respectively, events required for chromosome segregation and mitotic exit (London and Biggins, 2014b; Musacchio, 2015; Joglekar, 2016; Corbett, 2017). A weakened or an overactive SAC can lead to chromosome segregation errors and thereby to aneuploidy, a condition frequently associated with tumorigenesis (Michel et al, 2001; Iwanaga et al, 2007; Morais da Silva et al, 2013; Resende et al, 2018). To avoid such errors, the SAC must be tightly regulated: it must be strong enough so that a single unattached kinetochore can delay anaphase onset but also highly responsive in a way that mitotic exit is triggered as soon as all the kinetochores become attached to microtubules. This equilibrium between strength and responsiveness of the SAC results, in part, from the balance of kinases and phosphatases activities at kinetochores (Saurin, 2018; Moura and Conde, 2019). The ability to timely silence the SAC relies largely on the Protein Phosphatase 1 (PP1), a serine threonine phosphatase whose activity at metaphase kinetochores drives the dephosphorylation of Knl1 MELT motifs (London et al, 2012; Zhang et al, 2014; Nijenhuis et al, 2014) and of Mps1 T-loop (Moura et al, 2017). These events lead to limited accumulation of SAC proteins at kinetochores and to a complete inactivation of Mps1, which culminate in the switching off of the SAC and anaphase onset.

Despite its importance for the control of SAC signalling, it is still unclear how PP1 is regulated so that its SAC-antagonizing activity is low during early mitosis and elevated when biorientation is achieved. Aurora B has been shown to limit the recruitment of PP1 to unattached or tensionless kinetochores by phosphorylating PP1-receptor Knl1 on its RVxF and SILK motifs at unattached or tensionless kinetochores (Liu et al, 2010; Rosenberg et al, 2011; Bajaj et al, 2018; Nasa et al, 2018). However, nocodazole-treated cells expressing a phospho-mimetic version of Knl1 (DSILK and RVDF) are able to accumulate at kinetochores up to 50% of the PP1 levels that are detected when unattached kinetochores harbour unphosphorylatable Knl1 SILK and RVxF motifs (ASILK and RVAF) (Nijenhuis et al, 2014). This suggests that a significant pool of PP1 might already be present at kinetochores in prometaphase, thus leaving room to question whether additional

mechanisms are necessary to dampen PP1 activity during early mitosis and prevent premature SAC silencing. Additional layers for control of PP1 activity may involve the formation of specific holoenzymes through its association with regulatory interactors of protein phosphatase one (RIPPOs) (Bollen et al. 2010; Cao et al, 2022) and the phosphorylation of a conserved threonine residue in the disordered C-terminal tail of PP1. This threonine (T320 in PP1 α , T316 in PP1 β and T311 in PP1 γ) is targeted by Cdk2 and Cdk1 and has been associated *in vivo* with an inhibitory effect on PP1 during the G1/S transition and mitosis, respectively (Dohadwala et al, 1994; Yamano et al, 1994; Kwon et al, 1997; Liu et al, 1999; Wu et al, 2009; Grallert et al, 2015; Qian et al, 2015). Structural, semisynthetic and biochemical data support the hypothesis that phosphorylation of T320/316/311 inhibits PP1 function by mediating protein complex formation rather than by direct structural changes or substrate competition (Salvi et al, 2020). However, the mechanism through which phosphorylation of PP1 C-terminal tail controls holoenzyme assembly to reduce PP1 activity and its relevance for SAC signalling remains unclear. SAC silencing precedes APC/C-mediated ubiquitination of Cyclin B, thus implying that PP1-dependent dephosphorylation of Knl1 and Mps1 occurs when Cdk1 is still active. Furthermore, PP1 also triggers prompt APC/C-dependent degradation of Cyclin B downstream of the SAC through the dephosphorylation of Cdc20 N-terminus (Kim et al, 2017; Bancroft et al, 2020). This event contributes to accelerate the metaphase to anaphase transition and, as for the dephosphorylation of Knl1 and Mps1, takes place when the C-terminus threonine is phosphorylated in a significant fraction of cellular PP1 (Bancroft et al, 2020). These observations challenge the involvement of Cdk1:Cyclin B-mediated inhibition of PP1 in controlling timely anaphase onset.

Here, we set out to investigate the regulation of PP1 in mitosis by using *Drosophila* as a model organism. Fruit flies have four *loci* encoding PP1 isoforms, of which PP1 α 87B is the most abundant and responsible for the bulk of PP1 activity in mitosis (Axton et al, 1990; Dombrádi et al. 1990; Moura et al, 2017; Audett et al, 2022). Importantly, PP1 α 87B is inherently truncated in its C-terminal tail thereby lacking the regulatory Cdk1-targeted threonine. Hence, we took advantage of this naturally occurring simpler system to uncover whether and how additional mechanisms control PP1 activity in mitosis to ensure a timely SAC and accurate chromosome segregation.

RESULTS

Cdk1 and Aurora B-mediated mechanisms do not fully account for the control of PP1 α 87B activity during early mitosis

We started by testing in *Drosophila* S2 cells the relevance of PP1-antagonizing mechanisms previously described in other systems. PP1 is recruited to kinetochore through its interaction with RVxF and SILK motifs of Knl1. Aurora B phosphorylation of these motifs, which is maximal at unattached/tensionless kinetochores, decreases the binding affinity to PP1. This limits PP1 recruitment to kinetochores before these become stably attached to spindle microtubules (Liu et al, 2010; Rosenberg et al, 2011; Bajaj et al, 2018; Nasa et al, 2018). Several lines of evidence suggest that this mechanism also operates in S2 cells: Aurora B phosphorylates Spc105 (Kn1) PP1-binding motifs in the absence of intra-kinetochore stretch (Drpic et al, 2015; Barbosa et al, 2020); inhibition of Aurora B activity enhances the accumulation of PP1 α 87B at unattached kinetochores (Moura et al, 2017) and live-cell imaging of S2 cells stably expressing EGFP-PP1 α 87B shows an evident increase of PP1 α 87B recruitment to aligned kinetochores (Figure II.1A). Nevertheless, discrete puncta of EGFP-PP1 α 87B could be observed during early mitosis (Figure II.1 A) and immunofluorescence analysis confirmed that, albeit at lower levels, EGFP-PP1 α 87B also localizes to prometaphase kinetochores in S2 cells (Figure II.1B).

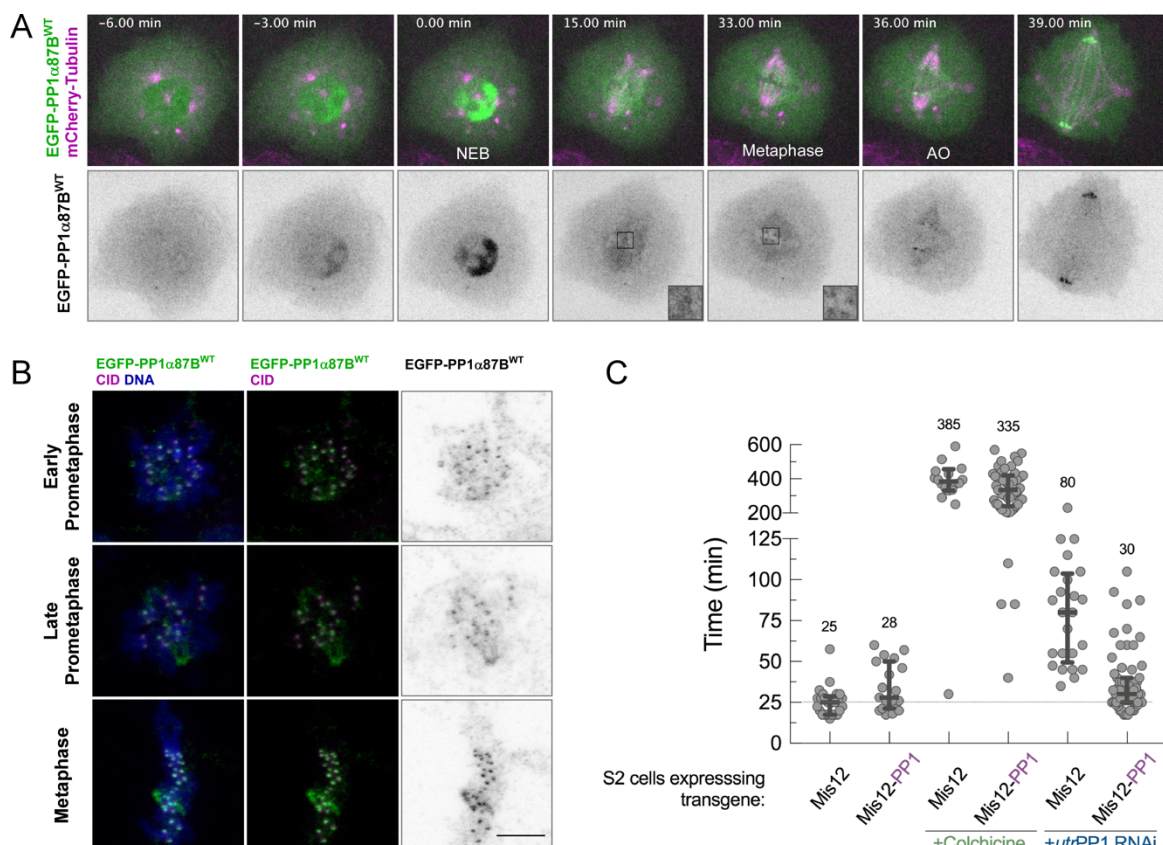


Figure II. 1 – Forced accumulation of PP1 α 87B at the kinetochore does not promote premature anaphase onset. (A) Mitotic progression of S2 cells expressing mCherry- α -Tubulin and EGFP-PP1 α 87B^{WT}. Mitotic progression was monitored by time-lapse microscopy. Nuclear envelope breakdown (NEB), Metaphase and Anaphase Onset (AO) are identified. Insets show the accumulation of EGFP-PP1 α 87B in discrete puncta. (B) Representative immunofluorescence images of EGFP-PP1 α 87B localization throughout mitosis in S2 cells expressing EGFP-PP1 α 87B^{WT}. Scale bar: 5 μ m. (C) Mitotic duration of S2 cells expressing the indicated transgenes in the indicated conditions. Mitotic progression was monitored by time-lapse microscopy. The mitotic duration was defined by the length of time measured between NEB and AO or NEB and chromatin decondensation in the case of colchicine-treated cells ($n \geq 20$ cells for each condition). Data information: data in (C) is shown as median plus interquartile range.

To test whether Aurora B-controlled recruitment of PP1 α 87B is sufficient to prevent premature SAC silencing, we constitutively tethered PP1 α 87B to the outer-kinetochore by fusing it to Mis12 and monitored mitotic progression by live cell imaging. Compared to Mis12-EGFP, expression of the Mis12-EGFP-PP1 α 87B transgene did not accelerate anaphase onset in asynchronous cultured cells and marginally affected the capacity of cells to arrest in mitosis following microtubule depolymerization induced by colchicine (Figure II.1C). Importantly, expression of Mis12-EGFP-PP1 α 87B restored timely mitotic exit in S2 cells depleted of endogenous PP1 α 87B, thus indicating that the kinetochore-tethered phosphatase is functional (Figure II.1C). These results show that bypassing the phosphoregulation of PP1-docking motifs on Spc105 is not sufficient to silence or significantly weaken the SAC, which suggests that additional mechanisms contribute to repress PP1 α 87B activity and consequently prevent premature dephosphorylation of SAC substrates.

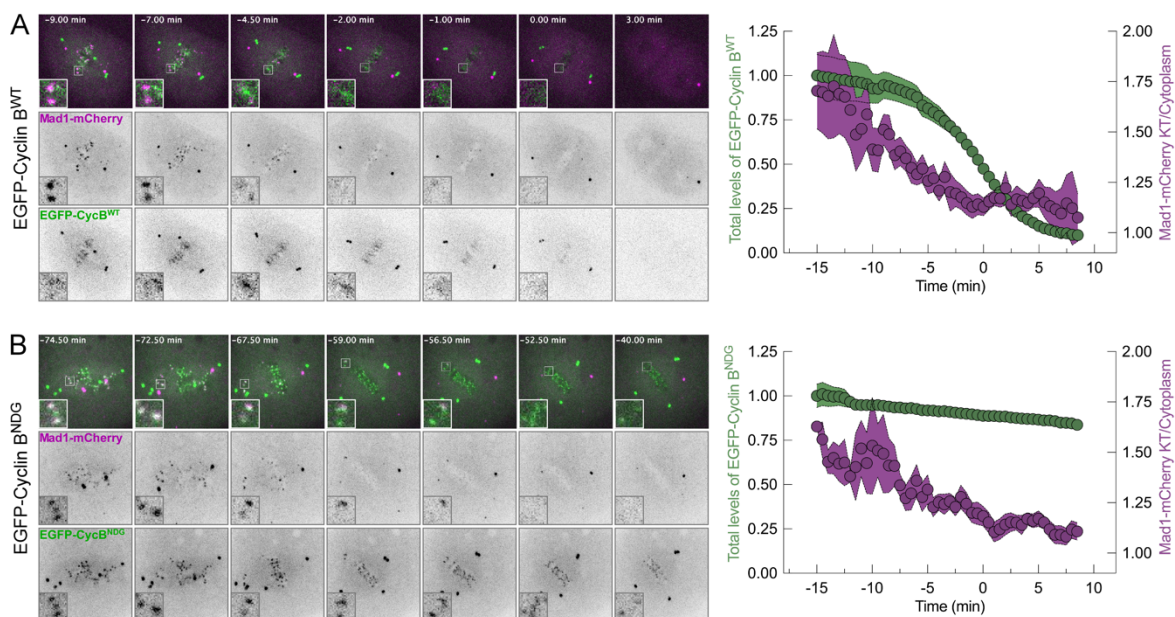


Figure II. 2 – The Spindle Assembly Checkpoint is silenced under high CyclinB:Cdk1 activity. Mitotic progression of S2 cells expressing Mad1-mCherry and EGFP-Cyclin B^{WT} (A) or EGFP-Cyclin B^{NDG} (Non degradable Cyclin B) (A,B). Mitotic progression was monitored by time-lapse microscopy. The fluorescence

intensities of Mad1-mCherry and EGFP-Cyclin B at the kinetochores were corrected to the cytoplasm and plotted against the time in mitosis. Time = 0min represents anaphase onset ($n \geq 8$ cells for each condition).

Phosphorylation of PP1 C-terminal tail by Cdk1 has been proposed to negatively regulate PP1 activity in mitosis (Wu et al, 2009; Grallert et al, 2015; Qian et al, 2015). This seems however implausible in S2 cells, as the naturally truncated PP1 α 87B lacks the regulatory Cdk-targeted threonine. Nevertheless, and despite inconspicuous mitotic roles, the *Drosophila* paralogues PP1 β 9C and PP1 α 96A do have disordered C-terminal tails harbouring conserved proline-rich PxTPP sequences (Figure II.2 – Supplementary figure 1), which prompted us to test whether Cdk1 could be contributing to reduce overall PP1 function in mitotic S2 cells. We started by establishing S2 cell lines stably co-expressing Mad1-mCherry and EGFP-tagged wild type (WT) or non-degradable (NDG) Cyclin B (Sigrist et al, 1995), and monitored the kinetochore localization of Mad1-mCherry as a readout for active SAC signalling. In EGFP-Cyclin B^{WT}-expressing cells, Mad1-mCherry signal at kinetochore pairs begins to fade as these align to the metaphase plate (~10 minutes before anaphase onset) and equals background intensity ~4 minutes prior to anaphase onset, when ~75% of EGFP-Cyclin B^{WT} levels are still present in the cell (Figure II.2A). This supports the notion that the SAC is switched off (Mad1 absent from kinetochores) under elevated Cdk1:Cyclin B activity (indirectly inferred from EGFP-Cyclin B^{WT} levels), a premise that was confirmed in cells expressing EGFP-Cyclin B^{NDG}, where the kinetics of Mad1-mCherry disappearance from bioriented kinetochores was completely unaffected by the lack of Cyclin B proteolysis (Figure II.2B).

To interrogate if SAC silencing in the presence of high Cdk1:Cyclin B relies on PP1 α 87B, we used a phospho-specific antibody (Jelluma et al, 2008a) to assess the phosphorylation of Mps1 T-loop (Mps1^{T490Ph}) at kinetochores, which we have previously established as a PP1 α 87B substrate and whose dephosphorylation during metaphase is required for Mps1 inactivation and SAC silencing in *Drosophila* cells (Moura et al, 2017). Immunofluorescence analysis demonstrated that expression of EGFP-Cyclin B^{NDG}, or treatment with MG132 to prevent proteasome-mediated degradation of Cyclin A and Cyclin B, did not preclude Mps1 inactivation in metaphase, as revealed by the substantial decrease in Mps1 T-loop autophosphorylation as cells progress from prometaphase to metaphase (Figure II.3). Dephosphorylation of Mps1^{T490Ph} at metaphase kinetochores was however prevented when PP1 α 87B was depleted. These data show that the dephosphorylation of Mps1 T-loop in MG132-treated and EGFP-Cyclin B^{NDG}-expressing cells mirrors the observed in untreated control cells and is mediated by PP1 α 87B (Figure II.3).

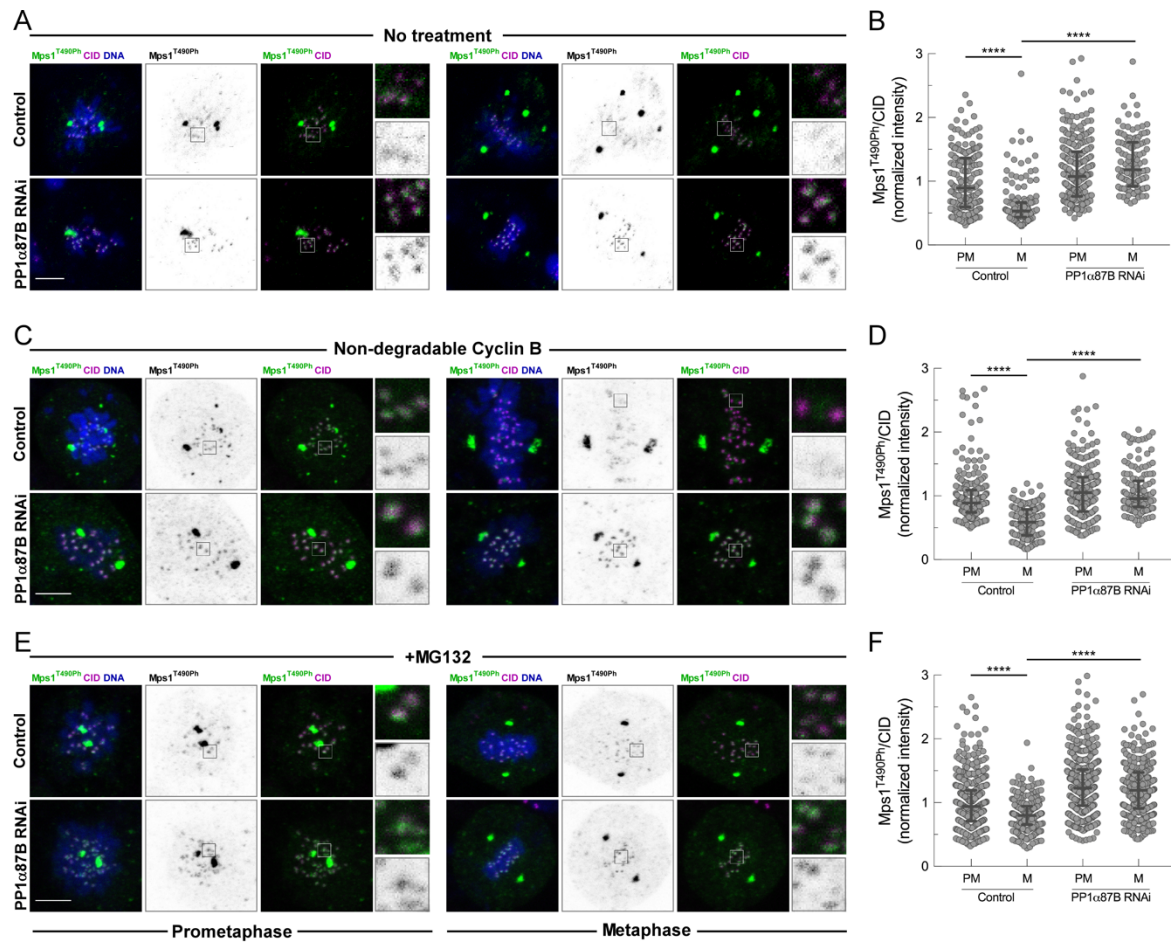


Figure II. 3 – PP1 α 87B-mediated dephosphorylation of Mps1 T-loop in metaphase occurs in the presence of Cyclin B or Cyclin A:Cdk1 activity. Representative immunofluorescence images and corresponding quantifications of Mps1^{T490Ph} levels at the kinetochores of control and PP1 α 87B depleted S2 cells in prometaphase and metaphase. Cells were either untreated, expressing a non-degradable version of Cyclin B or treated with MG132 for 2 hours, as indicated. Mps1^{T490Ph} fluorescence intensity was determined relative to CID signal. Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** p <0.0001 (Kruskal-Wallis, Dunn's multiple comparison test).

In line with Mps1 activation status, the phosphorylation of Mad1 C-terminus on T726 (T716 in human Mad1), which is catalysed by Mps1 (Figure II.4 – Supplementary figure 1) and rate-limiting for SAC signalling (Faesen et al, 2017; Ji et al, 2017; Ji et al, 2018; Allan et al, 2020; Fischer et al, 2022), also decreases at metaphase kinetochores of cells impaired in Cyclin A and Cyclin B degradation, unless PP1 α 87B is depleted (Figure II.4). Collectively, these results indicate that PP1 α 87B-mediated inactivation of Mps1 can still occur in the presence of Cyclin A and Cyclin B, thus showing that Cdk1 activity, at most, contributes marginally to prevent PP1 α 87B from prematurely silencing the SAC in *Drosophila* S2 cells.

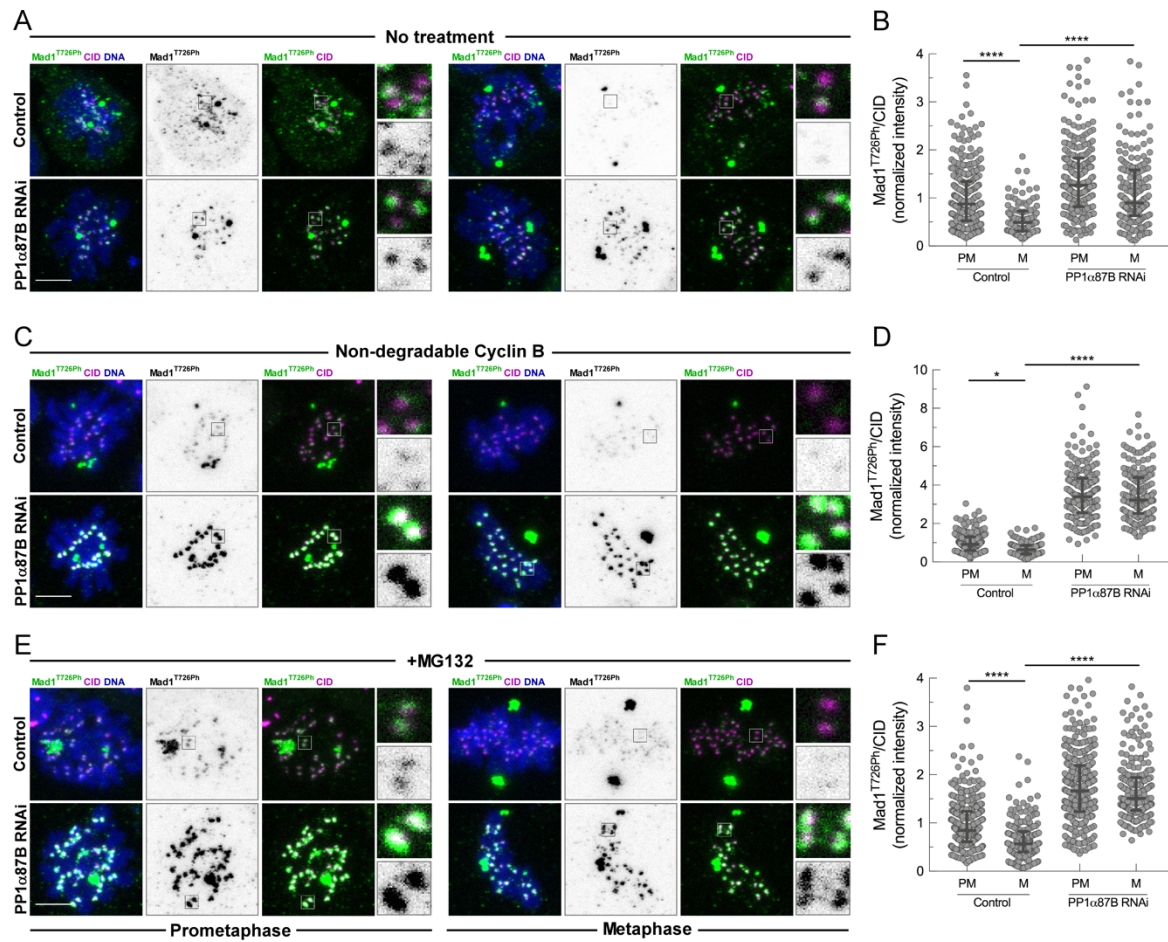


Figure II. 4 – PP1 α 87B-dependent dephosphorylation of Mad1^{T726} in metaphase occurs in the presence of Cyclin B or Cyclin A:Cdk1 activity. Representative immunofluorescence images and corresponding quantifications of Mad1^{T726Ph} levels at the kinetochores of control and PP1 α 87B depleted S2 cells in prometaphase and metaphase. Cells were either untreated, expressing a non-degradable version of Cyclin B or treated with MG132 for 2 hours, as indicated. Mad1^{T726Ph} fluorescence intensity was determined relative to CID signal. Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, * $p < 0.05$, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

Polo phosphorylates PP1 α 87B at unattached/tensionless kinetochores

PP1 α 87B is impervious to Cdk1, and yet, its forced accumulation at kinetochores (fusion with Mis12) fails to prematurely silence the SAC (Figure II.1 C). Therefore, we hypothesized that other mechanisms operate during prometaphase to restrain PP1 α 87B activity. The presence of a conserved consensus sequence for Polo/Plk1 phosphorylation at the C-terminus of PP1 α 87B (T286, Figure II.5 A) prompted us to interrogate whether Polo could directly regulate PP1 α 87B activity in mitosis. To address this, we first assessed Polo capacity to phosphorylate PP1 α 87B in S2 cells. We raised an antibody against phosphorylated T286 (T288 in human PP1 γ) and performed immunofluorescence analysis (Figure II.5). Phosphorylation of PP1 α 87B on T286 (T286Ph) was uniformly detected at

kinetochores of colchicine-treated cells (Figure II.5 B,C). However, kinetochore levels of T286Ph were severely reduced following RNAi-mediated depletion of Polo or pharmacological inhibition of its kinase activity with BI2536 (Figure II.5 C,E). Depletion of PP1 α 87B (Figure II.5 B,D) or replacing the endogenous protein with a kinetochore-tethered unphosphorylatable mutant for T286 also compromised T286Ph staining (Figure II.5 F,G), which further validates the specificity of the antibody. Collectively, these results support the notion that Polo phosphorylates PP1 α 87B T286 in a cellular context.

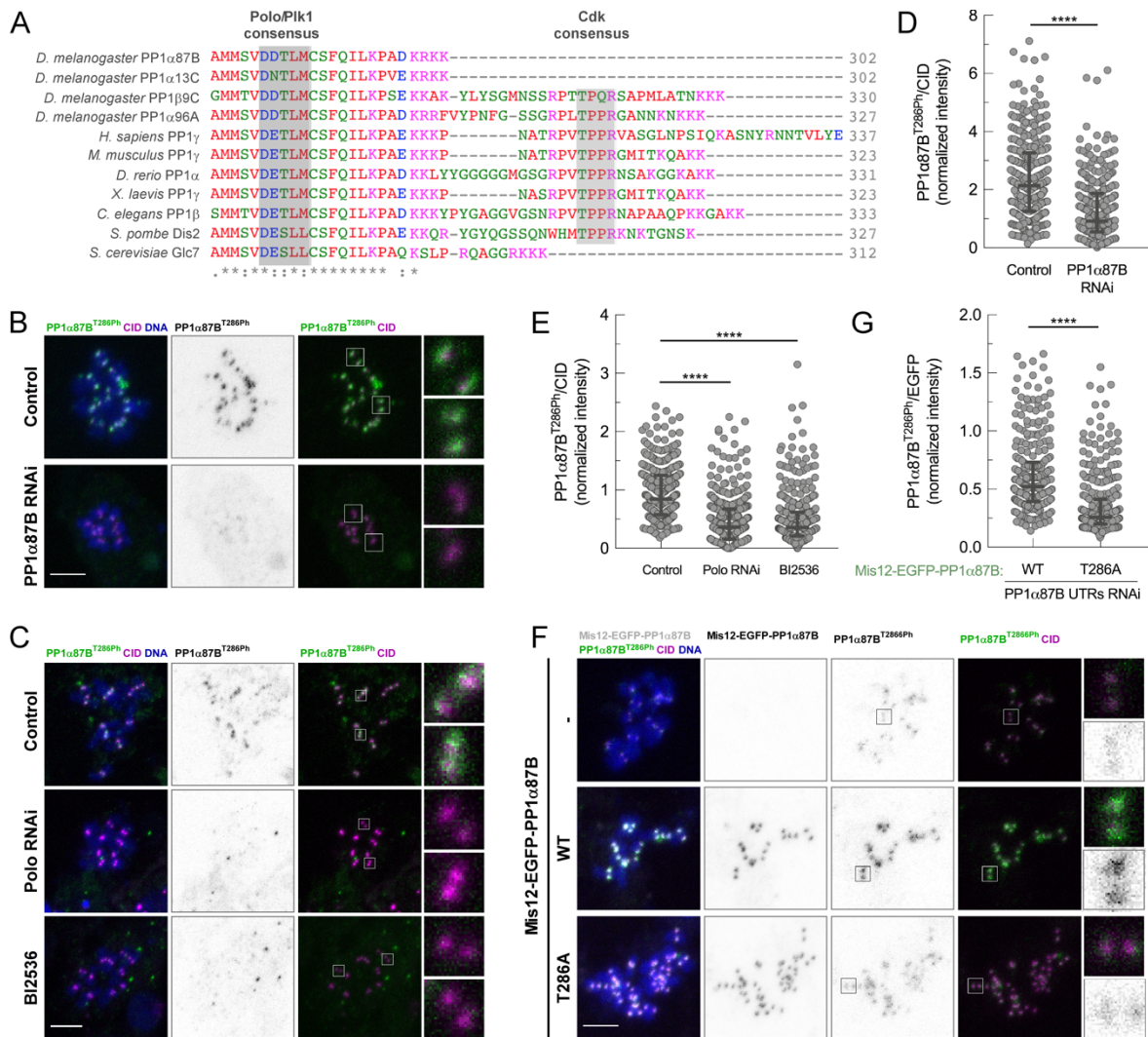


Figure II. 5 – Polo phosphorylates PP1 α 87B at unattached kinetochores. (A) Clustal W alignments of the C-terminus of indicated PP1 orthologues. The conserved Cdk and Polo/Plk1 consensus motifs are highlighted in grey shading. (B,D) Representative immunofluorescence images (B) and corresponding quantifications (D) of PP1 α 87B T286 phosphorylation (PP1 α 87B^{T286Ph}) at kinetochores of colchicine-treated cells in the indicated conditions. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to CID signal (n ≥ 307 kinetochores from at least 17 cells for each condition). (C,E) Representative immunofluorescence images (C) and corresponding quantifications (E) of PP1 α 87B T286 phosphorylation (PP1 α 87B^{T286Ph}) at kinetochores of colchicine-treated cells in the indicated conditions. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to CID signal (n ≥ 338 kinetochores from at least 19 cells for each condition). (F,G) Representative immunofluorescence images (F) and corresponding quantifications (G) of PP1 α 87B T286 phosphorylation (PP1 α 87B^{T286Ph}) at prometaphase kinetochores of cells in the indicated conditions. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to EGFP signal (n ≥ 286 kinetochores from at least 13 cells for each condition).

Scale bar: 5 μ m. Data information: data in D and G is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** p <0.0001 (Mann-Whitney U test); data in E is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** p <0.0001 (Kruskal-Wallis, Dunn's multiple comparison test).

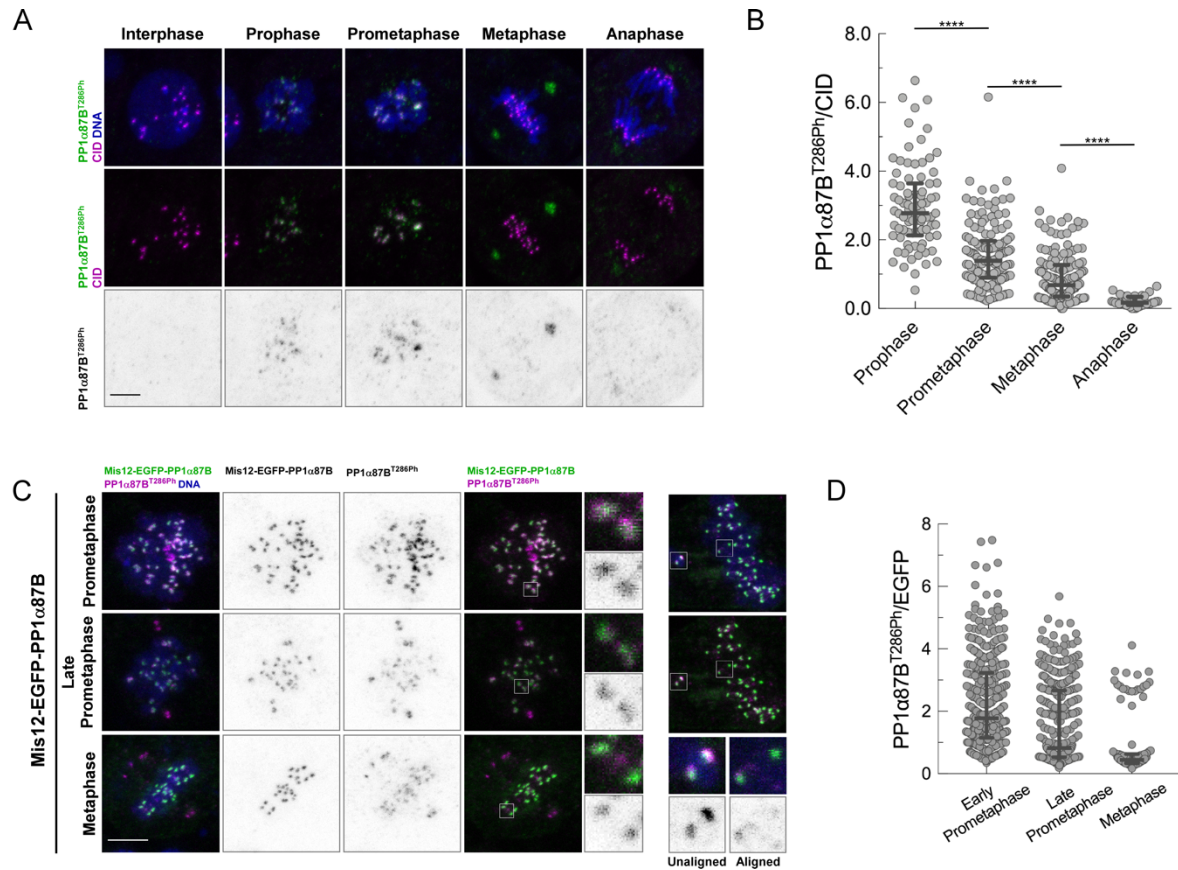


Figure II. 6 – PP1 α 87B T286 phosphorylation decreases with mitotic progression. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of PP1 α 87B^{T286Ph} levels at the kinetochores of S2 cells throughout mitosis. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to CID signal. (C,D) Representative immunofluorescence images (C) and corresponding quantifications (D) of PP1 α 87B^{T286Ph} levels at prometaphase and metaphase kinetochores of S2 cells expressing Mis12-EGFP-PP1 α 87B^{WT}. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to EGFP signal. Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** p <0.0001 (Kruskal-Wallis, Dunn's multiple comparison test).

We then assessed the phosphorylation of PP1 α 87B T286 in unperturbed mitosis of asynchronous cultured S2 cells. The kinetochore signal of T286Ph was readily detected in prophase and prometaphase cells, but becomes significantly reduced in metaphase and virtually undetectable in cells undergoing anaphase (Figure II.6 A,B). Because the kinetochore recruitment of PP1 α 87B increases as chromosome biorientation is established (Figure II.1 A,B, Audet et al, 2022; Moura et al, 2017), the decrease in the phosphorylation of T286 at metaphase kinetochores cannot be explained by reduced phosphatase levels at kinetochores. To confirm this, we assessed the phosphorylation of PP1 α 87B T286 in S2

cells expressing the outer kinetochore-tethered version of the phosphatase (Figure II.6 C,D). Kinetochore levels of Mis12-EGFP-PP1 α 87B remain constant throughout mitosis, thus allowing us to monitor PP1 α 87B^{T286Ph} relative to the amount of PP1 α 87B. Detailed analysis showed that, for any given cell, the phosphorylation of T286 is kept elevated at tensionless kinetochores of unaligned chromosomes, but was strikingly reduced at kinetochore pairs of chromosomes that have already congressed to the metaphase plate and exhibit inter-kinetochore tension (Figure II.6 C). These results indicate that PP1 α 87B T286 phosphorylation is regulated throughout mitosis and is responsive to the status of kinetochore attachment/chromosome alignment.

This phosphorylation pattern of PP1 α 87B in its T286 correlates with the activation status of Polo at kinetochores throughout mitosis (Conde et al, 2013; Barbosa et al, 2020). Notably, in contrast with S2 cells expressing endogenous Polo or a wild-type Polo transgene (Polo^{WT}-EGFP), preventing Polo inactivation through the expression of a constitutively active version of the kinase (Polo^{T182D}-EGFP) was sufficient to retain elevated levels of PP1 α 87B^{T286Ph} at metaphase kinetochores (Figure II.7). Collectively these data demonstrate that PP1 α 87B phosphorylation on its T286 is driven by Polo activity and suggests that this occurs preferentially at unattached kinetochores.

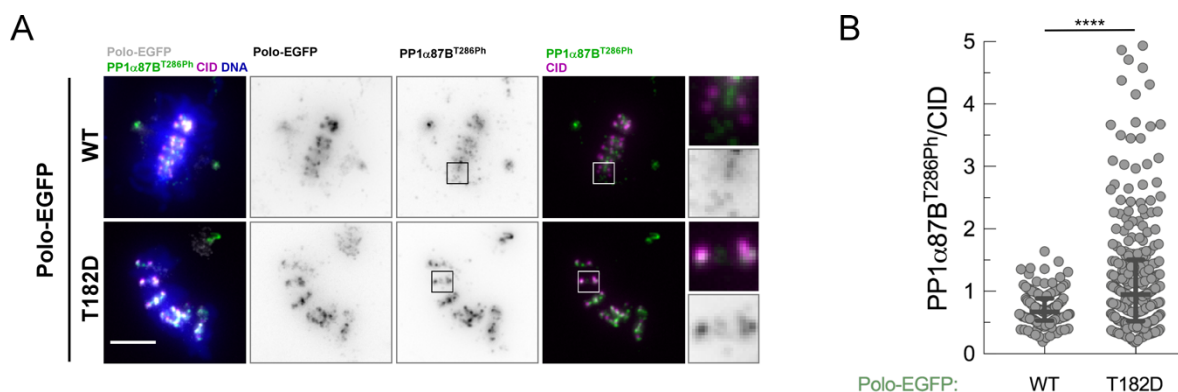


Figure II. 7 – Expression of constitutively active Polo maintains PP1 α 87B T286 phosphorylated at metaphase kinetochores. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of PP1 α 87B^{T286Ph} levels at metaphase kinetochores of S2 cells expressing Polo^{WT}-EGFP or Polo^{T182D}-EGFP. PP1 α 87B^{T286Ph} fluorescence intensity was determined relative to CID signal. Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, ****p<0.0001 (Mann-Whitney U test).

Phosphorylation of PP1 α 87B T286 is required for robust SAC signalling

To dissect the relevance of Polo-mediated phosphorylation of PP1 α 87B on T286, we established S2 cell lines expressing EGFP-tagged wild type (WT), unphosphorylatable (T286A) and phospho-mimetic (T286D) versions of PP1 α 87B. We first assessed the

activation of Mps1 in prometaphase cells. As expected, immunofluorescence analysis demonstrated that phosphorylation of Mps1 T-loop is enhanced at prometaphase kinetochores in PP1 α 87B depleted cells (Moura et al, 2017; Figure II.3; Figure II.8). This effect was rescued by the expression of EGFP-PP1 α 87B^{WT} and less efficiently by EGFP-PP1 α 87B^{T286D} (Figure II.8). Importantly, the kinetochore levels of Mps1^{T490Ph} were significantly reduced in EGFP-PP1 α 87B^{T286A}-expressing cells when compared to control parental cells or cells expressing the wild type transgene (Figure II.8), which indicates that preventing the phosphorylation of PP1 α 87B T286 renders Mps1 less active at prometaphase kinetochores.

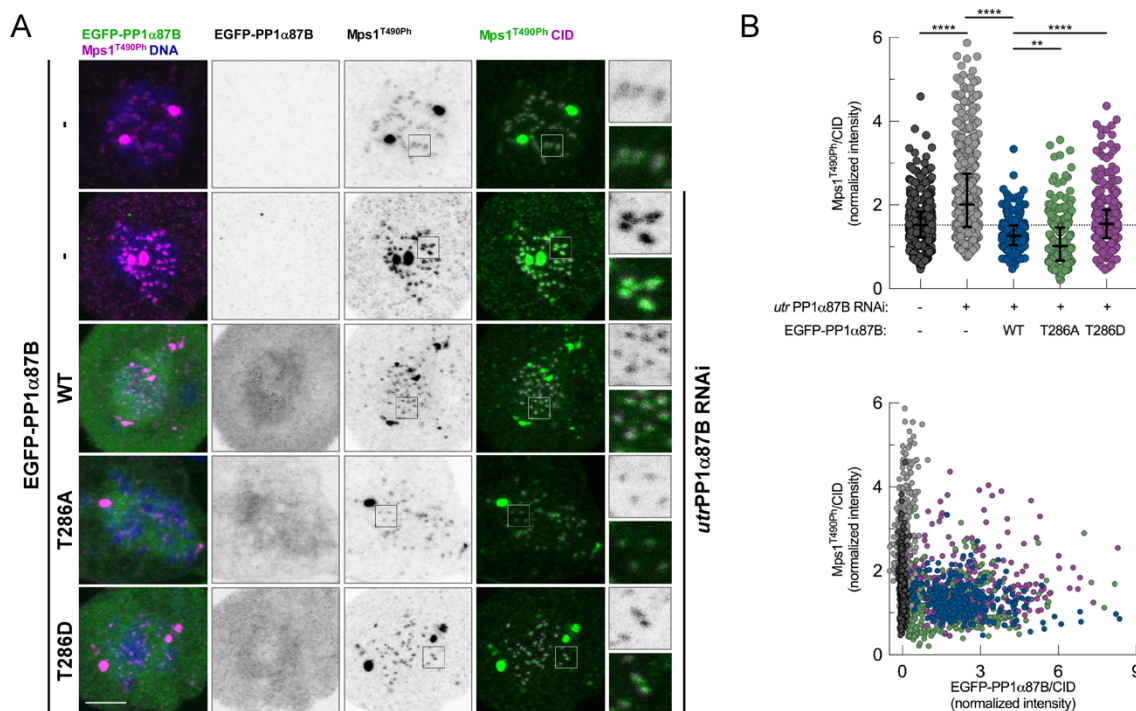


Figure II. 8 – Preventing PP1 α 87B T286 phosphorylation decreases Mps1 T-loop phosphorylation at prometaphase kinetochores. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mps1^{T490Ph} levels at the kinetochores of prometaphase S2 cells in the indicated conditions. Mps1^{T490Ph} and EGFP fluorescence intensities were determined relative to CID signal. ($n \geq 300$ kinetochores from at least 10 cells for each condition). Scale bar: 5 μ m. Data information: data in B is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, ** $p < 0.01$, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

Given the upstream role of Mps1 in SAC activation, we hypothesized that the phosphorylation of PP1 α 87B T286 could be required for proper SAC signalling. To address this, we examined by live cell imaging the effect of EGFP-PP1-87B^{T286A} on mitotic progression. Depletion of endogenous PP1 α 87B caused a significant extension of the time from nuclear envelope breakdown (NEB) to anaphase onset (AO) (Figure II.9). EGFP-PP1 α 87B^{T286A} and EGFP-PP1 α 87B^{WT} were indistinguishable in their ability to restore

normal mitotic duration as measured for control parental S2 cells (Figure II.9), thus suggesting that lack of PP1 α 87B T286 phosphorylation does not accelerate mitotic exit in otherwise unperturbed S2 cells.

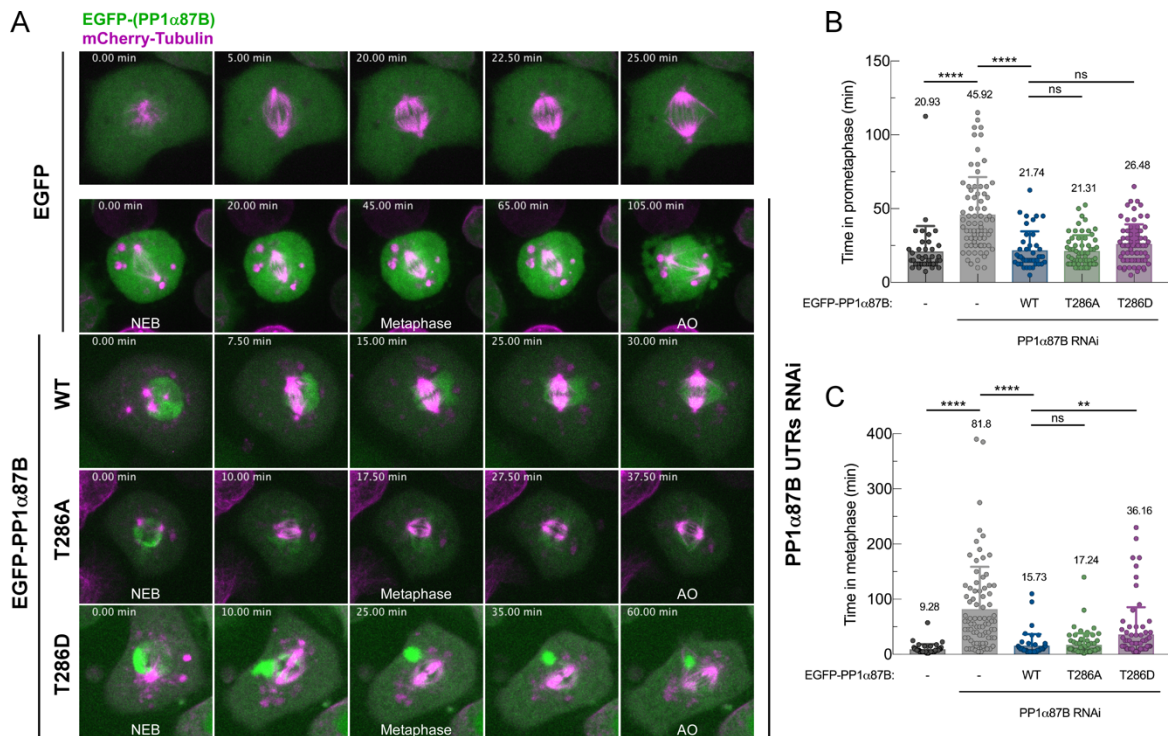


Figure II.9 – Mimicking constitutive phosphorylation of PP1 α 87B T286 extends the metaphase duration. (A-C) Mitotic progression (A) and prometaphase (B) and metaphase duration (C) of S2 cells expressing mCherry- α -Tubulin and EGFP or the indicated transgenes of EGFP-PP1 α 87B in an endogenous PP1 α 87B depleted background. Mitotic progression was monitored by time-lapse microscopy. The prometaphase duration was defined by the length of time measured between the nuclear envelope breakdown (NEB) and the first still in which all kinetochore pairs were perfectly aligned at the metaphase plate (Metaphase). The metaphase duration was defined by the length of time measured between Metaphase and anaphase onset (AO) ($n \geq 39$ cells for each condition, from at least two independent experiments). Data information: data in (B) and (C) is shown as mean plus standard deviation, mean value is shown above each column, asterisks indicate that differences between mean ranks are statistically significant, ** $p < 0.01$, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

To specifically evaluate the importance of PP1 α 87B T286 phosphorylation for SAC signalling, we assessed by live-cell imaging the capacity of PP1 α 87B phospho-mutants to arrest in mitosis when incubated with colchicine (Figure II.10 A). In line with SAC proficiency, cells expressing EGFP-PP1 α 87B^{WT} or EGFP-PP1 α 87B^{T286D} significantly delayed the transition to anaphase in the presence of colchicine (~469 min and ~454 min, respectively). In contrast, EGFP-PP1 α 87B^{T286A} cells, although able to halt mitotic progression to some extent in response to unattached kinetochores (~369 min), failed to sustain this arrest as efficiently as EGFP-PP1 α 87B^{WT} cells (Figure II.10 A). This weakened SAC correlates with the observed reduction in Mps1 activation (Figure II.10 B) and Mps1-

mediated phosphorylation of Mad1 T726 at unattached kinetochores (Figure II.10 C), which is critical for catalysis of MCC assembly and consequently for a functional checkpoint. Therefore, we concluded that the phosphorylation of PP1 α 87B T286 by Polo is required for efficient Mps1 activation at kinetochores and robust SAC signalling.

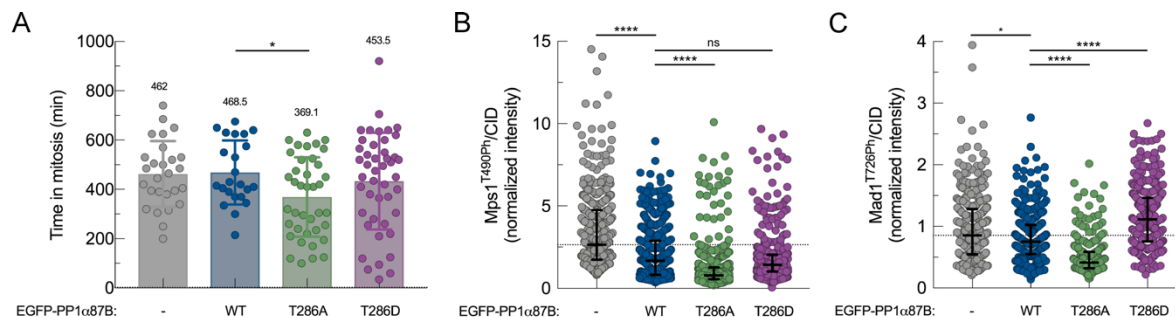


Figure II. 10 – Preventing the phosphorylation of PP1 α 87B T286 weakens Mps1 activation and SAC signalling at unattached kinetochores. (A) Mitotic duration of S2 cells incubated in colchicine and expressing the indicated transgenes. Mitotic timing was monitored by time-lapse microscopy. The mitotic duration was defined by the length of time measured between the nuclear envelope breakdown and the mitotic exit, defined as the still where the DNA starts to decondense ($n \geq 23$ cells for each condition). (B) Quantifications of Mps1^{T490Ph} levels at the kinetochores of S2 cells treated with colchicine for 7 hours and expressing the indicated transgenes. Mps1^{T490Ph} fluorescence intensity was determined relative to CID signal. ($n \geq 440$ kinetochores from at least 20 cells for each condition). (C) Quantifications of Mad1^{T726Ph} levels at the kinetochores of S2 cells incubated with colchicine for 7 hours and expressing the indicated transgenes. Mad1^{T726Ph} fluorescence intensity was determined relative to CID signal. ($n \geq 242$ kinetochores from at least 15 cells for each condition). Data information: (A) data is shown as mean plus standard deviation. Mean value is shown above each column. Asterisk indicates that differences between mean ranks are statistically significant, * $p < 0.05$ (Mann-Whitney U test). (B,C) data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, * $p < 0.05$, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

Mimicking constitutive phosphorylation of PP1 α 87B T286 delays SAC silencing

The above results imply that phosphorylation of PP1 α 87B on T286 attenuates its capacity to dephosphorylate Mps1 and possibly other SAC substrates. Therefore, we next assessed the impact of mimicking PP1 α 87B T286 permanently phosphorylated. Immunofluorescence analysis of metaphase kinetochores showed that expression of EGFP-PP1 α 87B^{WT} and EGFP-PP1 α 87B^{T286A} were able to rescue the increase of active Mps1 (Mps1^{T490Ph}) caused by the depletion of endogenous PP1 α 87B (Figure II.11). On the other hand, cells expressing the phospho-mimetic version EGFP-PP1 α 87B^{T286D} failed to efficiently dephosphorylate Mps1-T loop (Figure II.11). In line with permanently active Mps1, EGFP-PP1 α 87B^{T286D} expressing cells were delayed in the transition to anaphase (~36 min), when compared to cells expressing EGFP-PP1-87B^{WT} (~16 min) or EGFP-PP1-87B^{T286A} (~17 min), which fully rescued the prolonged metaphase duration caused by the depletion of endogenous PP1 α 87B (~82 min) (Figure II.9). Based on these results we concluded that mimicking PP1 α 87B constitutively phosphorylated on T286 impairs timely inactivation of

Mps1 and thereby delays SAC silencing and anaphase onset despite normal chromosome attachment and biorientation.

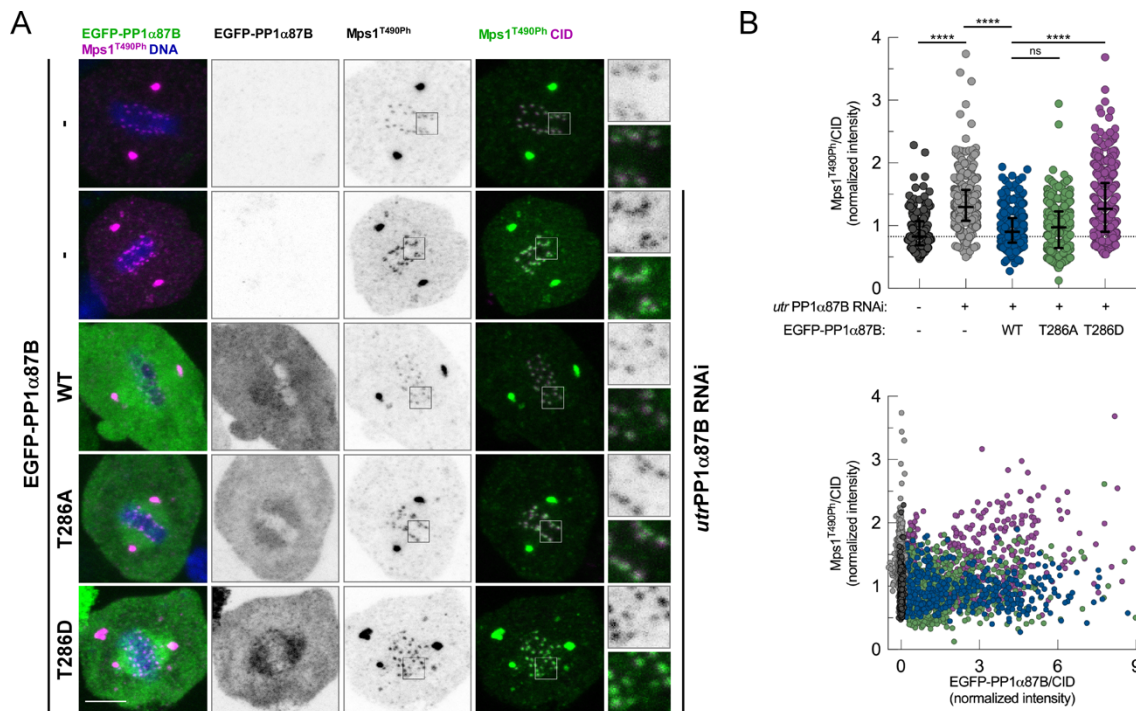


Figure II. 11 – Mimicking constitutive phosphorylation of PP1 α 87B T286 impairs Mps1 T-loop dephosphorylation at metaphase kinetochores. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mps1^{T490Ph} levels at the kinetochores of metaphase S2 cells in the indicated conditions. Mps1^{T490Ph} and EGFP fluorescence intensities were determined relative to CID signal. ($n \geq 229$ kinetochores from at least 10 cells for each condition). Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

Kinetochores-tethering of PP1 α 87B does not bypass the control exerted by T286 phosphorylation

Next, we set out to dissect how phosphorylation of T286 by Polo negatively affects PP1 α 87B-mediated inactivation of kinetochore-localized Mps1. We hypothesized that T286 phosphorylation might affect PP1 α 87B dynamics at kinetochores (turnover rate) in a way that precludes its interaction with Mps1 and other SAC substrates. To test that, we fused PP1 α 87B^{WT}, PP1 α 87B^{T286A} and PP1 α 87B^{T286D} with Mis12 to render all versions of PP1 α 87B constitutively tethered to the outer-kinetochore and in this way ensure similar phosphatase abundance and dynamics at kinetochores. Also under these conditions, and again contrasting with the wild type or unphosphorylatable Mis12-EGFP-PP1 α 87B^{T286A}, the phospho-mimetic version Mis12-EGFP-PP1 α 87B^{T286D} failed to efficiently dephosphorylate the T-loop of Mps1 and the C-terminus of Mad1 present on metaphase kinetochores (Figure II.12; Figure II.13).

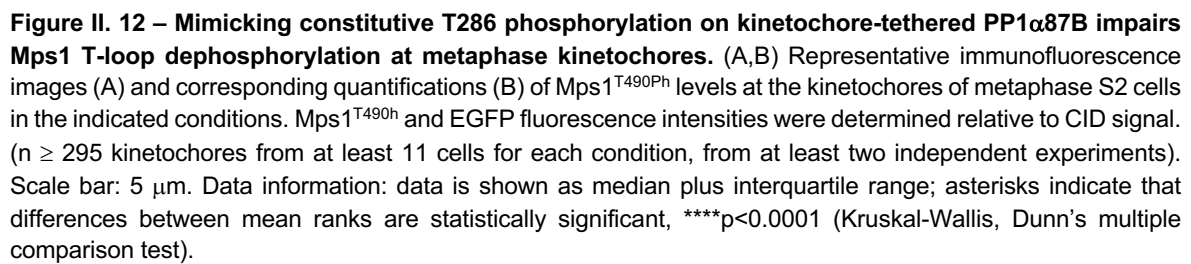


Figure II. 13 – Mimicking constitutive T286 phosphorylation on kinetochore-tethered PP1 α 87B impairs Mad1 T726 dephosphorylation at metaphase kinetochores. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mad1^{T726h} levels at the kinetochores of metaphase S2 cells in the indicated conditions. Mad1^{T726h} and EGFP fluorescence intensities were determined relative to CID signal. ($n \geq 373$ kinetochores from at least 13 cells for each condition, from at least two independent experiments). Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

Accordingly, S2 cells in which endogenous PP1 α 87B was replaced by Mis12-EGFP-PP1 α 87B^{T286D} exhibited a prolonged metaphase delay (~46 min), whereas expression of Mis12-EGFP-PP1 α 87B^{WT} or Mis12-EGFP-PP1 α 87B^{T286A} could promptly restore timely anaphase onset in a PP1 α 87B RNAi background. The metaphase delay observed in Mis12-EGFP-PP1 α 87B^{T286D}-cells was SAC-dependent, as it could be readily averted by depletion of Mps1 (Figure II.14).

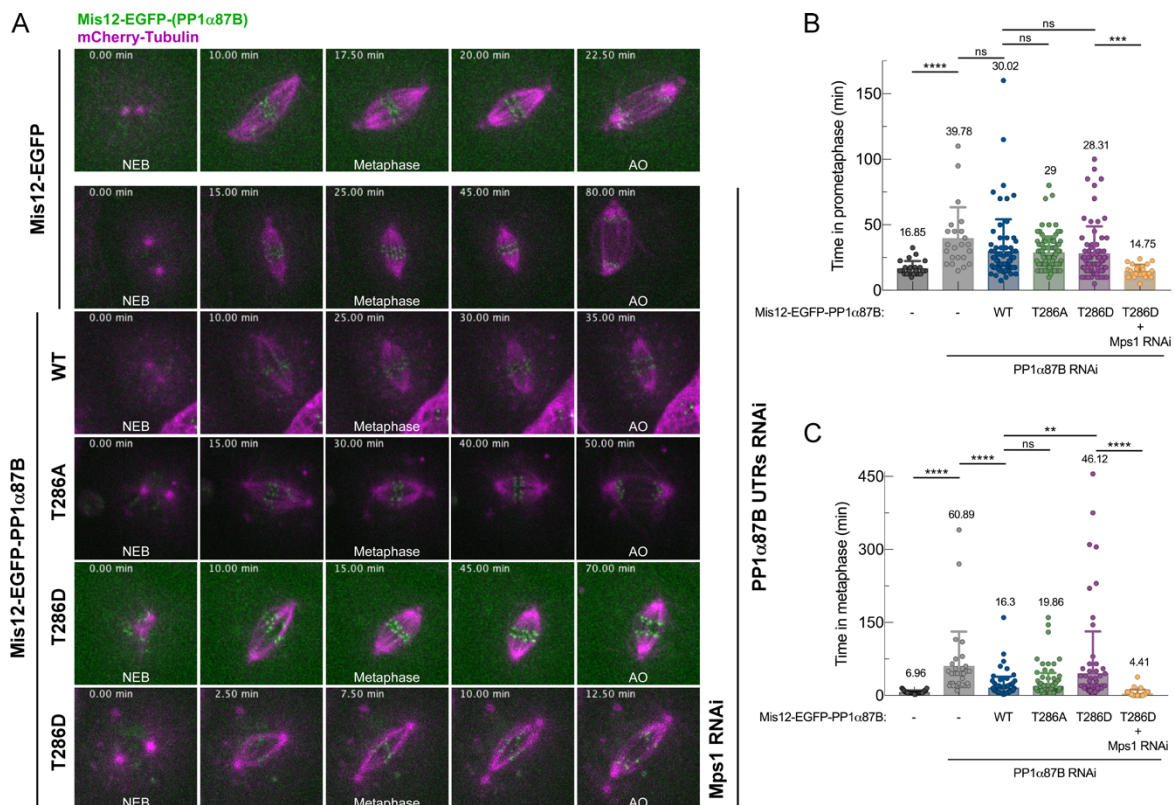


Figure II. 14 – Mimicking constitutive T286 phosphorylation on kinetochore-tethered PP1 α 87B delays S2 cells in metaphase. (A-C) Mitotic progression (A) and prometaphase (B) and metaphase (C) duration of S2 cells expressing mCherry- α -Tubulin and Mis12-EGFP or the indicated transgenes of Mis12-EGFP-PP1 α 87B in a PP1 α 87B endogenous depleted background. Mitotic progression was monitored by time-lapse microscopy. The prometaphase duration was defined by the length of time measured between the nuclear envelope breakdown (NEB) and the first still in which all kinetochore pairs were perfectly aligned at the metaphase plate (Metaphase). The metaphase duration was defined by the length of time measured between Metaphase and anaphase onset (AO) ($n \geq 23$ cells for each condition, from at least two independent experiments). Data information: data is shown as mean plus standard deviation, mean value is shown above each column, asterisks

indicate that differences between mean ranks are statistically significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

We then evaluated the impact of the kinetochore-tethered PP1 α 87B phospho-mutants on SAC strength by monitoring their ability to delay mitotic exit in the presence of colchicine. Although producing a less robust mitotic arrest than their untethered counterparts (Figure II.10), cells expressing Mis12-EGFP-PP1 α 87B^{WT} or MIS12-EGFP-PP1 α 87B^{T286D} were still able to significantly extend the mitotic duration (~353 min and ~412 min), thus retaining considerable SAC proficiency despite PP1 α 87B forced accumulation at unattached kinetochores (Figure II.15). On the other hand, preventing T286 phosphorylation of kinetochore-tethered PP1 α 87B (Mis12-EGFP-PP1 α 87B^{T286A}) significantly accelerated mitotic exit (~221 min), which was symptomatic of a compromised SAC. Hence, despite similar outer-kinetochore localization of the different Mis12-EGFP-PP1 α 87B versions at unattached kinetochores, Mps1 inactivation and subsequent SAC silencing, do not occur as long as T286 remains phosphorylatable or is rendered phospho-mimetic.

Collectively, the results obtained with the kinetochore-tethered transgenes strongly suggest that phosphorylation of T286 can inhibit PP1 α 87B-mediated SAC silencing through mechanisms other than a possible impact on PP1 α 87B kinetochore recruitment or dynamics.

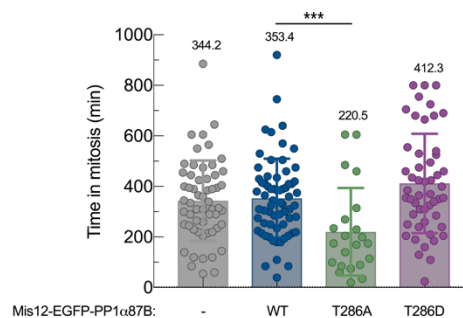


Figure II. 15 – Kinetochore-tethered PP1 α 87B harbouring a T286A mutation weakens the SAC. Mitotic duration of S2 cells expressing mCherry- α -Tubulin and the indicated transgenes and treated with Colchicine. Mitotic progression was monitored by time-lapse microscopy. The mitotic duration was defined by the length of time measured between the nuclear envelope breakdown and the mitotic exit, defined as the still where chromatin decondensation starts ($n \geq 23$ cells for each condition). Data information: data is shown as mean plus standard deviation; mean value is shown above each column; asterisks indicate that differences between mean ranks are statistically significant, *** $p < 0.001$ (Mann-Whitney U test).

Phospho-regulation of PP1 α 87B on T286 is required for genome stability *in vivo*

The results obtained so far with cultured S2 cells demonstrate that Polo phosphorylates PP1 α 87B at T286 during early mitosis to limit the phosphatase activity towards kinetochore-

associated Mps1 and thereby ensure a robust SAC signalling. Conversely, the phosphorylation status of PP1 α 87B T286 at kinetochores concomitantly decreases with chromosome biorientation to enable SAC silencing and timely anaphase onset. To investigate if this phospho-regulation occurs *in vivo* and whether it is relevant for genome stability, we analysed the impact of PP1 α 87B^{T286} phospho-mutants on dividing neuroblasts of 3rd instar larvae. All UAS-PP1 α 87B-HA transgenes (WT and phospho-mutants T286A and T286D) were expressed at similar levels in a *pp1 α 87B* knockdown background (*pp1 α 87B^{87Bg-3}/pp1 α 87B¹*) and Mps1 activation status assessed upon colchicine treatment. Similarly to what is observed for S2 cells, loss of endogenous *pp1 α 87B* led to an increase of Mps1-T-loop phosphorylation at unattached kinetochores, which was efficiently prevented by the expression of PP1 α 87B^{WT}-HA (Figure II.16 A,B). Notably, unphosphorylatable PP1 α 87B^{T286A}-HA reduced even further the kinetochore levels of Mps1^{T490Ph}, whereas phospho-mimetic PP1 α 87B^{T286D}-HA failed to rescue Mps1 hyper-activation (Figure II.16 A,B).

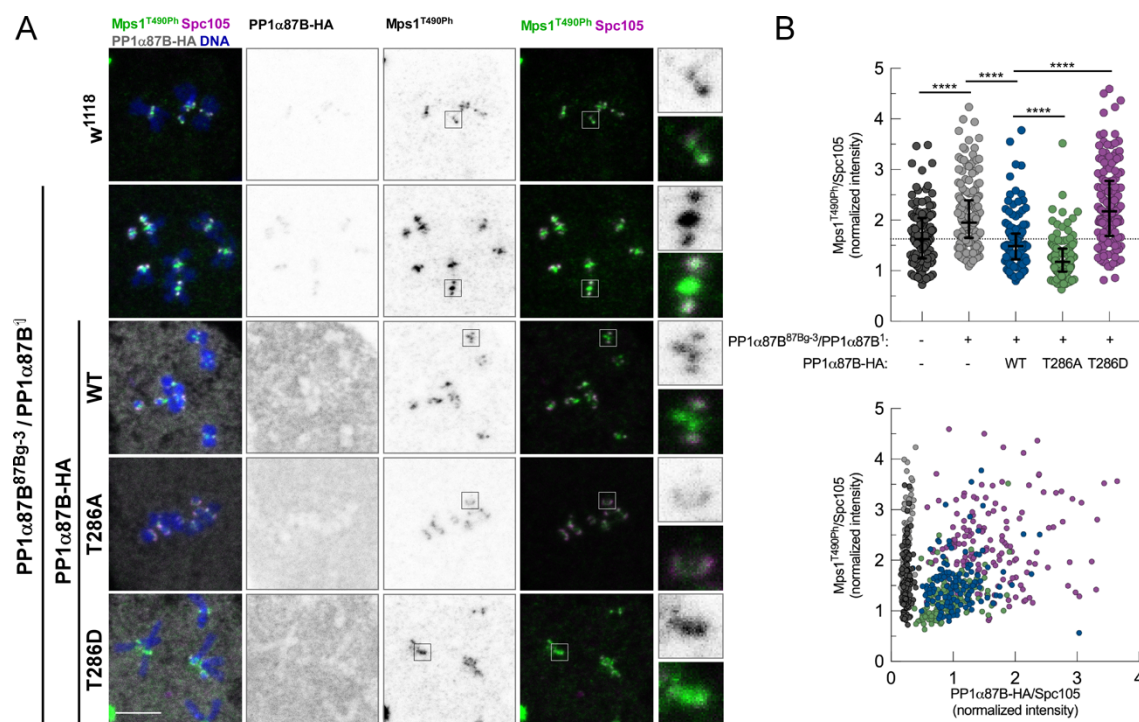


Figure II. 16 – Phospho-regulation of PP1 α 87B T286 is required to control Mps1 T-loop phosphorylation in *Drosophila* neuroblasts. (A,B) Representative immunofluorescence images (A) and corresponding quantifications (B) of Mps1^{T490Ph} levels at the kinetochores of 3rd instar larval neuroblasts treated with colchicine and in the indicated conditions. Mps1^{T490Ph} and HA fluorescence intensities were determined relative to Spc105 signal. ($n \geq 121$ kinetochores from at least 9 cells for each condition, from at least two independent experiments). Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Kruskal-Wallis, Dunn's multiple comparison test).

We then monitored by live imaging neuroblasts undergoing anaphase to evaluate the accuracy of genome partitioning. Approximately 55% of the trans-heterozygous *pp1 α 87b* mutant neuroblasts displayed chromosome segregation defects, which were readily averted if PP1 α 87B^{WT}-HA was expressed (Figure II.17 A). However, both phospho-mutants failed to restore the fidelity of chromosome segregation as efficiently as the wild-type transgene, with PP1 α 87B^{T286A}-HA neuroblasts exhibiting lagging chromosomes in approximately 27% of anaphases and asynchronous segregation of chromosomes being scored in 25% of PP1 α 87B^{T286D}-HA neuroblasts (Figure II.17 A). In line with the observed segregation defects, the % of aneuploid neuroblasts in the larvae brain increases significantly when the phospho-regulation of PP1 α 87B T286 is compromised (Figure II.17 B,C).

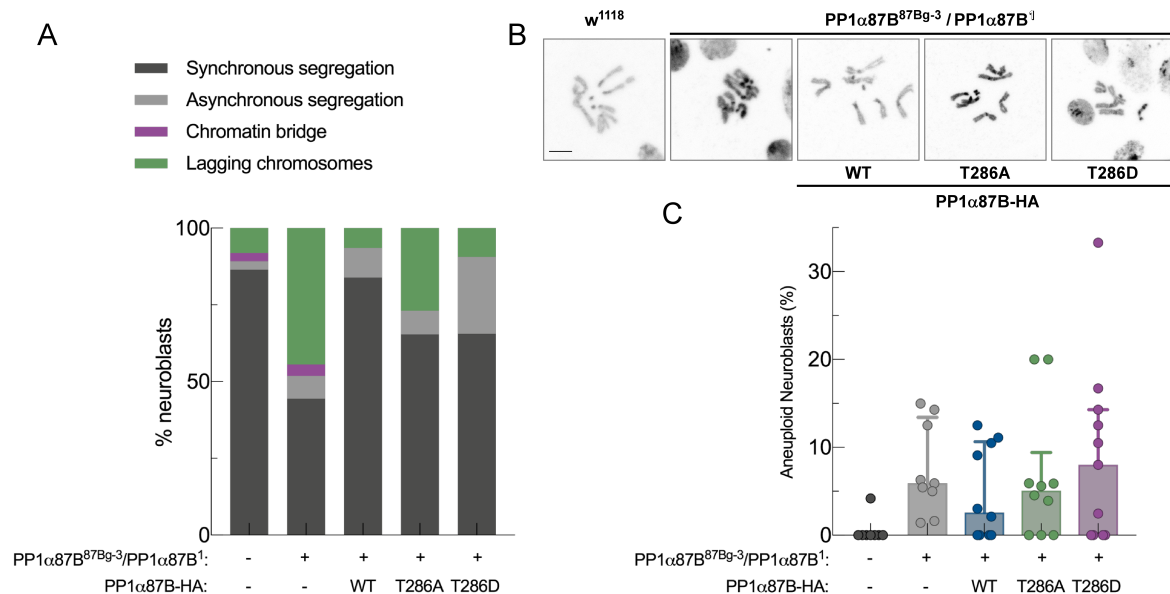


Figure II. 17 – Phospho-regulation of PP1 α 87B T286 is required for faithful chromosome segregation and genome stability in *Drosophila* neuroblasts (A) Quantifications of the percentage of neuroblasts that undergo anaphase with chromosome segregation errors in the indicated conditions ($n \geq 27$ cells for each condition). (B,C) Representative immunofluorescence images (B) and corresponding quantification (C) of the percentage of aneuploid neuroblasts in the indicated conditions. Each dot represents an independent experiment. A total of at least 184 neuroblasts from 14 brains were quantified for each condition. Scale bar: 5 μ m. Data information: data in (C) is shown as median plus interquartile range.

Collectively, these results strongly support that timely phosphorylation/ dephosphorylation of PP1 α 87B at T286 in mitosis is critical for faithful chromosome segregation, and consequently, for genome stability *in vivo*.

DISCUSSION

Protein Phosphatase 1 (PP1) plays a critical role in silencing the SAC to allow timely anaphase onset and mitotic exit. Knl1-bound PP1 promotes the dephosphorylation of the Knl1 MELT motifs, which drives the removal of SAC proteins from microtubule-attached kinetochores (Meadows et al, 2011; Rosenberg et al, 2011; Espeut et al, 2012; London et al, 2012; Nijenhuis et al, 2014; Zhang et al, 2014). Similarly, PP1 recruited to kinetochores via the Ska complex was shown to oppose Knl1 MELT phosphorylation at kinetochores of bioriented chromosomes (Sivakumar et al, 2016). Moreover, in *Drosophila*, the orthologue PP1 α 87B dephosphorylates the T-loop of Mps1, rendering the kinase inactive in metaphase cells (Moura et al, 2017). Hence, by switching-off the upstream SAC orchestrator (Mps1) and by antagonizing its downstream phosphorylations, PP1 ensures prompt SAC silencing at kinetochores following chromosome biorientation and thereby avoids unnecessary and potentially genome-menacing delays (Orth et al, 2012; Shindo et al, 2021; Jain and Tran, 2023) in cell cycle progression.

Additionally, and despite being dispensable for initial kinetochore-microtubule interactions, PP1 has been proposed to enhance the stability of end-on attachments (Posch et al, 2010; Wurzenberger et al, 2012; Conti et al, 2019). PP1 activity antagonizes the activating T-loop autophosphorylation of Aurora B (Murnion et al, 2001; Rosasco-Nitcher et al, 2008; Posch et al, 2010;) and enables the recruitment of the Ska complex to the KMN network (Sivakumar and Gorbsky 2017). Interestingly, the Knl1:PP1 γ holoenzyme does not seem to contribute to this process (Shrestha et al, 2017). Instead, it is a pool of PP1 γ delivered onto microtubule-end tethered kinetochores by Astrin that ensures the stabilisation of end-on attachments, as soon as they form (Conti et al, 2019). Additional PP1 γ :Kif18A and PP1 γ :CENP E holoenzymes have been identified at the outer-kinetochore as cells progress from prometaphase to metaphase, thus providing a localized delivery of PP1 that might also contribute to promote attachment stability and faithful chromosome segregation (Kim et al, 2010; Kim and Stumpff, 2018).

Despite the well-established importance of PP1 for genome stability, how its activity is regulated during mitosis had remained ill-defined. In this work, we specifically interrogated how PP1 is regulated so that SAC signalling is coordinated with kinetochore-microtubules attachment status. In our view, a unifying model of how PP1 activity is repressed during early mitosis to ensure robust SAC function and becomes increasingly active in metaphase to drive SAC silencing was still missing.

One possible mechanism concerns the controlled recruitment of PP1 to SAC substrates. At unattached or tensionless kinetochores, Aurora B phosphorylates Knl1 RVxF and SILK

motifs, which decreases the binding affinity for PP1 and thereby limits its accumulation (Liu et al, 2010; Rosenberg et al, 2011; Nasa et al, 2018). BubR1-bound PP2A:B56 continuously dephosphorylates these docking sites, thus rendering the recruitment of PP1 responsive to end-on microtubule attachments, in which Aurora B activity at the outer-kinetochore is reduced (Nijenhuis et al, 2014). Intriguingly however, structural studies show that PP1 and microtubules bind Knl1 via overlapping binding sites and co-sedimentation assays suggest that microtubules and PP1 binding to Knl1 is mutually exclusive, with preferential formation of the Knl1:PP1 holoenzyme in the presence of PP1, at least *in vitro* (Bajaj et al, 2018). Furthermore, a significant pool of PP1 can still be detected at prometaphase kinetochores of human HeLa (Nijenhuis et al, 2014) and *Drosophila* S2 cultured cells (Moura et al, 2017; Figure II.1 B) and in this work we show that premature and constitutive kinetochore accumulation of PP1 α 87B, achieved by fusing it to Mis12, fails to silence or dramatically weaken the SAC in S2 cells (Figure II.1 C). These observations suggest that, in addition to phospho-regulation of PP1-docking to Knl1/Spc105, other mechanisms must contribute to dampen PP1 α 87B activity at prometaphase kinetochores and consequently prevent untimely dephosphorylation of SAC substrates.

Phosphorylation of PP1 C-terminal tail by Cdk1 has been proposed to inhibit PP1 in mitosis (Dohadwala et al, 1994; Yamano et al, 1994; Kwon et al, 1997; Wu et al, 2009; Grallert et al, 2015; Qian et al, 2015). However, paradoxically, PP1-dependent SAC silencing typically occurs when cellular levels of Cyclin B are still elevated (Figure II.2A). Here, we confirmed that preventing Cyclin A or Cyclin B degradation in *Drosophila* S2 cells, fails to avert PP1 activity in metaphase (Figure II.2B; Figure II.3; Figure II.4). Furthermore, the main PP1 isoform in flies, PP1 α 87B, which lacks the C-terminal tail and consequently the Cdk phospho-site, is still kept in check throughout prophase/prometaphase. These observations suggest that inhibition of PP1 by Cdk1:Cyclin B plays a minor role in restraining PP1-mediated SAC silencing, which is somewhat expected, given that APC/C targeting of Cyclin B for proteolysis is only efficiently triggered following SAC inactivation, which itself requires PP1 activity.

Hence, in the present study we investigated whether alternative mechanisms operate during mitosis to control PP1 activity towards SAC substrates. We demonstrate in *Drosophila* S2 cells that phosphorylation by Polo kinase of a conserved residue (T286) close to the RVxF binding pocket of PP1 α 87B is essential to promote robust SAC signalling (Figure II.10) and dephosphorylation of this site is required for timely checkpoint silencing and anaphase onset (Figure II.9). Phosphorylation of this site hinders PP1 α 87B ability to efficiently antagonize Mps1 activation at kinetochores (Figure II.8; Figure II.11), thereby maintaining the downstream MCC-promoting phosphorylation of Mad1 C-terminus (T726)

elevated (Figure II.13). Polo-mediated phosphorylation of PP1 α 87B T286 is highest on kinetochores that are unattached or tensionless and becomes residual following chromosome biorientation in metaphase (Figure II.6). This pattern mirrors the activation status of Polo at kinetochores (Conde et al, 2013; Barbosa et al, 2020) and it is in line with the SAC-promoting function of PP1 α 87B T286 phosphorylation.

Despite our efforts, the mechanistic details of how T286 phosphorylation inhibits PP1 α 87B capacity to dephosphorylate Mps1 T-loop and switch off the SAC remain unclear. We first confirmed that phosphorylation of T286 does not directly impair the phosphatase catalytic activity. For that, we purified the wild-type and phospho-mutant versions of bacterially-produced PP1 α 87B; PP1 α (obtained from Mathieu Bollen) and PP1 γ (obtained from Jakob Nilsson) and assessed their capacity to dephosphorylate 8-difluoro-4-methylumbelliferone phosphate (DiFMUP), an artificial fluorogenic substrate lacking PP1-interacting SLiMs. We did not observe significant changes in DiFMUP dephosphorylation rate between the wild-type and the phospho-mimetic or phospho-defective versions of the phosphatases, nor a measurable impact caused by Plk1-mediated phosphorylation of T288 on the wild-type PP1 γ (data not shown). Conversely, all phosphatases were consistently inhibited by okadaic acid, a well-established inhibitor of PP1 and PP2A phosphatases (Garcia et al, 2002), thereby confirming that the purified recombinant proteins used in the assay contained phosphatase activity. These results show that the *in vitro* enzymatic activities of PP1 α 87B/PP1 are not affected by the phosphorylation of T286/T288. These results fit with the lack of obvious structural changes or catalytic impairments in PP1 α 87B/PP1 γ when T286/T288 is converted to aspartate (T286D/T288D) or to alanine (T286A/T288A), as predicted by AlphaFold2 (AF2) in the variant Colabfold (Jumper et al, 2021; Mirdita et al, 2022). We conclude that the phosphorylation of T286/T288 does not directly inhibit PP1 α 87B/PP1 catalysis, which is not unanticipated, considering that T286/T288 is located in a small loop at the surface of the protein and considerably distant from the catalytic center.

Because T286 is positioned at the entrance of the PP1 α 87B pocket that binds to RVxF motifs, and in some cases, directly contributes to the interaction (Ragusa et al, 2010; Fedoryshchak et al, 2020), we hypothesised that T286 phosphorylation could impair PP1 α 87B binding to Spc105 and thereby prevent its enrichment or dynamics at unattached kinetochores, known to be required for SAC silencing in *Drosophila* S2 cells (Audett et al, 2022). We find that unlikely, as constitutive tethering of phospho-mimetic PP1 α 87B T286D to the outer-kinetochore (fusion with Mis12) failed to rescue the SAC silencing defects caused by the loss of endogenous PP1 α 87B (Figure II.12; Figure II.13; Figure II.14). It is

however possible that fusing PP1 α 87B^{T286D} to Mis12 fails to deliver the phosphatase to Mps1 and to other SAC substrates as efficiently as the recruitment of PP1 α 87B to Spc105 N-terminus does. Nevertheless, cells expressing Mis12-PP1 α 87B^{WT} are able to dephosphorylate Mps1 T-loop and inactivate the SAC in a timely manner, thus suggesting that phosphorylation of T286 by Polo inhibits PP1 α 87B activity through mechanisms beyond, or in addition to, its potential impact on PP1 α 87B kinetochore recruitment or dynamics. At this point, one cannot exclude that T286 phosphorylation negatively impacts the ability of PP1 α 87B to interact with Spc105 and/or other SAC substrates, such as Mps1, in a way that precludes SAC inactivation. It is possible that phosphorylation of T286 by Polo increases/decreases PP1 α 87B affinity to specific RIPPPOs to control holoenzyme assembly and modulate substrate accessibility and/or phosphatase activity in mitosis (Bollen et al. 2010; Cao et al, 2022).

Previous studies have shown that Plk1 directly enhances Mps1 catalytic activity (von Schubert et al, 2015). Here we demonstrate that robust activation of Mps1 at unattached kinetochores further requires Polo-mediated inhibition of its antagonizing phosphatase. This two-pronged regulation of Mps1 by Polo is required for sustained SAC signalling, as indicated by the reduced capacity of S2 cells expressing unphosphorylatable PP1 α 87B T286A to prolong a mitotic arrest when treated with colchicine (Figure II.10A). Notably, artificially forcing the outer-kinetochore accumulation of unphosphorylatable PP1 α 87B T286A (Mis12-PP1 α 87B^{T286A}) weakened the SAC even further (Figure II.15), indicating that phosphorylation of PP1 α 87B T286 by Polo synergizes with Aurora B-mediated phosphorylation of Spc105 SILK and RVxF motifs to efficiently downregulate PP1 α 87B activity at unattached kinetochores and ensure robust SAC function. Yet, expression of PP1 α 87B^{T286A}, or of the tethered counterpart Mis12-PP1 α 87B^{T286A}, does not prematurely inactivate the SAC nor accelerate the transition to anaphase under, otherwise, unperturbed conditions (asynchronous cultured cells). This observation supports the notion that Polo-mediated phosphorylation of PP1 α 87B T286 promotes SAC robustness required for sustained checkpoint signalling, but is dispensable for SAC establishment at the onset of mitosis. Conversely, mimicking constitutive phosphorylation of T286 precludes efficient Mps1 inactivation at metaphase kinetochores and consequently delays SAC silencing and timely anaphase onset. Hence, “activation” of PP1 α 87B following dephosphorylation of T286 is not sufficient, per se, to completely switch off the SAC, but it is required to render it responsive to end-on microtubule attachments and enable prompt cell-cycle progression. The molecular mechanisms responsible for dephosphorylation of T286 remain elusive.

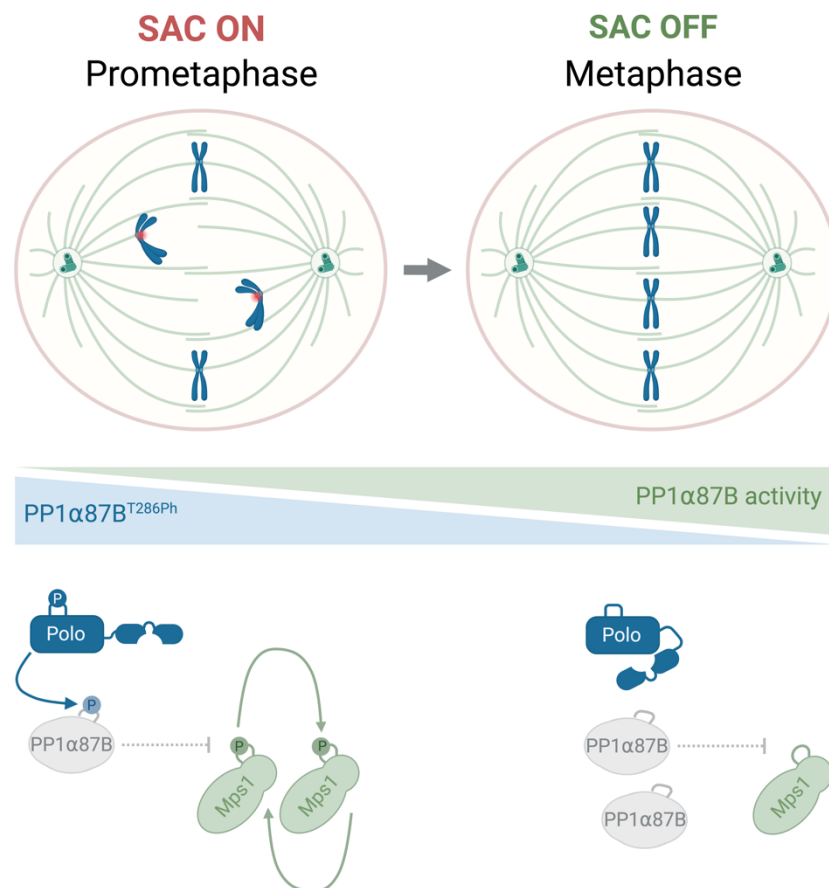


Figure II. 18 – Polo inhibits PP1 to ensure timely Spindle Assembly Checkpoint signalling. Proposed model for regulation of PP1 α 87B activity in mitosis. During prometaphase, Polo phosphorylates PP1 α 87B on its T286 to render the phosphatase less active towards Mps1 T-loop and allow robust SAC signalling. Once biorientation is achieved, Polo activity drops, which in combination with PP1 α 87B^{T286} dephosphorylation (through an unknown mechanism) results in increased phosphatase activity at metaphase kinetochores. This allows PP1 α 87B to efficiently dephosphorylate Mps1 T-loop (and possibly other SAC substrates), resulting in checkpoint silencing and prompt anaphase onset. Figure created with Biorender.com.

Thus, we propose a model in which phospho-regulation of T286 by Polo fine-tunes PP1 α 87B activity to ensure SAC robustness and responsiveness (Figure II.18). This mechanism operates in concert with Aurora B-controlled recruitment of PP1 α 87B to the outer-kinetochore, and we show here, to be required for genome stability *in vivo*. Preventing or constitutively mimicking T286 phosphorylation in dividing *Drosophila* neuroblasts leads to chromosome segregation errors and to a concomitant accumulation of aneuploid cells in larvae brains. Neuroblasts expressing unphosphorylatable PP1 α 87B^{T286A} exhibit a high frequency of anaphases with lagging chromosomes, whereas expression of PP1 α 87B^{T286D} causes asynchronous migration of segregating chromosomes towards the spindle poles. In light of these defects, we speculate that in the absence of Polo-mediated phosphorylation of T286, PP1 α 87B is not efficiently repressed during prometaphase and may therefore prematurely stabilize kinetochore-microtubule interactions. This can preclude efficient

correction of merotelic attachments, which often lead to lagging chromosomes in anaphase (Thompson and Compton, 2008; Thompson and Compton, 2011; Holland and Cleveland, 2012). Alternatively, and even though the mitotic timing is not affected when T286 is not phosphorylated, the weakened SAC in PP1 α 87B^{T286A} neuroblasts can enable anaphase onset before eventual syntelic and merotelic interactions are converted into amphitelic attachments. The underlying reason for asynchronous anaphases in PP1 α 87B^{T286D} neuroblasts is less clear. Whether these result from a prolonged metaphase duration imposed by SAC silencing defects, or from unstable kinetochore-microtubule attachments, are possibilities that need to be explored. Nevertheless, our *in vivo* analysis of proliferating larval brains demonstrates that timely phosphorylation/dephosphorylation of PP1 α 87B at T286 is critical for faithful chromosome segregation, and consequently, for the maintenance of genome stability.

ANNEXES

Figure II.2 – Supplementary figure 1

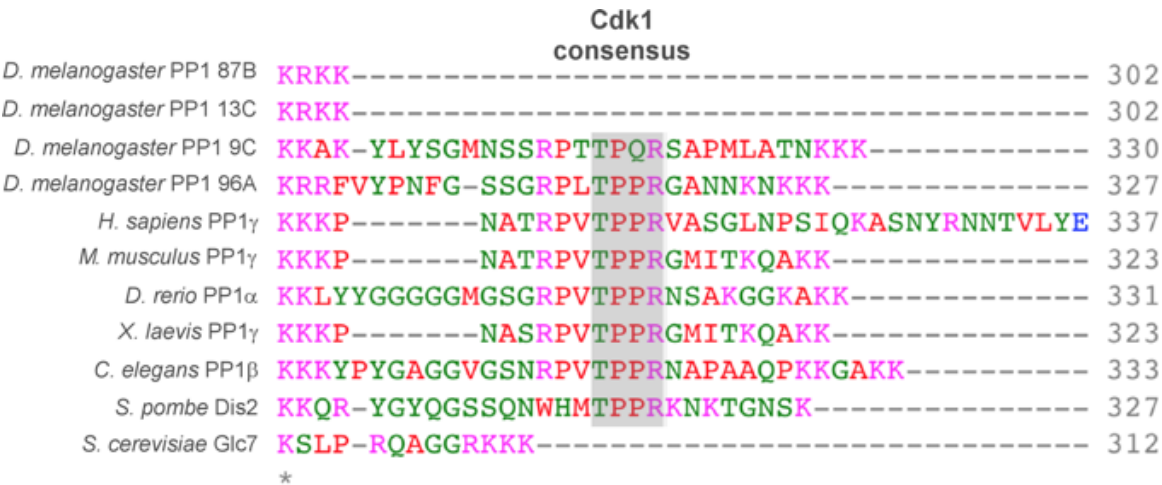


Figure II.2 – Supplementary figure 1 – A Cdk consensus site can be found in the majority of PP1 orthologues but not in *D. melanogaster* PP1α87B. Conservation among Cdk consensus motifs. Clustal W alignments of the C-terminus of indicated PP1 orthologues. The conserved Cdk consensus motif is highlighted in grey shading.

Figure II.4 – Supplementary figure 1

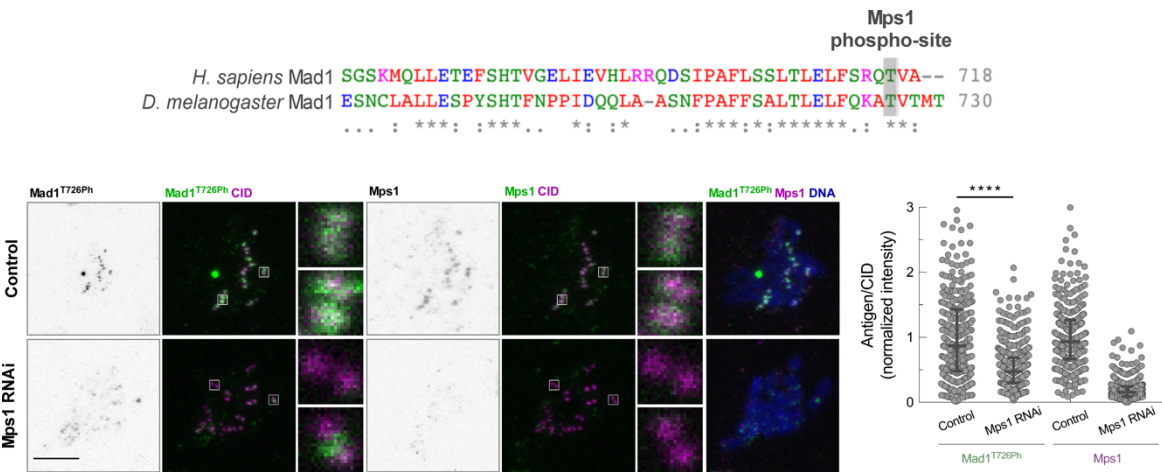


Figure II.4 – Supplementary figure 1 – Supplementary figure 1 – Mad1^{T726Ph} antibody validation in *D. melanogaster*. Conservation among Mps1 phospho-site residues. Clustal W alignments *H. sapiens* and *D. melanogaster* Mad1 orthologues. The conserved Mps1 phosphorylation threonine is highlighted in grey shading. Representative immunofluorescence images and corresponding quantifications of Mad1 T726 phosphorylation (Mad1^{T726Ph}) at prometaphase kinetochores of control or Mps1-depleted S2 cells. Mps1^{T490Ph} and Mps1 fluorescence intensities were determined relative to CID signal($n \geq 326$ kinetochores from at least 18 cells for each condition, from at least two independent experiments). Scale bar: 5 μ m. Data information: data is shown as median plus interquartile range; asterisks indicate that differences between mean ranks are statistically significant, **** $p < 0.0001$ (Mann-Whitney U test).

GENERAL DISCUSSION

To ensure accurate transfer of genetic information encoded in DNA in each cell division, eukaryotic cells rely on the Spindle Assembly Checkpoint (SAC). This surveillance mechanism delays the onset of anaphase until all kinetochores are bound to spindle microtubules. Unattached kinetochores catalyse the formation of the Mitotic Checkpoint Complex (MCC), a diffusible tetrameric complex, composed of Bub3, BubR1, Mad2 and Cdc20, which targets the APC/C, inhibiting its E3-ubiquitin ligase activity. Once all kinetochores are bound to spindle microtubules, the SAC is silenced and MCC production ceases, thus allowing APC/C activation and consequent degradation of Cyclin B and Securin, which respectively lead to inactivation of Cdk1 and Separase-mediated cleavage of the Cohesins complexes that hold sister chromatids together (Lara-Gonzalez et al, 2021; McAnish and Kops, 2023).

The balance between SAC robustness and responsiveness is pivotal for the maintenance of genome stability. A weak checkpoint can lead to a premature anaphase onset, which leads to segregation errors. On the other hand, a non-responsive or an overactive SAC delays cells in metaphase, which can cause the uncoordinated loss of sister chromatid cohesion prior to anaphase onset, a phenomenon known as “cohesion fatigue” (Daum et al, 2011; Stevens et al, 2011; Gorbsky, 2013). Hence, both a weak SAC or the inability to switch it off in a timely manner often result in aneuploidy, which is intimately associated with tumorigenesis (Michel et al, 2001; Iwanaga et al, 2007; Morais da Silva et al, 2013; Resende et al, 2018). Therefore, it is of utmost importance to understand how SAC signalling is initiated and maintained when kinetochores are unattached but also how it is silenced shortly after microtubule attachment.

The SAC is highly responsive to changes in Mps1 activity, the orchestrator of the signalling pathway (Faesen et al, 2017; Ji et al, 2017). At unattached kinetochores, Mps1 phosphorylates multiple targets to promote the recruitment of SAC proteins and MCC assembly (Figure 3). Mps1-mediated phosphorylation of Knl1 MELT motifs promotes the recruitment of the Bub3:Bub1 complex (London et al, 2012; Sheppard et al, 2012; Yamagishi et al, 2012), which in turn serve as a platform for the binding of additional checkpoint proteins: Bub3:BubR1 dimers, Mad1:Mad2 tetramers and Cdc20 (Joglekar, 2016; Musacchio, 2015; Pachis and Kops, 2018). To promote Mad1:Mad2 complex localization to the kinetochore, Mps1 phosphorylates Bub1, which enables its interaction with Mad1 (London and Biggins, 2014a; Zhang, G. et al, 2017; Fischer et al, 2021). Phosphorylation of Mad1 by Mps1 allows Cdc20 (bound to Bub1) to interact with Mad1, promoting a conformational change on the former necessary for its role in assisting the conversion of O-Mad2 to C-Mad2 (Ji et al, 2017; Faesen et al, 2017). In addition to the MCC assembled at unattached kinetochores, other APC/C inhibitory complexes are thought to

be generated during late G2 and prophase at the nuclear pores and in the cytoplasm (Sudakin et al, 2001; Lopes et al, 2005; Lince-Faria et al, 2009; Maciejowski et al, 2010; Schweizer et al, 2013; Rodriguez-Bravo et al, 2014; Cunha-Silva et al, 2020). This pre-mitotic pool of MCC gives the cell the minimum time of APC/C inhibition until newly assembled kinetochores are able to generate an efficient SAC response. Mps1 inhibition results in a substantial decrease in pre-mitotic MCC levels and a consequent reduction in Cyclin B levels already in G2 and prophase (Maciejowski et al, 2010; Rodriguez-Bravo et al, 2014). Given the relevance of Mps1 for the SAC, it is critical to dissect the molecular underpinnings of its regulation during mitosis.

Consistent with its role in assisting pre-mitotic MCC assembly, Mps1 activity starts to be detected in the nucleus 9-12min prior to nuclear envelope breakdown (NEB), in a kinetochore-dependent manner. After NEB it also starts to be detected at the cytoplasm (Kuijt et al, 2020). To become active, Mps1 is recruited to the outer kinetochore, where it dimerizes and is able to *trans*-autophosphorylate the T-loop. This recruitment is mediated by the calponin homology (CH) domains of Ndc80 and Nuf2, subunits of the Ndc80 complex (Hiruma et al, 2015; Ji et al, 2015). Mps1 inactivation at mitotic exit is thought to be associated with Mps1 removal from the kinetochores upon microtubule binding, which also interact with Ndc80 via CH domains (Hiruma et al, 2015; Ji et al, 2015). Despite the clear reduction on the kinetochore-localization of Mps1 in metaphase, a fraction of Mps1 is still retained at the kinetochore after the formation of end-on attachments (Vázquez-Novelle and Petronczki, 2010; Aravamudhan et al, 2015). Nonetheless, and although low levels of Mps1 were shown to be sufficient to delay anaphase onset (Herriott et al, 2012; Foijer et al, 2014), the residual pool of Mps1 at metaphase kinetochores does not prevent SAC silencing. On the other hand, the activity of Mps1 in the cytoplasmic pool is also required to generate and/or prevent the disassembly of MCC, further strengthening the idea that the removal of Mps1 from the kinetochore is insufficient to terminate SAC signalling efficiently. The activity of cytosolic Mps1 was shown to be critical to sustain the arrest in metaphase caused by Mad1 constitutively tethered to stably attached bioriented kinetochores that were stripped from Mps1 (Maldonado and Kapoor, 2011). Moreover, a truncated version of Mps1 that does not localize to the kinetochore was unable to promote the recruitment of Bub1 to unattached kinetochores but could still sustain the assembly of mitotic APC/C inhibitory complexes and delay anaphase onset (Maciejowski et al, 2010). Altogether, these data strongly suggest that other mechanisms must operate in metazoans to limit Mps1 activity, both at kinetochores and in the cytoplasm, and thus extinguish SAC signalling in metaphase.

In Chapter I of this Thesis, we present *in vitro* and cellular evidence that PP1 α 87B directly dephosphorylates Mps1 activating T-loop, which decreases the kinase activity at metaphase kinetochores and in the cytoplasm of *Drosophila* cells. Depleting PP1 α 87B or preventing it to interact with Mps1 increases the activity of a pool of Mps1 that persists at kinetochores of bioriented chromosomes (Figure I.4; Figure I.7), despite stable end-on attachments and inter-kinetochore tension (Figure I.2; Figure I.5). Moreover, preventing the dephosphorylation, and consequent inactivation of soluble Mps1 by PP1 α 87B is sufficient to significantly delay mitotic exit even in the absence of kinetochore-generated MCC (Figure I.12). In flies, PP1 α 87B binding to Mps1 relies on a [R/K]VxF motif present on the disordered N-terminus of the kinase (Figure I.7; Figure I.10). Although this motif is poorly conserved among Mps1 orthologues, other PP1-binding motifs, such as the SILK signature, can be found in the C-terminal region of human and mouse Mps1, which suggests that the dephosphorylation of Mps1 T-loop by PP1 is likely to be conserved, despite the diversification in type and localization of the PP1-docking motifs.

To allow timely SAC signalling, PP1 activity is regulated throughout mitosis, regarding both its recruitment to the kinetochore and its capacity to dephosphorylate substrates. PP1 binding to RVxF and SILK motifs of Knl1 is decreased by Aurora B phosphorylation at tensionless kinetochores (Liu et al, 2010; Rosenberg et al, 2011; Nasa et al, 2018) and enhanced by PP2A:B56 counteracting dephosphorylation as microtubules attach to kinetochores (Nijenhuis et al, 2014). However, this mechanism is unlikely sufficient to completely repress PP1 activity since a significant pool of the phosphatase still resides at prometaphase kinetochores (Nijenhuis et al, 2014) and its forced and premature accumulation at kinetochores does not significantly accelerate SAC silencing nor mitotic exit (Figure II.1). In addition, Cdk1 phosphorylates the C-terminus tail of PP1, decreasing its activity in mitosis (Dohadwala et al, 1994; Yamano et al, 1994; Kwon et al, 1997; Wu et al, 2009; Grallert et al, 2015; Qian et al, 2015). However, this regulatory mechanism albeit important for mitotic exit following anaphase onset, is not expected to contribute for SAC signalling, as the SAC must be silenced before Cdk1 inactivation. In fact, if Cyclin B degradation is prevented in *Drosophila* S2 cells, PP1 α 87B is still able to dephosphorylate Mps1 T-loop and promote SAC silencing (Figure II.3; Figure II.4). PP1 activity is further regulated by other regulatory interactors of protein phosphatase one (RIPPOs), which form specific holoenzymes with the phosphatase and act as substrate specifiers or modulate PP1 activity in different ways (Bollen et al 2010; Cao et al, 2022; Weith et al, 2018). However, the mechanisms controlling the association of PP1 with these RIPPOs during mitosis are still not fully understood.

The work reported in Chapter II of the present Thesis provides new knowledge to better understand how PP1 activity at kinetochores is coordinated with their attachment status in order to ensure a robust and timely SAC. We demonstrated that Polo-mediated phosphorylation of PP1 α 87B on T286 decreases the phosphatase ability to inactivate Mps1 and antagonize the checkpoint. In line with this inhibitory role on PP1 α 87B-driven SAC silencing, phosphorylation of T286 occurs predominantly at unattached kinetochores and at kinetochores of uncongressed chromosomes. In fact, replacing endogenous PP1 α 87B with a phospho-mimetic version prevents the inactivation of Mps1 causing a SAC-dependent metaphase delay. Conversely, expression of an unphosphorylatable PP1 α 87B T286A mutant significantly weakens the SAC response of S2 cells in the presence of colchicine (Figure II.9; Figure II.10; Figure II.11). These data show that phosphorylation of PP1 α 87B on T286 promotes SAC robustness, and its dephosphorylation is required for timely SAC silencing. Importantly, this phospho-regulation is critical to maintain genome stability of dividing cells *in vivo*. *Drosophila* larval neuroblasts expressing PP1 α 87B^{T286} phospho-mutants show an increased frequency in anaphases with chromosome segregation errors, which correlates with significant levels of aneuploidy in the developing brain (Figure II.17).

The mechanism by which T286 phosphorylation inhibits PP1 α 87B-mediated dephosphorylation of Mps1 T-loop remains unclear. Our *in vitro* assays with recombinant PP1 α 87B and human PP1 orthologues produced in bacteria showed that the phosphatase catalytic activity is not affected by T286/T288 phosphorylation or by the phospho-mimetic mutation to aspartate (data not shown).

Given that T286 is positioned at the entrance of the RVxF-binding pocket, it is possible that the phosphorylation impacts PP1 α 87B ability to interact with Mps1 RVxF or to bind to Spc105 and thus, limit the phosphatase recruitment to kinetochores. However, we performed pull-down experiments that show that, at least *in vitro*, the binding of PP1 α 87B to Mps1 and of PP1 γ to Knl1 RvxF is impervious to T286/T288 phosphorylation. Furthermore, tethering PP1 α 87B^{T286D} to the outer kinetochore does not restore its ability to dephosphorylate Mps1 (Figure II.12; Figure II.14), thus suggesting that phosphorylation of T286 by Polo inhibits PP1 α 87B activity through mechanisms beyond its potential impact on PP1 α 87B kinetochore recruitment or dynamics. One possibility is that phosphorylation of T286 may hinder PP1 α 87B activity or impair the interaction with SAC substrates in a cellular context by controlling holoenzyme assembly during mitosis.

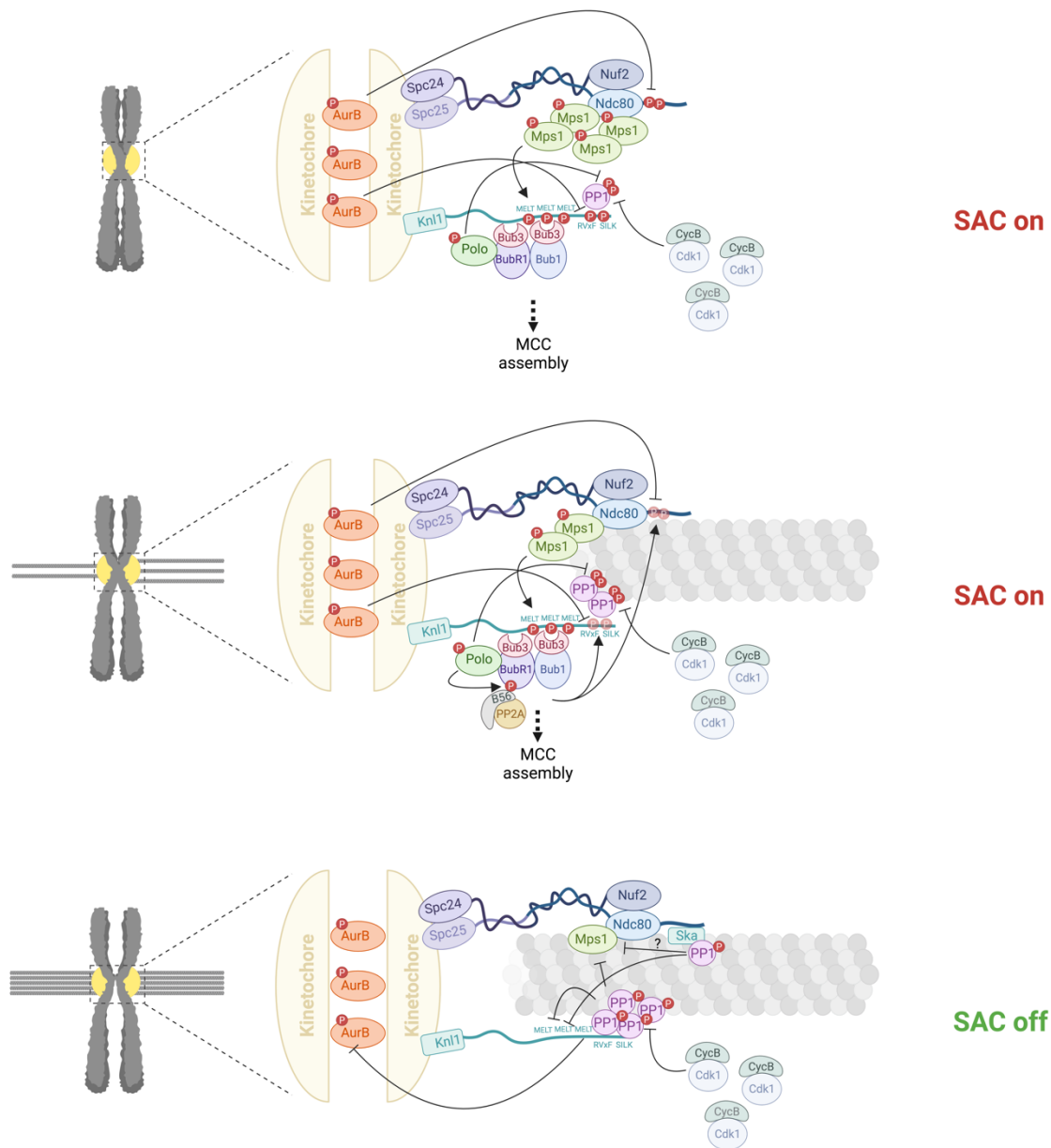


Figure 11 – Phospho-regulation of the Spindle Assembly Checkpoint. In the absence of microtubule attachments, Mps1 efficiently accumulates at kinetochores through direct binding to the Ndc80 complex, which enables Mps1 *trans*-autophosphorylation and consequent activation. This initiates SAC signalling, by phosphorylation of several MELT motifs on Knl1, which generates docking sites for the hierarchical recruitment of SAC proteins required for MCC assembly. Centromeric Aurora B catalyses the phosphorylation of Ndc80 N-terminal, counteracted by PP2A:B56 bound to BubR1. This phosphorylation is important for error correction mechanisms as it destabilizes the interaction between Ndc80 and the microtubules. Aurora B also phosphorylates Knl1 on RVxF and SILK motifs to decrease PP1 recruitment to the kinetochore. In coordination with the recruitment impairment, Polo and Cdk1 also phosphorylate PP1 to decrease its potential to dephosphorylate mitotic substrates, thus preventing premature mitotic exit. As microtubules occupancy increases, kinetochore tension or alterations on the kinetochore architecture place outer-kinetochore proteins further apart from the Aurora B zone of influence. This allows a shift on the (de)phosphorylation balance between Aurora B and PP2A:B56, which results in increasing stabilization of Ndc80-microtubule interactions and PP1 recruitment to the kinetochore. Increasing levels of PP1 bound to Knl1 further suppress Aurora B activity at kinetochores, hence enhancing the stability of microtubule attachments. Since Mps1 and microtubules compete for Ndc80 binding, the kinase is gradually removed from the kinetochore as kinetochore-microtubule attachments increase. The local accumulation of PP1 and the inactivation of Polo result in decreased PP1 T286

phosphorylation, which in turn results in increased Knl1 MELT motifs and Mps1 T-loop dephosphorylation and SAC silencing. Figure created with Biorender.com.

In addition to the roles that kinetochore-localized Polo/Plk1 plays on kinetochore-microtubule attachments, the kinase also acts as an important SAC regulator, alongside Mps1. Although the molecular mechanisms are poorly understood, Polo/Plk1 is important for Mps1 recruitment and activation at the kinetochore (Conde et al, 2013; von Schubert et al, 2015). Moreover, it has been shown that Plk1 shares some checkpoint substrates with Mps1, including Knl1 MELT motifs and phosphorylation sites on Mps1 (von Schubert et al, 2015). Polo-mediated phosphorylation of PP1 α 87B impairs the phosphatase ability to inactivate Mps1, at unattached kinetochores. Thus, this two-pronged regulation of Mps1 by Polo triggers a rapid and robust Mps1 and consequently SAC activation at mitotic commitment. When kinetochores become attached to microtubules, the kinetochore recruitment of Polo/Plk1 is decreased. Importantly, it was shown that PP1 and PP2A:B56 cooperate to promote Polo/Plk1 removal from the kinetochore, possibly by dephosphorylation of its receptors Bub1 and BubR1 (Cordeiro et al, 2020; Benzi and Piatti, 2020). In a SAC-induced mitotic arrest (e.g., upon microtubule depolymerization), kinetochore phosphatases are already partially active, specially PP2A:B56, which results in partial displacement of Polo/Plk1 from the kinetochores. This leaves Mps1 inactivation as the main trigger for SAC silencing and contributes for the responsiveness of the checkpoint. In addition, the kinase activity of Polo/Plk1 is also regulated along mitotic progression. Although the molecular mechanism remains elusive, PP1 was proposed as the Plk1 counteracting phosphatase, dephosphorylating the kinase activating loop *in vitro* and *in vivo* (Yamashiro et al, 2008; Bruinsma et al, 2012). This negative feedback loop allows prompt Mps1 and SAC inactivation following chromosome biorientation. Altogether, the mechanism we propose defines a molecular switch that ensures timely SAC activity and perfect coordination with mitotic progression.

REFERENCES

- Abad, M.A.; Medina, B.; Santamaria, A.; Zou, J.; Plasberg-Hill, C.; Madhumalar, A.; Jayachandran, U.; Redli, P.M.; Rappsilber, J.; Nigg, E.A.; et al. Structural basis for microtubule recognition by the human kinetochore Ska complex. *Nat. Commun.* 2014, 5: 2964.
- Afshar, K.; Werner, M.E.; Tse, Y.C.; Glotzer, M.; Gönczy, P. Regulation of cortical contractility and spindle positioning by the protein phosphatase 6 PPH-6 in one-cell stage *C. elegans* embryos. *Development* 2010, 137: 237–247.
- Agircan, F.G.; Schiebel, E.; Mardin, B.R. Separate to operate: Control of centrosome positioning and separation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 2014, 369: 20130461.
- Agostinis, P.; Derua, R.; Sarno, S.; Goris, J.; Merlevede, W. Specificity of the polycation-stimulated (type-2A) and ATP, Mg-dependent (type-1) protein phosphatases toward substrates phosphorylated by P34cdc2 kinase. *Eur. J. Biochem.* 1992, 205: 241–248.
- Agostinis, P.; Goris, J.; Pinna, L.A.; Marchiori, F.; Perich, J.W.; Meyer, H.E.; Merlevede, W. Synthetic peptides as model substrates for the study of the specificity of the polycation-stimulated protein phosphatases. *Eur. J. Biochem.* 1990, 189: 235–241.
- Akiyoshi, B.; Sarangapani, K.K.; Powers, A.F.; Nelson, C.R.; Reichow, S.L.; Arellano-Santoyo, H.; Gonen, T.; Ranish, J.A.; Asbury, C.L.; Biggins, S. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. 2010, 468: 576–579.
- Alessi, D.R.; Street, A.J.; Cohen, P.; Cohen, P.T. Inhibitor-2 functions like a chaperone to fold three expressed isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. *Eur. J. Biochem.* 1993, 213: 1055–1066.
- Allan, L.A.; Reis, M.C.; Ciossani, G.; Huis, P.J.; Wohlgemut, S.; Kops, G.J.P.L.; Musacchio A.; Saurin, A.T. Cyclin B1 scaffolds MAD1 at the kinetochore corona to activate the mitotic checkpoint. *EMBO J.* 2020, 39: e103180.
- Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. Protein tyrosine phosphatases in the human genome. *Cell* 2004, 117: 699–711.
- Althoff, F.; Karess, R.E.; Lehner C.F. Spindle checkpoint-independent inhibition of mitotic chromosome segregation by *Drosophila* Mps1. *Mol. Biol. Cell.* 2012, 23: 2275–2291.
- Alushin, G.M.; Ramey, V.H.; Pasqualato, S.; Ball, D.A.; Grigorieff, N.; Musacchio, A.; Nogales, E. The Ndc80 kinetochore complex forms oligomeric arrays along microtubules. *Nature* 2011, 467: 805–810.
- Álvarez-Fernández, M.; Sánchez-Martínez, R.; Sanz-Castillo, B.; Can, P.P.; Sanz-Flores, M.; Trakala, M.; Ruiz-Torres, M.; Lorca, T.; Castro, A.; Malumbres, M. Greatwall is essential to prevent mitotic collapse after nuclear envelope breakdown in mammals. *Proc. Natl. Acad. Sci. USA* 2013, 110: 17374–17379.
- Amin, M.A.; Itoh, G.; Iemura, K.; Ikeda, M.; Tanaka, K. CLIP-170 is required to recruit PLK1 to kinetochores during early mitosis for chromosome alignment. *J. Cell Sci.* 2014, jcs-150755.

- Andersen, J.N.; Jansen, P.G.; Echwald, S.M.; Mortensen, O.H.; Fukada, T.; Del Vecchio, R.; Tonks, N.K.; Møller, N.P. A genomic perspective on protein tyrosine phosphatases: Gene structure, pseudogenes, and genetic disease linkage. *FASEB J.* 2004, 18: 8–30.
- Andersen, J.N.; Mortensen, O.H.; Peters, G.H.; Drake, P.G.; Iversen, L.F.; Olsen, O.H.; Jansen, P.G.; Andersen, H.S.; Tonks, N.K.; Møller, N.P. Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol. Cell. Biol.* 2001, 21: 7117–7136.
- Andrésson, T.; Ruderman, J.V. The kinase Eg2 is a component of the *Xenopus* oocyte progesterone-activated signaling pathway. *EMBO J.* 1998, 17: 5627–5637.
- Antonin, W.; Neumann, H. Chromosome condensation and decondensation during mitosis. *Curr. Opin. Cell. Biol.* 2016, 40: 15–22.
- Aravamudhan, P.; Goldfarb, A.A.; Joglekar, A.P. The kinetochore encodes a mechanical switch to disrupt spindle assembly checkpoint signalling. *Nat. Cell Biol.* 2015, 17: 868–879.
- Archambault, V.; Zhao, X.; White-Cooper, H.; Carpenter, A.T.C.; Glover, D.M. Mutations in *Drosophila* Greatwall/Scant Reveal Its Roles in Mitosis and Meiosis and Interdependence with Polo Kinase. *PLoS Genet.* 2007, 3: e200.
- Aressy, B.; Ducommun, B. Cell cycle control by the CDC25 phosphatases. *Anticancer Agents Med. Chem.* 2008, 8: 818–824.
- Arino, J.; Woon, C.W.; Brautigan, D.L.; Miller, T.B.; Johnson, G.L. Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc. Natl. Acad. Sci. USA* 1988, 85: 4252–4256.
- Asencio, C.; Davidson, I.F.; Santarella-Mellwig, R.; Ly-Hartig, T.B.; Mall, M.; Wallenfang, M.R.; Mattaj, I.W.; Gorjánácz, M. Coordination of kinase and phosphatase activities by Lem4 enables nuclear envelope reassembly during mitosis. *Cell* 2012, 150: 122–135.
- Auckland, P.; Clarke, N.I.; Royle, S.J.; McAinsh, A.D. Congressing kinetochores progressively load Ska complexes to prevent force-dependent detachment. *J. Cell Biol.* 2017, 216: 1623–1638.
- Audett, M.R.; Johnson, E.L.; McGory, J.M.; Barcelos, D.M.; Szalai, E.O.; Przewloka, M.R.; Maresca, T.J. The microtubule- and PP1-binding activities of *Drosophila melanogaster* Spc105 control the kinetics of SAC satisfaction. *Mol Biol Cell.* 2022, 33(1): ar1.
- Axton, J.M.; Dombrádi, V.; Cohen, P.T.W.; Glover, D.M. One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell.* 1990, 63: 33–46.
- Bader, J.R.; Kasuboski, J.M.; Winding, M.; Vaughan, P.S.; Hinchcliffe, E.H.; Vaughan, K.T. Polo-like kinase 1 is required for recruitment of dynein to kinetochores during mitosis. *J. Biol. Chem.* 2011, jbc-M111.
- Bahe, S.; Stierhof, Y.D.; Wilkinson, C.J.; Leiss, F.; Nigg, E.A. Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *J. Cell Biol.* 2005, 171: 27–33.
- Bahmanyar, S.; Kaplan, D.D.; Deluca, J.G.; Giddings, T.H., Jr.; O'Toole, T.E.; Winey, M.; Salmon, E.D.; Casey, P.J.; Nelson, W.J.; Barth, A.I. beta-Catenin is a Nek2 substrate involved in centrosome separation. *Genes Dev.* 2008, 22: 91–105.
- Bajaj, R.; Bollen, M.; Peti, W.; Page, R. KNL1 Binding to PP1 and Microtubules Is Mutually Exclusive. *Structure* 2018, 26: 1327–1336.

- Bakhoun, S.F.; Genovese, G.; Compton, D.A. Deviant Kinetochore Microtubule Dynamics Underlie Chromosomal Instability. *Curr. Biol.* 2009, 19: 1937–1942.
- Bakhoun, S.F.; Thompson, S.L.; Manning, A.L.; Compton, D.A. Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. *Nat. Cell Biol.* 2009, 11: 27–35.
- Baldin, V.; Cans, C.; Knibiehler, M.; Ducommun, B. Phosphorylation of human CDC25B phosphatase by CDK1-cyclin A triggers its proteasome-dependent degradation. *J. Biol. Chem.* 1997, 272: 32731–32734.
- Ballister, E.R.; Riegman, M.; Lampson, M.A. Recruitment of Mad1 to metaphase kinetochores is sufficient to reactivate the mitotic checkpoint. *J. Cell Biol.* 2014, 204(6): 901–908.
- Bancroft, J.; Holder, J.; Gerachty, Z.; Alfonso-Pérez, T.; Murphy, D.; Barr, F.A.; Gruneberg, U. PP1 promotes cyclin B destruction and the metaphase-anaphase transition by dephosphorylating CDC20. *Mol Biol Cell.* 2020, 31(21): 2315–2330.
- Barbosa, J.; Martins, T.; Bange, T.; Tao, L.; Conde, C.; Sunkel, C. Polo regulates Spindly to prevent premature stabilization of kinetochore-microtubule attachments. *EMBO J.* 2020, 39: e100789.
- Barford, D.; Das, A.K.; Egloff, M.P. The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct.* 1998, 27: 133–164.
- Barford, D.; Flint, A.J.; Tonks, N.K. Crystal structure of human protein tyrosine phosphatase 1B. *Science* 1994, 263: 1397–1404.
- Barisic, M.; Aguiar, P.; Geley, S.; Maiato, H. Kinetochore motors drive congression of peripheral polar chromosomes by overcoming random arm-ejection forces. *Nat. Cell Biol.* 2014, 16: 1249–1256.
- Barisic, M.; Maiato, H. The tubulin code: A navigation system for chromosomes during mitosis. *Trends Cell Biol.* 2016, 26: 766–775.
- Barisic, M.; Sousa, R.S.; Tripathy, S.K.; Magiera, M.M.; Zaytsev, A.V.; Pereira, A.L.; Janke, C.; Grishchuk, E.L.; Maiato, H. Microtubule detyrosination guides chromosomes during mitosis. *Science* 2015, 348: 799–803.
- Barr, F.A.; Elliott, P.R.; Gruneberg, U. Protein phosphatases and the regulation of mitosis. *J. Cell Sci.* 2011, 124: 2323–2334.
- Barros, T.P.; Kinoshita, K.; Hyman, A.A.; Raff, J.W. Aurora A activates D-TACC-Msps complexes exclusively at centrosomes to stabilize centrosomal microtubules. *J. Cell Biol.* 2005, 170: 1039–1046.
- Bayliss, R.; Sardon, T.; Vernos, I.; Conti, E. Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol. Cell* 2003, 12: 851–862.
- Ben-Shahar, T.R.; Heeger, S.; Lehane, C.; East, P.; Flynn, H.; Skehel, M.; Uhlmann, F. Eco1-dependent cohesion acetylation during establishment of sister chromatid cohesion. *Science* 2008, 321: 563–566.
- Benzi, G.; Piatti, S. Silencing the spindle assembly checkpoint: Let's play Polo! *J Cell Biol.* 2020, 219(12): e202010053.

- Bergstralh, D.T.; Dawney, N.S.; St. Johnston, D. Spindle orientation: A question of complex positioning. *Development* 2017, 144: 1137–1145.
- Bieling, P.; Telley, I.A.; Surrey, T. A minimal midzone protein module controls formation and length of antiparallel microtubule overlaps. *Cell* 2010, 142: 420–432.
- Bishop, J.D.; Schumacher, J.M. Phosphorylation of the Carboxyl Terminus of Inner Centromere Protein (INCENP) by the Aurora B Kinase Stimulates Aurora B Kinase Activity. *J. Biol. Chem.* 2002, 277: 27577–27580.
- Blake-Hodek, K.A.; Williams, B.C.; Zhao, Y.; Castilho, P.V.; Chen, W.; Mao, Y.; Yamamoto, T.M.; Goldberg, M.L. Determinants for activation of the atypical AGC kinase Greatwall during M phase entry. *Mol. Cell. Biol.* 2012, 32: 1337–1353.
- Bloecher, A.; Tatchell, K. Defects in *Saccharomyces cerevisiae* protein phosphatase type I activate the spindle/kinetochore checkpoint. *Genes Dev.* 1999, 13: 517–522.
- Boens, S.; Szekér, K.; Van Eynde, A.; Bollen, M. Interactor-guided dephosphorylation by protein phosphatase-1. *Methods Mol. Biol.* 2013, 1053: 271–281.
- Bollen, M.; Peti, W.; Ragusa, M.J.; Beullens, M. The extended PP1 toolkit: Designed to create specificity. *Trends Biochem. Sci.* 2010, 35: 450–458.
- Booher, R.N.; Holman, P.S.; Fattaey, A. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *J. Biol. Chem.* 1997, 272: 22300–22306.
- Booth, D.G.; Takagi, M.; Sanchez-Pulido, L.; Petfalski, E.; Vargiu, G.; Samejima, K.; Imamoto, N.; Ponting, C.P.; Tollervey, D.; Earnshaw, W.C.; et al. Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *Elife* 2014, 3: e01641.
- Boutros, R.; Dozier, C.; Ducommun, B. The when and wheres of CDC25 phosphatases. *Curr. Opin. Cell Biol.* 2006, 18: 185–191.
- Boutros, R.; Lobjois, V.; Ducommun, B. CDC25 phosphatases in cancer cells: Key players? Good targets? *Nat. Rev. Cancer* 2007, 7: 495–507.
- Brautigan, D.L.; Shenolikar, S. Protein serine/threonine phosphatases: Keys to unlocking regulators and substrates. *Annu. Rev. Biochem.* 2018, 87: 921–964.
- Bruinsma, W.; Raaijmakers, J.A.; Medema, R.H. Switching Polo-like kinase-1 on and off in time and space. *Trends Biochem. Sci.* 2012, 37(12): 534–542.
- Buheitel, J.; Stemmann, O. Prophase pathway-dependent removal of cohesion from human chromosomes requires opening of the Smc3–Scc1 gate. *EMBO J.* 2013, 32: 666–676.
- Burgess, A.; Vigneron, S.; Brioudes, E.; Labbé, J.C.; Lorca, T.; Castro, A. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proc. Natl. Acad. Sci. USA.* 2010, 107: 12564–12569.
- Burgess, A.; Vuong, J.; Rogers, S.; Malumbres, M.; O'Donoghue, S.I. SnapShot: Phosphoregulation of Mitosis. *Cell* 2017, 169: 1358.
- Burgess, S.G.; Mukherjee, M.; Sabir, S.; Joseph, N.; Gutiérrez-Caballero, C.; Richards, M.W.; Huguenin-Dezot, N.; Chin, J.W.; Kennedy, E.J.; Pfuhl, M.; et al. Mitotic spindle association of TACC3 requires Aurora-A-dependent stabilization of a cryptic α -helix. *EMBO J.* 2018, 37: e97902.

- Cai, S.; O'connell, C.B.; Khodjakov, A.; Walczak, C.E. Chromosome congression in the absence of kinetochore fibres. *Nat. Cell Biol.* 2009, 11: 832–838.
- Caldas G.V., Lynch T.R., Anderson R., Afreen S., Varma D., DeLuca J.G. The RZZ complex requires the N-terminus of KNL1 to mediate optimal Mad1 kinetochore localization in human cells. *Open Biology.* 2015, 5: 150160.
- Campbell M.S.; Chan G.K.; Yen T.J. Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. *J. Cell Sci.* 2001, 114(Pt 5): 953–963.
- Cao, X.; Lemaire, S.; Bollen, M. Protein phosphatase 1: life-course regulation by SDS22 and Inhibitor-3. *FEBS J.* 2022, 289(11): 3072–3085.
- Caron, D.; Byrne, D.P.; Thebault, P.; Soulet, D.; Landry, C.R.; Eysers, P.A.; Elowe, S. Mitotic phosphotyrosine network analysis reveals that tyrosine phosphorylation regulates Polo-like kinase 1 (PLK1). *Sci. Signal.* 2016, 9: rs14.
- Castilho, P.V.; Williams, B.C.; Mochida, S.; Zhao, Y.; Goldberg, M.L. The M Phase Kinase Greatwall (Gwl) Promotes Inactivation of PP2A/B55, a Phosphatase Directed Against CDK Phosphosites. *Mol. Biol. Cell.* 2009, 20: 4777–4789.
- Cavazza, T.; Vernos, I. The Ran GTP Pathway: From Nucleo-Cytoplasmic Transport to Spindle Assembly and Beyond. *Front Cell Dev. Biol.* 2016, 3: 82.
- Cazales, M.; Schmitt, E.; Montembault, E.; Dozier, C.; Prigent, C.; Ducommun, B. CDC25B phosphorylation by Aurora A occurs at the G2/M transition and is inhibited by DNA damage. *Cell Cycle.* 2005, 4: 1233–1238.
- Ceulemans, H.; Stalmans, W.; Bollen, M. Regulator-driven functional diversification of protein phosphatase-1 in eukaryotic evolution. *Bioessays.* 2002, 24: 371–381.
- Chan, K.L.; Roig, M.B.; Hu, B.; Beckouët, F.; Metson, J.; Nasmyth, K. Cohesin's DNA exit gate is distinct from its entrance gate and is regulated by acetylation. *Cell.* 2012, 150: 961–974.
- Chan, Y.W.; Jeyaprakash, A.A.; Nigg, E.A.; Santamaria, A. Aurora B controls kinetochore–microtubule attachments by inhibiting Ska complex–KMN network interaction. *J. Cell Biol.* 2012, 196: 563–571.
- Chatterjee, A.; Zakian, S.; Hu, X.W.; Singleton, M.R. Structural insights into the regulation of cohesion establishment by Wpl1. *EMBO J.* 2013, 32: 677–687.
- Cheerambathur, D.K.; Prevo, B.; Hattersley, N.; Lewellyn, L.; Corbett, K.D.; Oegema, K.; Desai, A. Dephosphorylation of the Ndc80 tail stabilizes kinetochore-microtubule attachments via the Ska complex. *Dev. Cell.* 2017, 41: 424–437.
- Cheeseman, I.M.; Chappie, J.S.; Wilson-Kubalek, E.M.; Desai, A. The Conserved KMN Network Constitutes the Core Microtubule-Binding Site of the Kinetochore. *Cell* 2006, 127, 983–997.
- Chen, F.; Archambault, V.; Kar, A.; Lio', P.; D'Avino, P.P.; Sinka, R.; Lilley, K.; Laue, E.D., Deak, P.; Capalbo, L.; Glover, D.M. Multiple protein phosphatases are required for mitosis in *Drosophila*. *Current Biology.* 2007, 17: 293–303.
- Chen, H.Z.; Tsai, S.Y.; Leone, G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nat Rev Cancer.* 2009, 9: 785–797

- Chen, M.J.; Dixon, J.E.; Manning, G. Genomics and evolution of protein phosphatases. *Sci. Signal.* 2017, 10: eaag1796.
- Chen, M.S.; Ryan, C.E.; Piwnica-Worms, H. Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. *Mol. Cell. Biol.* 2003, 23: 7488–7497.
- Chen, R.H.; Waters, J.C.; Salmon, E.D.; Murray, A.W. Association of spindle assembly checkpoint component XMad2 with unattached kinetochores. *Science* 1996, 274: 242–246.
- Chen, W.; Wilborn, M.; Rudolph, J. Dual-specific Cdc25B phosphatase: In search of the catalytic acid. *Biochem.* 2000, 39: 10781–10789.
- Chen, Y.; Poon, R.Y. The multiple checkpoint functions of CHK1 and CHK2 in maintenance of genome stability. *Front Biosci.* 2008, 13: 5016–5029.
- Cheng, Y.L.; Chen, R.H. Assembly and quality control of protein phosphatase 1 holoenzyme involve Cdc48-Shp1 chaperone. *J. Cell Sci.* 2015, 128: 1180–1192.
- Cho, U.S.; Xu, W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature.* 2006, 445: 53–57.
- Choy, M.S.; Hieke, M.; Kumar, G.S.; Lewis, G.R.; Gonzalez-DeWhitt, K.R.; Kessler, R.P.; Stein, B.J.; Hessenberger, M.; Nairn, A.C.; Nairn Peti, W.; et al. Understanding the antagonism of retinoblastoma protein dephosphorylation by PNUTS provides insights into the PP1 regulatory code. *Proc. Natl. Acad. Sci. USA.* 2014, 111: 4097–4102.
- Ciferri, C.; Pasqualato, S.; Screpanti, E.; Varetto, G.; Santaguida, S.; Reis, G.; Maiolica, A.; Polka, J.; DeLuca, J.G.; de Wulf, P.; et al. Implications for Kinetochore-Microtubule Attachment from the Structure of an Engineered Ndc80 Complex. *Cell.* 2008, 133: 427–439.
- Ciliberto, A.; Hauf, S. Micromanaging checkpoint proteins. *Elife.* 2017, 6: e25001.
- Cimini, D.; Howell, B.; Maddox, P.; Khodjakov, A.; Degrossi, F.; Salmon, E.D. Merotelic Kinetochore Orientation Is a Major Mechanism of Aneuploidy in Mitotic Mammalian Tissue Cells. *J. Cell Biol.* 2001, 153: 517–527.
- Clarke, P.R.; Hoffmann, I.; Draetta, G.; Karsenti, E. Dephosphorylation of Cdc25-Cbyatype-2A protein phosphatase: Specific regulation during the cell cycle in *Xenopus* egg extracts. *Mol. Biol. Cell.* 1993, 4: 397–411.
- Clarke, P.R.; Zhang, C. Spatial and temporal coordination of mitosis by Ran GTPase. *Nat. Rev. Mol. Cell Biol.* 2008, 9: 464–477.
- Collin P., Nashchekina O., Walker R., Pines J. The spindle assembly checkpoint works like a rheostat rather than a toggle switch. *Nat. Cell Biol.* 2013, 15: 1378–1385.
- Conde, C.; Gassmann, R. Spindle checkpoint: trapped by the corona, cyclin B1 goes MAD. *EMBO J.* 2020, 39: e105279.
- Conde, C.; Osswald, M.; Barbosa, J.; Moutinho-Santos, T.; Pinheiro, D.; Guimarães, S.; Matos, I.; Maiato, H.; Sunkel, C.E. *Drosophila* Polo regulates the spindle assembly checkpoint through Mps1-dependent BubR1 phosphorylation. *EMBO J.* 2013, 32: 1761–1777.

- Conduit, P.T.; Wainman, A.; Raff, J.W. Centrosome function and assembly in animal cells. *Nat. Rev. Mol. Cell Biol.* 2015, 16: 611–624.
- Conti, D.; Gul, P.; Islam, A.; Martín-Durán, J.M.; Pickersgill, R.W.; Draviam, V.M. Kinetochores attached to microtubule-ends are stabilised by Astrin bound PP1 to ensure proper chromosome segregation. *eLife*. 2019, 8: e49325.
- Corbett, K.D. Molecular Mechanisms of Spindle Assembly Checkpoint Activation and Silencing. *Prog. Mol. Subcell. Biol.* 2017, 56: 429–455.
- Cordeiro, M.H.; Smith, R.J.; Saurin, A.T. Kinetochores suppress autonomous Polo-like kinase 1 activity to control the mitotic checkpoint. *J Cell Biol.* 2020, 219(12): e202002020.
- Craney, A.; Kelly, A.; Jia, L.; Fedrigo, I.; Yu, H.; Rape, M. Control of APC/C-dependent ubiquitin chain elongation by reversible phosphorylation. *Proc. Natl. Acad. Sci. USA.* 2016, 113: 1540–1545.
- Croasdale, R.; Ivins, F.J.; Muskett, F.; Daviter, T.; Scott, D.J.; Hardy, T.; Smerdon, S.J.; Fry, A.M.; Pfuhl, M. An undecided coiled coil: The leucine zipper of Nek2 kinase exhibits atypical conformational exchange dynamics. *J. Biol. Chem.* 2011, 286: 27537–27547.
- Cundell, M.J.; Bastos, R.N.; Zhang, T.; Holder, J.; Gruneberg, U.; Novak, B.; Barr, F.A. The BEG (PP2A-B55/ENSA/Greatwall) pathway ensures cytokinesis follows chromosome separation. *Mol. Cell.* 2013, 52: 393–405.
- Cundell, M.J.; Hutter, L.H.; Bastos, R.N.; Poser, E.; Holder, J.; Mohammed, S.; Novak, B.; Barr, F.A. A PP2A-B55 recognition signal controls substrate dephosphorylation kinetics during mitotic exit. *J. Cell Biol.* 2016, 214: 539–554.
- Cunha-Silva S.; Osswald M.; Goemann J.; Barbosa J.; Santos L.M.; Resende P.; Bange T.; Ferrás C.; Sunkel C.E.; Conde C. Mps1-mediated release of Mad1 from nuclear pores ensures the fidelity of chromosome segregation. *J. Cell Biol.* 2020, 219(3): 201906039.
- Cyphers, S.; Ruff, E.F.; Behr, J.M.; Chodera, J.D.; Levinson, N.M. A water-mediated allosteric network governs activation of Aurora kinase A. *Nat. Chem. Biol.* 2017, 13: 402–408.
- Dai, W.; Wang, Q.; Liu, T.; Swamy, M.; Fang, Y.; Xie, S.; Mahmood, R.; Yang, Y.M.; Xu, M.; Rao, C.V. Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Res.* 2004, 64, 440–445.
- Dancheck, B.; Ragusa, M.J.; Allaire, M.; Nairn, A.C.; Page, R.; Peti, W. Molecular Investigations of the Structure and Function of the Protein Phosphatase 1-Spinophilin-Inhibitor 2 Heterotrimeric Complex. *Biochemistry* 2011, 50: 1238–1246.
- Daum, J.R.; Potapova, T.A.; Sivakumar, S.; Daniel, J.J.; Flynn, J.N.; Rankin, S.; Gorbsky, G.J. Cohesion Fatigue Induces Chromatid Separation in Cells Delayed at Metaphase. *Curr Biol.* 2011, 21(12): 1018-1024.
- Davey, N.E.; Cyert, M.S.; Moses, A.M. Short linear motifs—ex nihilo evolution of protein regulation. *Cell Commun. Signal.* 2015, 13: 43.
- Davey, N.E.; Van Roey, K.; Weatheritt, R.J.; Toedt, G.; Uyar, B.; Altenberg, B.; Budd, A.; Diella, F.; Dinkel, H.; Gibson, T.J. Attributes of short linear motifs. *Mol. Biosyst.* 2012, 8: 268–281.

- De Antoni, A.; Pearson, C.G.; Cimini, D.; Canman, J.C.; Sala, V.; Nezi, L.; Mapelli, M.; Sironi, L.; Faretta, M.; Salmon, E.D.; Musacchio, A. The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. *Curr. Biol.* 2005, 15(3): 214–225.
- de Castro, I.J.; Gil, R.S.; Ligammari, L.; Di Giacinto, M.L.; Vagnarelli, P. CDK1 and PLK1 coordinate the disassembly and reassembly of the nuclear envelope in vertebrate mitosis. *Oncotarget* 2017, 9: 7763–7773.
- de Castro, I.J.; Gokhan, E.; Vagnarelli, P. Resetting a functional G1 nucleus after mitosis. *Chromosoma*. 2016, 125: 607–619.
- De Souza, C.P.; Ellem, K.A.; Gabrielli, B.G. Centrosomal and cytoplasmic Cdc2/cyclinB1 activation precedes nuclear mitotic events. *Exp. Cell Res.* 2000, 257: 11–21.
- De Wever, V.; Nasa, I.; Chamousset, D.; Lloyd, D.; Nimick, M.; Xu, H.; Trinkle-Mulcahy, L.; Moorhead, G.B. The human mitotic kinesin KIF18A binds protein phosphatase 1 (PP1) through a highly conserved docking motif. *Biochem. Biophys. Res. Commun.* 2014, 453: 432–437.
- Deana, A.D.; Marchiori, F.; Meggio, F.; Pinna, L.A. Dephosphorylation of synthetic phosphopeptides by protein phosphatase-T, a phosphothreonyl protein phosphatase. *J. Biol. Chem.* 1982, 257: 8565–8568.
- Deana, A.D.; Pinna, L.A. Identification of pseudo ‘phosphothreonyl-specific’ protein phosphatase T with a fraction of polycation-stimulated protein phosphatase 2A. *Biochim. Biophys. Acta* 1988, 968: 179–185.
- Deardorff, M.A.; Wilde, J.J.; Albrecht, M.; Dickinson, E.; Tennstedt, S.; Braunholz, D.; Mönnich, M.; Yan, Y.; Xu, W.; Gil-Rodríguez, M.C.; et al. RAD21 mutations cause a human cohesinopathy. *Am. J. Hum. Genet.* 2012, 90: 1014–1027.
- Decarreau, J.; Wagenbach, M.; Lynch, E.; Halpern, A.R.; Vaughan, J.C.; Kollman, J.; Wordeman, L. The tetrameric kinesin Kif25 suppresses pre-mitotic centrosome separation to establish proper spindle orientation. *Nat. Cell Biol.* 2017, 19: 384–390.
- DeLuca, J.G.; Gall, W.E.; Ciferri, C.; Cimini, D.; Musacchio, A.; Salmon, E.D. Kinetochore Microtubule Dynamics and Attachment Stability Are Regulated by Hec1. *Cell* 2006, 127: 969–982.
- Denu, J.M.; Dixon, J.E. A catalytic mechanism for the dual-specific phosphatases. *Proc.Natl.Acad.Sci.USA.* 1995, 92: 5910–5914.
- Denu, J.M.; Stuckey, J.A.; Saper, M.A.; Dixon, J.E. Formand function in protein dephosphorylation. *Cell.* 1996, 87: 361–364.
- di Pietro, F.; Echard, A.; Morin, X. Regulation of mitotic spindle orientation: An integrated view. *EMBO Rep.* 2016, 17: 1106–1130.
- Dick, A.E.; Gerlich, D.W. Kinetic framework of spindle assembly checkpoint signalling. *Nat. Cell Biol.* 2013, 15: 1370–1377.
- Diril, M.K.; Bisteau, X.; Kitagawa, M.; Caldez, M.J.; Wee, S.; Gunaratne, J.; Lee, S.H.; Kaldis, P. Loss of the Greatwall Kinase Weakens the Spindle Assembly Checkpoint. *PLoS Genet.* 2016, 12: e1006310.
- Dodson, C.A.; Bayliss, R. Activation of Aurora-A kinase by protein partner binding and phosphorylation are independent and synergistic. *J. Biol. Cell* 2012, 287: 1150–1157.

- Dohadwala, M.; e Silva, E.D.C.; Hall, F.L.; Williams, R.T.; Carbonaro-Hall, D.A.; Nairn, A.C.; Greengard, P.; Berndt, N. Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. *Proc. Natl. Acad. Sci. USA* 1994, 91: 6408–6412.
- Dombrádi, V.; Axton, M.; Brewis, N.D.; da Cruz e Silva, E.F.; Alphey, L.; Cohen, P.T.W. *Drosophila* contains three genes that encode distinct isoforms of protein phosphatase 1. *European Journal of Biochem.* 1990, 194(3): 739-745.
- Donzelli, M.; Squatrito, M.; Ganioth, D.; Hershko, A.; Pagano, M.; Draetta, G.F. Dual mode of degradation of Cdc25 A phosphatase. *EMBO J.* 2002, 21: 4875–4884.
- Dreier, M.R.; Bekier, M.E., 2nd.; Taylor, W.R. Regulation of sororin by Cdk1-mediated phosphorylation. *J. Cell Sci.* 2011, 124: 2976–2987.
- Drpic, D.; Pereira, A.J.; Barisic, M.; Maresca, T.; Maiato, H. Polar Ejection Forces Promote the Conversion from Lateral to End-on Kinetochore-Microtubule Attachments on Mono-oriented Chromosomes. *Cell Reports.* 2015, 13(3): 460-468.
- Du, Q.; Macara, I.G. Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. *Cell.* 2004, 119: 503–516.
- Duckworth, B.C.; Weaver, J.S.; Ruderman, J.V. G2 arrest in *Xenopus* oocytes depends on phosphorylation of Cdc25 by protein kinase A. *Proc. Natl. Acad. Sci. USA.* 2002, 99: 16794–16799.
- Dumitru, A.M.G.; Rusin, S.F.; Clark, A.E.; Kettenbach, A.N.; Compton, D.A. Cyclin A/Cdk1 modulates Plk1 activity in prometaphase to regulate kinetochore-microtubule attachment stability. *Elife.* 2017, 6: e29303.
- Dutertre, S.; Cazales, M.; Quaranta, M.; Froment, C.; Trabut, V.; Dozier, C.; Mirey, G.; Bouché, J.; Theis-Febvre, N.; Schmitt, E.; et al. Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G2–M transition. *J. Cell Sci.* 2004, 117: 2523–2531.
- Dynlacht, B.D.; Moberg, K.; Lees, J.A.; Harlow, E.; Zhu, L. Specific regulation of E2F family members by -dependent kinases. *Mol Cell Biol.* 1997, 17: 3867–3875.
- Egloff, M.P.; Cohen, P.T.; Reinemer, P.; Barford, D. Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* 1995, 254: 942–959.
- Egloff, M.P.; Johnson, D.F.; Moorhead, G.; Cohen, P.T.; Cohen, P.; Barford, D. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* 1997, 16: 1876–1887.
- Ehlen, A.; Martin, C.; Miron, S.; Julien, M.; Theillet, F.X.; Ropars, V.; Sessa, G.; Beaupere, R.; Boucherit, V.; Duchambon, P.; Marjou, A.E.; Zinn-Justin, S.; Carreira, A. Proper chromosome alignment depends on BRCA2 phosphorylation by PLK1. *Nat. Comm.* 2020, 11: 1819
- Eichinger, C.S.; Kurze, A.; Oliveira, R.A.; Nasmyth, K. Disengaging the Smc3/kleisin interface releases cohesin from *Drosophila* chromosomes during interphase and mitosis. *EMBO J.* 2013, 32: 656–665.
- Eiteneuer, A.; Seiler, J.; Weith, M.; Beullens, M.; Lesage, B.; Krenn, V.; Musacchio, A.; Bollen, M.; Meyer, H. Inhibitor-3 ensures bipolar mitotic spindle attachment by limiting

- association of SDS22 with kinetochore-bound protein phosphatase-1. *EMBO J.* 2014, 33: 2704–2720.
- Elowe, S.; Hümmer, S.; Uldschmid, A.; Li, X.; Nigg, E.A. Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore–microtubule interactions. *Genes Dev.* 2007, 21: 2205–2219.
- Espert, A.; Uluocak, P.; Bastos, R.N.; Mangat, D.; Graab, P.; Gruneberg, U. PP2A-B56 opposes Mps1 phosphorylation of Knl1 and thereby promotes spindle assembly checkpoint silencing. *J. Cell Biol.* 2014, 206: 833–842.
- Espeut, J.; Cheerambathur, D.K.; Krenning, L.; Oegema, K.; Desai, A. Microtubule binding by KNL-1 contributes to spindle checkpoint silencing at the kinetochore. *J. Cell Biol.* 2012, 196: 469–482.
- Espeut, J.; Lara-Gonzalez, P.; Sassine, M.; Shiau, A.K.; Desai, A.; Abrieu, A. Natural loss of Mps1 kinase in nematodes uncovers a role for polo-like kinase 1 in spindle checkpoint initiation. *Cell Rep.* 2015, 12: 58–65.
- Etemad B., Kops G.J. Attachment issues: kinetochore transformations and spindle checkpoint silencing. *Curr. Opin. Cell Biol.* 2016, 39: 101–108.
- Etemad B.; Vertesy A.; Kuijt T.E.F.; Sacristan C.; van Oudenaarden A.; Kops G. Spindle checkpoint silencing at kinetochores with submaximal microtubule occupancy. *J. Cell Sci.* 2019, 132(12): 231589
- Eto, M.; Elliot, E.; Prickett, T.D.; Brautigan, D.L. Inhibitor-2 regulates protein phosphatase-1 complexed with NimA-related kinase to induce centrosome separation. *J. Biol. Chem.* 2002, 277: 44013–44020.
- Eto, M.; Kirkbride, J.; Elliott, E.; Lo, S.H.; Brautigan, D.L. Association of the tensin N-terminal protein-tyrosine phosphatase domain with the α isoform of protein phosphatase-1 in focal adhesions. *J. Biol. Chem.* 2007, 282: 17806–17815.
- Eyers, P.A.; Erikson, E.; Chen, L.G.; Maller, J.L. A novel mechanism for activation of the protein kinase Aurora A. *Curr. Biol.* 2003, 13: 691–697.
- Faesen, A.C.; Thanasoula, M.; Maffini, S.; Breit, C.; Müller, F.; van Gerwen, S.; Bange, T.; Musacchio, A. Basis of catalytic assembly of the mitotic checkpoint complex. *Nature* 2017, 542: 498–502.
- Fang, G. Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. *Mol. Biol. Cell* 2002, 13: 755–766.
- Fang, G.; Yu, H.; Kirschner, M.W. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* 1998, 12: 1871–1883.
- Faragher, A.J.; Fry, A.M. Nek2 A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol. Biol. Cell* 2003, 14: 2876–2889.
- Fauman, E.B.; Cogswell, J.P.; Lovejoy, B.; Rocque, W.J.; Holmes, W.; Montana, V.G.; Piwnicka-Worms, H.; Rink, M.J.; Saper, M.A. Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A. *Cell* 1998, 93: 617–625.

- Favreau, C.; Worman, H.J.; Wozniak, R.W.; Frappier, T.; Courvalin, J.C. Cell cycle-dependent phosphorylation of nucleoporins and nuclear pore membrane protein Gp210. *Biochemistry* 1996, 35: 8035–8044.
- Fedoryshchak, R.O.; Přečková, M.; Butler, A.M.; Lee, R.; O'Reilly, N.; Flynn, H.R.; Snijders, A.P.; Eder, N.; Ultanir, S.; Mouilleron, S.; Treisman, R. Molecular basis for substrate specificity of the Phactr1/PP1 phosphatase holoenzyme. *Elife*. 2020, 9: e61509.
- Fernandez, A.G.; Piano, F. MEL-28 is downstream of the Ran cycle and is required for nuclear-envelope function and chromatin maintenance. *Curr. Biol.* 2006, 16: 1757–1763.
- Fischer, E.S.; Yu, C.H.; Bellini, D.; McLaughlin, S.H.; Orr, C.M.; Wagner, A.; Freund, S.M.V.; Barford, D. Molecular mechanism of Mad1 kinetochore targeting by phosphorylated Bub1. *EMRO Rep.* 2021, 22(7): e52242.
- Fischer, E.S.; Yu, C.W.H.; Hevler, J.F.; McLaughlin, S.H.; Maslen, S.L.; Heck, A.J.R.; Freund, S.M.V.; Barford, D. Juxtaposition of Bub1 and Cdc20 on phosphorylated Mad1 during catalytic mitotic checkpoint complex assembly. *Nat Commun* 2022, 13: 6381.
- Fischle, W.; Tseng, B.S.; Dormann, H.L.; Ueberheide, B.M.; Garcia, B.A.; Shabanowitz, J.; Hunt, D.F.; Funabiki, H.; Allis, C.D. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* 2005, 438: 1116–1122.
- Foijer F., Xie S.Z., Simon J.E., Bakker P.L., Conte N., Davis S.H., Kregel E., Jonkers J., Bradley A., Sorger P.K. Chromosome instability induced by Mps1 and p53 mutation generates aggressive lymphomas exhibiting aneuploidy-induced stress. *PNAS*. 2014, 111: 13427–13432.
- Foley, E.A.; Kapoor, T.M. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nat. Rev. Mol. Cell Biol.* 2013, 14: 25–37.
- Foley, E.A.; Maldonado, M.; Kapoor, T.M. Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nat. Cell Biol.* 2011, 13: 1265–1271.
- Franz, C.; Walczak, R.; Yavuz, S.; Santarella, R.; Gentzel, M.; Askjaer, P.; Galy, V.; Hetzer, M.; Mattaj, I.W.; Antonin, W. MEL-28/ELYS is required for the recruitment of nucleoporins to chromatin and postmitotic nuclear pore complex assembly. *EMBO Rep.* 2007, 8: 165–172.
- Friedel, A.M.; Pike, B.L.; Gasser, S.M. ATR/ Mec1: coordinating fork stability and repair. *Curr Opin Cell Biol.* 2009, 21: 237–244.
- Fry, A.M.; Arnaud, L.; Nigg, E.A. Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J. Biol. Chem.* 1999, 274, 16304–16310.
- Fry, A.M.; Mayor, T.; Meraldi, P.; Stierhof, Y.D.; Tanaka, K.; Nigg, E.A. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle regulated protein kinase Nek2. *Cell Biol.* 1998, 141, 1563–1574.
- Fung, T.K.; Poon, R.Y. A roller coaster ride with the mitotic cyclins. *Semin Cell Dev Biol.* 2005, 16: 335–342.
- Furnari, B.; Blasina, A.; Boddy, M.N.; McGowan, C.H.; Russell, P. Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. *Mol. Biol. Cell* 1999, 10, 833–845.

- Furnari, B.; Rhind, N.; Russell, P. Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. *Science* 1997, 277, 1495–1497.
- Gabrielli, B.G.; Clark, J.M.; McCormack, A.K.; Ellem, K.A.O. Ultraviolet light-induced G2 phase cell cycle checkpoint blocks cdc25-dependent progression into mitosis. *Oncogene* 1997, 15: 749–758.
- Gabrielli, B.G.; De Souza, C.P.; Tonks, I.D.; Clark, J.M.; Hayward, N.K.; Ellem, K.A. Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells. *J. Cell Sci.* 1996, 109: 1081–1093.
- Gaitanos, T.N.; Santamaria, A.; Jeyaprakash, A.A.; Wang, B.; Conti, E.; Nigg, E.A. Stable kinetochore–microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *EMBO J.* 2009, 28: 1442–1452.
- Galaktionov, K.; Beach, D. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: Evidence for multiple roles of mitotic cyclins. *Cell* 1991, 67: 1181–1194.
- Gallini, S.; Carminati, M.; De Mattia, F.; Pirovano, L.; Martini, E.; Oldani, A.; Asteriti, I.A.; Guarguaglini, G.; Mapelli, M. NuMA Phosphorylation by Aurora-A Orchestrates Spindle Orientation. *Curr. Biol.* 2016, 26: 458–469.
- Galy, V.; Askjaer, P.; Franz, C.; López-Iglesias, C.; Mattaj, I.W. MEL-28, a novel nuclear-envelope and kinetochore protein essential for zygotic nuclear-envelope assembly in *C. elegans*. *Curr. Biol.* 2006, 16: 1748–1756.
- Gandhi, R.; Gillespie, P.J.; Hirano, T. Human Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase. *Curr. Biol.* 2006, 16: 2406–2417.
- Garcia, L.; Garcia, F.; Llorens, F.; Unzeta, M.; Itarte, E.; Gómez, N. PP1/PP2A phosphatases inhibitors okadaic acid and calyculin A block ERK5 activation by growth factors and oxidative stress. *FEBS Letters*. 2002, 523: 90–94.
- Garner-Hamrick, P.A.; Fisher, C. Antisense phosphorothioate oligonucleotides specifically down-regulate cdc25B causing S-phase delay and persistent antiproliferative effects. *Int. J. Cancer* 1998, 76: 720–728.
- Gergely, F. Centrosomal TACCtics. *Bioessays* 2002, 24: 915–925.
- Gergely, F.; Draviam, V.M.; Raff, J.W. The ch-TOG/XMAP215 protein is essential for spindle pole organization in human somatic cells. *Genes Dev.* 2003, 17: 336–341.
- Gharbi-Ayachi, A.; Labbé, J.C.; Burgess, A.; Vigneron, S.; Strub, J.M.; Brioudes, E.; Van-Dorselaer, A.; Castro, A.; Lorca, T. The Substrate of Greatwall Kinase, Arpp19, Controls Mitosis by Inhibiting Protein Phosphatase 2A. *Science* 2010, 330: 1673–1677.
- Gheghiani, L.; Loew, D.; Lombard, B.; Mansfeld, J.; Gavet, O. PLK1 activation in late G2 sets up commitment to mitosis. *Cell Rep.* 2017, 19: 2060–2073.
- Ghosh, S.; Paweletz, N.; Schroeter, D. Cdc2-independent induction of premature mitosis by okadaic acid in HeLa cells. *Exp. Cell Res.* 1998, 242: 1–9.
- Gibbons, J.A.; Kozubowski, L.; Tatchell, K.; Shenolikar, S. Expression of human protein phosphatase-1 in *Saccharomyces cerevisiae* highlights the role of phosphatase isoforms in regulating eukaryotic functions. *J. Biol. Chem.* 2007, 282: 21838–21847.

- Giet, R.; McLean, D.; Descamps, S.; Lee, M.J.; Raff, J.W.; Prigent, C.; Glover, D.M. *Drosophila* Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. *J. Cell Biol.* 2002, 156: 437–451.
- Gillespie, P.J.; Khoudoli, G.A.; Stewart, G.; Swedlow, J.R.; Blow, J.J. ELYS/MEL-28 chromatin association coordinates nuclear pore complex assembly and replication licensing. *Curr. Biol.* 2007, 17, 1657–1662.
- Giménez-Abián, J.F.; Sumara, I.; Hirota, T.; Hauf, S.; Gerlich, D.; de la Torre, C.; Ellenberg, J.; Peters, J.M. Regulation of sister chromatid cohesion between chromosome arms. *Curr. Biol.* 2004, 14, 1187–1193.
- Glavy, J.S.; Krutchinsky, A.N.; Cristea, I.M.; Berke, I.C.; Boehmer, T.; Blobel, G.; Chait, B.T. Cell-cycle-dependent phosphorylation of the nuclear pore Nup107-160 subcomplex. *Proc. Natl. Acad. Sci. USA* 2007, 104, 3811–3816.
- Godfrey, M.; Touati, S.A.; Kataria, M.; Jones, A.; Snijders, A.P.; Uhlmann, F. PP2ACdc55 Phosphatase Imposes Ordered Cell-Cycle Phosphorylation by Opposing Threonine Phosphorylation. *Mol. Cell* 2017, 65, 393–402.
- Goldberg, J.; Huang, H.B.; Kwon, Y.G.; Greengard, P.; Nairn, A.C.; Kuriyan, J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* 1995, 376, 745–753.
- Gomes, J.E.; Tavernier, N.; Richaudeau, B.; Formstecher, E.; Boulín, T.; Mains, P.E.; Dumont, J.; Pintard, L. Microtubule severing by the katanin complex is activated by PPFR-1-dependent MEI-1 dephosphorylation. *J. Cell Biol.* 2013, 202, 431–439.
- Gorbsky, G.J. Cohesion fatigue. *Curr. Biol.* 2013, 23, 986–988.
- Gorbsky, G.J.; Ricketts, W.A. Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Biol.* 1993, 122: 1311–1321.
- Gorjánácz, M.; Klerkx, E.P.; Galy, V.; Santarella, R.; López-Iglesias, C.; Askjaer, P.; Mattaj, I.W. *Caenorhabditis elegans* BAF-1 and its kinase VRK-1 participate directly in post-mitotic nuclear envelope assembly. *EMBO J.* 2007, 26, 132–143.
- Grallert, A.; Boke, E.; Hagting, A.; Hodgson, B.; Connolly, Y.; Griffiths, J.R.; Smith, D.L.; Pines, J.; Hagan, I.M. A PP1–PP2A phosphatase relay controls mitotic progression. *Nature* 2015, 517, 94–98.
- Graser, S.; Stierhof, Y.D.; Nigg, E.A. Cep68 and Cep215 (Cdk5rap2) are required for centrosome cohesion. *J. Cell Sci.* 2007, 120, 4321–4331.
- Groen, A.C.; Cameron, L.A.; Coughlin, M.; Miyamoto, D.T.; Mitchison, T.J.; Ohi, R. XRHAMM functions in ran-dependent microtubule nucleation and pole formation during an astral spindle assembly. *Curr. Biol.* 2004, 14, 1801–1811.
- Groves, M.R.; Hanlon, N.; Turowski, P.; Hemmings, B.A.; Barford, D. The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. *Cell* 1999, 96, 99–110.
- Gruss, O.J.; Carazo-Salas, R.E.; Schatz, C.A.; Guarguaglini, G.; Kast, J.; Wilm, M.; Le Bot, N.; Vernos, I.; Karsenti, E.; Mattaj, I.W. Ran induces spindle assembly by reversing the inhibitory effect of importin α on TPX2 activity. *Cell* 2001, 104, 83–93.

- Gudimchuk, N.; Vitre, B.; Kim, Y.; Kiyatkin, A.; Cleveland, D.W.; Ataullakhanov, F.I.; Grishchuk, E.L. Kinetochore kinesin CENP-E is a processive bi-directional tracker of dynamic microtubule tips. *Nat. Cell Biol.* 2013, 15, 1079–1088.
- Guimarães, G.J.; Dong, Y.; McEwen, B.F.; DeLuca, J.G. Kinetochore-Microtubule Attachment Relies on the Disordered N-Terminal Tail Domain of Hec1. *Curr. Biol.* 2008, 18, 1778–1784.
- Gutiérrez-Caballero, C.; Cebollero, L.R.; Pendás, A.M. Shugoshins: From protectors of cohesion to versatile adaptors at the centromere. *Trends Genet.* 2012, 28, 351–360.
- Güttinger, S.; Laurell, E.; Kutay, U. Orchestrating nuclear envelope disassembly and reassembly during mitosis. *Nat. Rev. Mol. Cell Biol.* 2009, 10, 178–191.
- Haarhuis, J.H.; Elbatsh, A.M.O.; van den Broek, B.; Camps, D.; Erkan, H.; Jalink, K.; Medema, R.H.; Rowland, B.D. WAPL-mediated removal of cohesin protects against segregation errors and aneuploidy. *Curr. Biol.* 2013, 23, 2071–2077.
- Haarhuis, J.H.I.; Elbatsh, A.M.O.; Rowland, B.D. Cohesin and its regulation: On the logic of X-shaped chromosomes. *Dev. Cell* 2014, 31, 7–18.
- Haering, C.H.; Löwe, J.; Hochwagen, A.; Nasmyth, K. Molecular architecture of SMC proteins and the yeast cohesion complex. *Mol. Cell* 2002, 9, 773–788.
- Häfner, J.; Mayr, M.I.; Möckel, M.M.; Mayer, T.U. Pre-anaphase chromosome oscillations are regulated by the antagonistic activities of Cdk1 and PP1 on Kif18A. *Nat. Commun.* 2014, 5, 4397.
- Hames, R.S.; Wattam, S.L.; Yamano, H.; Bacchieri, R.; Fry, A.M. APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *EMBO J.* 2001, 20, 7117–7127.
- Han, X.; Gomes, J.E.; Birmingham, C.L.; Pintard, L.; Sugimoto, A.; Mains, P.E. The role of protein phosphatase 4 in regulating microtubule severing in the *Caenorhabditis elegans* embryo. *Genetics* 2009, 181, 933–943.
- Hanisch, A.; Silljé, H.H.; Nigg, E.A. Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1 and Ska2. *EMBO J.* 2006, 25, 5504–5515.
- Hara, M.; Abe, Y.; Tanaka, T.; Yamamoto, T.; Okumura, E.; Kishimoto, T. Greatwall kinase and cyclin B-Cdk1 are both critical constituents of M-phase-promoting factor. *Nat. Commun.* 2012, 3, 1059.
- Hardy, T.; Lee, M.; Hames, R.S.; Prosser, S.L.; Cheary, D.M.; Samant, M.D.; Schultz, F.; Baxter, J.E.; Rhee, K.; Fry, A.M. Multisite phosphorylation of C-Nap1 releases it from Cep135 to trigger centrosome disjunction. *J. Cell Sci.* 2014, 127, 2493–2506.
- Harel, A.; Chan, R.C.; Lachish-Zalait, A.; Zimmerman, E.; Elbaum, M.; Forbes, D.J. Importin beta negatively regulates nuclear membrane fusion and nuclear pore complex assembly. *Mol. Biol. Cell* 2003, 14, 4387–4396.
- Hattersley, N.; Cheerambathur, D.; Moyle, M.; Stefanutti, M.; Richardson, A.; Lee, K.Y.; Dumont, J.; Oegema, K.; Desai, A. A Nucleoporin Docks Protein Phosphatase 1 to Direct Meiotic Chromosome Segregation and Nuclear Assembly. *Dev. Cell* 2016, 38, 463–477.

- Hauf, S.; Roitinger, E.; Koch, B.; Dittrich, C.M.; Mechtler, K.; Peters, J.M. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. *PLoS Biol.* 2005, 3, e69.
- He, R.; Huang, N.; Bao, Y.; Zhou, H.; Teng, J.; Chen, J. LRRC45 is a centrosome linker component required for centrosome cohesion. *Cell Rep.* 2013, 4, 1100–1107.
- Heald, R.; McKeon, F. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 1990, 61, 579–589.
- Heim, A.; Konietzny, A.; Mayer, T.U. Protein phosphatase 1 is essential for Greatwall in activation at mitotic exit. *EMBO Rep.* 2015, 16, 1501–1510.
- Hein, J.B.; Hertz, E.P.T.; Garvanska, D.H.; Kruse, T.; Nilsson, J. Distinct kinetics of serine and threonine dephosphorylation are essential for mitosis. *Nat. Cell Biol.* 2017, 19, 1433–1440.
- Heinrich S.; Sewart K.; Windecker H.; Langeegger M.; Schmidt N.; Hustedt N.; Hauf S. Mad1 contribution to spindle assembly checkpoint signalling goes beyond presenting Mad2 at kinetochores. *EMBO Rep.* 2014, 15(3): 291–298.
- Helgeson, L.A.; Zelter, A.; Riffle, M.; MacCoss, M.J.; Asbury, C.L.; Davis, T.N. Human Ska complex and Ndc80 complex interact to form a load-bearing assembly that strengthens kinetochore–microtubule attachments. *Proc. Nat. Acad. Sci. USA* 2018, 115, 2740–2745.
- Helps, N.R.; Luo, X.; Barker, H.M.; Cohen, P.T. NIMA-related kinase2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem. J.* 2000, 349, 509–518.
- Hengeveld, R.C.; Vromans, M.J.; Vleugel, M.; Hadders, M.A.; Lens, S.M. Inner centromere localization of the CPC maintains centromere cohesion and allows mitotic checkpoint silencing. *Nat. Commun.* 2017, 8, 15542.
- Henley, S.A.; Dick, F.A. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Div* 2012, 7:10.
- Hermeking, H.; Benzinger, A. 14-3-3 proteins in cell cycle regulation. *Semin. Cancer Biol.* 2006, 16, 183–192.
- Heroes, E.; Lesage, B.; Görnemann, J.; Beullens, M.; Van Meervelt, L.; Bollen, M. The PP1 binding code: A molecular-lego strategy that governs specificity. *FEBS J.* 2013, 280, 584–595.
- Heroes, E.; Rip, J.; Beullens, M.; Van Meervelt, L.; De Gendt, S.; Bollen, M. Metals in the active site of native protein phosphatase-1. *J. Inorg. Biochem.* 2015, 149, 1–5.
- Herriott, A.; Sweeney, M.; Whitaker, M.; Taggart, M.; Huang, J.Y. Kinetochore localized Mad2 and Cdc20 is itself insufficient for triggering the mitotic checkpoint when Mps1 is low in *Drosophila melanogaster* neuroblasts. *Cell Cycle.* 2012, 11:4650–4660.
- Hertz, E.P.T.; Kruse, T.; Davey, N.E.; López-Méndez, B.; Sigurðsson, J.O.; Montoya, G.; Olsen, J.V.; Nilsson, J. A Conserved Motif Provides Binding Specificity to the PP2A-B56 Phosphatase. *Mol. Cell* 2016, 63, 686–695.
- Hewitt, L.; Tighe, A.; Santaguida, S.; White, A.M.; Jones, C.D.; Musacchio, A.; Green, S.; Taylor, S.S. Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. *J. Cell Biol.* 2010, 190, 25–34.

- Hinchcliffe, E.H.; Sluder, G. "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. *Genes Dev.* 2001, 15:1167–1181.
- Hirano, K.; Phan, B.C.; Hartshorne, D.J. Interactions of the subunits of smooth muscle myosin phosphatase. *J. Biol. Chem.* 1997, 272, 3683–3688.
- Hiruma, Y.; Sacristan, C.; Pachis, S.T.; Adamopoulos, A.; Kuijt, T.; Ubbink, M.; von Castelmur, E.; Perrakis, A.; Kops, G.J. CELL DIVISION CYCLE. Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling. *Science* 2015, 348, 1264–1267.
- Hitosugi, T.; Kang, S.; Vander Heiden, M.G.; Chung, T.W.; Elf, S.; Lythgoe, K.; Dong, S.; Lonial, S.; Wang, X.; Chen, G.Z.; et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2009, 2, ra73.
- Hoelz, A.; Debler, E.W.; Blobel, G. The structure of the nuclear pore complex. *Annu. Rev. Biochem.* 2011, 80, 613–643.
- Holland, A.J.; Cleveland, D.W. Chromoanagenesis and cancer: mechanisms and consequences of localized, complex chromosomal rearrangements. *Nat Med.* 2012, 18(11): 1630-8.
- Honda, R.; Korner, R.; Nigg, E.A. Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. *Mol. Biol. Cell* 2003, 14, 3325–3341.
- Hood, E.A.; Kettenbach, A.N.; Gerber, S.A.; Compton, D.A. Plk1 regulates the kinesin-13 protein Kif2b to promote faithful chromosome segregation. *Mol. Biol. Cell* 2012, 23, 2264–2274.
- Howell, B.J.; Moree, B.; Farrar, E.M.; Stewart, S.; Fang, G.; Salmon, E.D. Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr. Biol.* 2004, 14: 953–964.
- Hoyt, M.A.; Totis, L.; Roberts, B.T. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell.* 1991, 66:507-517.
- Huang, H.; Feng, J.; Famulski, J.; Rattner, J.B.; Liu, S.T.; Kao, G.D.; Muschel, R.; Chan, G.K.; Yen, T.J. Tripin/hSgo2 recruits MCAK to the inner centromere to correct defective kinetochore attachments. *J. Cell Biol.* 2007, 177, 413–424.
- Huang, H.; Hittle, J.; Zappacosta, F.; Annan, R.S.; Hershko, A.; Yen, T.J. Phosphorylation sites in BubR1 that regulate kinetochore attachment, tension, and mitotic exit. *J. Cell Biol.* 2008, 183, 667–680.
- Hurley, T.D.; Yang, J.; Zhang, L.; Goodwin, K.D.; Zou, Q.; Cortese, M.; Dunker, A.K.; DePaoli-Roach, A.A. Structural basis for regulation of protein phosphatase 1 by inhibitor-2. *J. Biol. Chem.* 2007, 282, 28874–28883.
- Hwang, L.H.; Lau, L.F.; Smith, D.L.; Mistrot, C.A.; Hardwick, K.G.; Hwang, E.S.; Amon, A.; Murray, A.W. Budding yeast Cdc20: a target of the spindle checkpoint. *Science* 1998, 279: 1041–1044.
- Iemura, K.; Tanaka, K. Chromokinesin Kid and kinetochore kinesin CENP-E differentially support chromosome congression without end-on attachment to microtubules. *Nat. Commun.* 2015, 6, 6447.
- Ikeda, M.; Tanaka, K. Plk1 bound to Bub1 contributes to spindle assembly checkpoint activity during mitosis. *Sci Rep.* 2017, 7, 8794.

- Imaoka, T.; Lynham, J.A.; Haslam, R.J. Purification and characterization of the 47,000-dalton protein phosphorylated during degranulation of human platelets. *J. Biol. Chem.* 1983, 258, 11404–11414.
- Ingebritsen, T.S.; Cohen, P. Protein phosphatases: Properties and role in cellular regulation. *Science* 1983, 221, 331–338.
- Itoh, G.; Ikeda, M.; Iemura, K.; Amin, M.A.; Kuriyama, S.; Tanaka, M.; Mizuno, N.; Osakada, H.; Haraguchi, T.; Tanaka, K. Lateral attachment of kinetochores to microtubules is enriched in prometaphase rosette and facilitates chromosome alignment and bi-orientation establishment. *Sci. Rep.* 2018, 8, 3888.
- Iwanaga, Y.; Chi, Y.H.; Miyazato, A.; Sheleg, S.; Haller, K.; Peloponese, J.M.Jr.; Li, Y.; Ward, J.M.; Benezra, R.; Jeang, K.T. Heterozygous deletion of mitotic arrest-deficient protein 1 (MAD1) increases the incidence of tumors in mice. *Cancer Res.* 2007, 67, 160–166.
- Jackman, M.; Marcozzi, C.; Barbiero, M.; Pardo, M.; Yu, L.; Tyson, A.L.; Choudhary, J.S.; Pines, J. Cyclin B1-Cdk1 facilitates MAD1 release from the nuclear pore to ensure a robust spindle checkpoint. *J. Cell Biol.* 2020, 219(6): 201907082.
- Jain, I.; Tran, P.T. Prolongation of mitosis is associated with enhanced endogenous DNA damage in fission yeast. *microPublication Biology.* 2023.
- Janczyk, P.Ł.; Skorupka, K.A.; Tooley, J.G.; Matson, D.R.; Kestner, C.A.; West, T.; Pornillos, O.; Stukenberg, P.T. Mechanism of Ska recruitment by Ndc80 complexes to kinetochores. *Dev. Cell* 2017, 41, 438–449.
- Janssens, V.; Goris, J. Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* 2001, 353, 417–439.
- Jelluma, N.; Brenkman, A.B.; McLeod, I.; Yates, J.R., 3rd.; Cleveland, D.W.; Medema, R.H.; Kops, G.J. Chromosomal instability by inefficient Mps1 auto-activation due to a weakened mitotic checkpoint and lagging chromosomes. *PLoS ONE* 2008, 3, e2415. (a)
- Jelluma, N.; Brenkman, A.B.; van den Broek, N.J.; Cruijssen, C.W.; van Osch, M.H.; Lens, S.M.; Medema, R.H.; Kops, G.J. Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. *Cell* 2008, 132, 233–246. (b)
- Jelluma, N.; Dansen, T.B.; Sliedrecht, T.; Kwiatkowski, N.P.; Kops, G.J. Release of Mps1 from kinetochores is crucial for timely anaphase onset. *The Journal of Cell Biology.* 2010, 191:281–290.
- Jeyapakash, A.A.; Santamaria, A.; Jayachandran, U.; Chan, Y.W.; Benda, C.; Nigg, E.A.; Conti, E. Structural and functional organization of the Ska complex, a key component of the kinetochore-microtubule interface. *Mol. Cell* 2012, 46, 274–286.
- Ji, W.; Luo, Y.; Ahmad, E.; Lio, S.-T. Direct interactions of mitotic arrest deficient 1 (MAD1) domains with each other and MAD2 conformers are required for mitotic checkpoint signaling. *J Cell Biol.* 2018, 293(2): 484–496.
- Ji, Z.; Gao, H.; Jia, L.; Li, B.; Yu, H. A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. *Elife* 2017, 6, e22513.

- Ji, Z.; Gao, H.; Yu, H. CELL DIVISION CYCLE. Kinetochore attachment sensed by competitive Mps1 and microtubule binding to Ndc80C. *Science* 2015, 348, 1260–1264.
- Jia, L.; Li, B.; Yu, H. The Bub1-Plk1 kinase complex promotes spindle checkpoint signalling through Cdc20 phosphorylation. *Nat. Commun.* 2016, 7, 10818.
- Jia, Z.; Barford, D.; Flint, A.J.; Tonks, N.K. Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science* 1995, 268, 1754–1758.
- Jiang, W.; Jimenez, G.; Wells, N.J.; Hope, T.J.; Wahl, G.M.; Hunter, T.; Fukunaga, R. PRC1: A human mitotic spindle-associated CDK substrate protein required for cytokinesis. *Mol. Cell* 1998, 2, 877–885.
- Jiang, Y.; Li, X.; Yang, W.; Hawke, D.H.; Zheng, Y.; Xia, Y.; Aldape, K.; Wei, C.; Guo, F.; Chen, Y.; et al. PKM2 regulates chromosome segregation and mitosis progression of tumor cells. *Mol. Cell* 2014, 53, 75–87.
- Jin, D.Y.; Spencer, F.; Jeang, K.T. Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell* 1998, 93: 81–91.
- Jinno, S.; Suto, K.; Nagata, A.; Igarashi, M.; Kanaoka, Y.; Nojima, H.; Okayama, H. Cdc25A is a novel phosphatase functioning early in the cell cycle. *EMBO J.* 1994, 13, 1549–1556.
- Joglekar, A.P. A Cell Biological Perspective on Past, Present and Future Investigations of the Spindle Assembly Checkpoint. *Biology* 2016, 5, 44.
- Joukov, V.; De Nicolo, A. Aurora-PLK1 cascades as key signaling modules in the regulation of mitosis. *Sci. Signal.* 2018, 11, eaar4195.
- Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S.A.A.; Ballard, A.J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstern, S.; Silver, D.; Vinyals, O.; Senior, A.W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021, 596: 583-589.
- Kabeche, L.; Compton, D.A. Cyclin A regulates kinetochore microtubules to promote faithful chromosome segregation. *Nature* 2013, 502, 110–113.
- Kakeno, M.; Matsuzawa, K.; Matsui, T.; Akita, H.; Sugiyama, I.; Ishidate, F.; Nakano, A.; Takashima, S.; Goto, H.; Inagaki, M.; et al. Plk1 phosphorylates CLIP-170 and regulates its binding to microtubules for chromosome alignment. *Cell Struct Funct.* 2014, 39, 45–59.
- Kalab, P.; Heald, R. The Ran GTP gradient — AGPS for the mitotic spindle. *J. Cell Sci.* 2008, 121, 1577–1586.
- Kallio, M.; Weinstein, J.; Daum, J.R.; Burke, D.J.; Gorbsky, G.J. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J. Cell Biol.* 1998, 141: 1393–1406.
- Kamenz J., Hauf S. Slow checkpoint activation kinetics as a safety device in anaphase. *Current Biology*. 2014, 24: 646–651.

- Kanemori, Y.; Uto, K.; Sagata, N. β -TrCP recognizes a previously undescribed non phosphorylated destruction motif in Cdc25A and Cdc25B phosphatases. *Proc. Natl. Acad. Sci. USA* 2005, 102, 6279–6284.
- Kang, J.; Chen, Y.; Zhao, Y.; Yu, H. Autophosphorylation-dependent activation of human Mps1 is required for the spindle checkpoint. *Proc. Natl. Acad. Sci. USA* 2007, 104, 20232–20237.
- Kapoor, T.M.; Lampson, M.A.; Hergert, P.; Cameron, L.; Cimini, D.; Salmon, E.D.; McEwen, B.F.; Khodjakov, A. Chromosomes Can Congress to the Metaphase Plate Before Biorientation. *Science* 2006, 311, 388–391.
- Karaïskou, A.; Jessus, C.; Brassac, T.; Ozon, R. Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J. Cell Sci.* 1999, 112, 3747–3756.
- Kaseda, K.; McAinsh, A.D.; Cross, R.A. Dual pathway spindle assembly increases both the speed and the fidelity of mitosis. *Biol. Open* 2012, 1, 12–18.
- Kelly, A.E.; Sampath, S.C.; Maniar, T.A.; Woo, E.M.; Chait, B.T.; Funabiki, H. Chromosomal Enrichment and Activation of the Aurora B Pathway Are Coupled to Spatially Regulate Spindle Assembly. *Dev. Cell* 2007, 12, 31–43.
- Kemmler S., Stach M., Knapp M., Ortiz J., Pfannstiel J., Ruppert T., Lechner J. Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling. *The EMBO Journal*. 2009, 28:1099–1110.
- Kettenbach, A.N.; Schlosser, K.A.; Lyons, S.P.; Nasa, I.; Gui, J.; Adamo, M.E.; Gerber, S.A. Global assessment of its network dynamics reveals that the kinase Plk1 inhibits the phosphatase PP6 to promote Aurora A activity. *Sci. Signal*. 2018, 11, eaaq1441.
- Khodjakov A., Pines J. Centromere tension: a divisive issue. *Nature Cell Biology*. 2010, 12:919–923.
- Kieffer, I.; Lorenzo, C.; Dozier, C.; Schmitt, E.; Ducommun, B. Differential mitotic degradation of the CDC25B phosphatase variants. *Oncogene* 2007, 26, 7847–7858.
- Kim, D.H.; Han, J.S.; Ly, P.; Ye, Q.; McMahon, M.A.; Myung, K.; Corbett, K.D.; Cleveland, D.W. TRIP13 and APC15 drive mitotic exit by turnover of interphase- and unattached kinetochore-produced MCC. *Nat Commun*. 2018, 19(9): 4354.
- Kim, H.; Stumpff, J.K. Kif18A promotes Hec1 dephosphorylation to coordinate chromosome alignment with kinetochore microtubule attachment. *bioRxiv* 2018.
- Kim, S.H.; Lin, D.P.; Matsumoto, S.; Kitazono, A.; Matsumoto, T. Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint. *Science* 1998, 279: 1045– 1047.
- Kim, S.J.; Ryu, S.E. Structure and catalytic mechanism of human protein tyrosine phosphatome. *BMB Rep*. 2012, 45, 693–699.
- Kim, T.; Lara-Gonzalez, P.; Prevo, B.; Meitinger, F.; Cheerambathur, D.K.; Oegema, K.; Desai, A. Kinetochores accelerate or delay APC/C activation by directing Cdc20 to opposing fates. *Genes Dev*. 2017, 31, 1089–1094.
- Kim, Y.; Holland, A.J.; Lan, W.; Cleveland, D.W. Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E. *Cell* 2010, 142, 444–455.

- Kinoshita, K.; Noetzel, T.L.; Pelletier, L.; Mechtler, K.; Drechsel, D.N.; Schwager, A.; Lee, M.; Raff, J.W.; Hyman, A.A. Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *J. Cell Biol.* 2005, 170, 1047–1055.
- Kitajima, T.S.; Hauf, S.; Ohsugi, M.; Yamamoto, T.; Watanabe, Y. Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization. *Curr. Biol.* 2005, 15, 353–359.
- Kitajima, T.S.; Sakuno, T.; Ishiguro, K.; Iemura, S.; Natsume, T.; Kawashima, S.A.; Watanabe, Y. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* 2006, 441, 46–52.
- Klebig C.; Korinth D.; Meraldi P. Bub1 regulates chromosome segregation in a kinetochore-independent manner. *J. Cell Biol.* 2009, 185(5): 841–858.
- Kotak, S.; Afshar, K.; Busso, C.; Gönczy, P. Aurora A kinase regulates proper spindle positioning in *C.elegans* and human cells. *J. Cell Sci.* 2016, 129, 3015–3025.
- Kotak, S.; Busso, C.; Gönczy, P. Cortical dynein is critical for proper spindle positioning in human cells. *J. Cell Biol.* 2012, 199, 97–110.
- Kotak, S.; Busso, C.; Gönczy, P. NuMA phosphorylation by CDK1 couples mitotic progression with cortical dynein function. *EMBO J.* 2013, 32, 2517–2529.
- Krek, W.; Xu, G.; Livingston, D.M. A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell.* 1995, 83:1149–1158.
- Kremmer, E.; Ohst, K.; Kiefer, J.; Brewis, N.; Walter, G. Separation of PP2A core enzyme and holoenzyme with monoclonal antibodies against the regulatory A subunit: Abundant expression of both forms in cells. *Mol. Cell. Biol.* 1997, 17, 1692–1701.
- Krenn V.; Overlack K.; Primorac I.; van Gerwen S.; Musacchio A. KI motifs of human Knl1 enhance assembly of comprehensive spindle checkpoint complexes around MELT repeats. *Curr. Biol.* 2014, 24(1): 29–39.
- Krenn, V.; Musacchio, A. The Aurora B kinase in chromosome bi-orientation and spindle checkpoint signaling. *Front. Oncol.* 2015, 5.
- Kristjánssdóttir, K.; Rudolph, J. Cdc25 phosphatases and cancer. *Chem. Biol.* 2004, 11, 1043–1051.
- Kruse T.; Larsen M.S.; Sedgwick G.G.; Sigurdsson J.O.; Streicher W.; Olsen J.V.; Nilsson J. A direct role of Mad1 in the spindle assembly checkpoint beyond Mad2 kinetochore recruitment. *EMBO Rep.* 2014, 15(3): 282–290.
- Kruse, T.; Zhang, G.; Larsen, M.S.Y.; Lischetti, T.; Streicher, W.; Nielsen, T.K.; Bjørn, S.P.; Nilsson, J. Direct binding between BubR1 and B56-PP2A phosphatase complexes regulate mitotic progression. *J. Cell Sci.* 2013, 126, 1086–1092.
- Kueng, S.; Hegemann, B.; Peters, B.H.; Lipp, J.J.; Schleiffer, A.; Mechtler, K.; Peters, J.M. Wapl controls the dynamic association of cohesion with chromatin. *Cell* 2006, 127, 955–967.
- Kufer, T.A.; Silljé, H.H.; Körner, R.; Gruss, O.J.; Meraldi, P.; Nigg, E.A. Human TPX2 is required for targeting Aurora-A kinase to the spindle. *J. Cell Biol.* 2002, 158, 617–623.

- Kuijt, T.E.F.; Lambers, M.L.A.; Weterings, S.; Ponsioen, B.; Bolhaqueiro, A.C.F.; Staijen, D.H.M.; Kops, G.J.P.L. A Biosensor for the Mitotic Kinase MPS1 Reveals Spatiotemporal Activity Dynamics and Regulation. *Current Biology*. 2020, 3862–3870.
- Kuilman, T.; Maiolica, A.; Godfrey, M.; Scheidel, N.; Aebersold, R.; Uhlmann, F. Identification of Cdk targets that control cytokinesis. *EMBO J*. 2015, 34, 81–96.
- Kumagai, A.; Dunphy, W.G. Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* 1996, 237, 1377–1380.
- Kumagai, A.; Guo, Z.; Emami, K.H.; Wang, S.X.; Dunphy, W.G. The *Xenopus* Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J. Cell Biol*. 1998, 142, 1559–1569.
- Kumagai, A.; Yakowec, P.S.; Dunphy, W.G. 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in *Xenopus* egg extracts. *Mol. Biol. Cell* 1998, 9, 345–354.
- Kumar, G.S.; Gokhan, E.; De Munter, S.; Bollen, M.; Vagnarelli, P.; Peti, W.; Page, R. The Ki-67 and RepoMan mitotic phosphatases assemble via an identical, yet novel mechanism. *Elife* 2016, 5, e16539.
- Kwon, Y.G.; Lee, S.Y.; Choi, Y.; Greengard, P.; Nairn, A.C. Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase. *Proc. Natl. Acad. Sci. USA* 1997, 94, 2168–2173.
- Laan, L.; Pavin, N.; Husson, J.; Romet-Lemonne, G.; van Duijn, M.; López, M.P.; Vale, R.D.; Jülicher, F.; Reck-Peterson, S.L.; Dogterom, M. Cortical dynein controls microtubule dynamics to generate pulling forces that position microtubule asters. *Cell* 2012, 148, 502–514.
- Labit, H.; Fujimitsu, K.; Bayin, N.S.; Takaki, T.; Gannon, J.; Yamano, H. Dephosphorylation of Cdc20 is required for its C-box-dependent activation of the APC/C. *EMBO J*. 2012, 31, 3351–3362.
- Lafont, A.L.; Song, J.; Rankin, S. Sororin cooperates with the acetyltransferase Eco2 to ensure DNA replication-dependent sister chromatid cohesion. *Proc. Natl. Acad. Sci. USA* 2010, 107, 20364–20369.
- LaJoie, D.; Ullman, K.S. Coordinated events of nuclear assembly. *Curr. Opin. Cell Biol*. 2017, 46, 39–45.
- Lampson, M.A.; Cheeseman, I.M. Sensing centromere tension: AuroraB and the regulation of kinetochore function. *Trends Cell Biol*. 2011, 21, 133–140.
- Lampson, M.A.; Renduchitala, K.; Khodjakov, A.; Kapoor, T.M. Correcting improper chromosome–spindle attachments during cell division. *Nat. Cell Biol*. 2004, 6, 232–237.
- Lan, W.; Cleveland, D.W. A chemical tool box defines mitotic and interphase roles for Mps1 kinase. *J. Cell Biol*. 2010, 190: 21–24.
- Landsverk, H.B.; Mora-Bermúdez, F.; Landsverk, O.J.; Hasvold, G.; Naderi, S.; Bakke, O.; Ellenberg, J.; Collas, P.; Syljuåsen, R.G.; Küntziger, T. The protein phosphatase 1 regulator PNUTS is a new component of the DNA damage response. *EMBO Rep*. 2010, 11, 868–875.
- Lara-Gonzalez, P.; Pines, J.; Desai, A. Spindle assembly checkpoint activation and silencing at kinetochores. *Semin Cell Dev Biol*. 2021, 117:86–98.

- Lattao, R.; Kovács, L.; Glover, D.M. The Centrioles, Centrosomes, Basal Bodies, and Cilia of *Drosophila melanogaster*. *Genetics* 2017, 206, 33–53.
- Laurell, E.; Beck, K.; Krupina, K.; Theerthagiri, G.; Bodenmiller, B.; Horvath, P.; Aebersold, R.; Antonin, W.; Kutay, U. Phosphorylation of Nup98 by multiple kinases is crucial for NPC disassembly during mitotic entry. *Cell* 2011, 144, 539–550.
- Lavecchia, A.; Di Giovanni, C.; Novellino, E. CDC25 phosphatase inhibitors: An update. *Mini Rev. Med. Chem.* 2012, 12, 62–73.
- Lee S.H., Sterling H.; Burlingame A.; McCormick F. Tpr directly binds to Mad1 and Mad2 and is important for the Mad1-Mad2-mediated mitotic spindle checkpoint. *Genes Dev.* 2008, 22(21): 2926–2931.
- Lee, B.H.; Schwager, F.; Meraldi, P.; Gotta, M. p37/UBXN2B regulates spindle orientation by limiting cortical NuMA recruitment via PP1/Repo-Man. *J. Cell Biol.* 2018, 217: 483–493.
- Lee, J.; Kitajima, T.S.; Tanno, Y.; Yoshida, K.; Morita, T.; Miyano, T.; Miyake, M.; Watanabe, Y. Unified mode of centromeric protection by shugoshin in mammalian oocytes and somatic cells. *Nat. Cell Biol.* 2008, 10: 42–52.
- Lee, M.J.; Gergely, F.; Jeffers, K.; Peak-Chew, S.Y.; Raff, J.W. Mps/XMAP215 interacts with the centrosomal protein D-TACC to regulate microtubule behavior. *Nat. Cell Biol.* 2001, 3: 643–649.
- Lee, S.J.; Rodriguez-Bravo, V.; Kim, H.; Datta, S.; Foley, E.A. The PP2AB56 phosphatase promotes the association of Cdc20 with APC/C in mitosis. *J. Cell Sci.* 2017, 130: 1760–1771.
- Lénárt, P.; Petronczki, M.; Steegmaier, M.; Di Fiore, B.; Lipp, J.J.; Hoffmann, M.; Rettig, W.J.; Kraut, N.; Peter, J.M. The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr. Biol.* 2007, 17: 304–315.
- Lera, R.F.; Potts, G.K.; Suzuki, A.; Johnson, J.M.; Salmon, E.D.; Coon, J.J.; Burkard, M.E. Decoding Polo-like kinase 1 signaling along the kinetochore–centromere axis. *Nat. Chem. Biol.* 2016, 12: 411–418.
- LeRoy, P.J.; Hunter, J.J.; Hoar, K.M.; Burke, K.E.; Shinde, V.; Ruan, J.; Bowman, D.; Galvin, K.; Ecsedy, J.A. Localization of human TACC3 to mitotic spindles is mediated by phosphorylation on Ser558 by Aurora A: A novel pharmacodynamic method for measuring Aurora A activity. *Cancer Res.* 2007, 67: 5362–5370.
- Lesage, B.; Beullens, M.; Pedelini, L.; Garcia-Gimeno, M.A.; Waelkens, E.; Sanz, P.; Bollen, M. A complex of catalytically inactive protein phosphatase-1 sandwiched between Sds22 and inhibitor-3. *Biochem.* 2007, 46: 8909–8919.
- Li X., Nicklas R.B. Mitotic forces control a cell-cycle checkpoint. *Nature.* 1995, 373: 630–632.
- Li, C.; Wen, A.; Shen, B.; Lu, J.; Huang, Y.; Chang, Y. FastCloning: a highly simplified, purification-free, sequence- and ligation-independent PCR cloning method. *BMC Biotechnol.* 2011, 11: 92.
- Li, H.; Liu, X.S.; Yang, X.; Wang, Y.; Wang, Y.; Turner, J.R.; Liu, X. Phosphorylation of CLIP-170 by Plk1 and CK2 promotes timely formation of kinetochore–microtubule attachments. *EMBO J.* 2010, 29, 2953–2965.

- Li, R.; Murray, A.W. Feedback control of mitosis in budding yeast. *Cell*. 1991; 66:519-531.
- Li, Y.; Yu, W.; Liang, Y.; Zhu, X. Kinetochore dynein generates a poleward pulling force to facilitate congression and full chromosome alignment. *Cell Res*. 2007, 17: 701–712.
- Lince-Faria, M.; Maffini, S.; Orr, B.; Ding, Y.; Florindo, C.; Sunkel, C.E.; Tavares, A.; Johansen, J.; Johansen, K.M.; Maiato H. Spatiotemporal control of mitosis by the conserved spindle matrix protein megator. *The Journal of Cell Biology*. 2009, 184:647–657.
- Linder, M.I.; Köhler, M.; Boersema, P.; Weberruss, M.; Wandke, C.; Marino, J.; Ashiono, C.; Picotti, P.; Antonin, W.; Kutay, U. Mitotic Disassembly of Nuclear Pore Complexes Involves CDK1- and PLK1-Mediated Phosphorylation of Key Interconnecting Nucleoporins. *Dev. Cell* 2017, 43, 141–156.
- Lindqvist, A.; Källström, H.; Lundgren, A.; Barsoum, E.; Rosenthal, C.K. Cdc25B cooperates with Cdc25 A to induce mitosis but has a unique role in activating cyclin B1–Cdk1 at the centrosome. *J. Cell Biol*. 2005, 171, 35–45.
- Lindqvist, A.; Rodríguez-Bravo, V.; Medema, R.H. The decision to enter mitosis: Feedback and redundancy in the mitotic entry network. *J. Cell Biol*. 2009, 185, 193–202.
- Liu, D.; Davydenko, O.; Lampson, M.A. Polo-like kinase-1 regulates kinetochore–microtubule dynamics and spindle checkpoint silencing. *J. Cell Biol*. 2012, 198, 491–499.
- Liu, D.; Vader, G.; Vromans, M.J.M.; Lampson, M.A.; Lens, S.M.A. Sensing Chromosome Bi-Orientation by Spatial Separation of Aurora B Kinase from Kinetochore Substrates. *Science* 2009, 323, 1350–1353.
- Liu, D.; Vleugel, M.; Backer, C.B.; Hori, T.; Fukagawa, T.; Cheeseman, I.M.; Lampson, M.A. Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase. *J. Cell Biol*. 2010, 188, 809–820.
- Liu, H.; Rankin, S.; Yu, H. Phosphorylation-enabled binding of SGO1-PP2A to cohesion protects sororin and centromeric cohesion during mitosis. *Nat. Cell Biol*. 2013, 15, 40–49.
- Liu, R.; Correll, R.N.; Davis, J.; Vagnozzi, R.J.; York, A.J.; Sargent, M.A.; Nairn, A.C.; Molkentin, J.D. Cardiac-specific deletion of protein phosphatase 1 promotes increased myofilament protein phosphorylation and contractile alterations. *J. Mol. Cell. Cardiol*. 2015, 87, 204–213.
- Liu, X.; Winey, M. The MPS1 family of protein kinases. *Annu. Rev. Biochem*. 2012, 81, 561–585.
- Liu, Y.; Bishop, A.; Witucki, L.; Kraybill, B.; Shimizu, E.; Tsien, J.; Ubersax, J.; Blethrow, J.; Morgan, D.O.; Shokat, K.M. Structural basis for selective inhibition of Src family kinases by PP1. *Chem Biol*. 1999, 6(9): 671-8.
- Llano, E.; Gómez, R.; Gutiérrez-Caballero, C.; Herrán, Y.; Sánchez-Martín, M.; Vázquez-Quñones, L.; Hernández, T.; de Alava, E.; Cuadrado, A.; Barbero, J.L.; et al. Shugoshin-2 is essential for the completion of meiosis but not for mitotic cell division in mice. *Genes Dev*. 2008, 22, 2400–2413.
- Lobjois, V.; Froment, C.; Braud, E.; Grimal, F.; Burlet-Schiltz, O.; Ducommun, B.; Bouche, J.P. Study of the docking-dependent PLK1 phosphorylation of the CDC25B phosphatase. *Biochem. Biophys. Res. Commun*. 2011, 410, 87–90.

- Lobjois, V.; Jullien, D.; Bouché, J.P.; Ducommun, B. The polo-like kinase 1 regulates CDC25B-dependent mitosis entry. *Biochim. Biophys. Acta* 2009, 1793, 462–468.
- Lombillo, V.A.; Nislow, C.; Yen, T.J.; Gelfand, V.I.; McIntosh, J.R. Antibodies to the kinesin motor domain and CENP-E inhibit microtubule depolymerization-dependent motion of chromosomes in vitro. *J. Cell Biol.* 1995, 128, 107–115.
- London, N.; Biggins, S. Mad1 kinetochore recruitment by Mps1-mediated phosphorylation of Bub1 signals the spindle checkpoint. *Genes Dev.* 2014, 28(2): 140–152. (a)
- London, N.; Biggins, S. Signalling dynamics in the spindle checkpoint response. *Nat. Rev. Mol. Cell. Biol.* 2014, 15, 736–747. (b)
- London, N.; Ceto, S.; Ranish, J.A.; Biggins, S. Phosphoregulation of Spc105 by Mps1 and PP1 regulates Bub1 localization to kinetochores. *Curr. Biol.* 2012, 22, 900–906.
- Lopes, C.S.; Sampaio, P.; Williams, B.; Goldberg, M.; Sunkel C.E. The *Drosophila* Bub3 protein is required for the mitotic checkpoint and for normal accumulation of γ -H2A during G2 and early stages of mitosis. *Journal of Cell Science.* 2005, 118:187–198.
- Lorca, T.; Bernis, C.; Vigneron, S.; Burgess, A.; Brioudes, E.; Labbé, J.C.; Castro, A. Constant regulation of both the MPF amplification loop and the Greatwall-PP2A pathway is required for metaphase II arrest and correct entry into the first embryonic cell cycle. *J. Cell Sci.* 2010, 123, 2281–2291.
- Losada, A.; Hirano, M.; Hirano, T. Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev.* 2002, 16, 3004–3016.
- Ma, S.; Vigneron, S.; Robert, P.; Strub, J.M.; Cianferani, S.; Castro, A.; Lorca, T. Greatwall dephosphorylation and inactivation upon mitotic exit is triggered by PP1. *J. Cell Sci.* 2016, 129, 1329–1339.
- Macaulay, C.; Meier, E.; Forbes, D.J. Differential mitotic phosphorylation of proteins of the nuclear pore complex. *J. Biol. Chem.* 1995, 270, 254–262.
- Maciejowski J., George K.A., Terret M.E., Zhang C., Shokat K.M., Jallepalli P.V. Mps1 directs the assembly of Cdc20 inhibitory complexes during interphase and mitosis to control M phase timing and spindle checkpoint signaling. *The Journal of Cell Biology.* 2010, 190:89–100.
- Magidson, V.; O'Connell, C.B.; Lončarek, J.; Paul, R.; Mogilner, A.; Khodjakov, A. The spatial arrangement of chromosomes during prometaphase facilitates spindle assembly. *Cell* 2011, 146, 555–567.
- Maia, A.R.; Garcia, Z.; Kabeche, L.; Barisic, M.; Maffini, S.; Macedo-Ribeiro, S.; Cheeseman, I.M.; Compton, D.A.; Kaverina, I.; Maiato, H. Cdk1 and Plk1 mediate a CLASP2 phospho-switch that stabilizes kinetochore–microtubule attachments. *J. Cell Biol.* 2012, 199, 285–301.
- Mailand, N.; Podtelejnikov, A.V.; Groth, A.; Mann, M.; Bartek, J.; Lukas, J. Regulation of G2/M events by Cdc25A through phosphorylation-dependent modulation of its stability. *EMBO J.* 2002, 21, 5911–5920.

- Maldonado M., Kapoor T.M. Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation. *Nature Cell Biology*. 2011, 13: 475–482.
- Manning, G.; Whyte, D.B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* 2002, 298, 1912–1934.
- Mansfeld, J.; Güttinger, S.; Hawryluk-Gara, L.A.; Panté, N.; Mall, M.; Galy, V.; Haselmann, U.; Mühlhäusser, P.; Wozniak, R.W.; Mattaj, I.W.; et al. The conserved transmembrane nucleoporin NDC1 is required for nuclear pore complex assembly in vertebrate cells. *Mol. Cell* 2006, 22, 93–103.
- Mapelli, M.; Musacchio, A. MAD contortions: conformational dimerization boosts spindle checkpoint signaling. *Curr. Opin. Struct. Biol.* 2007, 17(6): 716–725.
- Mardin, B.R.; Agircan, F.G.; Lange, C.; Schiebel, E. Plk1 controls the Nek2A-PP1 antagonism in centrosome disjunction. *Curr. Biol.* 2011, 21, 1145–1151.
- Mardin, B.R.; Lange, C.; Baxter, J.E.; Hardy, T.; Scholz, S.R.; Fry, A.M.; Schiebel, E. Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nat. Cell Biol.* 2010, 12, 1166–1176.
- Maresca, T.J.; Salmon, E.D. Intrakinetochores stretch is associated with changes in kinetochore phosphorylation and spindle assembly checkpoint activity. *J. Cell Biol.* 2009, 184, 373–381.
- Maresca, T.J.; Salmon, E.D. Welcome to a new kind of tension: Translating kinetochore mechanics into a wait-anaphase signal. *J. Cell Sci.* 2010, 123, 825–835.
- Margalit, A.; Brachner, A.; Gotzmann, J.; Foisner, R.; Gruenbaum, Y. Barrier-to-auto integration factor — A BAF fling little protein. *Trends Cell Biol.* 2007, 17, 202–208.
- Margolis, S.S.; Perry, J.A.; Weitzel, D.H.; Freel, C.D.; Yoshida, M.; Haystead, T.A.; Kornbluth, S. A role for PP1 in the Cdc2/Cyclin B-mediated positive feedback activation of Cdc25. *Mol. Biol. Cell* 2006, 17, 1779–1789.
- Margolis, S.S.; Walsh, S.; Weiser, D.C.; Yoshida, M.; Shenolikar, S.; Kornbluth, S. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. *EMBO J.* 2003, 22, 5734–5745.
- Marsh, J.A.; Dancheck, B.; Ragusa, M.J.; Allaire, M.; Forman-Kay, J.D.; Peti, W. Structural diversity in free and bound states of intrinsically disordered protein phosphatase 1 regulators. *Structure* 2010, 18, 1094–1103.
- Martin-Granados, C.; Philp, A.; Oxenham, S.K.; Prescott, A.R.; Cohen, P.T. Depletion of protein phosphatase 4 in human cells reveals essential roles in centrosome maturation, cell migration and the regulation of Rho GTPases. *Int. J. Biochem. Cell Biol.* 2008, 40, 2315–2332.
- Martin-Lluesma, S.; Stucke, V.M.; Nigg, E.A. Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. *Science* 2002, 297: 2267–2270.
- Martino, L.; Morchoisne-Bolhy, S.; Cheerambathur, D.K.; Van Hove, L.; Dumont, J.; Joly, N.; Desai, A.; Doye, V.; Pintard, L. Channel Nucleoporins Recruit PLK-1 to Nuclear Pore Complexes to Direct Nuclear Envelope Breakdown in *C. elegans*. *Dev. Cell* 2017, 43, 157–171.

- Matsumura, S.; Hamasaki, M.; Yamamoto, T.; Ebisuya, M.; Sato, M.; Nishida, E.; Toyoshima, F. ABL1 regulates spindle orientation in adherent cells and mammalian skin. *Nat. Commun.* 2012, 3, 626.
- Mattison, C.P.; Old, W.M.; Steiner, E.; Huneycutt, B.J.; Resing, K.A.; Ahn, N.G.; Winey, M. Mps1 activation loop autophosphorylation enhances kinase activity. *J. Biol. Chem.* 2007, 282, 30553–30561.
- Mayer-Jaekel, R.E.; Ohkura, H.; Ferrigno, P.; Andjelkovic, N.; Shiomi, K.; Uemura, T.; Glover, D.M.; Hemmings, B.A. *Drosophila* mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of p34cdc2. *J. Cell Sci.* 1994, 107, 2609–2616.
- McAnish, A.D.; Kops, G.J.P.L. Principles and dynamics of spindle assembly checkpoint signalling. 2023, 24(8): 543-559.
- McCain, D.F.; Catrina, I.E.; Hengge, A.C.; Zhang, Z.Y. The catalytic mechanism of Cdc25A phosphatase. *J. Biol. Chem.* 2002, 277, 11190–11200.
- McCloy, R.A.; Parker, B.L.; Rogers, S.; Chaudhuri, R.; Gayevskiy, V.; Hoffman, N.J.; Ali, N.; Watkins, D.N.; Daly, R.J.; James, D.E.; et al. Global Phosphoproteomic Mapping of Early Mitotic Exit in Human Cells Identifies Novel Substrate Dephosphorylation Motifs. *Mol. Cell Proteom.* 2015, 14, 2194–2212.
- McGuinness, B.E.; Hirota, T.; Kudo, N.R.; Peters, J.M.; Masmyth, K. Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells. *PLoS Biol.* 2005, 3, e86.
- McNall, S.J.; Fischer, E.H. Phosphorylase phosphatase. Comparison of active forms using peptide substrates. *J. Biol. Chem.* 1988, 263, 1893–1897.
- McNally, F. Capturing a ring of samurai. *Nat. Cell Biol.* 2000, 2, E4–E7.
- McNally, F.J.; Vale, R.D. Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell* 1993, 75, 419–429.
- Meadows, J.C.; Shepperd, L.A.; Vanoosthuyse, V.; Lancaster, T.C.; Sochaj, A.M.; Buttrick, G.J.; Hardwick, K.G.; Millar, J.B. Spindle checkpoint silencing requires association of PP1 to both Spc7 and kinesin-8 motors. *Dev. Cell* 2011, 20, 739–750.
- Mehsen, H.; Boudreau, V.; Garrido, D.; Bourouh, M.; Larouche, M.; Maddox, P.S.; Swan, A.; Archambault, V. PP2A-B55 promotes nuclear envelope reformation after mitosis in *Drosophila*. *J. Cell Biol.* 2018, 217, 4106–4123.
- Meiselbach, H.; Sticht, H.; Enz, R. Structural analysis of the protein phosphatase 1 docking motif: Molecular description of binding specificities identifies interacting proteins. *Chem. Biol.* 2006, 13, 49–59.
- Mennella, V.; Agard, D.A.; Huang, B.; Pelletier, L. Amorphous no more: Subdiffraction view of the pericentriolar material architecture. *Trends Cell Biol.* 2014, 24, 188–197.
- Meppelink, A.; Kabeche, L.; Vromans, M.J.; Compton, D.A.; Lens, S.M. Shugoshin-1 balances Aurora B kinase activity via PP2A to promote chromosome bi-orientation. *Cell Rep.* 2015, 11, 508–515.
- Meraldi, P.; Nigg, E.A. Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *J. Cell Sci.* 2001, 114, 3749–3757.

- Meunier, S.; Vernos, I. Acentrosomal Microtubule Assembly in Mitosis: The Where, When, and How. *Trends Cell Biol.* 2016, 26, 80–87.
- Mi, J.; Guo, C.; Brautigan, D.L.; Larner, J.M. Protein phosphatase-1 α regulates centrosome splitting through Nek2. *Cancer Res.* 2007, 67, 1082–1089.
- Michel, L.S.; Liberal, V.; Chatterjee, A.; Kirchwegger, R.; Pasche, B.; Gerald, W.; Dobles, M.; Sorger, P.K.; Murty, V.V.; Benezra, R. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* 2001, 409, 355–359.
- Miller, S.A.; Johnson, M.L.; Stukenberg, P.T. Kinetochore Attachments Require an Interaction between Unstructured Tails on Microtubules and Ndc80/Hec1. *Curr. Biol.* 2008, 18, 1785–1791.
- Mirchenko L., Uhlmann F. Sli15(INCENP) dephosphorylation prevents mitotic checkpoint reengagement due to loss of tension at anaphase onset. *Current Biology.* 2010, 20:1396–1401.
- Mirdita, M.; Schütze, K.; Moriwaki, Y.; Heo, L.; Ovchinnikov, S.; Steinegger, M. ColabFold: making protein folding accessible to all. *Nature Methods.* 2022, 19: 679-682.
- Mishima, M.; Pavicic, V.; Grüneberg, U.; Nigg, E.A.; Glotzer, M. Cell cycle regulation of central spindle assembly. *Nature* 2004, 430, 908–913.
- Mitić, N.; Miraula, M.; Selleck, C.; Hadler, K.S.; Uribe, E.; Pedroso, M.M.; Schenk, G. Catalytic mechanisms of metallohydrolases containing two metal ions. *Adv. Protein Chem. Struct. Biol.* 2014, 97, 49–81.
- Mochida, S.; Hunt T. Protein phosphatases and their regulation in the control of mitosis. *EMBO Reports.* 2012, 13: 197–203.
- Mochida, S.; Hunt, T. Calcineurin is required to release *Xenopus* egg extracts from meiotic M phase. *Nature* 2007, 449, 336–340.
- Mochida, S.; Ikeo, S.; Gannon, J.; Hunt, T. Regulated activity of PP2A-B55 is crucial for controlling entry into and exit from mitosis in *Xenopus* egg extracts. *EMBO J.* 2009, 28, 2777–2785.
- Mochida, S.; Maslen, S.L.; Skehel, M.; Hunt, T. Greatwall Phosphorylates an Inhibitor of Protein Phosphatase 2A That Is Essential for Mitosis. *Science* 2010, 330, 1670–1673.
- Molinari, M.; Mercurio, C.; Dominguez, J.; Goubin, F.; Draetta, G.F. Human Cdc25A inactivation in response to S phase inhibition and its role in preventing premature mitosis. *EMBO Rep.* 2000, 1, 71–79.
- Mollinari, C.; Kleman, J.P.; Jiang, W.; Schoehn, G.; Hunter, T.; Margolis, R.L. PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J. Cell Biol.* 2002, 157, 1175–1186.
- Monda, J.K.; Cheeseman, I.M. The kinetochore–microtubule interface at a glance. *J. Cell Sci.* 2018, 131, jcs214577.
- Monda, J.K.; Whitney, I.P.; Tarasovets, E.V.; Wilson-Kubalek, E.; Milligan, R.A.; Grishchuk, E.L.; Cheeseman, I.M. Microtubule Tip Tracking by the Spindle and Kinetochore Protein Ska1 Requires Diverse Tubulin-Interacting Surfaces. *Curr. Biol.* 2017, 27, 3666–3675.

- Mora-Santos, M.D.; Hervas-Aguilar, A.; Sewart, K.; Lancaster, T.C.; Meadows, J.C.; Millar, J.B. Bub3-Bub1 Binding to Spc7/KNL1 Toggles the Spindle Checkpoint Switch by Licensing the Interaction of Bub1 with Mad1-Mad2. *Curr. Biol.* 2016, 26, 2642–2650.
- Morais da Silva, S.; Moutinho-Santos, T.; Sunkel, C.E. A tumor supressor role of the Bub3 spindle checkpoint protein after apoptosis inhibition. *J Cell Biol.* 2013, 201(3): 385-393.
- Morales, C.; Losada, A. Establishing and dissolving cohesion during the vertebrate cell cycle. *Curr.Opin. Cell Biol.* 2018, 52, 51–57.
- Morgan, D.O. Principles of CDK regulation. *Nature* 1995, 374,131–134.
- Morin, V.; Prieto, S.; Melines, S.; Hem, S.; Rossignol, M.; Lorca, T.; Espeut, J.; Morin, N.; Abrieu, A. CDK-dependent potentiation of MPS1 kinase activity is essential to the mitotic checkpoint. *Curr. Biol.* 2012, 22, 289–295.
- Moura, M.; Conde, C. Phosphatases in Mitosis: Roles and Regulation. 2019, 9(2): 55.
- Moura, M.; Osswald, M.; Leça, N.; Barbosa, J.; Pereira, A.J.; Maiato, H.; Sunkel, C.E.; Conde, C. Protein Phosphatase 1 inactivates Mps1 to ensure efficient Spindle Assembly Checkpoint silencing. *Elife* 2017, 6, e25366.
- Moutinho-Santos, T.; Conde, C.; Sunkel, C.E. POLO ensures chromosome bi-orientation by preventing and correcting erroneous chromosome–spindle attachments. *J. Cell Sci.* 2012, 125, 576–583.
- Moyle M.W.; Kim T.; Hattersley N.; Espeut J.; Cheerambathur D.K.; Oegema K.; Desai A. Bub1-Mad1 A. interaction targets the Mad1-Mad2 complex to unattached kinetochores to initiate the spindle checkpoint. *J. Cell Biol.* 2014, 204(5): 647–657.
- Mumby, M.C.; Russell, K.L.; Garrard, L.J.; Green, D.D. Cardiac contractile protein phosphatases. Purification of two enzyme forms and their characterization with subunit-specific antibodies. *J. Biol. Chem.* 1987, 262, 6257–6265.
- Murnion, M.E.; Adams,R.R.; Callister,D.M.; Allis,C.D.; Earnshaw,W.C.; Swedlow,J.R. Chromatin-associated protein phosphatase 1 regulates aurora-B and histone H3 phosphorylation. *J. Biol. Chem.* 2001, 276, 26656–26665.
- Musacchio, A. The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *Curr. Biol.* 2015, 25, 1002–1018.
- Musacchio, A., Salmon E.D. The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology.* 2007, 8:379–393.
- Musacchio, A.; Desai, A. A molecular view of kinetochore assembly and function. *Biology* 2017, 6, 5.
- Nagata, A.; Igarashi, M.; Jinno, S.; Suto, K.; Okayama, H. An additional homolog of the fission yeast *cdc25+* gene occurs in humans and is highly expressed in some cancer cells. *New Biol.* 1991, 3, 959–968.
- Nakajima, H.; Toyoshima-Morimoto,F.; Taniguchi,E.; Nishida,E. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J. Biol. Chem.* 2003, 278, 25227–25280.
- Nam, H.J.; vanDeursen,J.M. CyclinB2 and p53 control proper timing of centrosome separation. *Nat. Cell Biol.* 2014, 16, 538–549.

- Nasa, I.; Kettenbach, A.N. Coordination of Protein Kinase and Phosphoprotein Phosphatase Activities in Mitosis. *Front. Cell Dev. Biol.* 2018, 6, 30.
- Nasa, I.; Rusin, S.F.; Kettenbach, A.N.; Moorhead, G.B. Aurora B opposes PP1 function in mitosis by phosphorylating the conserved PP1-binding RVxF motif in PP1 regulatory proteins. *Sci. Signal.* 2018, 11, eaai8669.
- Nasmyth, K.; Haering, C.H. Cohesin: Its roles and mechanisms. *Annu. Rev. Genet.* 2009, 43, 525–558.
- Neef, R.; Gruneberg, U.; Kopajtich, R.; Li, X.; Nigg, E.A.; Sillje, H.; Barr, F.A. Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1. *Nat. Cell Biol.* 2007, 9, 436–444.
- Nelson, D.A.; Krucher, N.A.; Ludlow, J.W. High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *J Biol Chem.* 1997, 272:4528–4535.
- Nguyen-Ngoc, T.; Afshar, K.; Gönczy, P. Coupling of cortical dynein and Gα proteins mediates spindle positioning in *Caenorhabditis elegans*. *Nat. Cell Biol.* 2007, 9, 1294–1302.
- Nichols, R.J.; Wiebe, M.S.; Traktman, P. The vaccinia-related kinases phosphorylate the N-terminus of BAF, regulating its interaction with DNA and its retention in the nucleus. *Mol. Biol. Cell* 2006, 17, 2451–2464.
- Nicklas, R.B. How cells get the right chromosomes. *Science* 1997, 275, 632–637.
- Nicklas, R.B.; Ward, S.C.; Gorbsky, G.J. Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell. Biol.* 1995, 130: 929–939.
- Nijenhuis, W.; Vallardi, G.; Teixeira, A.; Kops, G.J.P.L.; Saurin, A.T. Negative feedback at kinetochores underlies a responsive spindle checkpoint signal. *Nat. Cell Biol.* 2014, 16, 1257–1264.
- Nijenhuis, W.; von Castelmur, E.; Littler, D.; DeMarco, V.; Tromer, E.; Vleugel, M.; van Osch, M.H.; Snel, B.; Perrakis, A.; Kops, G.J. A TPR domain-containing N-terminal module of MPS1 is required for its kinetochore localization by Aurora B. *J. Cell Biol.* 2013, 201, 217–231.
- Nilsson, J. Protein phosphatases in the regulation of mitosis. *J. Cell Biol.* 2018, 218, 395–409.
- Nishino, M.; Kurasawa, Y.; Evans, R.; Lin, S.H.; Brinkley, B.R.; Yu-Lee, L.Y. NudC is required for Plk1 targeting to the kinetochore and chromosome congression. *Curr. Biol.* 2006, 16, 1414–1421.
- Nishiyama, T.; Ladurner, R.; Schmitz, J.; Kreidl, E.; Schleiffer, A.; Bhaskara, V.; Bando, M.; Shirahige, K.; Hyman, A.A.; Mechtler, K.; et al. Sororin mediates sister chromatid cohesion by antagonizing Wapl. *Cell* 2010, 143, 737–749.
- Nishiyama, T.; Sykora, M.M.; Huis, P.J.; Mechtler, K.; Peters, J.M. Aurora B and Cdk1 mediate Wapl activation and release of acetylated cohesin from chromosomes by phosphorylating Sororin. *Proc. Natl. Acad. Sci. USA* 2013, 110, 13404–13409.
- O’Connell, N.; Nichols, S.R.; Heroes, E.; Beullens, M.; Bollen, M.; Peti, W.; Page, R. The molecular basis for substrate specificity of the nuclear NIPP1: PP1 holoenzyme. *Structure* 2012, 20, 1746–1756.

- O'Connor, A.; Maffini, S.; Rainey, M.D.; Kaczmarczyk, A.; Gaboriau, D.; Musacchio, A.; Santocanale, C. Requirement for PLK1 kinase activity in the maintenance of a robust spindle assembly checkpoint. *Biol. Open* 2016, 5, 11–19.
- O'Connor, C. Cell Division: Stages of Mitosis. *Nature Education*. 2008, 1(1):188.
- O'Farrell, P.H. Triggering the all-or-nothing switch into mitosis. *Trends Cell Biol.* 2001, 11, 512–519.
- Okano, K.; Heng, H.; Trevisanato, S.; Tyers, M.; Varmuza, S. Genomic organization and functional analysis of the murine protein phosphatase 1c (Ppp1cc) gene. *Genomic organization and functional analysis of the murine protein phosphatase 1c (Ppp1cc) gene. Genomics* 1997, 45, 211–215.
- Oliveira, R.A.; Hamilton, R.S.; Pauli, A.; Davis, I.; Nasmyth, K. Cohesin cleavage and Cdk inhibition trigger formation of daughter nuclei. *Nature Cell Biology*. 2010, 12:185–192.
- Olsen, J.V.; Blagoev, B.; Gnäd, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 2006, 127, 635–648.
- Olsen, J.V.; Vermeulen, M.; Santamaria, A.; Kumar, C.; Miller, M.L.; Jensen, L.J.; Gnäd, F.; Cox, J.; Jensen, T.S.; Nigg, E.A.; et al. Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal.* 2010, 3, ra3.
- Orth, J.D.; Loewer, A.; Lahav, G.; Mitchison, T.J. Prolonged mitotic arrest triggers partial activation of apoptosis, resulting in DNA damage and p53 induction. *Mol Biol Cell*. 2012, 23(4): 567–576.
- Ouyang, Z.; Zheng, G.; Song, J.; Borek, D.M.; Otwinowski, Z.; Brautigam, C.A.; Tomchick, D.R.; Rankin, S.; Yu, H. Structure of the human cohesin inhibitor Wapl. *Proc. Natl. Acad. Sci. USA* 2013, 110, 11355–11360.
- Overlack, K.; Primorac, I.; Vleugel, M.; Krenn, V.; Maffini, S.; Hoffmann, I.; Kops, G.J.; Musacchio, A. A molecular basis for the differential roles of Bub1 and BubR1 in the spindle assembly checkpoint. *Elife* 2015, 4, e05269.
- Ozlu, N.; Srayko, M.; Kinoshita, K.; Habermann, B.; O'toole, E.T.; Müller-Reichert, T.; Schmalz, N.; Desai, A.; Hyman, A.A. An essential function of the *C. elegans* ortholog of TPX2 is to localize activated aurora A kinase to mitotic spindles. *Dev. Cell* 2005, 9, 237–248.
- Pachis, S.T.; Kops, G.J.P.L. Leader of the SAC: Molecular mechanisms of Mps1/TTK regulation in mitosis. *Open Biol.* 2018, 8, 180109.
- Parker, L.L.; Piwnicka-Worms, H. Inactivation of the p34 cdc2-cyclinB complex by the human WEE1 tyrosine kinase. *Science* 1992, 257, 1955–1957.
- Peng, C.Y.; Graves, P.R.; Ogg, S.; Thoma, R.S.; Byrnes, M.J.3rd; Wu, Z.; Stephenson, M.T.; Piwnicka-Worms, H. C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell Growth Differ.* 1998, 9, 197–208.
- Peng, C.Y.; Graves, P.R.; Thoma, R.S.; Wu, Z.; Shaw, A.S.; Piwnicka-Worms, H. Mitotic and G2 Checkpoint Control: Regulation of 14-3-3 Protein Binding by Phosphorylation of Cdc25C on Serine-216. *Science* 1997, 277, 1501–1505.

- Pereira A.J., Aguiar P., Belsley M., Maiato H. Inducible fluorescent speckle microscopy. *The Journal of Cell Biology*. 2016, 212:245–255.
- Pereira, G.; Schiebel, E. Mitotic exit: Determining the PP2A dephosphorylation program. *J. Cell Biol.* 2016, 214, 499–501.
- Peset, I.; Seiler, J.; Sardon, T.; Bejarano, L.A.; Rybina, S.; Vernos, I. Function and regulation of Maskin, aTACC family protein, in microtubule growth during mitosis. *J. Cell Biol.* 2005, 170, 1057–1066.
- Peset, I.; Vernos, I. The TACC proteins: TACC-ling microtubule dynamics and centrosome function. *Trends Cell Biol.* 2008, 18, 379–388.
- Peter, M.; Nakagawa, J.; Dorée, M.; Labbé, J.C.; Nigg, E.A. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 1990, 61, 591–602.
- Peters, J.M.; Tedeschi, A.; Schmitz, J. The cohesion complex and its roles in chromosome biology. *Genes Dev.* 2008, 22, 3089–3114.
- Petronczki, M.; Glotzer, M.; Kraut, N.; Peters, J.M. Polo-like kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the Rho GEF Ect2 to the central spindle. *Dev. Cell* 2007, 12, 713–725.
- Piano V.; Alex A.; Stege P.; Maffini S.; Stoppiello G.A.; Huis In 't Veld P.J.; Vetter I.R.; Musacchio A. CDC20 assists its catalytic incorporation in the mitotic checkpoint complex. *Science*. 2021, 371(6524): 67–71.
- Pines, J.; Hunter, T. Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* 1989, 58, 833–846.
- Pinna, L.A.; Donella, A.; Clari, G.; Moret, V. Preferential dephosphorylation of protein bound phosphorylthreonine and phosphorylserine residues by cytosol and mitochondrial “casein phosphatase”. *Biochem. Biophys. Res. Commun.* 1976, 70, 1308–1315.
- Pinoyl, R.; Scrofani, J.; Vernos, I. The role of NEDD1 phosphorylation by Aurora A in chromosomal microtubule nucleation and spindle function. *Curr. Biol.* 2013, 23, 143–149.
- Pinsky, B.A.; Kung, C.; Shokat, K.M.; Biggins, S. The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. *Nat. Cell Biol.* 2006, 8, 78–83.
- Pinsky, B.A.; Nelson, C.R.; Biggins, S. Protein phosphatase 1 regulates exit from the spindle checkpoint in budding yeast. *Curr. Biol.* 2009, 19, 1182–1187.
- Poon, R.Y.C.. Cell Cycle Control: A System of Interlinking Oscillators. *Methods Mol Biol.* 2016, 1342, 352.
- Porter, I.M.; Schleicher, K.; Porter, M.; Swedlow, J.R. Bod1 regulates protein phosphatase 2A at mitotic kinetochores. *Nat. Commun.* 2013, 4, 2677.
- Posch, M.; Khoudoli, G.A.; Swift, S.; King, E.M.; DeLuca, J.G.; Swedlow, J.R. Sds22 regulates auroraB activity and microtubule–kinetochore interactions at mitosis. *J. Cell Biol.* 2010, 131, 61–74.
- Primorac, I.; Weir, J.R.; Chirolì, E.; Gross, F.; Hoffmann, I.; van Gerwen, S.; Ciliberto, A.; Musacchio, A. Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling. *Elife* 2013, 2, e01030.

- Qi, F.; Chen, Q.; Chen, H.; Yan, H.; Chen, B.; Xiang, X.; Liang, C.; Yi, Q.; Zhang, M.; Cheng, H.; et al. WAC Promotes Polo-like Kinase 1 Activation for Timely Mitotic Entry. *Cell Rep.* 2018, 24, 546–556.
- Qian, J.; Beullens, M.; Huang, J.; De Munter, S.; Lesage, B.; Bollen, M. Cdk1 orders mitotic events through coordination of a chromosome-associated phosphatase switch. *Nat. Commun.* 2015, 6, 10215.
- Qian, J.; Beullens, M.; Lesage, B.; Bollen, M. Aurora B defines its own chromosomal targeting by opposing the recruitment of the phosphatase scaffold Repo-Man. *Curr. Biol.* 2013, 23, 1136–1143.
- Qian, J.; García-Gimeno, M.A.; Beullens, M.; Manzione, M.G.; VanderHoeven, G.; Igual, J.C.; Heredia, M.; Sanz, P.; Gelens, L.; Bollen, M. An Attachment-Independent Biochemical Timer of the Spindle Assembly Checkpoint. *Mol. Cell* 2017, 68, 715–730.
- Qian, J.; Lesage, B.; Beullens, M.; Van Eynde, A.; Bollen, M. PP1/Repo-man dephosphorylates mitotic histone H3 at T3 and regulates chromosomal aurora B targeting. *Curr. Biol.* 2011, 21, 766–773.
- Qian, Y.W.; Erikson, E.; Maller, J.L. Purification and cloning of a protein kinase that phosphorylates and activates the polo-like kinase Plx1. *Science* 1998, 282, 1701–1704.
- Queralt, E.; Uhlmann, F. Cdk-counteracting phosphatases unlock mitotic exit. *Curr. Opin. Cell Biol.* 2008, 20, 661–668.
- Raaijmakers, J.A.; Tanenbaum, M.E.; Maia, A.F.; Medema, R.H. RAMA1 is a novel kinetochore protein involved in kinetochore-microtubule attachment. *J. Cell Sci.* 2009, 122, 2436–2445.
- Ragusa, M.J.; Dancheck, B.; Critton, D.A.; Nairn, A.C.; Page, R.; Peti, W. Spinophilin directs protein phosphatase 1 specificity by blocking substrate binding sites. *Nat. Struct. Mol. Biol.* 2010, 17, 459–464.
- Rasala, B.A.; Orjalo, A.V.; Shen, Z.; Briggs, S.; Forbes, D.J. ELYS is a dual nucleoporin/kinetochore protein required for nuclear pore assembly and proper cell division. *Proc. Natl. Acad. Sci. USA* 2006, 103, 17801–17806.
- Rattani A., Vinod P.K., Godwin J., Tachibana-Konwalski K., Wolna M., Malumbres M., Novák B., Nasmyth K. Dependency of the spindle assembly checkpoint on Cdk1 renders the anaphase transition irreversible. *Curr. Biol.* 2014, 24:630–637.
- Redli, P.M.; Gasic, I.; Meraldi, P.; Nigg, E.A.; Santamaria, A. The Ska complex promotes AuroraB activity to ensure chromosome biorientation. *J. Cell Biol.* 2016, 215, 77–93.
- Rellos, P.; Ivins, F.J.; Baxter, J.E.; Pike, A.; Nott, T.J.; Parkinson, D.M.; Das, S.; Howell, S.; Fedorov, O.; Shen, Q.Y.; et al. Structure and regulation of the human Nek2 centrosomal kinase. *J. Biol. Chem.* 2007, 282, 6833–6842.
- Ren, D.; Fisher, L.A.; Zhao, J.; Wang, L.; Williams, B.C.; Goldberg, M.L.; Peng, A. Cell cycle-dependent regulation of Greatwall kinase by protein phosphatase 1 and regulatory subunit 3B. *J. Biol. Chem.* 2017, 292, 10026–10034.
- Resende, L.P.; Monteiro, A.; Brás, R.; Lopes, T.; Sunkel, C.E. Aneuploidy in intestinal stem cells promotes gut dysplasia in *Drosophila*. *J Cell Biol.* 2018, 217(11): 3930-3946.

- Reynolds, R.A.; Yem, A.W.; Wolfe, C.L.; Deibel, M.R.Jr.; Chidester, C.G.; Watenpaugh, K.D. Crystal structure of the catalytic subunit of Cdc25B required for G2/M phase transition of the cell cycle. *J. Mol. Biol.* 1999, 293, 559–568.
- Riedel, C.G.; Katis, V.L.; Katou, Y.; Mori, S.; Itoh, T.; Helmhart, W.; Gálová, M.; Petronczki, M.; Gregan, J.; Cetin, B.; et al. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* 2006, 441, 53–61.
- Rieder, C.L.; Cole, R.W.; Khodjakov, A.; Sluder, G. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J. Cell Biol.* 1995, 130, 941–948.
- Rieder, C.L.; Schultz, A.; Cole, R.; Sluder, G. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.* 1994, 127: 1301–1310.
- Rivera, T.; Ghenoïu, C.; Rodríguez-Corsino, M.; Mochida, S.; Funabiki, H.; Losada, A. Xenopus Shugoshin 2 regulates the spindle assembly pathway mediated by the chromosomal passenger complex. *EMBO J.* 2012, 31, 1467–1479.
- Rivera, T.; Losada, A. Shugoshin regulates cohesion by driving relocalization of PP2A in Xenopus extracts. *Chromosoma* 2009, 118, 223–233.
- Rodríguez-Bravo, V.; Maciejowski, J.; Corona, J.; Buch, H.K.; Collin, P.; Kanemaki, M.T.; Shah, J.V.; Jallepalli, P.V. Nuclear pores protect genome integrity by assembling a premitotic and Mad1-dependent anaphase inhibitor. *Cell.* 2014, 156:1017–1031.
- Rogers, S.; Fey, D.; McCloy, R.A.; Parker, B.L.; Mitchell, N.J.; Payne, R.J.; Daly, R.J.; James, D.E.; Caldon, C.E.; Watkins, D.N.; Croucher, D.R.; Burgess, A. PP1 initiates the dephosphorylation of MASTL, triggering mitotic exit and bistability in human cells. *J. Cell Sci.* 2016, 129, 1340–1354.
- Rosasco-Nitcher, S.E.; Lan, W.; Khorasanizadeh, S.; Stukenberg, P.T. Centromeric Aurora-B activation requires TD-60, microtubules, and substrate priming phosphorylation. *Science* 2008, 319, 469–472.
- Rosenberg, J.S.; Cross, F.R.; Funabiki, H. KNL1/Spc105 recruits PP1 to silence the spindle assembly checkpoint. *Curr. Biol.* 2011, 21, 942–947.
- Roshak, A.K.; Capper, E.A.; Imburgia, C.; Fornwald, J.; Scott, G.; Marshall, L.A. The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. *Cell Signal.* 2000, 12, 405–411.
- Rotem, A.; Gruber, R.; Shorer, H.; Shaulov, L.; Klein, E.; Harel, A. Importin beta regulates the seeding of chromatin with initiation sites for nuclear pore assembly. *Mol. Biol. Cell* 2009, 20, 4031–4042.
- Rowland, B.D.; Roig, M.B.; Nishino, T.; Kurze, A.; Uluocak, P.; Mishra, A.; Beckouët, F.; Underwood, P.; Metson, J.; Imre, R.; et al. Building sister chromatid cohesion: Smc3 acetylation counteracts an antiestablishment activity. *Mol. Cell* 2009, 33, 763–774.
- Roy B.; Han S.J.; Fontan A.N.; Joglekar A.P. The copy-number and varied strengths of MELT motifs in Spc105 balance the strength and responsiveness of the spindle assembly checkpoint. *Elife.* 2020, 9:55096.

- Roy, J.; Cyert, M.S. Cracking the phosphatase code: Docking interactions determine substrate specificity. *Sci. Signal.* 2009, 2, re9.
- Rudolph, J. Catalytic mechanism of Cdc25. *Biochemistry* 2002, 41:14613–14623.
- Rudolph, J. Cdc25 phosphatases: Structure, specificity, and mechanism. *Biochem.* 2007, 46: 3595–3604.
- Rudolph, J.; Epstein, D.M.; Parker, L.; Eckstein, J. Specificity of natural and artificial substrates for human Cdc25A. *Anal. Biochem.* 2001, 289, 43–51.
- Ruff, E.F.; Muretta, J.M.; Thompson, A.R.; Lake, E.W.; Cyphers, S.; Albanese, S.K.; Hanson, S.M.; Behr, J.M.; Thomas, D.D.; Chodera, J.D.; Levinson, N.M. A dynamic mechanism for allosteric activation of Aurora kinase A by activation loop phosphorylation. *eLife* 2018, 7, e32766.
- Ruppert, F.G.; Samejima, K.; Platani, M.; Molina, O.; Kimura, H.; Jeyaprakash, A.A.; Ohta, S.; Earnshaw, W.C. HP1 α targets the chromosomal passenger complex for activation at heterochromatin before mitotic entry. *EMBO J.* 2018, e97677.
- Rusin, S.F.; Schlosser, K.A.; Adamo, M.E.; Kettenbach, A.N. Quantitative phosphoproteomics reveals new roles for the protein phosphatase PP6 in mitotic cells. *Sci. Signal.* 2015, 8, rs12.
- Sacco, F.; Perfetto, L.; Castagnoli, L.; Cesareni, G. The human phosphatase interactome: An intricate family portrait. *FEBS Lett.* 2012, 586, 2732–2739.
- Sacristan, C.; Kops, G.J. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. *Trends in Cell Biology.* 2015, 25:21–28.
- Sadhu, K.; Reed, S.I.; Richardson, H.; Russell, P. Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. *Proc. Natl. Acad. Sci. USA* 1990, 87, 5139–5143.
- Salvi, F.; Hoermann, B.; García, J.P.; Fontanillo, M.; Derua, R.; Beullens, M.; Bollen, M.; Barabas, O.; Köhn, M. Towards Dissecting the Mechanism of Protein Phosphatase-1 Inhibition by its C-Terminal Phosphorylation. *ChemBioChem.* 2020, 22(5): 834-838.
- Sana, S.; Keshri, R.; Rajeevan, A.; Kapoor, S.; Kotak, S. Plk1 regulates spindle orientation by phosphorylating NuMA in human cells. *Life Sci. Alliance* 2018, 1, e201800223.
- Sanchez, Y.; Wong, C.; Thoma, R.S.; Richman, R.; Wu, Z.; Piwnicka-Worms, H.; Elledge, S.J. Conservation of the Chk1 Checkpoint Pathway in Mammals: Linkage of DNA Damage to Cdk Regulation Through Cdc25. *Science* 1997, 277, 1497–1501.
- Santaguida S., Tighe A., D'Alise A.M., Taylor S.S., Musacchio A. Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine. *The Journal of Cell Biology.* 2010, 190: 73–87.
- Santaguida, S.; Vernieri, C.; Villa, F.; Ciliberto, A.; Musacchio, A. Evidence that AuroraB is implicated in spindle checkpoint signalling independently of error correction. *EMBO J.* 2011, 30, 1508–1519.
- Santamaria, A.; Neef, R.; Eberspächer, U.; Eis, K.; Husemann, M.; Mumberg, D.; Prechtel, S.; Schulze, V.; Siemeister, G.; Wortmann, L.; et al. Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis. *Mol. Biol. Cell* 2007, 18, 4024–4036.

- Sassoon, I.; Severin, F.F.; Andrews, P.D.; Taba, M.R.; Kaplan, K.B.; Ashford, A.J.; Stark, M.J.; Sorger, P.K.; Hyman, A.A. Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. *Genes Dev.* 1999, 13, 545–555.
- Saurin, A.T. Kinase and phosphatase cross-talk at the kinetochore. *Front. Cell Dev. Biol.* 2018, 6, 62.
- Saurin, A.T.; van der Waal, M.S.; Medema, R.H.; Lens, S.M.; Kops, G.J. Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis. *Nat. Commun.* 2011, 2, 316.
- Schellhaus, A.K.; De Magistris, P.; Antonin, W. Nuclear Reformation at the End of Mitosis. *J. Mol. Biol.* 2016, 428, 1962–1985.
- Schittenhelm R.B., Chaleckis R., Lehner C.F. Intrakinetochore localization and essential functional domains of *Drosophila* Spc105. *The EMBO Journal.* 2009, 28:2374–2386.
- Schlaitz, A.L.; Srayko, M.; Dammermann, A.; Quintin, S.; Wielsch, N.; MacLeod, I.; de Robillard, Q.; Zinke, A.; Yates, J.R.3rd; Müller-Reichert, T.; Shevchenko, A.; Oegema, K.; Hyman, A.A. The *C. elegans* RSA complex localizes protein phosphatase 2A to centrosomes and regulates mitotic spindle assembly. *Cell* 2007, 128, 115–127.
- Schleicher, K.; Porter, M.; ten Have, S.; Sundaramoorthy, R.; Porter, I.M.; Swedlow, J.R. The Ndc80 complex targets Bod1 to human mitotic kinetochores. *Open Biol.* 2017, 7, 170099.
- Schmidt, J.C.; Arthanari, H.; Boeszoermenyi, A.; Dashkevich, N.M.; Wilson-Kubalek, E.M.; Monnier, N.; Markus, M.; Oberer, M.; Milligan, R.A.; Bathe, M.; et al. The kinetochore-bound Ska1 complex tracks depolymerizing microtubules and binds to curved protofilaments. *Dev. Cell* 2012, 23, 968–980.
- Schmitz, M.H.A.; Held, M.; Janssens, V.; Hutchins, J.R.A.; Hudecz, O.; Ivanova, E.; Goris, J.; Trinkle-Mulcahy, L.; Lamond, A.I.; Poser, I.; et al. Live-cell imaging RNAi screen identifies PP2A-B55 and importin-1 as key mitotic exit regulators in human cells. *Nat. Cell Biol.* 2010, 12, 886–893.
- Schvartzman, J.M.; Sotillo, R.; Benezra, R. Mitotic chromosomal instability and cancer: Mouse modelling of the human disease. *Nat. Rev. Cancer* 2010, 10, 102–115.
- Schweizer, N.; Ferrás, C.; Kern, D.M.; Logarinho, E.; Cheeseman, I.M.; Maiato, H. Spindle assembly checkpoint robustness requires Tpr-mediated regulation of Mad1/Mad2 proteostasis. *The Journal of Cell Biology.* 2013, 203:883– 893.
- Scrofani, J.; Sardon, T.; Meunier, S.; Vernos, I. Microtubule nucleation in mitosis by a RanGTP-dependent protein complex. *Curr. Biol.* 2015, 25, 131–140.
- Seki, A.; Copping, J.A.; Jang, C.Y.; Yates, J.R.; Fang, G. Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. *Science* 2008, 320, 1655–1658.
- Seldin, L.; Macara, I. Epithelial spindle orientation diversities and uncertainties: Recent developments and lingering questions. *F1000Research* 2017, 6, 984.
- Seshacharyulu, P.; Pandey, P.; Datta, K.; Batra, S.K. Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Lett.* 2013, 335, 9–18.

- Sessa, F.; Mapelli, M.; Ciferri, C.; Tarricone, C.; Areces, L.B.; Schneider, T.R.; Stukenberg, P.T.; Musacchio, A. Mechanism of Aurora B Activation by INCENP and Inhibition by Hesperadin. *Mol. Cell* 2005, 18, 379–391.
- Shah, J.V.; Botvinick, E.; Bonday, Z.; Furnari, F.; Berns, M.; Cleveland, D.W. Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. *Curr. Biol.* 2004, 14: 942–952.
- Shao, H.; Huang, Y.; Zhang, L.; Yuan, K.; Chu, Y.; Dou, Z.; Jin, C.; Garcia-Barrio, M.; Liu, X.; Yao, X. Spatiotemporal dynamics of Aurora B-PLK1-MCAK signaling axis orchestrates kinetochore bi-orientation and faithful chromosome segregation. *Sci. Rep.* 2015, 5, 12204.
- Sharma, K.; D'Souza, R.C.; Tyanova, S.; Schaab, C.; Wiśniewski, J.R.; Cox, J.; Mann, M. Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep.* 2014, 8, 1583–1594.
- Shepperd, L.A.; Meadows, J.C.; Sochaj, A.M.; Lancaster, T.C.; Zou, J.; Buttrick, G.J.; Rappsilber, J.; Hardwick, K.G.; Millar, J.B. Phosphodependent recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 kinase maintains the spindle checkpoint. *Curr. Biol.* 2012, 22, 891–899.
- Shi, Y. Serine/Threonine Phosphatases: Mechanism through Structure. *Cell* 2009, 139, 468–484.
- Shindo, N.; Otsuki, M.; Uchida, K.S.K.; Hirota, T. Prolonged mitosis causes separase deregulation and chromosome nondisjunction. *Cell Reports*. 2021, 34(3): 108652.
- Shrestha, R.L.; Conti, D.; Tamura, N.; Braun, D.; Ramalingam, R.A.; Cieslinski, K.; Ries, J.; Draviam, V.M. Aurora-B kinase pathway controls the lateral to end-on conversion of kinetochore-microtubule attachments in human cells. *Nat. Commun.* 2017, 8, 150.
- Shrestha, R.L.; Draviam, V.M. Lateral to end-on conversion of chromosome-microtubule attachment requires kinesins CENP-E and MCAK. *Curr. Biol.* 2013, 23, 1514–1526.
- Sigrist, S.; Jacobs, H.; Stratmann, R.; Lehner, C.F. Exit from mitosis is regulated by *Drosophila* fizzy and the sequential destruction of cyclins A, B and B3. *EMBO J.* 1995, 14(19): 4827–4838.
- Silió V., McAinsh A.D., Millar J.B. KNL1-Bubs and RZZ provide two separable pathways for checkpoint activation at human kinetochores. *Developmental Cell*. 2015, 35:600–613.
- Silkworth, W.T.; Nardi, I.K.; Paul, R.; Mogilner, A.; Cimini, D. Timing of centrosome separation is important for accurate chromosome segregation. *Mol. Biol. Cell* 2012, 23, 401–411.
- Siller, K.H.; Doe, C.Q. Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* 2009, 11, 365–374.
- Simonetta, M.; Manzoni, R.; Mosca, R.; Mapelli, M.; Massimiliano, L.; Vink, M.; Novak, B.; Musacchio, A.; Ciliberto, A. The influence of catalysis on mad2 activation dynamics. *PLoS Biol.* 2009, 7(1): 10.
- Sinha, N.; Pilder, S.; Vijayaraghavan, S. Significant expression levels of transgenic PPP1CC2 in testis and sperm are required to overcome the male infertility phenotype of Ppp1cc null mice. *PLoS ONE* 2012, 7, e47623.

- Sinha, N.; Puri, P.; Nairn, A.C.; Vijayaraghavan, S. Selective ablation of Ppp1cc gene in testicular germ cells causes oligo-teratozoospermia and infertility in mice. *Biol. Reprod.* 2013, 89, 128.
- Sivakumar, S.; Gorbsky, G.J. Phosphatase-regulated recruitment of the spindle- and kinetochore-associated (Ska) complex to kinetochores. *Biol Open.* 2017, 6(11): 1672-1679.
- Sivakumar, S.; Janczyk, P.Ł.; Qu, Q.; Brautigam, C.A.; Stukenberg, P.T.; Yu, H.; Gorbsky, G.J. The human SKA complex drives the metaphase-anaphase cell cycle transition by recruiting protein phosphatase 1 to kinetochores. *Elife* 2016, 5, e12902.
- Slupe, A.M.; Merrill, R.A.; Strack, S. Determinants for substrate specificity of protein phosphatase 2A. *Enzyme Res.* 2011, 2011, 398751.
- Smurnyy Y., Toms A.V., Hickson G.R., Eck M.J., Eggert U.S. Binucleine 2, an isoform-specific inhibitor of *Drosophila* Aurora B kinase, provides insights into the mechanism of cytokinesis. *ACS Chemical Biology.* 2010, 5: 1015–1020.
- Sohn, J.; Buhrman, G.; Rudolph, J. Kinetic and structural studies of specific protein-protein interactions in substrate catalysis by Cdc25B phosphatase. *Biochem.* 2007, 46, 807–818.
- Sohn, J.; Kristjánsdóttir, K.; Safi, A.; Parker, B.; Kiburz, B.; Rudolph, J. Remote hot spots mediate protein substrate recognition for the Cdc25 phosphatase. *Proc. Natl. Acad. Sci. USA* 2004, 101, 16437–16441.
- Srayko, M.; Kaya, A.; Stamford, J.; Hyman, A.A. Identification and characterization of factors required for microtubule growth and nucleation in the early *C. elegans* embryo. *Dev. Cell* 2005, 9, 223–226.
- Stearns, T.; Kirschner, M. In vitro reconstitution of centrosome assembly and function: The central role of γ -tubulin. *Cell* 1994, 76, 623–637.
- Steen, R.L.; Beullens, M.; Landsverk, H.B.; Bollen, M.; Collas, P. AKAP149 is a novel PP1 specifier required to maintain nuclear envelope integrity in G1 phase. *J. Cell Sci.* 2003, 116, 2237–2246.
- Steen, R.L.; Martins, S.B.; Tasken, K.; Collas, P. Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly. *J. Cell Biol.* 2000, 150, 1251–1262.
- Stevens, D.; Gassmann, R.; Oegema, K.; Desai, A. Uncoordinated loss of chromatid cohesion is a common outcome of extended metaphase arrest. *PLoS One.* 2011, 6(8): e22969.
- Stucke, V.M.; Baumann, C.; Nigg, E.A. Kinetochore localization and microtubule interaction of the human spindle checkpoint kinase Mps1. *Chromosoma* 2004, 113: 1–15.
- Subramanian, R.; Wilson-Kubalek, E.M.; Arthur, C.P.; Bick, M.J.; Campbell, E.A.; Darst, S.A.; Milligan, R.A.; Kapoor, T.M. Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. *Cell* 2010, 142, 433–443.
- Sudakin, V.; Chan, G.K.; Yen, T.J. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.* 2001, 154: 925–936.

- Suijkerbuijk, S.J.; van Dam, T.J.; Karagoz, G.E.; von Castelmur E.; Hubner, N.C.; Duarte, A.M.; Vleugel, M.; Perrakis, A.; Rudiger, S.G.; Snel, B.; Kops, G.J. The vertebrate mitotic checkpoint protein BUBR1 is an unusual pseudokinase, *Dev. Cell.* 2012, 22:1321-1329. (a)
- Suijkerbuijk, S.J.; Vleugel, M.; Teixeira, A.; Kops, G.J. Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. *Dev. Cell* 2012, 23, 745–755. (b)
- Sumara, I.; Vorlaufer, E.; Stukenberg, P.T.; Kelm, O.; Redemann, N.; Nigg, E.A.; Peters, J.M. The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol. Cell* 2002, 9, 515–525.
- Sumiyoshi, E.; Sugimoto, A.; Yamamoto, M. Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in *C. elegans*. *J. Cell Sci.* 2002, 115, 1403–1410.
- Sunkel, C.E.; Gomes, R.; Sampaio, P.; Perdigo, J.; González, C. Gamma-tubulin is required for the structure and function of the microtubule organizing centre in *Drosophila* neuroblasts. *EMBO J.* 1995, 14, 28–36.
- Sutani, T.; Kawaguchi, T.; Kanno, R.; Itoh, T.; Shirahige, K. Budding yeast Wpl1 (Rad61)-Pds5 complex counteracts sister chromatid cohesion-establishing reaction. *Curr. Biol.* 2009, 19, 492–497.
- Suzuki, A.; Gupta, A.; Long, S.K.; Evans, R.; Badger, B.L.; Salmon, E.D.; Biggings, S.; Bloom, K.A Kinesin-5, Cin8, Recruits Protein Phosphatase 1 to Kinetochores and Regulates Chromosome Segregation. *Curr. Biol.* 2018, 28, 2697–2704.
- Tanaka, K.; Mukae, N.; Dewar, H.; Breugel, M.; James, E.K.; Prescott, A.R.; Anthony, A.; Tanaka, T.U. Molecular mechanisms of kinetochore capture by spindle microtubules. *Nature* 2005, 434, 987–994.
- Tanaka, T.; Fuchs, J.; Loidl, J.; Nasmyth, K. Cohesin ensures bipolar attachment of microtubules to sister centromeres and resists their precocious separation. *Nat. Cell Biol.* 2000, 2, 492–499.
- Tanaka, T.U.; Rachidi, N.; Janke, C.; Pereira, G.; Galova, M.; Schiebel, E.; Stark, M.J.R.; Nasmyth, K. Evidence that the Ipl1-Sli15 (Aurora Kinase-INCENP) Complex Promotes Chromosome Bi-orientation by Altering Kinetochore-Spindle Pole Connections. *Cell* 2002, 108, 317–329.
- Tanenbaum, M.E.; Medema, R.H. Mechanisms of centrosome separation and bipolar spindle assembly. *Dev. Cell* 2010, 19, 797–806.
- Tang, N.H.; Toda, T. Alp7/TACC recruits kinesin-8-PP1 to the Ndc80 kinetochore protein for timely mitotic progression and chromosome movement. *J. Cell Sci.* 2015, 128, 354–363.
- Tang, Z.; Bharadwaj, R.; Li, B.; Yu, H. Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. *Dev. Cell* 2001, 1: 227–237.
- Tang, Z.; Shu, H.; Qi, W.; Mahmood, N.A.; Mumby, M.C.; Yu, H. PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. *Dev. Cell* 2006, 10, 575–585.

- Tanno, Y.; Kitajima, T.S.; Honda, T.; Ando, Y.; Ishiguro, K.; Watanabe, Y. Phosphorylation of mammalian Sgo2 by Aurora B recruits PP2A and MCAK to centromeres. *Genes Dev.* 2010, 24, 2169–2179.
- Taylor, S.S.; Ha, E.; McKeon, F. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J. Cell Biol.* 1998; 142, 1–11.
- Tedeschi, A.; Wutz, G.; Huet, S.; Jaritz, M.; Wuensche, A.; Schirghuber, E.; Davidson, I.F.; Tang, W.; Cisneros, D.A.; Bhaskara, V.; et al. Wapl is an essential regulator of chromatin structure and chromosome segregation. *Nature* 2013, 501, 564–568.
- Terrak, M.; Kerff, F.; Langsetmo, K.; Tao, T.; Dominguez, R. Structural basis of protein phosphatase 1 regulation. *Nature* 2004, 429, 780–784.
- Terry-Lorenzo, R.T.; Elliot, E.; Weiser, D.C.; Prickett, T.D.; Brautigan, D.L.; Shenolikar, S. Neurabins recruit protein phosphatase-1 and inhibitor-2 to the actin cytoskeleton. *J. Biol. Chem.* 2002, 277, 46535–46543.
- Thawani, A.; Kadzik, R.S.; Petry, S. XMAP215 is a microtubule nucleation factor that functions synergistically with the γ -tubulin ring complex. *Nat. Cell Biol.* 2018, 20, 575–585.
- Thompson, L.J.; Bollen, M.; Fields, A.P. Identification of protein phosphatase 1 as a mitotic lamin phosphatase. *J. Biol. Chem.* 1997, 272, 29693–29697.
- Thompson, S.L.; Compton, D.A. Chromosome missegregation in human cells arises through specific types of kinetochore–microtubule attachment errors. *Proc. Natl Acad. Sci. USA* 2011, 108, 17974–17978.
- Thompson, S.L.; Compton, D.A. Examining the link between chromosomal instability and aneuploidy in human cells. *J Cell Biol.* 2008, 180(4): 665-72.
- Tiganis, T.; Bennett, A.M. Protein tyrosine phosphatase function: The substrate perspective. *Biochem. J.* 2007, 402, 1–15.
- Tonks, N.K. Protein tyrosine phosphatases: From genes, to function, to disease. *Nat. Rev. Mol. Cell. Biol.* 2006, 7, 833–846.
- Totsukawa, G.; Yamakita, Y.; Yamashiro, S.; Hosoya, H.; Hartshorne, D.J.; Matsumura, F. Activation of myosin phosphatase targeting subunit by mitosis-specific phosphorylation. *J. Cell Biol.* 1999, 144, 735–744.
- Tournebise, R.; Popov, A.; Kinoshita, K.; Ashford, A.J.; Rybina, S.; Pozniakovsky, A.; Mayer, T.U.; Walczak, C.E.; Karsenti, E.; Hyman, A.A. Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in *Xenopus* egg extracts. *Nat. Cell Biol.* 2000, 2, 13–19.
- Toyo-oka, K.; Mori, D.; Yano, Y.; Shiota, M.; Iwao, H.; Goto, H.; Inagaki, M.; Hiraiwa, N.; Muramatsu, M.; Wynshaw-Boris, A.; Yoshiki, A.; Hirotsune, S. Protein phosphatase 4 catalytic subunit regulates Cdk1 activity and microtubule organization via NDEL1 dephosphorylation. *J. Cell Biol.* 2008, 180, 1133–1147.
- Toyo-Oka, K.; Sasaki, S.; Yano, Y.; Mori, D.; Kobayashi, T.; Toyoshima, Y.Y.; Tokuoka, S.M.; Ishii, S.; Shimizu, T.; Muramatsu, M.; Hiraiwa, N.; Yoshiki, A.; Wynshaw-Boris, A.; Hirotsune, S. Recruitment of katanin p60 by phosphorylated NDEL1, an LIS1 interacting protein, is essential for mitotic cell division and neuronal migration. *Hum. Mol. Genet.* 2005, 14, 3113–3128.

- Toyoshima-Morimoto, F.; Taniguchi, E.; Nishida, E. Plk1 promotes nuclear translocation of human Cdc25C during prophase. *EMBO Rep.* 2002, 3, 341–348.
- Trinkle-Mulcahy, L.; Andersen, J.; Lam, Y.W.; Moorhead, G.; Mann, M.; Lamond, A.I. Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. *J. Cell. Biol.* 2006, 172, 679–692.
- Trinkle-Mulcahy, L.; Lamond, A.I. Mitotic phosphatases: No longer silent partners. *Curr. Opin. Cell Biol.* 2006, 18, 623–631.
- Tsai, M.Y.; Wiese, C.; Cao, K.; Martin, O.; Donovan, P.; Ruderman, J.; Prigent, C.; Zheng, Y. A Ran signalling pathway mediated by the mitotic kinase Aurora A in spindle assembly. *Nat. Cell Biol.* 2003, 5, 242–248.
- Tyler, R.K.; Chu, M.L.; Johnson, H.; McKenzie, E.A.; Gaskell, S.J.; Eysers, P.A. Phosphoregulation of human Mps1 kinase. *Biochem. J.* 2009, 417, 173–181.
- Uchida, K.S.; Takagaki, K.; Kumada, K.; Hirayama, Y.; Noda, T.; Hirota, T. Kinetochore stretching inactivates the spindle assembly checkpoint. *J. Cell Biol.* 2009, 184, 383–390.
- Ünal, E.; Heidinger-Pauli, J.M.; Kim, W.; Guacci, V.; Onn, I.; Gygi, S.P.; Koshland, D.E. A molecular determinant for the establishment of sister chromatid cohesion. *Science* 2008, 321, 566–569.
- Ungrecht, R.; Kutay, U. Mechanisms and functions of nuclear envelope remodelling. *Nat. Rev. Mol. Cell Biol.* 2017, 18, 229–245.
- Vagnarelli, P.; Hudson, D.F.; Ribeiro, S.A.; Trinkle-Mulcahy, L.; Spence, J.M.; Lai, F.; Farr, C.J.; Lamond, A.I.; Earnshaw, W.C. Condensin and Repo-Man-PP1 co-operate in the regulation of chromosome architecture during mitosis. *Nat. Cell. Biol.* 2006, 8, 1133–1142.
- Vagnarelli, P.; Ribeiro, S.; Sennels, L.; Sanchez-Pulido, L.; de Lima Alves, F.; Verheyen, T.; Kelly, D.A.; Ponting, C.P.; Rappsilber, J.; Earnshaw, W.C. Repo-Man coordinates chromosomal reorganization with nuclear envelope reassembly during mitotic exit. *Dev. Cell* 2011, 21, 328–342.
- Vallardi, G.; Allan, L.A.; Crozier, L.; Saurin, A.T. A molecular labour between PP2A-B56 isoforms at the centromere and kinetochore. *eLife.* 2019. 8: e42619.
- Vallardi, G.; Cordeiro, M.H.; Saurin, A.T. A kinase-phosphatase network that regulates kinetochore-microtubule attachments and the SAC. *Prog. Mol. Subcell. Biol.* 2017, 56, 457–484.
- van der Waal, M.S.; Saurin, A.T.; Vromans, M.J.; Vleugel, M.; Wurzenberger, C.; Gerlich, D.W.; Medema, R.H.; Kops, G.J.; Lens, S.M. Mps1 promotes rapid centromere accumulation of Aurora B. *EMBO Rep.* 2012, 13, 847–854.
- van Vugt, M.A.; Brás, A.; Medema, R.H. Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. *Mol. Cell* 2004, 15, 799–811.
- Vanoosthuyse, V.; Hardwick, K.G. A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism. *Curr. Biol.* 2009, 19, 1176–1181.
- Vázquez-Novelle, M.D.; Petronczki, M. Relocation of the chromosomal passenger complex prevents mitotic checkpoint engagement at anaphase. *Curr. Biol.* 2010, 20: 1402–1407.

- Vázquez-Novelle, M.D.; Sansregret, L.; Dick, A.E.; Smith, C.A.; McAinsh, A.D.; Gerlich, D.W.; Petronczki, M. Cdk1 inactivation terminates mitotic checkpoint surveillance and stabilizes kinetochore attachments in anaphase. *Curr. Biol.* 2014, 24, 638–645.
- Verbinnen, I.; Ferreira, M.; Bollen, M. Biogenesis and activity regulation of protein phosphatase 1. *Biochem. Soc. Trans.* 2017, 45, 89–99.
- Vigneron, S.; Gharbi-Ayachi, A.; Raymond, A.A.; Burgess, A.; Labbé, J.C.; Labesse, G.; Monsarrat, B.; Lorca, T.; Castro, A. Characterization of the mechanisms controlling Greatwall activity. *Mol. Cell. Biol.* 2011, 31, 2262–2275.
- Vigneron, S.; Prieto, S.; Bernis, C.; Labbé, J-C; Castro, A.; Lorca, T. Kinetochore localization of spindle checkpoint proteins: who controls whom? *Mol. Biol. Cell* 2004, 15: 4584–4596.
- Vigneron, S.; Sundermann, L.; Labbé, J.C.; Pintard, L.; Radulescu, O.; Castro, A.; Lorca, T. Cyclin A-Cdk1-dependent phosphorylation of Bora is the triggering factor promoting mitotic entry. *Dev. Cell* 2018, 45, 637–650.
- Vleugel M.; Tromer E.; Omerzu M.; Groenewold V.; Nijenhuis W.; Snel B.; Kops G.J. Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation. *J. Cell Biol.* 2013, 203(6): 943–955.
- Vleugel, M.; Hoogendoorn, E.; Snel, B.; Kops, G.J.P.L. Evolution and Function of the Mitotic Checkpoint. 2012, 23(2): 239-250.
- Vleugel, M.; Omerzu, M.; Groenewold, V.; Hadders, M.A.; Lens, S.M.A.; Kops, G.J.P.L. Sequential multisite phospho-regulation of KNL1-BUB3 interfaces at mitotic kinetochores. *Mol. Cell* 2015, 57, 824–835.
- Vlijm, R.; Li, X.; Panic, M.; Rüttnick, D.; Hata, S.; Herrmannsdörfer, F.; Kuner, T.; Heilemann, M.; Engelhardt, J.; Hell, S.W.; et al. STED nanoscopy of the centrosome linker reveals a CEP68-organized, periodic rootletin network anchored to a C-Nap1 ring at centrioles. *Proc. Natl. Acad. Sci. USA* 2018, 115, E2246–E2253.
- Voets, E.; Wolthuis, R.M. MASTL is the human orthologue of Greatwall kinase that facilitates mitotic entry, anaphase and cytokinesis. *Cell Cycle* 2010, 9, 3591–3601.
- von Schubert, C.; Cubizolles, F.; Bracher, J.M.; Slidrecht, T.; Kops, G.J.P.L.; Nigg, E.A. Plk1 and Mps1 Cooperatively Regulate the Spindle Assembly Checkpoint in Human Cells. *Cell Rep.* 2015, 12, 66–78.
- Vorozhko, V.V.; Emanuele, M.J.; Kallio, M.J.; Stukenberg, P.T.; Gorbsky, G.J. Multiple mechanisms of chromosome movement in vertebrate cells mediated through the Ndc80 complex and dynein/dynactin. *Chromosoma* 2008, 117, 169–179.
- Wakula, P.; Beullens, M.; Ceulemans, H.; Stalmans, W.; Bollen, M. Degeneracy and function of the ubiquitous RVXF-motif that mediates binding to protein phosphatase-1. *J. Biol. Chem.* 2003, 278, 18817–18823.
- Walczak, C.E.; Shaw, S.L. AMAP for bundling microtubules. *Cell* 2010, 142, 364–367.
- Walter, A.O.; Seghezzi, W.; Korver, W.; Sheung, J.; Lees, E. The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation. *Oncogene* 2000, 19, 4906–4916.

- Walther, T.C.; Askjaer, P.; Gentzel, M.; Habermann, A.; Griffiths, G.; Wilm, M.; Mattaj, I.W.; Hetzer, M. RanGTP mediates nuclear pore complex assembly. *Nature* 2003, 424, 689–694.
- Wan, X.; O'Quinn, R.P.; Pierce, H.L.; Joglekar, A.P.; Gall, W.E.; DeLuca, J.G.; Carroll, C.W.; Liu, S.; Yen, T.J.; McEwen, B.F.; Stukenberg, P.T.; Desai, A.; Salmon, E.D. Protein Architecture of the Human Kinetochore Microtubule Attachment Site. *Cell* 2009, 137, 672–684.
- Wang, E.; Ballister, E.R.; Lampson, M.A. Aurora B dynamics at centromeres create a diffusion-based phosphorylation gradient. *J. Cell Biol.* 2011, 194, 539–549.
- Wang, G.; Jiang, Q.; Zhang, C. The role of mitotic kinases in coupling the centrosome cycle with the assembly of the mitotic spindle. *J. Cell Sci.* 2014, 127, 4111–4122.
- Wang, J.; Wang, Z.; Yu, T.; Yang, H.; Virshup, D.M.; Kops, G.J.; Lee, S.H.; Zhou, W.; Li, X.; Xu, W.; et al. Crystal structure of a PP2A B56-BubR1 complex and its implications for PP2A substrate recruitment and localization. *Protein Cell* 2016, 7, 516–526.
- Wang, L.H.; Mayer, B.; Stemmann, O.; Nigg, E.A. Centromere DNA decatenation depends on cohesin removal and is required for mammalian cell division. *J. Cell Sci.* 2010, 123, 806–813.
- Wang, W.; Yang, Y.; Gao, Y.; Xu, Q.; Wang, F.; Zhu, S.; Old, W.; Resing, K.; Ahn, N.; Lei, M.; Liu, X. Structural and mechanistic insights into Mps1 kinase activation. *J. Cell. Mol. Med.* 2009, 13, 1679–1694.
- Wang, X.; Bajaj, R.; Bollen, M.; Peti, W.; Page, R. Expanding the PP2A interactome by defining a B56-specific SLiM. *Structure* 2016, 24, 2174–2181.
- Watanabe, N.; Arai, H.; Nishihara, Y.; Taniguchi, M.; Watanabe, N.; Hunter, T.; Osada, H. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCF-TrCP. *Proc. Natl. Acad. Sci. USA* 2004, 101, 4419–4424.
- Wee, P.; Shi, H.; Jiang, J.; Wang, Y.; Wang, Z. EGF stimulates the activation of EGF receptors and the selective activation of major signaling pathways during mitosis. *Cell Signal.* 2015, 27, 638–651.
- Wei, R.R.; Al-Bassam, J.; Harrison, S.C. The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat. Struct. Mol. Biol.* 2006, 14, 54–59.
- Weith, M.; Seiler, J.; van den Boom, J.; Kracht, M.; Hülsmann, J.; Primorac, I.; Garcia, J.P.; Kaschani, F.; Kaiser, M.; Musacchio, A.; et al. Ubiquitin-independent disassembly by a p97 AAA-ATPase complex drives PP1 holoenzyme formation. *Mol. Cell* 2018, 72, 766–777.
- Welburn, J.P.; Grishchuk, E.L.; Backer, C.B.; Wilson-Kubalek, E.M.; YatesIII, J.R.; Cheeseman, I.M. The human kinetochore Ska1 complex facilitates microtubule depolymerization-coupled motility. *Dev. Cell* 2009, 16, 374–385.
- Welburn, J.P.; Vleugel, M.; Liu, D.; Yates, J.R.; Lampson, M.A.; Fukagawa, T.; Cheeseman, I.M. Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. *Mol. Cell* 2010, 38, 383–392.
- Wilborn, M.; Free, S.; Ban, A.; Rudolph, J. The C-terminal tail of the dual-specificity Cdc25B phosphatase mediates modular substrate recognition. *Biochem.* 2001, 40, 14200–14206.

- Williams, B.C.; Filter, J.J.; Blake-Hodek, K.A.; Wadzinski, B.E.; Fuda, N.J.; Shalloway, D.; Goldberg, M.L. Greatwall-phosphorylated Endosulfine is both an inhibitor and a substrate of PP2A-B55 heterotrimers. *Elife* 2014, 3, e01695.
- Wittmann, T.; Hyman, A.; Desai, A. The spindle: A dynamic assembly of microtubules and motors. *Nat. Cell Biol.* 2001, 3, E28–E34.
- Wittmann, T.; Wilm, M.; Karsenti, E.; Vernos, I. TPX2, A novel xenopus MAP involved in spindle pole organization. *J. Cell Biol.* 2000, 149, 1405–1418.
- Wolfe, B.A.; Takaki, T.; Petronczki, M.; Glotzer, M. Polo-like kinase 1 directs assembly of the HsCdk-4 RhoGAP/Ect2 RhoGEF complex to initiate cleavage furrow formation. *PLoS Biol.* 2009, 7, e1000110.
- Woodruff, J.B.; Wueseke, O.; Hyman, A.A. Pericentriolar material structure and dynamics. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 2014, 369, 20130459.
- Wu, C.G.; Chen, H.; Guo, F.; Yadav, V.K.; McIlwain, S.J.; Rowse, M.; Choudhary, A.; Lin, Z.; Li, Y.; Gu, T.; et al. PP2A-B0 holoenzyme substrate recognition, regulation and role in cytokinesis. *Cell Discov.* 2017, 3, 17027.
- Wu, J.Q.; Guo, J.Y.; Tang, W.; Yang, C.S.; Freel, C.D.; Chen, C.; Nairn, A.C.; Kornbluth, S. PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation. *Nat. Cell Biol.* 2009, 11, 644–651.
- Wurzenberger, C.; Gerlich, D.W. Phosphatases: Providing safe passage through mitotic exit. *Nat. Rev. Mol. Cell Biol.* 2011, 12, 469–482.
- Wurzenberger, C.; Held, M.; Lampson, M.A.; Poser, I.; Hyman, A.A.; Gerlich, D.W. Sds22 and Repo-Man stabilize chromosome segregation by counteracting Aurora B on anaphase kinetochores. *J. Cell Biol.* 2012, 198, 173–183.
- Xie, Y.; Jüschke, C.; Esk, C.; Hirotsune, S.; Knoblich, J.A. The phosphatase PP4c controls spindle orientation to maintain proliferative symmetric divisions in the developing neocortex. *Neuron* 2013, 79, 254–265.
- Xing, Y.; Xu, Y.; Chen, Y.; Jeffrey, P.D.; Chao, Y.; Lin, Z.; Li, Z.; Strack, S.; Stock, J.B.; Shi, Y. Structure of Protein Phosphatase 2A Core Enzyme Bound to Tumor-Inducing Toxins. *Cell* 2006, 127, 341–353.
- Xu, P.; Raetz, E.A.; Kitagawa, M.; Virshup, D.M.; Lee, S.H. BUBR1 recruits PP2A via the B56 family of targeting subunits to promote chromosome congression. *Biol. Open* 2013, 2, 479–486.
- Xu, Y.; Chen, Y.; Zhang, P.; Jeffrey, P.D.; Shi, Y. Structure of a Protein Phosphatase 2A Holoenzyme: Insights into B55-Mediated Tau Dephosphorylation. *Mol. Cell* 2008, 31, 873–885.
- Xu, Y.; Xing, Y.; Chen, Y.; Chao, Y.; Lin, Z.; Fan, E.; Yu, J.W.; Strack, S.; Jeffrey, P.D.; Shi, Y. Structure of the protein phosphatase 2A holoenzyme. *Cell* 2006, 127, 1239–1251.
- Xu, Z.; Cetin, B.; Anger, M.; Cho, U.S.; Helmhart, W.; Nasmyth, K.; Xu, W. Structure and function of the PP2A-shugoshin interaction. *Mol. Cell* 2009, 35, 426–441.
- Yamagishi, Y.; Yang, C.H.; Tanno, Y.; Watanabe, Y. MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components. *Nat. Cell Biol.* 2012, 14, 746–752.

- Yamano H., Ishii K., Yanagida M. Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus and its potential role in cell cycle regulation. *EMBO J.* 1994, 13:5310–5318.
- Yamashiro, S.; Yamakita, Y.; Totsukawa, G.; Goto, H.; Kaibuchi, K.; Ito, M.; Hartshorne, D.J.; Matsumura, F. Myosin phosphatase-targeting subunit 1 regulates mitosis by antagonizing polo-like kinase 1. *Dev. Cell* 2008, 14, 787–797.
- Yang, J.; Adamian, M.; Li, T. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. *Mol. Biol. Cell* 2006, 17, 1033–1040.
- Yang, J.; Liu, X.; Yue, G.; Adamian, M.; Bulgakov, O.; Li, T. Rootletin, a novel coiled-coil protein, is a structural component of the ciliary rootlet. *J. Cell Biol.* 2002, 159, 431–440.
- Yang, Z.; Tulu, U.S.; Wadsworth, P.; Rieder, C.L. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr. Biol.* 2007, 17, 973–980.
- Yasui, Y.; Urano, T.; Kawajiri, A.; Nagata, K.; Tatsuka, M.; Saya, H.; Furukawa, K.; Takahashi, T.; Izawa, I.; Inagaki, M. Autophosphorylation of a newly identified site of Aurora-B is indispensable for cytokinesis. *J. Biol. Chem.* 2004, 279, 12997–13003.
- Ye A.A., Cane S., Maresca T.J. Chromosome biorientation produces hundreds of piconewtons at a metazoan kinetochore. *Nat. Commun.* 2016, 7: 13221.
- Yu H. Structural activation of Mad2 in the mitotic spindle checkpoint: the two-state Mad2 model versus the Mad2 template model. *J. Cell Biol.* 2006, 173(2): 153–157.
- Yu, J.; Fleming, S.L.; Williams, B.; Williams, E.V.; Li, Z.; Somma, P.; Rieder, C.L.; Goldberg, M.L. Greatwall kinase: A nuclear protein required for proper chromosome condensation and mitotic progression in *Drosophila*. *J. Cell Biol.* 2004, 164, 487–492.
- Yu, J.; Zhao, Y.; Li, Z.; Galas, S.; Goldberg, M.L. Greatwall kinase participates in the Cdc2 autoregulatory loop in *Xenopus* egg extracts. *Mol. Cell* 2006, 22, 83–91.
- Yuvaniyama, J.; Denu, J.M.; Dixon, J.E.; Saper, M.A. Crystal structure of the dual specificity protein phosphatase VHR. *Science* 1996, 272, 1328–1331.
- Zaytsev, A.V.; Grishchuk, E.L. Basic mechanism for biorientation of mitotic chromosomes is provided by the kinetochore geometry and indiscriminate turnover of kinetochore microtubules. *Mol. Biol. Cell* 2015, 26, 3985–3998.
- Zaytsev, A.V.; Mick, J.E.; Maslennikov, E.; Nikashin, B.; DeLuca, J.G.; Grishchuk, E.L. Multisite phosphorylation of the NDC80 complex gradually tunes its microtubule-binding affinity. *Mol. Biol. Cell* 2015, 26, 1829–1844.
- Zaytsev, A.V.; Segura-Peña, D.; Godzi, M.; Calderon, A.; Ballister, E.R.; Stamatov, R.; Mayo, A.M.; Peterson, L.; Black, B.E.; Ataullakhanov, F.I.; Lampson, M.A.; Grishchuk, E.L. Bistability of a coupled Aurora B kinase-phosphatase system in cell division. *Elife* 2016, 5: e10644.
- Zaytsev, A.V.; Sundin, L.J.R.; DeLuca, K.F.; Grishchuk, E.L.; DeLuca, J.G. Accurate phosphoregulation of kinetochore–microtubule affinity requires unconstrained molecular interactions. *J. Cell Biol.* 2014, 206, 45–59.

- Zeng, K.; Bastos, R.N.; Barr, F.A.; Gruneberg, U. Protein phosphatase 6 regulates mitotic spindle formation by controlling the T-loop phosphorylation state of Aurora A bound to its activator TPX2. *J. Cell Biol.* 2010, 191, 1315–1332.
- Zeng, Y.; Forbes, K.C.; Wu, Z.; Moreno, S.; Piwnica-Worms, H.; Enoch, T. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature* 1998, 395, 507–510.
- Zhang, C.; Hutchins, J.R.; Mühlhäusser, P.; Kutay, U.; Clarke, P.R. Role of importin-beta in the control of nuclear envelope assembly by Ran. *Curr. Biol.* 2002, 12, 498–502.
- Zhang, G.; Kelstrup, C.D.; Hu, X.W.; Kaas Hansen, M.J.; Singleton, M.R.; Olsen, J.V.; Nilsson, J. The Ndc80 internal loop is required for recruitment of the Ska complex to establish end-on microtubule attachment to kinetochores. *J. Cell Sci.* 2012, 125, 3243–3253.
- Zhang, G.; Kruse, T.; López-Méndez, B.; Sylvestersen, K.B.; Garvanska, D.H.; Schopper, S.; Nielsen, M.L.; Nilsson, J. Bub1 positions Mad1 close to KNL1 MELT repeats to promote checkpoint signalling. *Nat. Commun.* 2017, 8, 15822.
- Zhang, G.; Lischetti, T.; Nilsson, J. A minimal number of MELT repeats supports all the functions of KNL1 in chromosome segregation. *J. Cell Sci.* 2014, 127, 871–884.
- Zhang, J.; Shi, X.; Li, Y.; Kim, B.J.; Jia, J.; Huang, Z.; Yang, T.; Fu, X.; Jung, S.Y.; Wang, Y.; Zhang, P.; Kim, S.T.; Pan, X.; Qin, J. Acetylation of Smc3 by Eco1 is required for S phase sister chromatid cohesion in both human and yeast. *Mol. Cell* 2008, 31, 143–151.
- Zhang, N.; Panigrahi, A.K.; Mao, Q.; Pati, D. Interaction of Sororin protein with Polo-like kinase 1 mediates resolution of chromosomal arm cohesion. *J. Biol. Chem.* 2011, 286, 41826–41837.
- Zhang, Q.; Chen, Y.; Yang, L.; Liu, H. Multitasking Ska in Chromosome Segregation: Its Distinct Pools Might Specify Various Functions. *Bioessays* 2018, 40, 1700176.
- Zhang, Q.; Sivakumar, S.; Chen, Y.; Gao, H.; Yang, L.; Yuan, Z.; Yu, H.; Liu, H. Ska3 Phosphorylated by Cdk1 Binds Ndc80 and Recruits Ska to Kinetochores to Promote Mitotic Progression. *Curr. Biol.* 2017, 27, 1477–1484.
- Zhang, Y.; Foreman, O.; Wigle, D.A.; Kosari, F.; Vasmatazis, G.; Salisbury, J.L.; van Deursen, J.; Galardy, P.J. USP44 regulates centrosome positioning to prevent aneuploidy and suppress tumorigenesis. *J. Clin. Investig.* 2012, 122, 4362–4374.
- Zhao, Y.; Haccard, O.; Wang, R.; Yu, J.; Kuang, J.; Jesus, C.; Goldberg, M.L.L. Roles of Greatwall Kinase in the Regulation of Cdc25 Phosphatase. *Mol. Biol. Cell* 2008, 19, 1317–1327.
- Zheng, Z.; Wan, Q.; Meixiong, G.; Du, Q. Cell cycle-regulated membrane binding of NuMA contributes to efficient anaphase chromosome separation. *Mol. Biol. Cell* 2014, 25, 606–619.
- Zhu, C.; Jiang, W. Cell cycle-dependent translocation of PRC1 on the spindle by Kif4 is essential for midzone formation and cytokinesis. *Proc. Natl. Acad. Sci. USA* 2005, 102, 343–348.

Zhu, C.; Lau, E.; Schwarzenbacher, R.; Bossy-Wetzel, E.; Jiang, W. Spatiotemporal control of spindle midzone formation by PRC1 in human cells. *Proc. Natl. Acad. Sci. USA* 2006, 103, 6196–6201.

Zorba, A.; Buosi, V.; Kutter, S.; Kern, N.; Pontiggia, F.; Cho, Y.J.; Kern, D. Molecular mechanism of AuroraA kinase autophosphorylation and its allosteric activation by TPX2. *Elife* 2014, 3, e02667.