

Investigating the role of phosphatases in the regulation of mitosis



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All work in this thesis that is not solely the candidate's own work is presented with the permission of those it was done in collaboration with.

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Abstract

The equal segregation of sister chromatids into progeny cells during mitosis is essential for preserving genomic integrity and supporting normal cellular functions across generations. Many aspects of mitosis including the spindle assembly checkpoint (SAC), the anaphase promoting complex/cyclosome (APC/C) and formation of stable kinetochore-microtubule attachments are regulated by dynamic and reversible phosphorylation reactions carried out by kinases and phosphatases. Protein phosphatase 1 (PP1), of which there are three catalytic isoforms in mammalian cells, is essential for control of mammalian cell division and has been implicated in the control of the SAC, the APC/C and kinetochore-microtubule attachment formation. However, its precise roles during mitosis are not yet fully understood, especially the potential of distinct isoform specific functions. This thesis explores the effect of an acute PP1 degradation of the mitosis-associated PP1 α and PP1 γ catalytic isoforms to analyse cell cycle progression in the absence of PP1. Concurrent acute degradation of PP1 α and PP1 γ results in delayed chromosome alignment as well as a delay at the metaphase to anaphase transition. During chromosome congression, there is an increase in the frequency of polar localised chromosomes in the absence of PP1 which leads to prolonged SAC signalling. Biochemically overriding the SAC alleviates the chromosome alignment, but not the anaphase onset delay, suggesting that PP1 has SAC-dependent and independent functions in mitosis. The proposed phospho-regulation of the SAC protein p31^{comet} is also explored as a potential PP1 substrate for timely anaphase onset.

This study also demonstrates the role of PLK1 activity in the initial recruitment of the phosphatase PP2A-B56 to kinetochores using a fluorescently labelled B56 CRISPR cell line. Additional tools for investigating the role of PLK1 in the formation of stable kinetochore-microtubule attachments are also generated and preliminary experiments carried out.

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List of Abbreviations

ABBA motif	Cyclin A, BUBR1, BUB1 and Acm1 motif
ADP	Adenosine diphosphate
ANA	Anaphase
ANKRD	Ankyrin repeat domain
ANOVA	Analysis of variance
APC/C	Anaphase promoting complex/cyclosome
Arpp19	cAMP-regulated phosphoprotein 19
ATP	Adenosine triphosphate
BEG	PP2A-B55 α /ENSA/Greatwall
BUB1	Budding uninhibited by benzimidazoles 1
BUB3	Budding uninhibited by benzimidazoles 3
BUBR1	Budding uninhibited by benzimidazoles related 1
C-MAD2	Closed mitotic arrest deficient 2
CAK	CDK-activating kinase
CD1	Conserved domain 1
CDC20	Cell division control protein 20
CDC25	Cell division control protein 25
Cdh1	CDC20 homolog 1
CDK	Cyclin dependent kinase
CDT1	Chromatin licensing and DNA replication factor 1
CENP	Centromere protein
CENP-C	Centromere protein C
CENP-E	Centromere protein E/Kinesin-7
Chk1	Checkpoint Kinase 1
CPC	Chromosomal passenger complex
CRBN	Cereblon
CRISPR	Clustered regularly interspaced short palindromic repeats
Ctrl	Control
D-box	Destruction box
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DSB	Double strand break
DSP	Dual-specificity phosphatase
EDTA	Ethylenediaminetetraacetic acid
Emi1	Early mitotic inhibitor 1
ENSA	Alpha endosulfine
FCP1	transcription factor 2F-associating C-terminal domain phosphatase 1
GF	Growth factor
GFP	Green fluorescent protein
GLEBS	Gle2-binding sequence
H3pThr3	Histone H3 phospho-threonine 3
HAD	Haloacid dehydrogenase
HORMA	Hop1p, Rev7p and MAD2 domain
INCENP	Inner centromere protein
kb	Kilobases
kDa	Kilodalton
KIF18A	Chromosome-associated kinesin 18A
KMN	KNL1-MIS12-NDC80
KNL1	Kinetochore null protein 1
M18BP1	MIS18-binding protein 1
MAD1	Mitotic arrest deficient 1
MAD2	Mitotic arrest deficient 2
MAPS	Microtubule-associated proteins
MASTL	Microtubule-associated serine/threonine protein kinase-like
MCC	Mitotic checkpoint complex
MCPH1	Microcephalin
MDa	Megadalton
MELT	Methionine-glutamine-leucine-threonine
MIS12	MIND kinetochore complex component
MPS1	Monopolar spindle 1
mScarlet	Monomeric scarlet (Red fluorescent protein)
MTOC	Microtubule-organising centre
MYPT1	Myosin phosphatase target subunit 1

NDC80	Nuclear division cycle 80
NEBD	Nuclear envelope breakdown
noc	Nocodazole
O-MAD2	Open mitotic arrest deficient 2
PAGE	Polyacrylamide gel electrophoresis
PBD	Polo-box domain
PCM	Pericentriolar material
PCR	Polymerase chain reaction
PLK1	Polo-like kinase 1
PNUTS	Phosphatase 1 NUclear Targeting Subunit
PP1	Phosphoprotein phosphatase 1
PP1 α	PP1 alpha
PP1 β	PP1 beta
PP1 γ	PP1 gamma
PP2A	Phosphoprotein phosphatase 2A
PP6	Phosphoprotein phosphatase 6
PPM	Protein phosphatase metal-dependent phosphatase
PPP	Phosphoprotein phosphatase
PRC1	Protein regulator of cytokinesis 1
Pro	Proline
ProTAME	pro-tosyl-L-arginine methyl ester
PTM	Post translational modification
Rb	Retinoblastoma
RIF1	Replication Timing Regulatory Factor 1
RING	Really Interesting New Gene
RLK	Arginine-Leucine-Lysine
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SAC	Spindle assembly checkpoint
SAPS	SIT4 phosphatase-associated protein
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine

siRNA	Small interfering ribonucleic acid
Thr	Threonine
TPX2	Targeting protein for Xklp2
TRIP13	Thyroid hormone receptor interacting protein 13
Tyr	Tyrosine
VHL	Von Hippel-Lindau
WT	Wild type
γ -TuRC	Gamma tubulin ring complex

Chapter 1: Introduction

1.1 The Mammalian Cell Cycle

1.1.1 Overview of the stages of the cell cycle

The mammalian cell cycle is a complex sequence of tightly controlled molecular events. It consists of five distinct stages: G_0 , a period of the cell cycle in which the cells are in a quiescent or resting state, and G_1 and G_2 which is when the majority of RNA and protein synthesis takes place; S-phase when DNA is replicated in preparation of the next cell division; and finally M-phase which is when the replicated DNA is physically separated and the cell divides into two new daughter cells (Figure 1.1). The G phases (G_0 , G_1 and G_2) together with S-phase are collectively known as interphase.

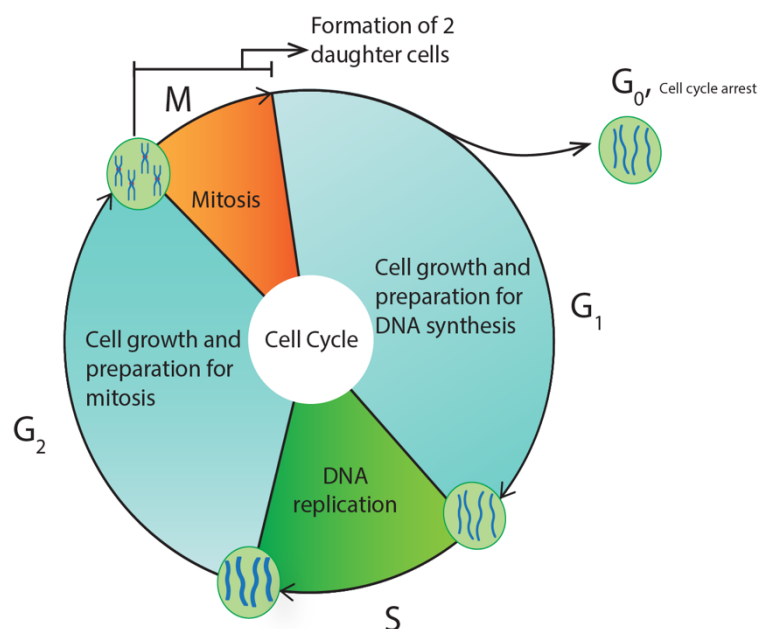


Figure 1.1: Overview of the stages of the cell cycle.

Arrows indicate the directionality of the cell cycle. See text for full details.

The distinct phases of the mammalian cell cycle are coordinated by the activities of the proline-directed serine/threonine (Ser/Thr) kinase family, Cyclin dependent kinases (CDKs). In association with their activating Cyclin subunits, CDK:Cyclin complexes control the order of events in the cell cycle. In addition to CDKs, growth factors (GFs) are also involved in controlling entry into the cell cycle. GFs are extracellular diffusible signalling glycoproteins that bind to cell surface receptors, such as those of the receptor tyrosine kinase (RTK) family. When GFs bind to RTKs, they initiate cellular signalling cascades that interact with each other to modulate gene expression at the transcriptional level. Through controlling the transcription of genes within the cell, GFs regulate cellular functions and are essential for entry into the cell cycle (Wang, 2021).

As their name suggests, the Cyclin proteins are cyclically expressed and degraded throughout the cell cycle (Evans et al., 1983, Pines and Hunter, 1989). Different Cyclins follow different synthesis and degradation patterns. CDKs, however, are expressed constitutively throughout the cell cycle. CDKs can bind and therefore be activated by different Cyclins over the course of the mammalian cell cycle at specific stages to coordinate cell cycle events (Figure 1.2). CDK4 and CDK6 are specific to D-type Cyclins only. CDK4/6:Cyclin D complexes govern cell cycle events in early G₁ by phosphorylating the tumour suppressor protein retinoblastoma (Rb) to promote the dissociation of Rb from the E2F transcription factors, which in turn promotes the transcription of multiple genes including the CDK2 activators, Cyclin A and Cyclin E (Kim et al., 2022). Unlike CDK4 and CDK6, CDK1 and CDK2 can bind to multiple Cyclins, although they have preferences for Cyclin B and Cyclin A Cyclins respectively. The CDK2:Cyclin

E complex triggers the transcription of genes required for DNA replication including chromatin licensing and DNA replication factor 1 (CDT1) and hence S-phase entry (Fagundes and Teixeira, 2021). Following this, Cyclin A in complex with either CDK1 or CDK2 is active during S-phase and G₂ phase where its targets include CDC6 to conclude S-phase (Petersen et al., 1999). CDK1:Cyclin B is the principal driver of mitosis and maintains the mitotic state (Nigg, 1995). CDK1:Cyclin B has a number of key targets including lamins, condensins and motor proteins.

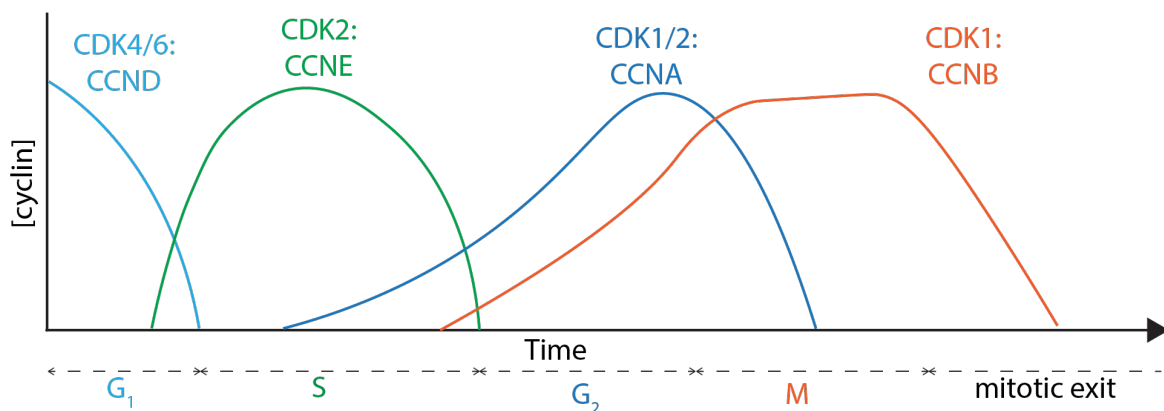


Figure 1.2: CDK:Cyclin complexes over the course of the cell cycle.

Cyclin levels oscillate during the cell cycle. Cyclins bind to and active Cyclin dependent kinases (CDKs).

A further Cyclin protein, Cyclin H, forms a complex with CDK7. This CDK7:Cyclin H complex is a CDK-activating kinase (CAK) which phosphorylates CDK1 and CDK2 kinases on residues Thr161 and Thr160, respectively, to promote the activation of CDK1:Cyclin complexes (Russo et al., 1996, Desai et al., 1995). Activation of CDK1:Cyclin complexes is needed as CDK subunits on their own have no detectable kinase activity (Lees, 1995). Activation occurs in two steps.

Firstly, when the Cyclin protein binds to the CDK subunit, the CDK undergoes a conformational change which creates a more favourable orientation for adenosine triphosphate (ATP) to be utilised within the catalytic domain (Connell-Crowley et al., 1993). The second activating event is the phosphorylation of the 'T-loop' of CDK by the CAK, which increases CDK activity (Fisher and Morgan, 1994, Jeffrey et al., 1995, Desai et al., 1995, Russo et al., 1996).

1.1.2 Cell cycle checkpoints safeguard cell growth

Correct order and control of cell cycle events is crucial for proper cell growth. Cell cycle progression is controlled by a series of surveillance mechanisms or "checkpoints", ensuring that the stages of the cell cycle occur in the correct sequence. The existence of cell cycle checkpoints was first shown by Hartwell and Weinert in yeast, in which they demonstrated that yeast cells undergo a cell cycle arrest in response to faulty DNA replication. This arrest could be overcome by mutations in specific proteins, known as "relief of inhibition mutations" (Hartwell and Weinert, 1989, Weinert and Hartwell, 1988).

Since this pioneering work in the 1980s, it has been established that three principal checkpoints exist in mammalian cells to control cell cycle progression. The first is the G₁/S transition, also known as the "restriction point" which regulates commitment to cell cycle progression in response to external stimuli. During G₁, external GFs activate both the Ras/Erk and the PI3K/Akt signalling pathways which converge to promote the transcription of Cyclin D1, required for cell cycle progression through G₁ (Balmain and Cook, 1999, Lavoie et al.,

1996). Once cells go beyond the restriction point into S-phase, they are committed to DNA synthesis and no longer need extracellular GFs to progress through the cell cycle (Pardee, 1974, Matsushime et al., 1991).

The second transition that is tightly monitored is the G₂/M transition, also known as the DNA damage checkpoint. If any DNA damage has occurred during DNA replication, the checkpoint halts the cells before mitotic entry to allow time for these errors to be corrected (Stark and Taylor, 2006).

The third principal checkpoint in the cell cycle is the spindle assembly checkpoint (SAC). This checkpoint functions during mitosis in which it monitors the attachment status of kinetochores, large multiprotein structures located on chromosomes, to microtubules that emanate from opposing spindle poles within the mitotic cell. The formation of stable kinetochore-microtubule attachments with the correct geometry needs to be achieved before cells exit mitosis, as it is these attachments that ensure sister chromatids are equally physically segregated into two genetically identical daughter cells (Lara-Gonzalez et al., 2012, Musacchio, 2015, Li and Murray, 1991, Hoyt et al., 1991). The SAC will be discussed further in Chapter 1.4.

Mistakes in any of the cell cycle checkpoints can have catastrophic effects on cell viability. For example, cells without a functional restriction point can prematurely enter the cell cycle regardless of the external stimuli, or lack thereof, it is receiving. This is a common phenotype among malignant cancer cells where it can cause increased growth rate (Pardee, 1974, Pardee, 1989). Furthermore,

faults at the G₂/M transition that result in cells prematurely entering mitosis despite the presence of DNA damage, such as double strand breaks (DSBs), can lead to chromosome segregation errors, aneuploidy and genome instability (Mankouri et al., 2013).

Aneuploidy, a term used to describe cells with an abnormal number of chromosomes, is also a phenotype that can occur due to a faulty SAC as these cells may enter anaphase before all kinetochores are correctly attached to microtubules and therefore the sister chromatids are not equally segregated into the two new daughter cells. Therefore, aneuploid cells can have aberrant protein expression due to having lost or gained full chromosomes. Chromosomal instability (CIN) is defined as frequent loss and gain of whole chromosomes which can further promote aneuploidy as it promotes chromosome missegregation. During mitosis, partial loss of SAC signalling can increase the chance of aneuploidy and promote escape from cell death as a weak checkpoint stills means that the cells can partially align their chromosomes and enter anaphase while not triggering apoptosis (Weaver and Cleveland, 2005). Aneuploidy is a hallmark and driving force of many cancers (Kops et al., 2005). In fact, more than 90% of solid human tumours are aneuploid, and aneuploid tumours display increased resistance to chemotherapy (Replogle et al., 2020, Lukow et al., 2021). Therefore, better understanding of the cellular mechanisms that can result in aneuploidy will be important for future cancer therapeutic developments.

1.2 Mitosis

1.2.1 Overview of the stages of mitosis

Mitosis occurs in all eukaryotic organisms and is the final stage of the cell cycle, in which two daughter cells are formed from one parent cell. Mitosis can be further divided into six distinct stages: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. These stages are categorised based on the architecture of the mitotic spindle and the chromosomes (McIntosh, 2016). In the early stages of mitosis, DNA that has been previously duplicated in S-phase recruits the condensin complexes, condensin I and condensin II, to promote the condensation of chromatin in preparation for chromosome segregation (Thadani et al., 2012). Condensin II initiates chromosome condensation during prophase, whereas condensin I can only access the chromatin after the nuclear envelope has broken down in prometaphase (Ono et al., 2004). It has recently been shown that CDK-mediated phosphorylation of MIS18-binding protein 1 (M18BP1) and microcephalin (MCPH1), two binding partners of condensin II, at mitotic entry may promote the localisation of condensin II to the chromatin, thus promoting its role in chromosome condensation (Borsellini et al., 2024). This chromatin condensation process continues until metaphase. Additionally, in prophase, the two pairs of centrioles migrate towards opposite poles of the cell and the mitotic spindle begins to form (Hannaford and Rusan, 2024). Prometaphase is characterised by the breakdown of the nuclear envelope, after which the microtubules of the mitotic spindle can access the chromosomes that were previously restricted within the nucleus. During prometaphase, microtubules emanating from opposing spindle poles become attached to sister chromatids via

proteinaceous structures called kinetochores that reside on the DNA in a stochastic 'search and capture' mechanism (Monda and Cheeseman, 2018, Kirschner and Mitchison, 1986). As these attachments are forming, the sister chromatids are pulled by the forces transmitted through the microtubules from opposite ends of the cell. The tension this creates results in the chromosomes aligning at the cell equator.

The alignment of chromosomes at the cell equator is tightly coupled with the process of forming kinetochore-microtubule attachments. The arrangement of microtubules and chromosomes where kinetochores of sister chromatids are attached to microtubules emanating from opposite spindle poles and all the chromosomes are found on the plane equidistant from both spindle poles, termed the metaphase plate, is known as metaphase (McIntosh, 2016).

Following metaphase, cells proceed into anaphase. In order for cells to enter anaphase, all kinetochores must be stably bound to microtubules in a bioriented arrangement (Figure 1.3A), and the SAC (discussed in detail in Chapter 1.4) must terminate signalling to release the cells from the metaphase arrest. Once these requirements are fulfilled, cells will enter anaphase during which sister chromatids are pulled apart towards opposite ends of the cell by the spindle microtubules (McAinsh and Kops, 2023). For the initiation of anaphase, two key molecules, Cyclin B and securin, have to be ubiquitinated on multiple lysine residues by the critical ubiquitin E3 ligase, anaphase promoting complex/cyclosome (APC/C). Once these proteins have been polyubiquitinated with K48 and K11 linkages, they are recognised by the 26S proteasome and

hence targeted for proteasomal degradation (Holder et al., 2019, Reddy et al., 2007, Alfieri et al., 2016). In some cases, the poly-ubiquitination alone can disrupt protein complexes, as has been suggested for the MCC (Reddy et al., 2007). Cyclin B degradation leads to a reduction in CDK1:Cyclin B kinase activity, important for mitotic exit (Clute and Pines, 1999). Securin is an inhibitor of the enzyme separase. Through the degradation of securin, separase becomes active and cleaves the cohesin molecules that keep sister chromatids together (Sivakumar and Gorbsky, 2015, Jallepalli et al., 2001, Uhlmann et al., 1999, Uhlmann et al., 2000). The sister chromatids are then physically pulled apart by the shortening of kinetochore-bound microtubule bundles. Separated sister chromatids reach the spindle poles in telophase and start to decondense from their tightly packaged mitotic structures to the much more loosely packaged interphase conformations in a process largely regulated by protein phosphatases (Gil and Vagnarelli, 2019). The nuclear envelope reforms around the two sets of chromosomes, creating two genetically identical nuclei. Cytokinesis follows on from telophase. This is where the cytoplasm divides, resulting in the formation of the two new daughter cells (McIntosh, 2016).

1.2.2 The importance of kinetochore-microtubule attachment geometries

Chromosome separation in anaphase only proceeds once all kinetochores have stably bound microtubules emanating from opposing spindle poles. These stable end-on microtubule attachments are known as amphitelic attachments which result in an orientation known as chromosome biorientation (Figure 1.3B). Only this orientation promotes correct, and equal, sister chromatids separation into the

daughter cells. However, during prometaphase, sister chromatids become attached to the mitotic spindle through a “search and capture” method (Kirschner and Mitchison, 1986) and many other orientations aside from biorientation can occur in the process. For example, merotelic, syntelic and monotelic attachments may arise (Tanaka, 2010), see Figure 1.3. Merotelic attachments are where a single kinetochore is attached to microtubules from spindle poles at both ends of the cell. Merotelic attachments are the most likely to evade SAC detection as the kinetochores are attached and tension may be present across the kinetochores, which may promote SAC silencing (Gegan et al., 2011). If this persists, chromosomes can be mis-segregated in anaphase, leading to aneuploidy. Syntelic attachment describes a situation in which the kinetochores on a pair of sister chromatids are attached to the same spindle pole, which can also prevent correct chromosome segregation. Thirdly, monotelic attachments only have one kinetochore of a sister chromatid pair attached to microtubules which means that there is not sufficient tension applied through the microtubules to properly align these chromatids at the metaphase plate and separate them correctly into the daughter cells (Tanaka, 2010).

Kinetochores can also interact with the edges of microtubules, rather than attach end-on, which is known as a lateral attachment. Lateral attachments are prevalent in early prometaphase where these attachments are important for moving the chromosomes towards the metaphase plate in a dynein-dependent manner to subsequently be converted into end-on microtubule attachments (Rieder and Alexander, 1990, Gassmann, 2023, Shrestha and Draviam, 2013, Shrestha et al., 2017). The SAC monitors the attachment status of all

kinetochores in the cell to ensure that cells only enter anaphase once all kinetochores have correctly bioriented and are stably end-on attached to microtubules.

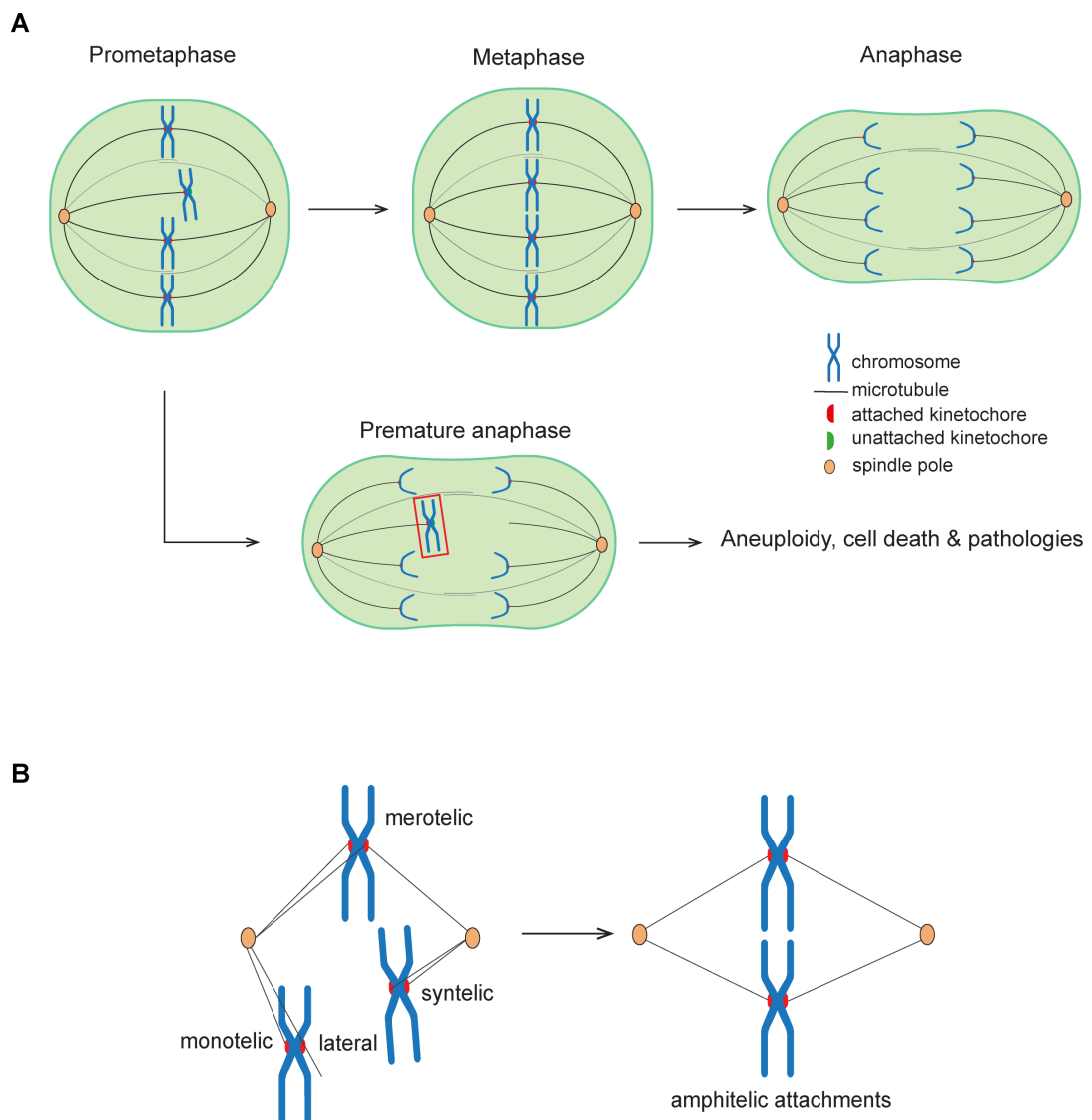


Figure 1.3: Attachment geometries in mitosis.

(A) Illustration of the mitotic stages prometaphase, metaphase and anaphase. Kinetochore become attached to microtubules and become aligned at the equator of the cell in metaphase. Once correctly bioriented, cells enter anaphase and chromosomes are equally segregated. Premature anaphase before all kinetochores are correctly attached leads to chromosome segregation errors. (B) Illustration of the array of attachment geometries that can exist.

In the process of forming amphitelic attachments, these unstable merotelic, syntelic or monotelic attachments are often created and need to be corrected

before anaphase onset to ensure equal segregation of chromosomes into the daughter cells. This process of detecting and correcting these incorrect attachments is termed 'error correction' (Nicklas and Ward, 1994, Nicklas, 1988, Nicklas et al., 1993). Interestingly, it has been suggested that cancer cells with chromosomal instability (CIN), defined as frequent loss and gain of whole chromosomes, have more stable kinetochore-microtubule attachments than a control diploid RPE1 cell line that is chromosomally stable, thus reducing error correction efficiency and further promoting CIN and aneuploidy (Bakhoun et al., 2009, Thompson et al., 2010).

Microtubules are very dynamic and are constantly undergoing cycles of polymerisation (growth) and depolymerisation (shrinkage) (Kollman et al., 2011). The dynamic nature of microtubules is crucial for the mitotic spindle's 'search and capture' method of kinetochores, aligning the chromosomes at the metaphase plate and for the physical separation of sister chromatids into two daughter cells during mitosis (McIntosh, 2016). Microtubule-associated proteins (MAPs) such as astrin are important for regulating microtubule attachments to kinetochores and stabilising end-on kinetochore-microtubule attachments to safeguard chromosome segregation (Conti et al., 2019, Dunsch et al., 2011). Additionally, Centromere protein E (CENP-E/Kinesin-7) is a plus-end directed motor protein that is important for congressing spindle pole localised chromosomes onto the metaphase plate (Kim et al., 2008, Kapoor et al., 2006). The frequency of polar localised chromosomes increases if CENP-E is inhibited or removed (Wood et al., 1997, Putkey et al., 2002, Yao et al., 2000, Yucel et al., 2000).

1.2.3 Kinases orchestrating mitosis

During mitosis a large number of molecular changes to chromatin, microtubules and cellular organelles have to be initiated and coordinated to allow the condensation of chromatin into sister chromatids and subsequent segregation of these by the mitotic spindle. At a molecular level, these changes are triggered by the actions of several key mitotic kinases, including CDK1:Cyclin B, Polo-like kinase 1 (PLK1), the kinases of the Aurora kinase family, Aurora A and B, as well as MPS1.

As mentioned previously, CDK1 in complex with its coactivator Cyclin B, is the principal mitotic kinase that drives cells into mitosis by phosphorylating a wide range of substrates to bring about whole cell changes including nuclear envelope breakdown (NEBD), chromosome condensation and SAC signalling (Hayward et al., 2019b, Nigg, 1995). For example, CDK1 phosphorylates nuclear lamins which leads to the disassembly of the nuclear envelope in early mitosis (Jeong et al., 2022). CDK1 activity also promotes MPS1 localisation to kinetochores to initiate SAC signalling (Hayward et al., 2019b). In this way, CDK1:Cyclin B maintains the mitotic state until all kinetochores are correctly attached to microtubules.

The control of CDK1 activity is crucial for proper mitotic progression. A bistable switch of CDK1:Cyclin B activity at the G₂/M transition provides a robust and irreversible commitment to mitosis (Mochida et al., 2016). This sharp, all-or-nothing, transition is important for maintaining the fidelity of cell division as the cells cannot return to the previous G₂ stage.

Cyclin B synthesis occurs during late S/G₂ and associates with CDK1. This alone is not sufficient to trigger the kinase activity of CDK1; it also needs to be phosphorylated by the CAK on Thr161 of the T-loop of CDK1 (Russo et al., 1996, Desai et al., 1995). However, in G₂, the CDK1:Cyclin B kinase is kept inactive through inhibitory phosphorylations on Thr14 and Tyr15 of CDK1, carried out by Wee1 and Myt1 (Morla et al., 1989, Gould and Nurse, 1989, Kellogg, 2003). CDK1:Cyclin B complexes are kept inactive until Cyclin B levels exceed a certain threshold where CDK1:Cyclin B can then promote its own activation in two ways (Mochida et al., 2016). Firstly, it phosphorylates Wee1 in an inhibitory manner, preventing it from inhibiting CDK1:Cyclin B (Morgan, 1995). CDK1:Cyclin B also phosphorylates the phosphatase Cell division control protein 25 (CDC25) to promote CDC25 activity and therefore increase the removal rate of the inhibitory phosphorylations from the inactive CDK1:Cyclin B complex (Gabrielli et al., 1997, Lammer et al., 1998, Hoffmann et al., 1993) (Figure 1.4). In this way, CDK1:Cyclin B creates a positive feedback loop to promote its own activation.

An additional layer of regulation exists on CDC25 in which it is phosphorylated by Checkpoint Kinase 1 (Chk1) in response to DNA damage, which targets CDC25 for proteasomal degradation and prevents interaction between CDC25 and CDK1:Cyclin B (Chen et al., 2003). Together, these regulatory processes allow CDK1:Cyclin B activity to be tightly controlled to create a bistable switch at the G₂/M transition.

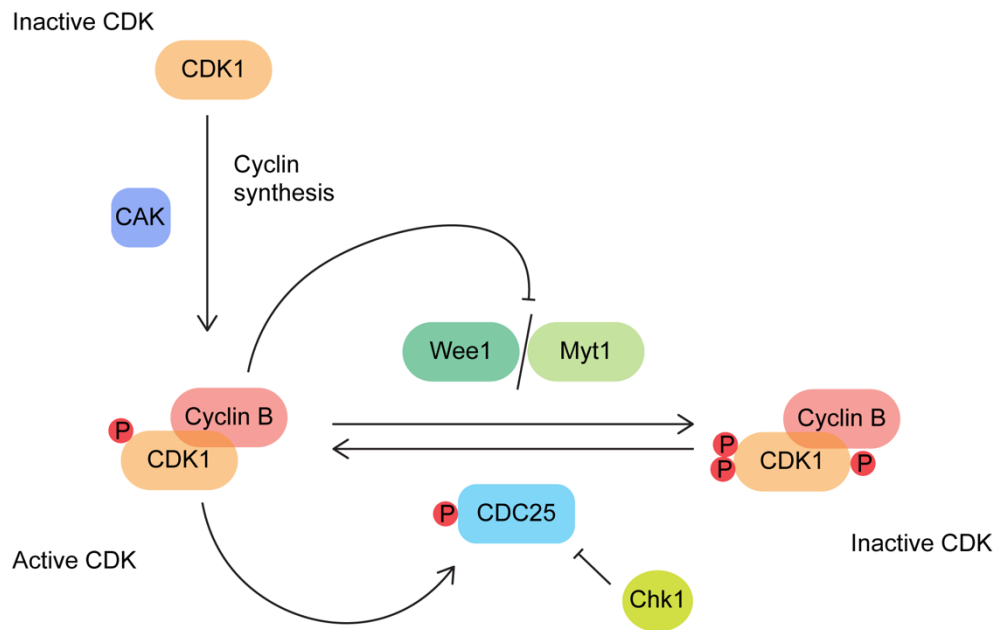


Figure 1.4: Regulation of CDK1 activity.

CDK1 is activated by the CDK-activating kinase (CAK). Additionally, CDK1:Cyclin B inhibits Wee1/Myt1 kinases and promotes CDC25 phosphatase to further promote its own activation. Chk1 kinase prevents CDC25 function in response to DNA damage.

While CDK1 is one of the main mitotic kinases, the Polo-like kinase (PLK) family and the Aurora kinase family also contribute to the global increase in phosphorylation of mitotic cells and play important roles in orchestrating mitotic events. Indeed, the drastic increase in phosphorylation levels brought about by these kinases is a defining feature of mitotic entry and the early stages of mitosis. Large scale phospho-proteomic studies have identified phosphorylation sites on over 5000 proteins in mitotic cells (Petrone et al., 2016, Dephoure et al., 2008, Olsen et al., 2010). Together, CDK1, PLK and Aurora kinases work to initiate mitotic events including NEBD, SAC activation and the inhibition of anaphase.

Polo kinase was first discovered through genetic screens in *Drosophila* (polo) and *S. Cerevisiae* (CDC5) by screening for factors that caused mitotic defects (Llamazares et al., 1991, Sunkel and Glover, 1988, Kitada et al., 1993). Subsequent studies have shown that this kinase is conserved in *Xenopus* (Plxs) and mammals (Polo-like kinases (PLKs)) (de Carcer et al., 2011b). In human cells, there are five members of the PLK family, PLK1 through PLK5. PLK1 is the most well studied of the PLK family and has roles in mitosis including centrosome maturation and kinetochore-microtubule attachments (Lane and Nigg, 1996, Haren et al., 2009, Chen et al., 2021). PLK2 and PLK4 are both implicated in centriole duplication (Kleylein-Sohn et al., 2007, Warnke et al., 2004). PLK3 supports the increase of Cyclin E at the G₁/S transition which in turn promotes DNA replication (Zimmerman and Erikson, 2007). PLK5 is less well understood, although research suggests it has a role in neurons (de Carcer et al., 2011a).

Members of the PLK family all have similar N-terminal catalytic kinase domains, and at least one polo-box domain (PBD) near the C-terminus which is important for regulating the dynamic localisation of PLK (Korns et al., 2022). Phosphorylation of the sequence Ser-(pThr/pSer)-(Pro/X), where X is any amino acid, often by a kinase other than PLK, creates a docking site for the PBD, and therefore the recruitment of PLK to its binding partners (Elia et al., 2003, Yun et al., 2009).

PLK1 activity peaks in mitosis, during which its localisation is very dynamic. The dynamic nature of its localisation is controlled by the phosphorylation state of its binding partners. PLK1 localises to centrosomes in late G₂ and prophase. At this

stage of the cell cycle, identified PLK1 substrates include CDC25, Myt1 and Wee1 highlighting its role at the G₂/M transition (Kumagai and Dunphy, 1996, Inoue and Sagata, 2005, Watanabe et al., 2004, Nakajima et al., 2003). By localising to centrosomes, PLK1 facilitates the recruitment of pericentriolar material (PCM) and promotes nucleation of microtubules (Lane and Nigg, 1996). PLK1 has also been implicated in the phosphorylation of SA2, a cohesin subunit, which promotes dissociation of cohesin from chromosome arms in prophase in the so-called 'prophase pathway', independent of proteolytic cleavage that removes cohesin from centromeric regions at the metaphase to anaphase transition (Hauf et al., 2005, Sumara et al., 2002). The prophase pathway is essential for the generation of mitotic chromosomes in which two sister chromatids are only held together by centromeric cohesion (Haarhuis et al., 2014). PLK1 localises to kinetochores in prometaphase and metaphase, through association with Budding uninhibited by benzimidazoles 1 (BUB1) and BUB-related 1 (BUBR1) (Chen et al., 2021, Singh et al., 2021, Elowe et al., 2007). During anaphase, PLK1 relocates to the midzone through binding to the protein regulator of cytokinesis 1 (PRC1), where it is critical for the regulation of cytokinesis (Neef et al., 2007).

The Aurora kinase family is a family of Ser/Thr kinases that is important for orchestrating cell division. In humans, the Aurora kinase family consists of three members, namely Aurora A, Aurora B and Aurora C. First discovered in *Drosophila* in 1995 where a loss of function mutation resulted in failure to generate a bipolar spindle, the Aurora kinases have since been shown to have several roles in mitosis (Willems et al., 2018, Glover et al., 1995). They share

similar protein structures with highly conserved kinase domains and similar kinase activity; however, they display distinct localisation within the cell. Despite having similar catalytic domains, the Aurora kinases have different substrates in vivo, owing to, at least to some extent, their different cellular localisations. Aurora B and Aurora C are paralogs; Aurora B expression is ubiquitous, whereas Aurora C expression is mainly within meiotic germ cells (Willems et al., 2018).

Aurora A localises to the centrosome at the G₂/M transition and on spindle poles and microtubules during prometaphase and metaphase (Barretta et al., 2016, Barr and Gergely, 2007). This localisation is mediated through the interaction with key binding partners, most prominently CEP192 at centrosomes and TPX2 at spindle poles and microtubules (Holder et al., 2024, Kufer et al., 2002). It recruits several proteins to the microtubule-organising centre (MTOC), including gamma tubulin ring complex (γ -TuRC), which promotes centrosome maturation through microtubule elongation and nucleation (Wang et al., 2014a, Toji et al., 2004). Aurora A also has roles in bipolar spindle formation through phosphorylation of the kinesin Eg5 which is responsible for regulating the anti-parallel forces of spindle microtubules to drive spindle pole separation (Giet et al., 1999).

The Aurora B kinase is the enzymatic component of a complex called the chromosomal passenger complex (CPC), which also contains the proteins INCENP, survivin and borealin. This complex with active Aurora B is crucial for early mitotic events including chromosome condensation and the formation of stable kinetochore-microtubule amphitelic attachments, as well as the execution of cytokinesis (Carmena, 2012). Importantly, research shows that Aurora B can

phosphorylate the N-terminal tail of the nuclear division cycle 80 (NDC80) complex of the KMN network at kinetochores (Welburn et al., 2010, Cheeseman et al., 2006, DeLuca et al., 2006). The KMN network is named after its components, the KNL1, MIS12 and NDC80 complexes, which itself is a component of the kinetochore linking centromeric DNA to the plus-end of spindle microtubules (Cheeseman and Desai, 2008). In vitro biochemistry presents evidence that the phosphorylation of NDC80 by Aurora B prevents the formation of stable kinetochore-microtubule attachments, likely due to the increase in negative charge of NDC80 which interrupts electrostatic interactions between kinetochores and microtubules (Welburn et al., 2010, Cheeseman et al., 2006, DeLuca et al., 2006, Ciferri et al., 2008). However, more recent work shows that Aurora A is the principal kinase that phosphorylates NDC80 at microtubule-attached kinetochores (Sobajima et al., 2023).

It has also been reported that Aurora B has a role in negatively regulating protein phosphatase 1 (PP1) recruitment to the kinetochore. PP1 is implicated in the regulation of various mitotic events and its localisation to kinetochores is crucial for several of these (PP1 will be discussed in detail in Chapter 1.3). Aurora B controls PP1 localisation to kinetochores by phosphorylating the PP1 binding site on KNL1 which prevents its binding (Nasa et al., 2018). In this way, Aurora B prevents premature PP1-mediated dephosphorylation of PP1 substrates during mitosis.

In a similar way to CDK1, the kinase activity of PLK1, Aurora A and Aurora B are all dependent on the phosphorylation of their T-loops. The principal activator of

PLK1 is Aurora A, which phosphorylates PLK1 on T210 of its T-loop (Bruinsma et al., 2014). Aurora A, Aurora B and Aurora C are phosphorylated on T281, T232 and T195 respectively (Bayliss et al., 2003, Willems et al., 2018). Additionally, full activation of Aurora A and Aurora B involves interaction of the kinase with their coactivators, the targeting protein for Xklp2 (TPX2) and INCENP, respectively (Carmena et al., 2009).

Another essential kinase for proper cell cycle control is MPS1. MPS1 was originally discovered for its role in spindle pole body duplication in budding yeast; it was given its name due to the striking phenotype in mutant cells which fail spindle pole body duplication and therefore have a monopolar spindle when they enter mitosis (Winey et al., 1991). Its role in mitotic checkpoint function was shown in later studies (Hardwick et al., 1996, Weiss and Winey, 1996). During mitosis, MPS1 localises to unattached kinetochores where it promotes its own activation through trans-autophosphorylation of residues Thr676 and Thr686 in the T-loop of the kinase (Kang et al., 2007, Wang et al., 2009). Once active, MPS1 initiates a phosphorylation signalling cascade that results in the formation of a tetrameric complex known as the mitotic checkpoint complex (MCC) (Musacchio, 2015). The MCC inhibits anaphase onset (Liu and Zhang, 2016). MPS1 itself needs to be phosphorylated by CDK1 on its Ser281 residue. This phosphorylation promotes MPS1's localisation to unattached kinetochores to mount a robust spindle checkpoint response (Hayward et al., 2019a).

1.3 The Role of Phosphatases in Mitosis

1.3.1 The discovery of protein phosphatases

Post translational modifications (PTMs) play an important role in regulating cellular processes. Phosphorylation is one of the more common types of PTM; over 32,000 phosphorylation and dephosphorylation events occur during mitosis alone (Burgess et al., 2017). 25 years ago, it was estimated that around 30% of proteins in the eukaryotic cell were phosphorylated (Cohen, 1999) but more recently it was shown that up to 70% of proteins are subject to phosphorylation (Olsen et al., 2010). Phosphatases, the group of enzymes that remove phosphate groups from molecules, were discovered several decades before kinases, the enzymes that add the phosphate group, through a series of studies in the early 20th century (Cori and Green, 1943, Cohen, 1994). At this time, they were known as “prosthetic group” removal or PR-enzymes as it was not yet known what inorganic “prosthetic group” was being removed. It is now well understood that these enzymes catalyse the removal of phosphate groups. Perhaps the most significant advancement in our understanding of the role of cellular phosphorylation events came from Krebs and Fischer in the 1950s. In a study of glycogen phosphorylase, they discovered that reversible phosphorylation could influence enzyme activity (Fischer and Krebs, 1955, Krebs and Fischer, 1955). This work led to them earning the Nobel Prize in Physiology or Medicine in 1992 for discoveries concerning “reversible protein phosphorylation as a biological regulatory mechanism”.

There are around 500 kinases and 189 phosphatases identified in humans (Manning et al., 2002, Chen et al., 2017). Given that there are many more kinases than phosphatases, it was thought for a long time that phosphatases were simply “housekeeping” enzymes that non-specifically removed the more imperative phosphorylation modifications from proteins. However, this view has now changed significantly and the role of phosphatases in coordinating cellular processes is much more widely acknowledged and studied, although not yet fully understood.

Kinases and phosphatases are two opposing groups of enzymes that have many differences, both structurally and in their mechanism of action. Kinases are structurally similar with a shared catalytic domain that transfers the terminal phosphate group of an ATP molecule to either the hydroxyl group of a serine (Ser), threonine (Thr), or tyrosine (Tyr) amino acid residue forming a phosphoester bond (Miller and Turk, 2018, Wang and Cole, 2014). Phosphatases reverse phosphorylation modifications by catalysing the hydrolysis of phosphate esters, replacing the phosphate group on the substrate with a hydroxyl group from a water molecule (Figure 1.5A) (Cheng et al., 2011).

1.3.2 Categorisation of phosphatases

Phosphatases are a diverse group of enzymes that can be categorised into distinct families based on their catalytic mechanisms and their substrate specificity. They are typically categorised into three groups: serine/threonine (Ser/Thr)-specific, Tyr-specific, and dual-specificity phosphatases (DSP). Phosphatases that dephosphorylate Ser and Thr residues can be separated

further into three groups: phosphoprotein phosphatases (PPPs), protein phosphatase metal-dependent (PPM) and haloacid dehydrogenase (HAD) (Figure 1.5B) (Shi, 2009, Barr et al., 2011). Ser/Thr residues constitute around 98% of the phosphorylated residues in the cell (Olsen et al., 2006). The PPP family of phosphatases consists of the protein phosphatases PP1, PP2A, PP2B, PP4, PP5, PP6, PP7. PP1 and PP2A are among some of the most abundantly expressed enzymes in many cells. Indeed, over 90% of all dephosphorylation reactions in eukaryotic cells are carried out by members of the PPP family (Heroes et al., 2013). As the catalytic subunits of the PPP family are very similar, many of them can be inhibited by the toxin okadaic acid. However, the sensitivity varies between the individual members of the family. For example, PP2A is 100 times more sensitive to okadaic acid induced inhibition than PP1 (Xing et al., 2006). Inhibition of the PPPs with okadaic acid brings about morphological changes associated with mitosis, including the condensation of chromatin, diffusion of nuclear lamins and fragmentation of the Golgi apparatus (Yamashita et al., 1990, Lucocq et al., 1991) indicating that inhibition of PPPs is required for mitotic entry.

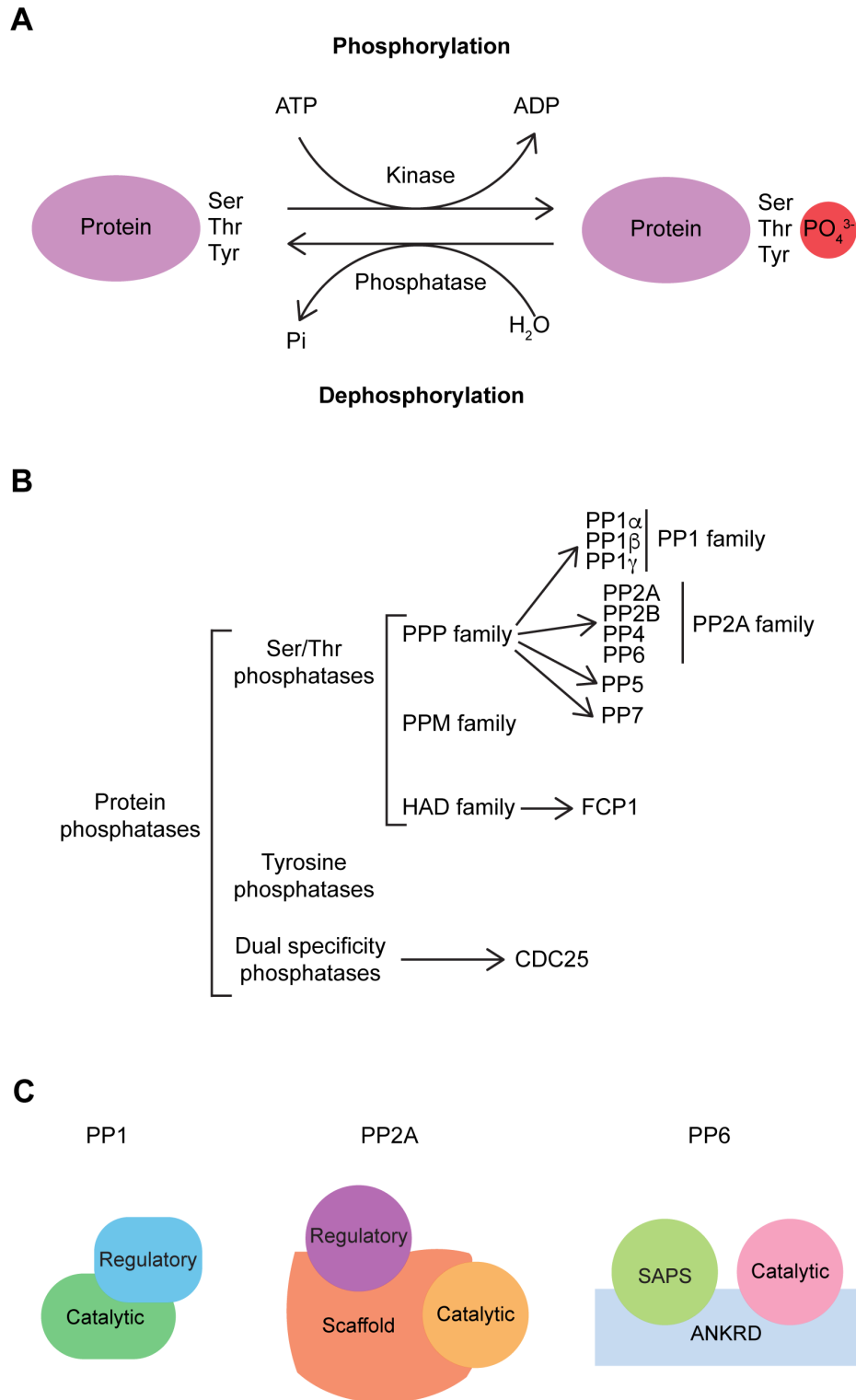


Figure 1.5: Protein phosphatases.

(A) Schematic of phosphorylation and dephosphorylation reactions, carried out by kinases and phosphatases respectively. (B) Classification of protein phosphatases. PPP = phosphoprotein phosphatases. PPM = phosphatase metal-dependent. HAD = haloacid dehydrogenase. The PP1 family has three distinct catalytic isoforms (PP1 alpha (PP1 α), PP1 beta (PP1 β), and PP1 gamma (PP1 γ)) in mammalian cells. See text for full details. (C) Illustration of PP1, PP2A and PP6 phosphatase complexes.

PPPs share a common ancestor and thus the same catalytic mechanism. They have a similar catalytic core consisting of two metal ions at the active site (often $\text{Zn}^{2+}:\text{Fe}^{2+}$) (positioned 3.3Å apart in PP1) which interact with the oxygen of a water molecule (Goldberg et al., 1995, Egloff et al., 1995). The water molecule is activated to act as a nucleophile for the nucleophilic attack on the phosphorous atom of the substrate. This reaction occurs in one step to hydrolyse the phosphoester bond and dephosphorylate the substrate (Cheng et al., 2011). Despite being remarkably similar in catalytic activity, members of the PPP family have distinct functions as a result of associating with different regulatory subunits to direct their phosphatase activity towards the desired substrates (Bollen et al., 2010).

1.3.3 Control of phosphatase activity through holoenzyme formation and CDK1-mediated inhibition

PP1 consists of a single catalytic subunit which binds to PP1-interacting proteins (PIPs), of which over 200 have been identified (Bollen et al., 2010). In human cells, there are three distinct catalytic subunits of PP1. Using one or more of these PIPs, PP1 becomes a highly specific holoenzyme towards its substrates. Since PP1 can dephosphorylate such a wide range of proteins, this targeting of the catalytic subunits by its PIPs may have evolved so that subcellular pools of PP1 exist with a much higher local concentration which are active towards its substrates. This differs from the activity of kinases in that kinases are very specific and recognise short consensus motifs within the amino acid sequence of their substrates, whereas PP1 has less underlying specificity and relies on PIPs to confer this specificity towards its targets (Miller and Turk, 2018, Barr et al., 2011).

In contrast to PP1, PP2A and PP6 both form trimeric complexes (Figure 1.5C). The PP2A holoenzyme consists of the catalytic (C) domain, a scaffold (A) domain and a regulatory (B) domain. There are four regulatory domains that can associate with PP2A, namely B/B55, B'/B56, B'' and B''''. Through binding to one of these regulatory (B) subunits, substrate specificity of the catalytic (C) domain is defined. The regulatory (B) subunits have little sequence homology but recognise similar regions within the scaffold (A) subunit (Janssens and Goris, 2001, Barr et al., 2011, Xing et al., 2006, Xu et al., 2006). The PP6 holoenzyme is also a heterotrimeric complex consisting of a catalytic subunit, an ankyrin repeat domain-containing subunit (ANKRD) and a SIT4 phosphatase associated protein domain (SAPS) (Barr et al., 2011, Stefansson and Brautigan, 2006, Stefansson et al., 2008).

The activity of the PPP family phosphatases throughout mitosis needs to be tightly controlled in a temporal manner to ensure the accurate dephosphorylation of substrates. PP1 and PP2A-B55 are both inhibited by CDK1 during mitosis (Figure 1.6) (Holder et al., 2019). The function of this is to keep global phosphatase activity low during early mitosis, to prevent the premature dephosphorylation of key mitotic regulators. However, at anaphase onset when the Cyclin B levels begin to drop as a result of APC/C activation, and thus CDK1 kinase activity is reduced, the global level of phosphatase activity in turn increases. Unlike the PPP family, little is known about the cell cycle dependent regulation of the activity of the HAD and PPM phosphatases (Shi, 2009).

CDK1:Cyclin B-mediated phosphorylation of a conserved C-terminal Thr in PP1 directly inhibits its phosphatase activity (Goldberg et al., 1995, Kwon et al., 1997, Dohadwala et al., 1994, Wu et al., 2009). At the metaphase to anaphase transition, this inhibitory phosphorylation needs to be removed to allow active PP1 to carry out its functions. In vitro data suggests that PP1 is able to auto-dephosphorylate this residue, however this ability has not yet been demonstrated in vivo in human cells (Dohadwala et al., 1994, Cundell et al., 2013, Cundell et al., 2016, Wu et al., 2009, Yamano et al., 1994).

Despite the global levels of PP1 phosphatase activity being low during mitosis, certain locally active pools of PP1 within the cell bound to PIPs may exist. For example, it has been suggested that PP1 opposes Aurora phosphorylation of the plus-end directed microtubule motor protein CENP-E to dynamically regulate CENP-E motor activity during mitosis (Kim et al., 2010). Additionally, PP1 plays an important role in reducing chromosome oscillations to promote metaphase plate thinning, an important step prior to anaphase onset, by opposing CDK1 at residues Ser674 and Ser684 of Chromosome-associated kinesin 18A (KIF18A) (De Wever et al., 2014, Hafner et al., 2014). Furthermore, an astrin-bound pool of PP1 stabilises end-on kinetochore-microtubule attachments which in turn supports astrin enrichment at attached kinetochores (Conti et al., 2019, Song et al., 2021). CENP-E, KIF18A and astrin all have mitotic roles prior to anaphase, and need to be dynamically regulated during this time to reflect the changing landscape of the mitotic cell. Together, the regulation of these proteins highlights the need for locally active pools of PP1 during a time where the global phosphatase activity levels are kept low through CDK1-mediated inhibition.

While PP1 is directly inhibited by CDK1:Cyclin B, PP2A-B55 is indirectly inhibited by CDK1:Cyclin B via the B55-ENSA-Greatwall (BEG) pathway (Cundell et al., 2013, Vigneron et al., 2009, Castilho et al., 2009, Burgess et al., 2010, Alvarez-Fernandez et al., 2013). In this phosphorylation pathway, CDK1 phosphorylates the Greatwall/MASTL kinase to partially activate the kinase. At this point, Greatwall/MASTL then autophosphorylates its own C-terminus to fully activate itself (Blake-Hodek et al., 2012). Greatwall/MASTL then goes on to phosphorylate α -endosulfine (ENSA) and cAMP-regulated phosphoprotein 19 (Arpp19) (Gharbi-Ayachi et al., 2010, Mochida et al., 2010). ENSA and Arpp19 are small proteins that bind to PP2A-B55 in their phosphorylated form and inhibit PP2A-B55. When Cyclin B is degraded during mitotic exit, ENSA and Arpp19 are slowly dephosphorylated, which eventually results in active PP2A-B55 (Mochida et al., 2010, Blake-Hodek et al., 2012, Gharbi-Ayachi et al., 2010, Williams et al., 2014). Combined with the suggestion from work in *Xenopus* that PP1 is involved in the dephosphorylation of ENSA and Arpp19 (Heim et al., 2015, Ma et al., 2016), this could explain the temporal control of reactivating different families of phosphatases at distinct times during mitotic exit. PP1 reactivates rapidly as CDK1 activity drops, whereas PP2A-B55 reactivation takes longer and only becomes active after PP1 activation.

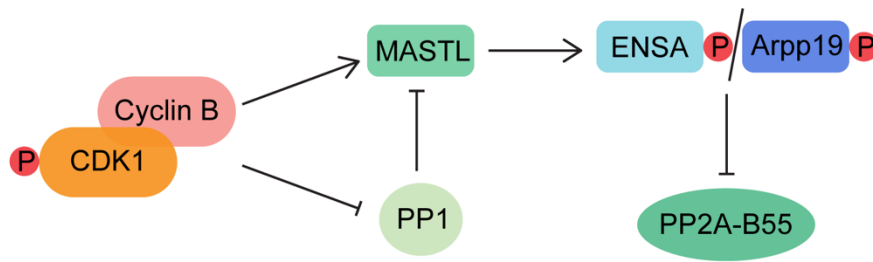


Figure 1.6: Regulation of CDK1-inhibited phosphatases during mitosis.

CDK1:Cyclin B-mediated inhibition of PP1 and PP2A-B55 is required for entry into mitosis. CDK1:Cyclin B inhibits PP1 directly, whereas PP2A-B55 is inhibited by CDK1:Cyclin B indirectly through the BEG pathway. CDK1:Cyclin B activates MASTL. MASTL then phosphorylates ENSA and Arpp19, which inhibit PP2A-B55. When PP1 is active, it is able to inactivate MASTL, therefore promoting the activation of PP2A-B55.

Unlike PP1 and PP2A-B55, PP2A-B56 is not known to be temporally controlled in mitosis. Rather, PP2A-B56 is active throughout mitosis and is known to play important roles in the formation of kinetochore-microtubule attachments (Kruse et al., 2013, Suijkerbuijk et al., 2012) and the SAC signalling pathway (Espert et al., 2014, Nijenhuis et al., 2014). Additionally, PP2A-B56 is recruited to the centromere by shugoshin where it opposes PLK1 and Aurora B to prevent cohesin removal from the centromere and thus maintain centromere cohesion during mitosis (Kitajima et al., 2006, Hauf et al., 2005, Riedel et al., 2006, Tang et al., 2006). Regulation and targeting of PP2A-B56 activity is controlled spatially through recruitment to binding partners via a short binding motif, LxxIxE, where x is any amino acid (Hertz et al., 2016). Phosphorylation of this binding motif by CDK1 has been shown to promote PP2A-B56 recruitment to binding partners (Hertz et al., 2016, Wang et al., 2016a, Wang et al., 2016b). These binding partners can be PP2A-B56 substrates themselves, such as Repo-Man (Qian et

al., 2013), a PP1 binding partner and regulator of chromatin decondensation (Vagnarelli et al., 2006), or direct PP2A-B56 to a subcellular localisation such as BUBR1 which recruits PP2A-B56 to the kinetochore (Suijkerbuijk et al., 2012). The BUBR1-bound pool of PP2A-B56 has been shown to dynamically regulate MPS1 activity by dephosphorylating the MPS1 T-loop in mammalian cells, important for the 'error correction' pathway during prometaphase (Hayward et al., 2019c).

Similarly to PP2A-B56, PP1 is often spatially regulated through binding to its substrates via short linear motifs (or SLiMs) that are found within the PIPs (introduced above). The RVxF motif, which generally conforms to the consensus sequence [K/R][K/R][V/I][x][F/W], where x is any residue other than Phe, Ile, Met, Tyr, Asp, or Pro, is one such SLiM that is found in around 90% of all known PIPs (Bollen et al., 2010, Peti et al., 2013). Some PIPs are PP1 substrates themselves, while others can inhibit PP1, target it to subcellular locations or mediate the interaction between PP1 and its targets. For example, Repo-Man targets PP1 to chromatin to promote the dephosphorylation of histone H3 Thr3, important for chromatin decondensation during anaphase (Trinkle-Mulcahy et al., 2006, Qian et al., 2011, Vagnarelli et al., 2011). Other PP1 docking sites in addition to the RVxF motif have been identified but are much less common (Bollen et al., 2010, Cohen, 2002). Importantly, the binding of PIPs to PP1 does not influence PP1 enzymatic activity, but it can change the accessibility of some substrates to the catalytic site. For instance, binding of the neuronal protein spinophilin to PP1 occludes a substrate binding site on PP1, but does not alter the active site (Allen et al., 1997, Ragusa et al., 2010, Hirschi et al., 2010). The RVxF motif of PIPs

binds to hydrophobic grooves on PP1 located approximately 20Å from the PP1 active site. Other members of the PPP family do not contain the hydrophobic grooves of the RVxF binding site as the residues creating them are not conserved from PP1 to PP2A and PP2B, indicating why PIPs with RVxF motifs do not bind other PPPs (Watanabe et al., 2001).

PP1 is a highly conserved and widely expressed Ser/Thr phosphatase that regulates many cellular processes, not limited to mitotic processes. In fact, PP1 has roles in DNA replication (Hiraga et al., 2017, Gnan et al., 2021, Jenkinson and Zegerman, 2022), RNA transcription (Estell et al., 2023, Estell and West, 2024, Cortazar et al., 2019), glycogen metabolism (Adeva-Andany et al., 2016) and neuronal signalling (Foley et al., 2021), and has been implicated in over half of all the Ser/Thr dephosphorylation events in the cell (Bollen et al., 2010). In DNA replication, PP1 is recruited to chromatin through its interaction with Replication Timing Regulatory Factor 1 (RIF1) where it regulates the replication timing by promoting DNA origin licensing, prior to replication initiation (Hiraga et al., 2017, Gnan et al., 2021). During RNA transcription, PP1 in association with its targeting subunit Phosphatase 1 Nuclear Targeting Subunit (PNUTS) dephosphorylates the C-terminal domain of RNA polymerase II thereby facilitating transcription termination (Cortazar et al., 2019, Estell et al., 2023). The highly conserved nature of PP1 is highlighted by the fact that human PP1 can rescue PP1 loss in budding yeast (Gibbons et al., 2007). This high degree of conservation across species highlights the importance of these enzymes in cellular processes. In mammalian cells, PP1 has three distinct, but similar, catalytic subunits, referred to PP1 alpha (PP1 α), PP1 beta (PP1 β) and PP1 gamma (PP1 γ) with a high level

of sequence homology between them, differing mostly at the extreme C- and N-terminal regions. They are often referred to as isoforms but are perhaps better described as paralogs as they are encoded by three different genes found on three different chromosomes. These paralogs only evolved relatively recently. In fact, budding yeast only has one catalytic subunit of PP1.

PP1 α , PP1 β and PP1 γ interact with PIPs to generate different PP1 holoenzymes, each characterised by unique subcellular localisation and substrate specificity (Egloff et al., 1997, Cohen, 2002, Bollen et al., 2010). During interphase, PP1 γ is included in the nucleoli whereas PP1 α is excluded (Trinkle-Mulcahy et al., 2001, Trinkle-Mulcahy et al., 2003, Andreassen et al., 1998). PP1 β is also enriched in the nucleoli due to the presence of an N-terminal arginine residue found in both PP1 β and PP1 γ , but not PP1 α , that is suggested to be important for interaction with nucleolar targeting subunits (Lesage et al., 2005). In metaphase, both PP1 α and PP1 γ localise to kinetochores through several RVxF-containing PIPs including the microtubule associated protein astrin (Conti et al., 2019), the kinesin family members CENP-E (Kim et al., 2010) and KIF18A (De Wever et al., 2014, Hafner et al., 2014) as well as the kinetochore component KNL1 (Nijenhuis et al., 2014, Liu et al., 2010, Rosenberg et al., 2011, Shepperd et al., 2012, Espeut et al., 2012).

In addition to PIPs defining PP1 subcellular localisation, phosphorylation of RVxF motifs can also modulate PP1 recruitment. In vitro evidence suggests that PP1 preferentially associates with non-phosphorylated RVxF motifs (Nasa et al., 2018). Indeed, Aurora B mediated phosphorylation of the KNL1 RVxF motif

negatively regulates PP1 γ localisation to kinetochores (Cohen, 2002, Welburn et al., 2010, Liu et al., 2010, Nasa et al., 2018). Some PIPs bind to PP1 isoforms in an isoform specific manner, as is the case with myosin phosphatase target subunit 1 (MYPT1) which binds to PP1 β and recruits it to kinetochores (Terrak et al., 2004, Yamashiro et al., 2008). This isoform specific binding of PIPs to PP1 can be most logically explained by the fact that the PP1 isoforms, while very similar in sequence, diverge at their C- and N-terminal regions and therefore differ in affinity for interacting target proteins. This is indeed true for MYPT1 that contains eight ankyrin repeats that bind to the C-terminus of PP1 β (Terrak et al., 2004). This phenomenon is also seen with Repo-Man which specifically interacts with PP1 γ during anaphase to promote chromatin decondensation through the dephosphorylation of histone H3 (Qian et al., 2011, Trinkle-Mulcahy et al., 2006).

The PPP family of phosphatases has long been implicated in mitotic regulation (Yamashita et al., 1990, Lucocq et al., 1991), but their roles are not fully understood. Additionally, the relative contributions of the three PP1 isoforms remain unclear. Evidence suggests that PP1 α and PP1 γ have some overlapping and some distinct functions (Trinkle-Mulcahy et al., 2006, Liu et al., 2010, Zeng et al., 2010a). For example, knock down of PP1 γ results in changes to cell shape and microtubule cytoskeleton, whereas a PP1 α knock down has no observable effect (Zeng et al., 2010a). In contrast, PP1 β has distinct roles from PP1 α and PP1 γ . Depletion of PP1 β results in nuclear deformation, a phenotype similar to that observed when MYTP1, its canonical binding partner, is depleted (Zeng et al., 2010a, Takaki et al., 2017). However, depletion of PP1 β or MYPT1 reduces control of actomyosin contraction in interphase cells, leading to genome

instability, and therefore this nuclear deformation phenotype is not thought to be a mitotic one (Takaki et al., 2017).

1.3.4 Phosphatases play an important role at the metaphase to anaphase transition

At the metaphase to anaphase transition of mitosis, the SAC is silenced, as all kinetochores are stably bound to microtubules, and subsequently the APC/C is activated to permit mitotic exit by promoting the degradation of Cyclin B and securin. This transition is associated with a global decrease in protein phosphorylation. In budding yeast, the dual-specificity phosphatase Cdc14 is critical for mitotic exit, through its role in removing CDK1-mediated phosphorylations (Stegmeier and Amon, 2004). However, the vertebrate Cdc14 orthologues, Cdc14A and Cdc14B, do not seem to have these same essential functions demonstrated by the fact that human cells with Cdc14 knocked out can progress through anaphase normally (Berdougo et al., 2008).

The Ser/Thr-specific phosphatases, PP1 and PP2A, however, are important for the regulation of mitosis in mammalian cells. It has been suggested that kinetochore-localised PP1 opposes Aurora B (Liu et al., 2010, Francisco and Chan, 1994, Emanuele et al., 2008), CDK1 (Barr et al., 2011, Bancroft et al., 2020) and MPS1 (Nijenhuis et al., 2014, Moura et al., 2017) activities. Furthermore, kinetochore-localised PP1 is a key candidate phosphatase at the metaphase to anaphase transition as it is a critical phosphatase in yeast SAC silencing and progression to anaphase (Pinsky et al., 2009, Vanoosthuyse and Hardwick, 2009, Nijenhuis et al., 2014, Meadows et al., 2011). However, there

are conflicting studies on the exact contributions of PP1 in SAC silencing and APC/C activation. In SAC silencing, proposed PP1 substrates include the CDK1 site on BUB1 (Ser459) required for MAD1 recruitment (Holder et al., 2020), the KNL1 MELT motifs (Nijenhuis et al., 2014) and the MPS1 T-loop (Moura et al., 2017). However, PP2A-B56 has also been implicated in the dephosphorylation of KNL1 MELT motifs and the MPS1 T-loop (Qian et al., 2017, Espert et al., 2014, Hayward et al., 2019c). These two sites require dynamic phosphorylation during mitosis to rapidly respond to changes in kinetochore attachment state. Therefore, the individual roles of PP1 and PP2A-B56 in dephosphorylating KNL1 and MPS1 are not fully understood.

Another necessary protein for the metaphase to anaphase transition is Cell division control protein 20 (CDC20), a coactivator of the APC/C. CDC20 is phosphorylated by CDK1 in mitosis and needs to be dephosphorylated at the metaphase to anaphase transition to promote incorporation into the APC/C and thus APC/C activation (Hein et al., 2017, Yudkovsky et al., 2000, D'Angiolella et al., 2003). In mammalian cells, PP2A-B55 (Hein et al., 2017, Mochida and Hunt, 2012), PP1 (Bancroft et al., 2020) and transcription factor 2F-associating C-terminal domain phosphatase 1 (FCP1) (Visconti et al., 2012) have all been identified as CDC20-directed phosphatases, as well as PP2A-B56 in *Xenopus* (Fujimitsu and Yamano, 2020). Depletion of PP2A-B55 does not affect mitotic timing into anaphase, which relies on active APC/C, and therefore dephosphorylated CDC20, unlike PP1 or FCP1 depletion which causes a mitotic delay (Bancroft et al., 2020, Visconti et al., 2012, Della Monica et al., 2015). Additionally, PP2A-B55 only becomes activated following APC/C activation as its

activity is controlled through the BEG pathway (Figure 1.6) (Cundell et al., 2013, Cundell et al., 2016). Together, this suggests that PP1 may be important for initiating CDC20 dephosphorylation, and therefore APC/C activation at the metaphase to anaphase transition, while PP2A-B55 can further promote APC/C activation at a later stage in anaphase.

Target	PP1	PP2A-B55	PP2A-B56
BUB1	✓		
KNL1 MELT motifs	✓		✓
MPS1 T-loop	✓		✓
Histone H3 Thr3	✓		
CDC20	✓	✓	✓
MPS1 S281		✓	
Repo-Man S893			✓
CENP-E T422	✓		
KIF18A	✓		

Figure 1.7: A number of mitotic players and their suggested associated phosphatases.

1.4 The Spindle Assembly Checkpoint (SAC) and the Metaphase to Anaphase Transition

1.4.1 The anaphase promoting complex/cyclosome (APC/C)

The cellular decision to enter anaphase is irreversible. It is therefore crucial for the maintenance of genomic integrity that the metaphase to anaphase transition of mitosis is tightly regulated to safeguard chromosome segregation. At a molecular level, this involves a signalling pathway that monitors the attachment status of kinetochores to spindle microtubules, known as the spindle assembly checkpoint (SAC), which is largely conserved from yeast to vertebrates (Musacchio and Salmon, 2007, Lara-Gonzalez et al., 2012, Musacchio, 2015). In response to unattached kinetochores, the SAC produces a diffusible inhibitor of anaphase called the mitotic checkpoint complex (MCC) which inhibits the anaphase promoting complex/cyclosome (APC/C) E3 ligase thus preventing the degradation of Cyclin B and securin and impeding anaphase onset (Alfieri et al., 2016).

Discovered close to 30 years ago, the APC/C is a large 1.2MDa protein complex that is a member of the cullin-RING family of E3 ligases (Sudakin et al., 1995, King et al., 1995). One of its key roles is the regulation of the metaphase to anaphase transition of mitosis (Irniger et al., 1995, King et al., 1995, Sudakin et al., 1995, Tugendreich et al., 1995, Cohen-Fix et al., 1996, Clute and Pines, 1999). The APC/C consists of 14 different subunits, 5 of which are present in duplicate thereby the number of subunits that make up the APC/C totals 19 (Schreiber et al., 2011). Additionally, the APC/C requires the association of one

of its WD40 domain containing coactivators, CDC20 or CDC20 homolog 1 (Cdh1), for its activity, referred to as APC/C^{CDC20} and APC/C^{Cdh1} respectively (da Fonseca et al., 2011, Buschhorn et al., 2011). CDC20 is also known as Fizzy (*Drosophila*) (Dawson et al., 1993), p53^{CDC} (mammals) (Kallio et al., 1998) and Slp1p (*S. pombe*) (Kim et al., 1998), while Cdh1 is known as Fizzy-related (*Drosophila*) (Sigrist and Lehner, 1997), Hct1p (*S. cerevisiae*) (Schwab et al., 1997) and Ste9p/Srw1 (*S. pombe*) (Blanco et al., 2000). The affinity of CDC20 and Cdh1 for the APC/C changes throughout the cell cycle, owing to the dynamic phosphorylation states of the proteins, and thus they temporally coordinate APC/C activity from mitosis until the end of G₁ of the next cell cycle (Zhang et al., 2016, Fujimitsu et al., 2016, Kramer et al., 2000, Zachariae et al., 1998, Qiao et al., 2016). For example, during mitosis, the APC/C subunit APC3/CDC27 is hyperphosphorylated by CDK1:Cyclin B which is required for CDC20 association with the core APC/C complex (Fujimitsu et al., 2016, Qiao et al., 2016). These phosphorylations are removed during mitotic exit allowing Cdh1 to bind and activate APC/C (Zhang et al., 2016).

The APC/C is a key component of mitotic regulation as it controls the stability of the anaphase inhibitors, Cyclin B and securin, through ubiquitination which targets them for ubiquitin-mediated proteasomal degradation (Meyer and Rape, 2011, Barford, 2020, Matsumoto et al., 2010). Cyclin B is a coactivator of the principal mitotic kinase CDK1. Therefore, degradation of Cyclin B leads to a reduction in CDK1 activity and shifts the global kinase-phosphatase balance within the cell to permit mitotic exit (Nigg, 2001). Secondly, degradation of securin relieves inhibition of separase, thus promoting the cleavage of sister chromatid

cohesion as the forces transferred via the kinetochore-attached spindle microtubules are able to physically separate the sister chromatids (Uhlmann et al., 1999, Nicklas and Ward, 1994, Hornig et al., 2002). The APC/C uses the E2 ligases Ube2C and UBE2S to initiate and elongate respectively the ubiquitin chains on target substrates (Yamano, 2019). UBE2S primarily creates K-11 linked ubiquitin chains but can also form K-48 linked chains (Garnett et al., 2009).

In mitosis, the APC/C is hyperphosphorylated on almost 150 sites (Zhang et al., 2016) which promotes its association with CDC20 (Fujimitsu et al., 2016, Qiao et al., 2016). The formation of APC/C^{CDC20} does not occur in early mitosis however, despite the core APC/C being phosphorylated. This is due to the N-terminal of CDC20 also being phosphorylated by CDK1:Cyclin B which reduces its binding efficiency to APC/C (Kramer et al., 2000, Yudkovsky et al., 2000). At the metaphase to anaphase transition, CDC20 is dephosphorylated which promotes its incorporation into the phosphorylated APC/C. In mammalian cells, evidence suggests that PP1 (Bancroft et al., 2020), PP2A-B55 (Hein et al., 2017, Mochida and Hunt, 2012) and FCP1 (Visconti et al., 2012) are all able to dephosphorylate CDC20. At this time point of mitosis, APC/C^{CDC20} is primed to ubiquitinate the substrates securin and Cyclin B to drive anaphase onset. The activation of APC/C^{CDC20} is a defining feature of anaphase onset.

Cdh1-mediated activation of APC/C is prevented until late mitosis. During S-phase and G₂, the early mitotic inhibitor 1 (Emi1) inhibits the APC/C by sequestering Cdh1 which allows for accumulation of mitotic Cyclins and progression into mitosis (Miller et al., 2006, Hsu et al., 2002). Emi1 is degraded

upon NEBD in a process that requires Emi1 to be phosphorylated, thus relieving its inhibition of the APC/C (Moshe et al., 2004). However, during early mitosis, the formation of APC/C^{Cdh1} is prevented by CDK1:Cyclin B mediated phosphorylation of Cdh1 which hinders its binding to APC/C (Kramer et al., 2000, Zachariae et al., 1998). During mitotic exit, Cdh1 is dephosphorylated and thus APC/C^{Cdh1} can form.

In addition to control of the APC/C by phosphorylation events on the APC/C itself and its coactivators, the APC/C is also regulated by the MCC (Alfieri et al., 2016). The MCC is the product of the SAC signalling pathway, initiated at unattached kinetochores, that consists of the checkpoint proteins mitotic arrest deficient 2 (MAD2), budding uninhibited by benzimidazoles 3 (BUB3), BUB related 1 (BUBR1), and cell division control protein 20 (CDC20) (Musacchio, 2015, Faesen et al., 2017). This tetrameric complex is able to inhibit APC/C activity. To facilitate the binding of the MCC to the APC/C, the BUBR1:BUB3 complex within the MCC utilises its degron motifs, a D-box, a KEN box and two ABBA motifs. Together, these degrons loop around the coactivator molecule of CDC20 in the APC/C and acts as a pseudo-substrate, blocking the substrate binding site of Cyclin B and securin (Izawa and Pines, 2015, Herzog et al., 2009, Di Fiore et al., 2016, Yamaguchi et al., 2016, Sewart and Hauf, 2017, Alfieri et al., 2016).

In mitosis, the APC/C also binds to a molecule of CDC20 to form the APC/C^{CDC20} complex. Therefore, when the SAC is turned on during mitosis and MCC, which contains CDC20, is actively being generated, the APC/C E3 ligase complex contains two distinct CDC20 molecules (Izawa and Pines, 2015, Alfieri et al.,

2016). MCC generation is a continuous process that occurs while unattached kinetochores persist in the cell. CDC20, both a coactivator of the APC/C and a component of the MCC, is continuously degraded and synthesised during mitosis, and this is coupled to the rapid turnover of MCC (Mansfeld et al., 2011). This is an important feature of the SAC to be prepared for rapid SAC silencing immediately once all kinetochores are stably bound to microtubules.

1.4.2 Spindle assembly checkpoint activity is initiated at unattached kinetochores

Kinetochores act as catalytic platforms for the generation of MCC (Faesen et al., 2017). They are large proteinaceous structures that self-assemble on DNA, consisting of an inner kinetochore that is tightly bound to centromeric nucleosomes, and the outer kinetochore that facilitates the interaction of the chromosome to microtubules (Cheeseman et al., 2006). The KNL1, MIS12 and NDC80 complexes, together known as the KMN network, are located at the outer kinetochore. In humans, there are around 250 copies of NDC80 which interact with spindle microtubules (Suzuki et al., 2015). Fully attached kinetochores in human cells have on average 10 microtubules per kinetochore (O'Toole et al., 2020, Kiewisz et al., 2022).

Activation of the SAC occurs at unattached kinetochores, which act autonomously as one single unattached kinetochore is enough to stop cells entering anaphase (Rieder et al., 1995, Li and Nicklas, 1995). The SAC is widely regulated by reversible phosphorylation reactions (Musacchio, 2015, Saurin, 2018, Nilsson, 2019). The principal checkpoint kinase MPS1 localises to

unattached kinetochores where it phosphorylates Met-Glu-Leu-Thr (MELT) motifs on the KMN component, KNL1, and initiates a phosphorylation signalling cascade (Abrieu et al., 2001, Ji et al., 2017, Pachis and Kops, 2018, Jallepalli et al., 2001, Yamagishi et al., 2012, Shepperd et al., 2012, London et al., 2012). The KNL1 phospho-MELT motifs recruit the BUB1 and BUB3 proteins. BUB1 and BUB3 exist as a dimer, with BUB3 binding to the Gle2-binding sequence (GLEBS) motif of BUB1. The BUB3:BUB1 complex docks at the phosphorylated MELT motifs of KNL1 and in turn recruits the BUB3:BUBR1 complex, CDC20 and the MAD1:C-MAD2 dimer (Musacchio, 2015, Musacchio and Salmon, 2007, Ji et al., 2017). BUB1 and BUBR1 are paralogues that are able to dimerise (Overlack et al., 2015). Recruitment of CDC20 occurs through the ABBA (Cyclin A, BUBR1, BUB1, Acm1) binding domain of BUB1 (Di Fiore et al., 2015). BUB1 also helps to recruit the MAD proteins to unattached kinetochores (Moyle et al., 2014). CDK1 phosphorylates Ser459 of BUB1 which subsequently promotes phosphorylation of Ser461 in an MPS1-mediated manner, stimulating the interaction between BUB1 and the Arg-Leu-Lys (RLK) motif of MAD1 (Qian et al., 2017, Zhang et al., 2017, Ji et al., 2017). This phosphorylation signalling cascade results in the formation of the MCC, consisting of the SAC proteins BUB3, BUBR1, MAD2 and the protein CDC20, which together inhibit the activity of the APC/C (Sudakin et al., 2001, Fang et al., 1998) (Figure 1.8). The MAD proteins and the BUB proteins were first identified in budding yeast through genetic screens studying mutants that were unable to arrest in mitosis after spindle disruption. Although the functions of these proteins are conserved from yeast to mammalian cells, it should be noted that the SAC proteins are non-essential in yeast whereas they

are thought to be essential in mammalian cells (Li and Murray, 1991, Hoyt et al., 1991).

Fundamental to the formation of the MCC is the ability of MAD2 to exist in two conformations (Luo and Yu, 2008, Mapelli and Musacchio, 2007, Musacchio, 2015, Mapelli et al., 2006). MAD2 is a HORMA (Hop1p, Rev7p and MAD2) domain protein that can exist in an open (O) or closed (C) conformation. O-MAD2

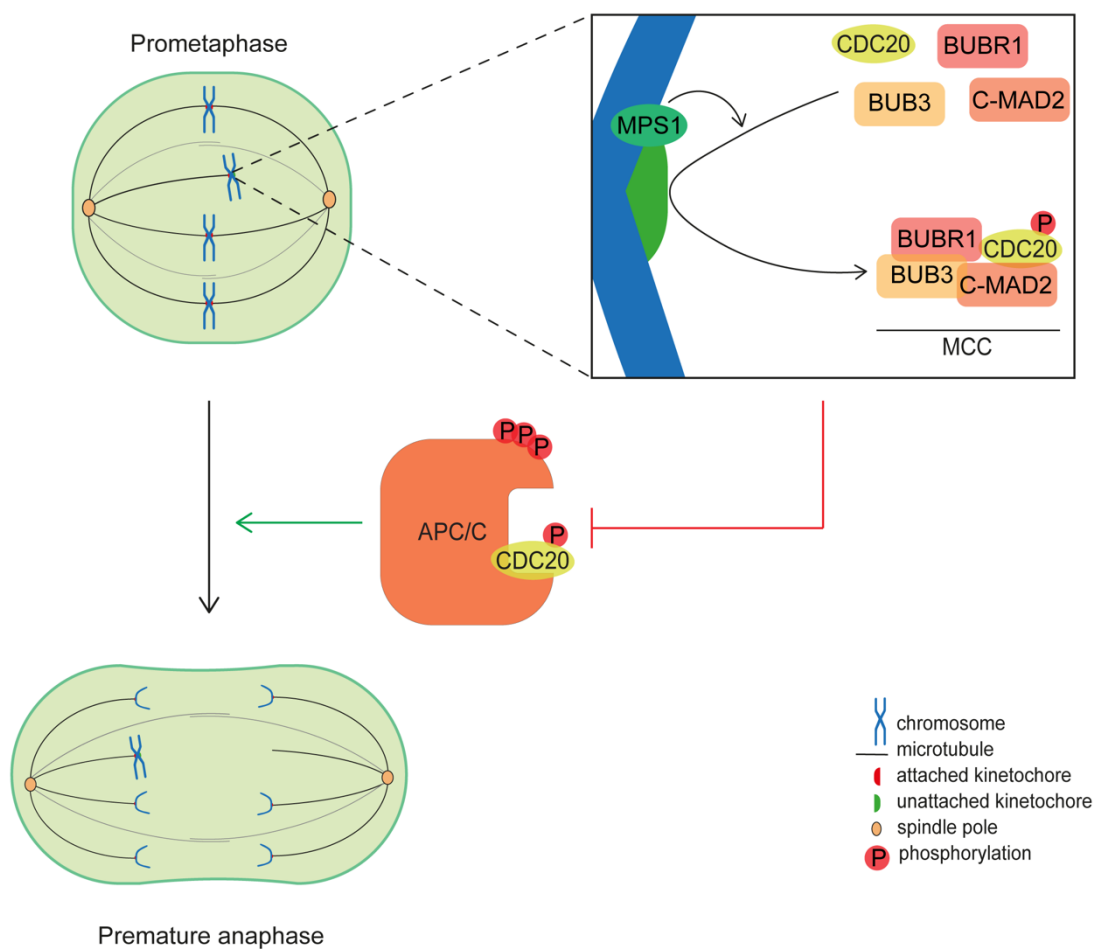


Figure 1.8: Spindle assembly checkpoint overview.

MPS1 localises to unattached kinetochores where it initiates a phosphorylation signalling cascade that results in the generation of the mitotic checkpoint complex (MCC). MCC inhibits the anaphase promoting complex/cyclosome (APC/C), to prevent premature anaphase while unattached kinetochores persist in the cell.

exists in the cytosol and is inactive in the SAC whereas C-MAD2 is active and found bound to its ligands MAD1 and CDC20 (De Antoni et al., 2005, Luo et al., 2000, Luo et al., 2004, Westhorpe et al., 2011). Structural interconversion of MAD2 at unattached kinetochores to the active C-MAD2 conformation is in fact the rate limiting step in MCC formation (Liu and Zhang, 2016, De Antoni et al., 2005). The MAD1:C-MAD2 dimer recruited via BUB1 to unattached kinetochores itself recruits inactive O-MAD2 molecules through O-MAD2:C-MAD2 dimerization and catalyses its activation and transfer to its ligands (Mapelli and Musacchio, 2007).

1.4.3 Spindle assembly checkpoint silencing leads to mitotic exit

In order for comprehensive SAC silencing at the completion of stable kinetochore-microtubule attachment formation to be achieved, there are several cellular processes that take place to allow the cell to enter anaphase. These processes include the removal of SAC proteins, such as MAD2, from the kinetochore to halt the phosphorylation signalling cascade and terminate the rolling production of MCC (Howell et al., 2001, Bader and Vaughan, 2010, Silva et al., 2014, Espert et al., 2014, Nijenhuis et al., 2014, Cordeiro et al., 2020, Lara-Gonzalez et al., 2021). Additional processes dissociate existing MCC complexes (Mansfeld et al., 2011, Uzunova et al., 2012, Foster and Morgan, 2012, Kim et al., 2018). Together, the SAC silencing processes facilitate rapid activation of the APC/C^{CDC20} at the metaphase to anaphase transition to permit APC/C dependent ubiquitination of Cyclin B and securin. Untimely anaphase entry as a result of defective SAC signalling can result in aneuploidy and chromosome instability, hallmarks of many

cancers and therefore it is of interest to understand the origins of aneuploidy more completely (Negrini et al., 2010, Santaguida and Amon, 2015, Kops et al., 2005).

At the metaphase to anaphase transition, many CDK1- and MPS1-dependent phosphorylations are removed by distinct phosphatases of the phosphoprotein phosphatase family, including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A)-B55, and PP2A-B56 (London et al., 2012, Hayward et al., 2019b, Espert et al., 2014, Barr et al., 2011, Rosenberg et al., 2011, Meadows et al., 2011, Holder et al., 2020, Pinsky et al., 2009, Vanoosthuyse and Hardwick, 2009, Nijenhuis et al., 2014). Many of these dephosphorylation events contribute to efficient checkpoint silencing at attached kinetochores and activation of the APC/C. Indeed, PP1 is the principal phosphatase involved in checkpoint silencing in yeast where it opposes both MPS1 and Aurora B phosphorylations (Pinsky et al., 2009, Vanoosthuyse and Hardwick, 2009, Nijenhuis et al., 2014, Meadows et al., 2011). One identified PP1 substrate is Spc7 (KNL1 in fission yeast) (Meadows et al., 2011), however in mammalian cells both PP1 (Nijenhuis et al., 2014) and PP2A-B56 (Espert et al., 2014) have been suggested as KNL1 directed phosphatases.

As MPS1 is an upstream regulator of the SAC, an important aspect of SAC silencing is the termination of localised MPS1 activity at the kinetochores to prevent it from phosphorylating its substrates, including KNL1 MELT motifs. A “competition model” was proposed to explain how MPS1 is lost from attached kinetochores in which microtubule binding to kinetochores displaces MPS1 and thus would nicely explain how localised MPS1 activity is prevented when

kinetochores become attached (Hiruma et al., 2015, Ji et al., 2015). However, recent research in both yeast and mammalian cells suggests that MPS1 binding to kinetochores is not competitive with microtubules and is instead governed by the balance of Aurora B and PP2A-B56 activities, at least in human cells (Hayward et al., 2022, Pleuger et al., 2024, Parnell et al., 2024, Zahm and Harrison, 2024). MPS1 also phosphorylates BUB1 and MAD1 which promotes their incorporation into the MCC (Ji et al., 2017, Faesen et al., 2017, Qian et al., 2017, Zhang et al., 2017), and MPS1 autophosphorylates itself (Koch et al., 2019, Wang et al., 2014c, Dou et al., 2011, Jelluma et al., 2008). In order to silence the SAC, these MPS1 substrates are dephosphorylated by MPS1-opposing phosphatases. Additionally, MPS1 activity at kinetochores depends on autophosphorylation of Thr676, a residue within the activation loop, or the “T-loop” of MPS1, a short polypeptide region located near the active site (Jelluma et al., 2008). PP2A-B56 dephosphorylates this activation loop, thereby terminating MPS1-mediated recruitment of SAC proteins and halting the signalling cascade (Hayward et al., 2019c). Another phosphoprotein phosphatase PP1, has been shown to dephosphorylate the activation loop of MPS1 in flies, but this activity is not conserved in mammalian cells (Hayward et al., 2019c, Moura et al., 2017).

PP2A-B56 has also been shown to oppose MPS1-mediated phosphorylations on KNL1, thus contributing to SAC silencing by preventing SAC protein recruitment to kinetochores (Espert et al., 2014). PP2A-B56 associates with BUBR1 in mammalian cells so is recruited alongside BUBR1 to the kinetochore when the SAC is active. In this way, a negative feedback loop is generated as MPS1 activity recruits a phosphatase that counteracts its own activity (Espert et al., 2014). In

addition to PP2A-B56, PP1 has been suggested to contribute to the dephosphorylation of KNL1 MELT motifs (Nijenhuis et al., 2014), although subsequent literature reported that the mitotic delay in PP1 depleted cells was more likely due to a defect in APC/C activation rather than in spindle checkpoint silencing (Bancroft et al., 2020). The exact contributions of PP1 and PP2A-B56 in dephosphorylating KNL MELT motifs to silence the SAC requires further study.

In addition to removal of SAC proteins from kinetochores as a result of dephosphorylation, many SAC proteins are also removed in a dynein-mediated manner. Dynein is a cytoskeletal motor protein that moves cargoes away from kinetochores towards the minus end of microtubules. The SAC proteins MAD1, MAD2, BUBR1 and BUB1 have all been identified as dynein cargoes that are removed from kinetochores when kinetochore-microtubule attachments form (Howell et al., 2001, Bader and Vaughan, 2010, Silva et al., 2014). Despite this, upon dynein depletion, SAC proteins are not retained at kinetochores once correct attachments to microtubules have formed, indicating that dynein-mediated stripping of SAC proteins is not the only mechanism of SAC silencing (Silva et al., 2014).

Furthermore, SAC silencing is promoted by preventing the interconversion of O-MAD2 to C-MAD2 at kinetochores. The HORMA domain protein p31^{comet} (MAD2L1BP), which binds MAD2 throughout the cell cycle, plays a role here (Figure 1.9) (Westhorpe et al., 2011, Xia et al., 2004, Habu et al., 2002, Hagan et al., 2011, Mapelli et al., 2006). When cells are depleted of p31^{comet}, anaphase entry is delayed (Habu et al., 2002). Structural studies have shown that p31^{comet}

binds directly at the juncture of the MAD2 dimer through “structural mimicry” which led to the proposal of the ‘capping model’ (Yang et al., 2007). When the SAC is active, the MAD1:C-MAD2 dimer located at the kinetochore activates an additional MAD2 molecule. p31^{comet} competes with O-MAD2 for binding to C-MAD2 thus preventing O-MAD2 recruitment and limiting MCC production (Mapelli et al., 2006, Westhorpe et al., 2011). Consistent with the ‘capping model’ of p31^{comet} activity, p31^{comet} localisation is MAD2-dependent. p31^{comet} localises to the nuclear envelope during interphase and to kinetochores during prometaphase, the same localisation as is seen with MAD1 and MAD2 during the cell cycle (Westhorpe et al., 2011). p31^{comet} localisation to kinetochores is significantly reduced in the absence of MAD2 which supports that idea that p31^{comet} forms an inhibitory “cap” on MAD2 (Westhorpe et al., 2011). However, it is surprising to note that a protein involved in SAC silencing such as p31^{comet} is associated with MAD2 when MAD2 is active. How p31^{comet} is regulated so that it is not permanently inhibiting MAD2 activation is not fully understood.

In addition to stopping the initiation cascade in order to prevent further MCC generation, the existing MCC needs to be either disassembled and/or degraded to relieve inhibition of APC/C. p31^{comet} has also been implicated in MCC disassembly, along with its partner, Thyroid hormone receptor interacting protein 13 (TRIP13) (Westhorpe et al., 2011). TRIP13 is an AAA protein that uses the energy of ATP hydrolysis to drive conformational changes. p31^{comet} acts as a TRIP13 ‘adaptor protein’ so that TRIP13 can catalyse the inactivation of C-MAD2 that forms a part of the soluble MCC (Alfieri et al., 2018, Ye et al., 2015). This conformational change of MAD2 from the closed to the open conformation

promotes the disassembly of the MCC. Interestingly, TRIP13 is not only important for SAC silencing; depletion causes major defects in SAC activation (Ma and Poon, 2016). This observation indicates that TRIP13 could be active during interphase to maintain a pool of O-MAD2 which is then important for activation of the checkpoint at the onset of mitosis (Kim et al., 2018, Ma and Poon, 2016, Ma and Poon, 2018).

TRIP13 overexpression is observed in many cancers and has been shown to

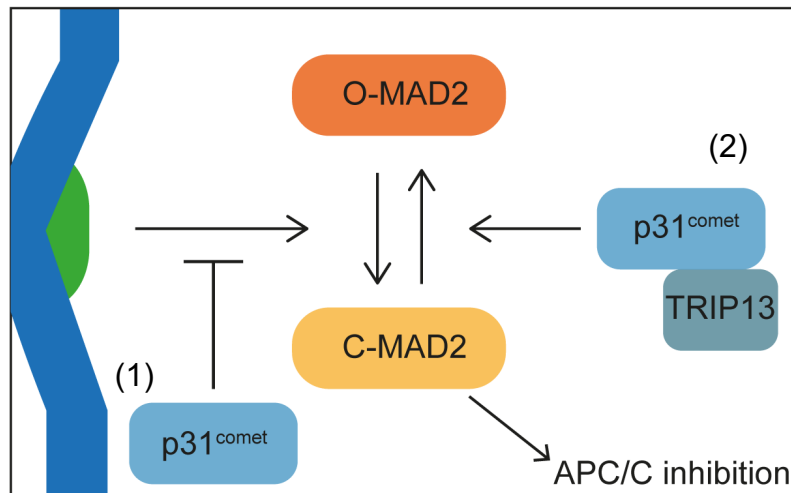


Figure 1.9: p31^{comet} in spindle assembly checkpoint (SAC) silencing.

The two proposed roles of p31^{comet} at kinetochores. (1) p31^{comet} prevents the activation of O-MAD2 to C-MAD2 to block SAC signalling. (2) p31^{comet} utilises TRIP13 to catalyse the inactivation of C-MAD2 to O-MAD2.

increase cell proliferation and drive tumorigenesis, so further understanding of its role in the SAC could be very important for the development of novel therapeutics (Wang et al., 2014b, Lu et al., 2019, Cai et al., 2020). In the absence of TRIP13, MAD2 exists solely in the closed conformation, which results in it not being able to be recruited to the MAD1:C-MAD2 dimer at kinetochores (Ma and Poon, 2016), illustrating the importance of TRIP13 in regenerating the O-MAD2 pool. Despite

this, research shows that neither TRIP13 nor p31^{comet} are essential for unperturbed mitosis (Ma and Poon, 2016) which supports the theory that there are multiple mechanisms of SAC silencing that function simultaneously to ensure rapid and well-timed anaphase onset. Additionally, although p31^{comet} is conserved in animals and plants, a p31^{comet} homologue has yet to be identified in lower eukaryotes, such as yeast.

The MCC associated with the APC/C^{CDC20} also requires dissociation and disassembling for timely anaphase onset. APC15, a subunit of the APC/C complex, promotes a more open conformation of the APC/C-MCC complex in which the catalytic site is exposed. Ubch10, the E2 ubiquitin ligase, is then able to bind to its binding sites on APC2 and APC11, two subunits of APC/C. Once here, the APC/C^{CDC20} complex is primed to ubiquitinate the CDC20 molecule within the APC/C-bound MCC (Foster and Morgan, 2012, Mansfeld et al., 2011, Uzunova et al., 2012, Alfieri et al., 2016). The ubiquitination targets this CDC20 molecule for proteasomal degradation which causes the APC/C-MCC complex to be disassembled and the APC/C^{CDC20} to return to its active state. As long as there are unattached kinetochores with active SAC signalling, more MCC will be produced so the APC/C^{CDC20} will be re-inhibited.

1.5 Thesis Aims

Mitosis is a fundamental process for life. Since the initial description of chromosome movements during mitosis by Walther Flemming in the late 19th century, it has become clear that progression through mitosis is tightly controlled to safeguard genomic integrity. In particular, kinases and phosphatases have been identified as key players in the dynamic regulation of cell cycle events. However, a key area of ongoing research in the field focuses on discovering additional layers of phospho-regulation as their roles in mitosis are not fully understood. A challenge in accurately defining the roles of phosphatases in mitosis lies in their broad responsibilities for cell viability beyond mitosis. Prolonged disruption of key phosphatase activity outside of mitosis may lead to deleterious secondary effects. Taking this into account, this thesis aims contribute to understanding the phospho-regulation of mitosis by achieving the following:

- Generate PP1 dTag cell lines for rapid targeted protein degradation for the study of PP1 specifically in mitosis.
- Compare the mitotic roles of PP1 catalytic isoforms in diploid and aneuploid cells.
- Investigate candidate PP1 targets at the metaphase to anaphase transition.
- Generate novel reagents to investigate the role of PLK1 in recruiting PP2A-B56 for the formation of K-fibres.

Chapter 2: The dTag system for rapid targeted protein degradation

2.1 Addressing the challenges of mitosis-specific PP1 function investigation

In mammalian cells, PP1 exists as three highly conserved catalytic isoforms: PP1 alpha (PP1 α), PP1 beta (PP1 β), and PP1 gamma (PP1 γ). The catalytic subunits are highly conserved across species, and within species have a high degree of sequence homology themselves, differing mostly at their extreme N- and C-terminal regions (Figure 2.1B). These isoforms are ubiquitously expressed (Verbinnen et al., 2017), but their specific roles are not yet fully understood. PP1 exerts its functions through forming holoenzymes, comprising a PP1 catalytic subunit and at least one regulatory protein (Bollen et al., 2010, Cohen, 2002, Peti et al., 2013). Around 90% of known PP1-interacting proteins contain an RVxF motif to facilitate binding to PP1 (Bollen et al., 2010, Roy and Cyert, 2009). Many studies exploit the RVxF motif-dependent PP1 binding and use RVxF motif mutants in which PP1 does not bind to prevent PP1 recruitment to particular subcellular localisation, such as kinetochores, to study its role (Conti et al., 2019, Trinkle-Mulcahy et al., 2006, Liu et al., 2010).

PP1 is responsible for dephosphorylating approximately half of all Ser/Thr-phosphorylated residues in cells, contributing to a wide range of cellular functions including DNA replication (Hiraga et al., 2017), transcription (Estell et al., 2023,

Estell and West, 2024), pre-mRNA splicing (Shi et al., 2006) and mitotic progression (Bancroft et al., 2020, Holder et al., 2019, Holder et al., 2020, Wu et al., 2009). More specifically in mitosis, PP1 is involved in several processes, including SAC silencing in both yeast and mammalian cells (Vanoosthuysen and Hardwick, 2009, Pinsky et al., 2009, Meadows et al., 2011, Nijenhuis et al., 2014), and timely activation of the APC/C (Bancroft et al., 2020). However, there are still many poorly understood aspects of PP1 action in mitosis. This can be attributed, at least in part, to there being a limited number of tools available to precisely and acutely manipulate its behaviour in the cell.

The role of PP1 in mitosis has largely been elucidated by using RNAi-mediated knock down against the catalytic or regulatory subunits. However, as PP1 is involved in essential processes during interphase including DNA replication (Hiraga et al., 2017), activation of the DNA damage checkpoint as well as regulating components of the DNA repair machinery in response to DNA damage (Campos and Clemente-Blanco, 2020), a long-term depletion of PP1 could be introducing confounding variables. For example, in the situation where PP1 has been depleted for multiple rounds through the cell cycle, errors in, or lack of, DNA repair may have occurred which could subsequently affect mitosis. In fact, DNA damage has been shown to delay mitotic entry (Harrison and Haber, 2006, Sancar et al., 2004), a phenotype that may be exacerbated in the long-term absence of PP1. Another non-mitotic cellular process that PP1 is involved in is transcription. PP1, along with its binding partner PNUTS, is important for control of RNA polymerase II elongation speed and transcriptional termination, ensuring that transcripts do not continue into independently regulated genes located

downstream of the target gene (Estell et al., 2023, Cortazar et al., 2019). The potential for PP1 depletion to affect cell cycle processes beyond mitosis, which may indirectly impact mitosis, complicates the ability to attribute observed mitotic phenotypes specifically to mitotic functions of PP1, making it challenging to define its precise role in mitosis. Therefore, a system in which PP1 catalytic isoforms could be acutely degraded in mitosis, or just before mitotic onset, would be beneficial as the specific mitotic role of PP1 could be further elucidated without the possibility of introducing unknown deleterious effects.

In recent years, targeted protein degradation systems such as the HaloTag and degradation tag (dTag) systems have gained popularity for their ability to precisely manipulate protein levels within cells by leveraging the cells' native degradation pathways (Bekes et al., 2022, Zhang et al., 2022). In the case of the dTag system, the protein of interest (POI) is fused to a mutant of the FKBP12 protein (FKBP12^{F36V}) (Nabet et al., 2018), often achieved using CRISPR/Cas9 technology. Endogenous WT FKBP12 is a 12kDa FK506-binding protein that is important for protein folding and transcription (Bonner and Boulianne, 2017, Tong and Jiang, 2015). The FKBP12^{F36V} mutation introduces a hydrophobic 90Å³ cavity not present in the WT protein, allowing dTag reagents to be designed to exclusively bind the mutant version, thereby minimizing side effects (Clackson et al., 1998). dTag reagents are cell permeable heterobifunctional degraders with a ligand that binds FKBP12^{F36V}, a linker and a ligand that recruits an E3 ligase. Upon addition of the dTag reagent, a ternary complex forms between the E3 ligase, the dTag molecule and the CRISPR tagged POI. Recruitment of the E3 ligase to the POI results in ubiquitination and subsequent proteasomal

degradation via the cell's endogenous proteasomal degradation pathway (Figure 2.1A). Different dTag reagents, such as dTag1, dTag7, and dTag13, recruit distinct E3 ligases—dTag1 recruits von Hippel-Lindau (VHL), while dTag7 and dTag13 recruit cereblon (CRBN) (Nabet et al., 2018, Nabet et al., 2020). Compared to small molecule inhibitors, degradation systems provide greater specificity and fewer off-target effects. Additionally, lower concentrations of dTag reagents can often be used since the degrader is recycled after the POI is degraded, and this also reduces the need for strong binding to the active site (Bekes et al., 2022).

The three different catalytic isoforms of PP1 in mammalian cells, PP1 α , PP1 β and PP1 γ , are thought to have largely overlapping functions due to being structurally similar Ser/Thr directed phosphatases (Peti et al., 2013). However, recent studies have presented evidence that PP1 isoforms have some distinct functions (Qian et al., 2011, Zeng et al., 2010a, Trinkle-Mulcahy et al., 2006). Taken together with the knowledge that the isoforms display distinct localisations during the cell cycle (Andreassen et al., 1998, Trinkle-Mulcahy et al., 2003), these data emphasise a need to further investigate the individual roles of PP1 isoforms as well as their combined functions. PP1 β , which localises to kinetochores via its interaction with MYPT1 (Terrak et al., 2004), has been shown to negatively regulate PLK1 activity through dephosphorylation (Yamashiro et al., 2008, Kiss et al., 2019). However, unlike PP1 α and PP1 γ which are important for proper mitotic timing, RNAi-induced PP1 β depletion does not cause a mitotic delay (Bancroft et al., 2020) suggesting that it is less essential for mitotic regulation. Therefore, PP1 β is not investigated further in this thesis.

As mentioned above, degradation systems are a powerful way to rapidly remove target proteins from cells. In order to circumvent the possible introduction of confounding variables due to faulty DNA replication caused by an extended depletion of PP1, cell lines were generated in which the PP1 α and PP1 γ catalytic isoforms were fused to the FKBP12^{F36V} protein of the dTag degradation system. The generation and characterisation of these cell lines will be discussed in this chapter. This approach facilitates investigation into the mitosis specific functions of PP1. The PP1 catalytic isoforms differ in sequence predominantly in the 30 amino acids at the C-terminus (Figure 2.1B). Furthermore, the C-terminal tail of PP1 is a disordered region that is not part of the enzyme's catalytic core (Salvi et al., 2021). Therefore, CRISPR/Cas9 knock-in constructs were designed to target the C-terminal region so as to have minimal effect on protein folding and enzyme function. An illustration of the CRISPR design for PP1 γ is displayed in Figure 2.1C. The integration cassette within the homology directed repair (HDR) construct contains a 12 amino acid (aa) linker to provide structural flexibility between the endogenous protein and the tag. Adjacent to the 12 aa linker is a GFP fluorescent protein immediately followed by the mutant FKBP12^{F36V} (FKBP-V). A P2A ribosomal skipping site links the FKBP12^{F36V} to a blasticidin (BSD) resistance gene which is used for selection of positive clones.

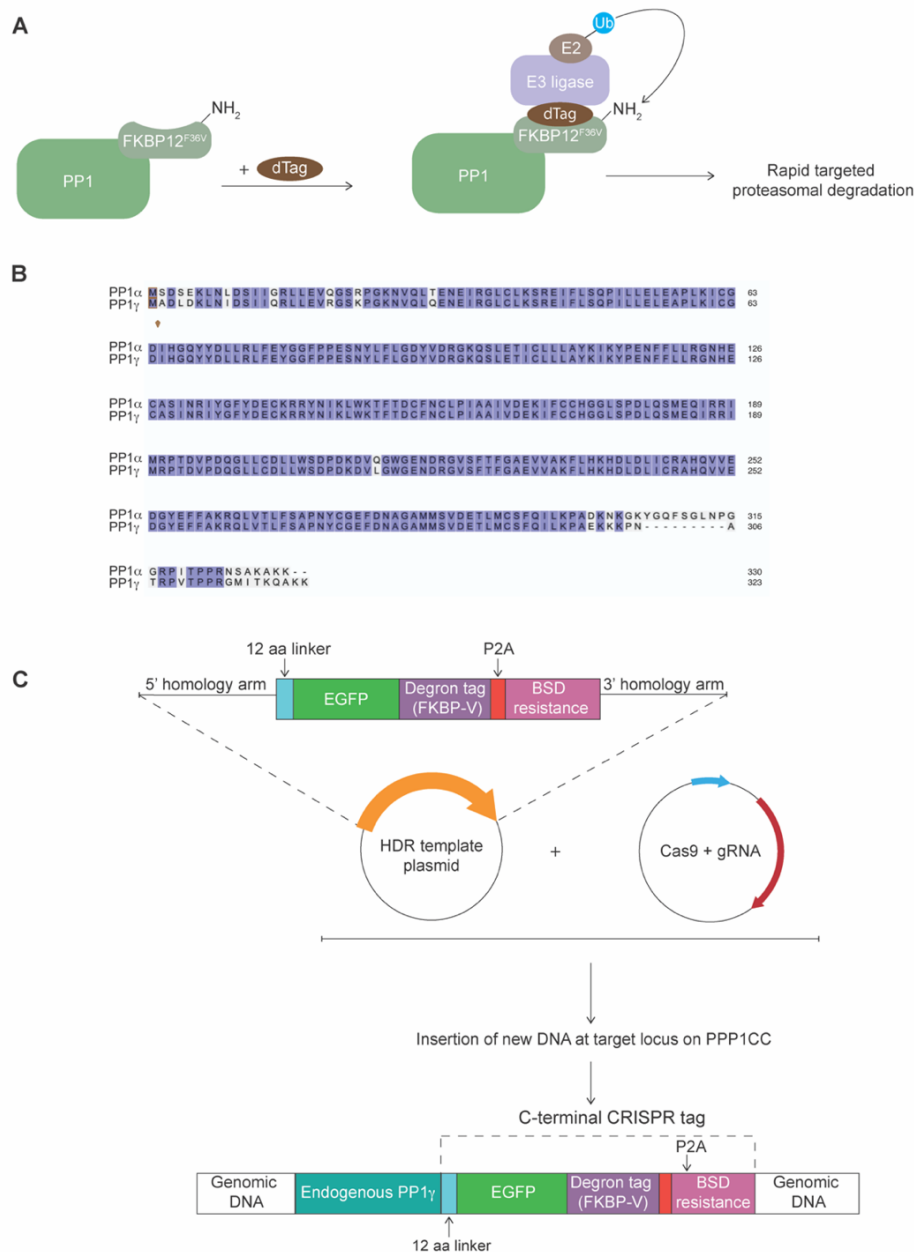


Figure 2.1: The dTag system is a targeted protein degradation system for rapid and precise degradation of a protein of interest.

(A) Illustration of the mechanism by which the dTag system results in proteasomal degradation using the cell's endogenous proteasomal degradation pathway. Ub = ubiquitin. (B) Sequence alignment of canonical forms of PP1α (top) and PP1γ (bottom). Sequences obtained from NCBI and aligned using Clustal Omega Program (version 1.2.4). (C) Schematic of the CRISPR design and tag inserted at the C-terminus of PP1γ. EGFP = enhanced green fluorescent protein. BSD = Blasticidin. P2A = ribosomal skipping site. HDR = homology directed repair. FKBP-V = FKBP12^{F36V}.

2.2 dTag1 and dTag13 reagents do not interfere with mitotic timings in plain HeLa cells

Prior to the generation of dTag cell lines, it was important to confirm that the addition of dTag reagent to cells would not interfere with normal mitotic progression. The dTag reagent is a heterobifunctional molecule, meaning it consists of two distinct functional ligands separated by a flexible linker that are designed to interact with different targets. One ligand selectively binds the non-native FKBP12^{F36V} that is fused to the POI, the other ligand is optimised to recruit an E3 ligase, thereby bringing the POI into close proximity to the E3 ligase (Nabet et al., 2018, Nabet et al., 2020). This promotes the formation of a ternary complex in which the POI is ubiquitinated and targeted for proteasomal degradation. A range of dTag reagents exist which all have the same FKBP12^{F36V}-binding ligand but can recruit different E3 ligases due to containing distinct E3 ligase-binding ligands; dTag1 engages VHL (Figure 2.2A), whereas dTag13 employs CRBN (Figure 2.2B). The solubility of the different available dTag reagents in DMSO differ slightly, with dTag1 being more soluble than dTag13 (Nabet et al., 2018, Nabet et al., 2020).

To confirm that the dTag reagent does not influence cell behaviour in mitosis when the cells do not contain FKBP12^{F36V} mutant protein, dTag1 (100nM) or dTag13 (500nM) was added to plain HeLa cells and imaged on an EVOS M7000 microscope imaging system. The time taken for cells to progress from NEBD to ANA was measured (Figure 2.2C,D). There was no significant difference between the control and +dTag conditions. Therefore, any difference in mitotic timings

observed in future experiments (Chapter 3) when dTag is added to the PP1 dTag CRISPR cells can be explained by the degradation, and thus absence, of PP1 rather than simply the presence of dTag reagent in the cells.

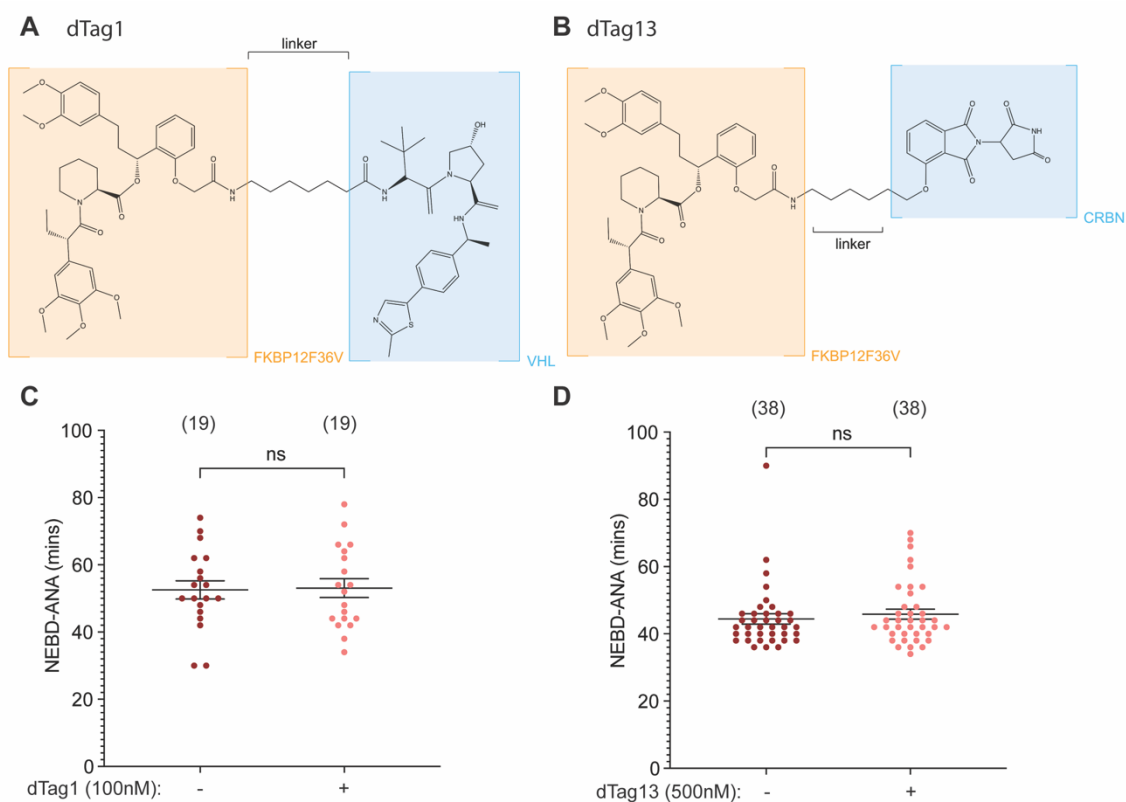


Figure 2.2: dTag1 and dTag13 reagents do not interfere with mitotic timings in plain HeLa cells.

(A,B) Structure of dTag1 (A) and dTag13 (B) reagents, indicating the domain that binds specifically to the FKBP12^{F36V} dTag protein (orange), the linker region, and the domain that recruits the E3 ligase (blue). The FKBP12^{F36V} region is identical in both dTag1 and dTag13. The structures differ in their E3 ligase recruiting domain. (C, D) Plain HeLa cells were incubated with dTag1 (100nM) (C) or dTag13 (500nM) (D) and imaged on the EVOS M7000 microscope at 2 min intervals. dTag reagent was added 2 hours before imaging started. SiR-DNA (50nM) was used to visualise the DNA. Nuclear envelope breakdown (NEBD) to anaphase (ANA) (mins) time is plotted. Graphs display mean $\pm$ SEM. Conditions were compared using a Two-tailed unpaired T-test. $P > 0.05$ = not significant (ns).

2.3 Generating HeLa PP1 α dTag and PP1 γ dTag cell lines

Generation of PP1 dTag cell lines was first carried out in HeLa cells. PCR screening of the generated CRISPR clones after blasticidin selection was performed by amplifying across the expected insertion site of the CRISPR tag (Figure 2.3A). PP1 γ was tagged with both EGFP and FKBP12^{F36V}, whereas PP1 α was tagged with FKBP12^{F36V} alone and did not have a fluorescent tag. Homozygous clones are essential when using degron systems to ensure that all protein expression is ablated upon the addition of the dTag reagent. A heterozygous clone in this study would result in only a proportion of PP1 being degraded via the proteasomal degradation pathway and enough catalytic PP1 could persist in the cell to carry out its full function. This could obscure any of the phenotype observed. To confirm successful homozygous cell line generation, both western blot and PCR screening was carried out (Figure 2.3B-F) which confirmed that the PP1 α dTag and PP1 γ cell lines were proteomically and genetically homozygous for the desired insertion.

The modification of an endogenous protein by CRISPR/Cas9 mediated knock-in can alter protein function or localisation due to a change in protein folding brought about by the insertion of the CRISPR tag. Therefore, this needs to be taken into consideration when tagging endogenous proteins, and the function of the tagged protein should be tested against the parental cell line to ensure that no change in normal cellular behaviour has taken place. In the case of the PP1 dTag cell lines generated in this study, live cell imaging was used to functionally examine whether the FKBP12^{F36V} tag interfered with normal mitotic conduct. An EVOS M7000 microscope imaging system was used to image the PP1 dTag cell lines

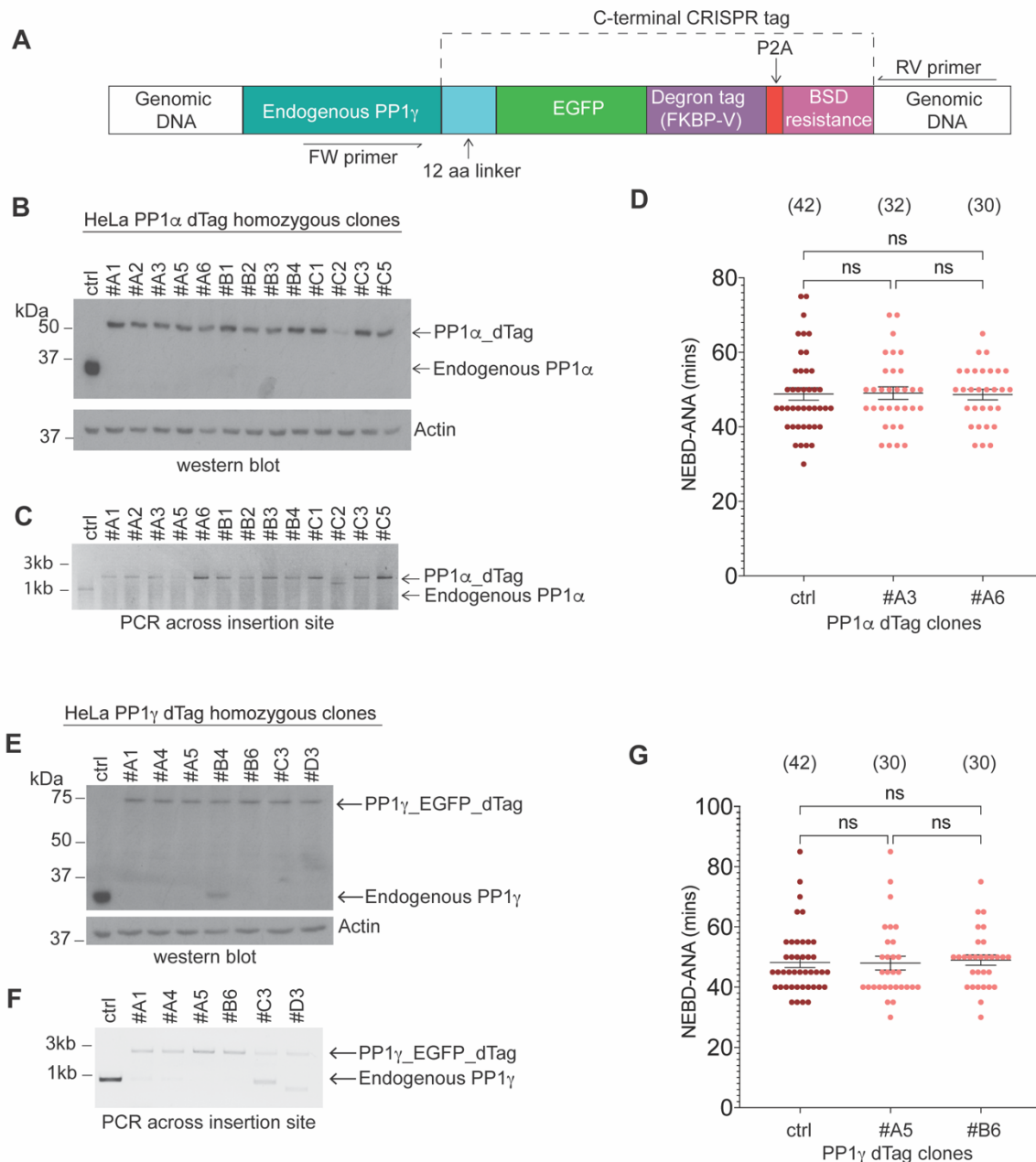


Figure 2.3: Generating HeLa PP1 α dTag and PP1 γ dTag cell lines

(A) Schematic of the CRISPR tag inserted at the C-terminus of PP1 γ showing the forward (FW) and reverse (RV) primers used for PCR screening. The location of the PCR primers for screening PP1 α are in the corresponding regions of the PP1 α tag. (B, C) Western blot (B) and PCR (C) screens confirm homozygous PP1 α dTag clones. (D) Quantification of nuclear envelope breakdown (NEBD) to anaphase (ANA) (mins) from live cell imaging of homozygous PP1 α dTag clones #A3 and #A6. (E, F) Western blot (E) and PCR (F) screens confirm homozygous PP1 γ dTag clones. (G) Quantification of NEBD-ANA (mins) from live cell imaging of homozygous PP1 γ dTag clones #A5 and #B6. For both (D) and (G), cells were incubated with 50nM SiR-DNA to facilitate visualisation of DNA. Ctrl = plain HeLa cells. Graphs display mean $\pm$ SEM. Conditions were compared using a Two-tailed unpaired T-test. $P > 0.05$ = not significant (ns). Expected sizes of protein bands in western blots: PP1 α ~ 35kDa. PP1 γ ~ 35kDa. PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

as they progressed through mitosis, and the time taken for cells to progress from

NEBD to ANA was measured. The CRISPR clones were compared to the parental HeLa cells used for the creation of the CRISPR cell lines. There was no significant difference in the time taken for the PP1 CRISPR clones to progress through mitosis in both the PP1 α and PP1 γ clones (Figure 2.2D,G), and no observable difference in the behaviour of the cells during their mitosis suggesting that the CRISPR tags did not interfere with normal PP1 mitotic function.

2.4 Generating HeLa PP1 α / γ dTag cell lines

PP1 has largely been studied as one entity, grouping the different catalytic subunits together. They are largely believed to have some overlapping biological functions, are very structurally similar and associate with many of the same regulatory subunits (Peti et al., 2013). However, there is recent evidence (Qian et al., 2011, Bancroft et al., 2020, Zeng et al., 2010a) to suggest that the PP1 catalytic isoforms could have distinct roles in mitosis. Therefore, as well as generating PP1 dTag cell lines for the individual PP1 α and PP1 γ isoforms, a double CRISPR cell line was created with both PP1 α and PP1 γ tagged with FKBP12^{F36V} in the same cells, to facilitate investigation into both the individual and combined roles of PP1. To do this, the antibiotic resistance marker for the PP1 α CRISPR HDR construct was swapped for puromycin. CRISPR/Cas9 technology was then used to tag PP1 α with FKBP12^{F36V} in the PP1 γ EGFP dTag cells. Puromycin was used to select for positive clones.

Western blot and PCR screening confirmed the generation of homozygous PP1 α / γ dTag cell lines (Figure 2.4A,B). As before with the individual PP1 α and PP1 γ cell lines, to confirm that the tag did not interfere with function, live cell imaging of PP1 α / γ dTag CRISPR clones was carried out on an EVOS M7000 microscope imaging system. The time taken for cells to progress from NEBD to ANA was measured. The CRISPR clones were compared to parental HeLa cells as a control. No difference was observed in the length of mitosis, suggesting that the CRISPR tagged PP1 catalytic isoforms did not behave differently to the untagged endogenous protein.

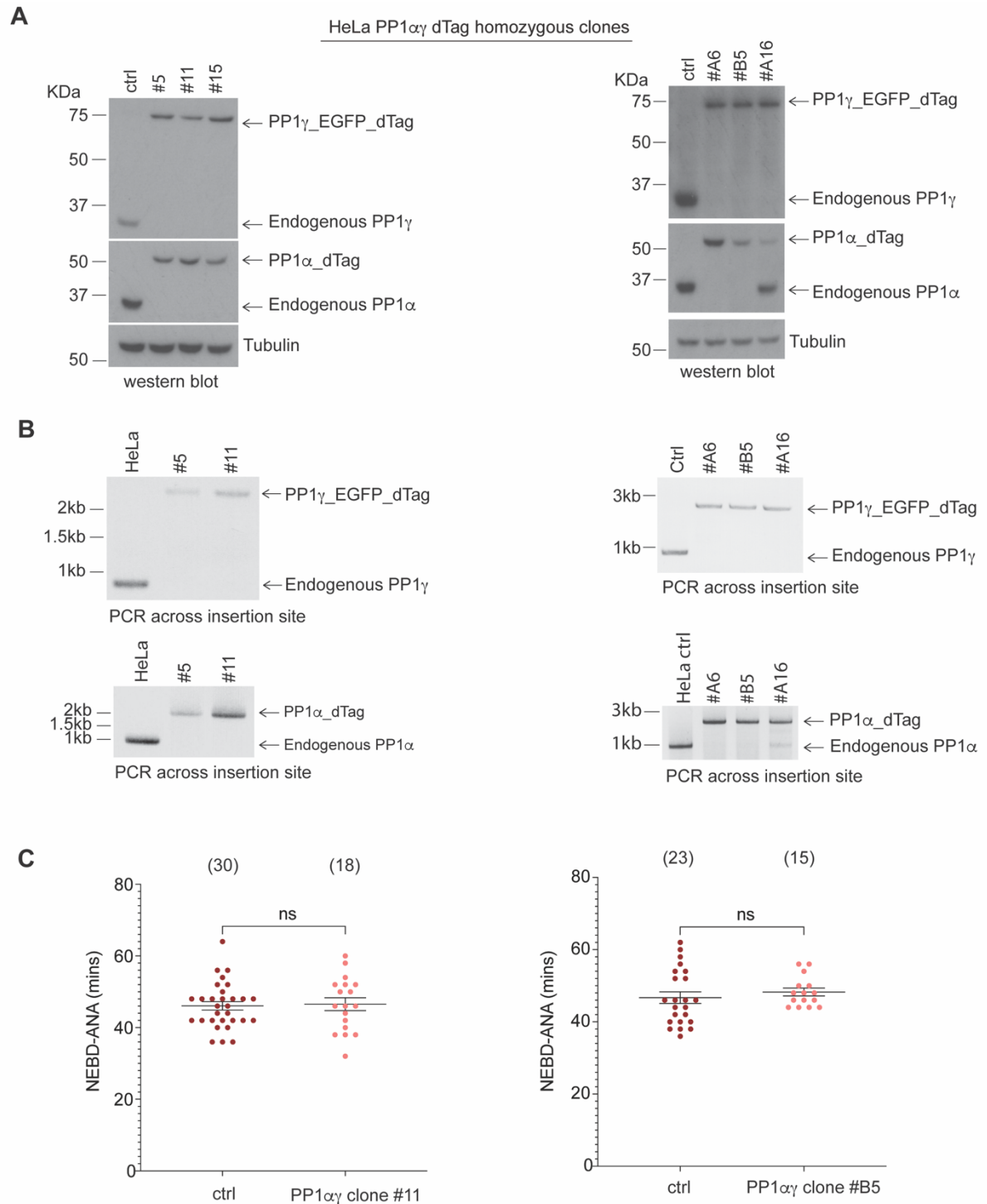


Figure 2.4: Generating HeLa PP1 α γ dTag cell lines

(A-B) Western blot (A) and PCR (B) screens confirm homozygous PP1 α γ dTag CRISPR cells. (C) Live cell imaging of homozygous PP1 α γ dTag clones #11 and #B5. Cells were incubated with 50nM SiR-DNA. Images were taken at 2min intervals. Ctrl = plain HeLa cells. Quantification of nuclear envelope breakdown (NEBD) to anaphase (ANA) (mins). Graphs display mean $\pm$ SEM. Conditions were compared using a Two-tailed unpaired T-test. $P > 0.05$ = not significant (ns). Expected sizes of protein bands in western blots: PP1 α ~ 35kDa. PP1 γ ~ 35kDa. PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

2.5 Generating HCT116 PP1 dTag cell lines

The PP1 dTag CRISPR cell line generation carried out thus far was in a HeLa cell background. HeLa cells are derived from cervical cancer cells and like many other cancer cells are aneuploid. HeLa cells carry three copies of both PP1 α and PP1 γ genes. However, a normal human somatic cell is diploid and has two copies of each of the PP1 catalytic subunits. The difference in the number of copies of PP1 in a cell may alter the expression of the enzyme across the different ploidies and possibly affect the dependence of the cell on the enzyme too. Therefore, it was of interest to investigate potential differences between cell lines of different ploidy.

To this end, PP1 dTag HCT116 cells were made in addition to the PP1 dTag HeLa cells generated above. HCT116 are a diploid human colorectal carcinoma cell line that contains two copies of PP1 α and two of PP1 γ . HCT116 parental cells were transfected with the same PP1 gRNA Cas9 constructs and HDR constructs that were used to generate the HeLa PP1 dTag cell lines before being placed under blasticidin selection. Cells were screened by western blot and PCR to confirm successful generation of homozygous PP1 α dTag clones and PP1 γ dTag clones (Figure 2.5A,B).

To produce HCT116 cells with both PP1 α and PP1 γ fused to the FKBP12^{F36V} protein, PP1 γ dTag cells were subjected to a second round of CRISPR to tag PP1 α using the HDR construct that had been swapped to puromycin from blasticidin. This is the same CRISPR tagging protocol that was used to create the HeLa PP1 PP1 α/γ dTag clones (Figure 2.4). HCT116 PP1 α/γ dTag clones were screened by western blot (Figure 2.5C). The upshift on the western blots and loss

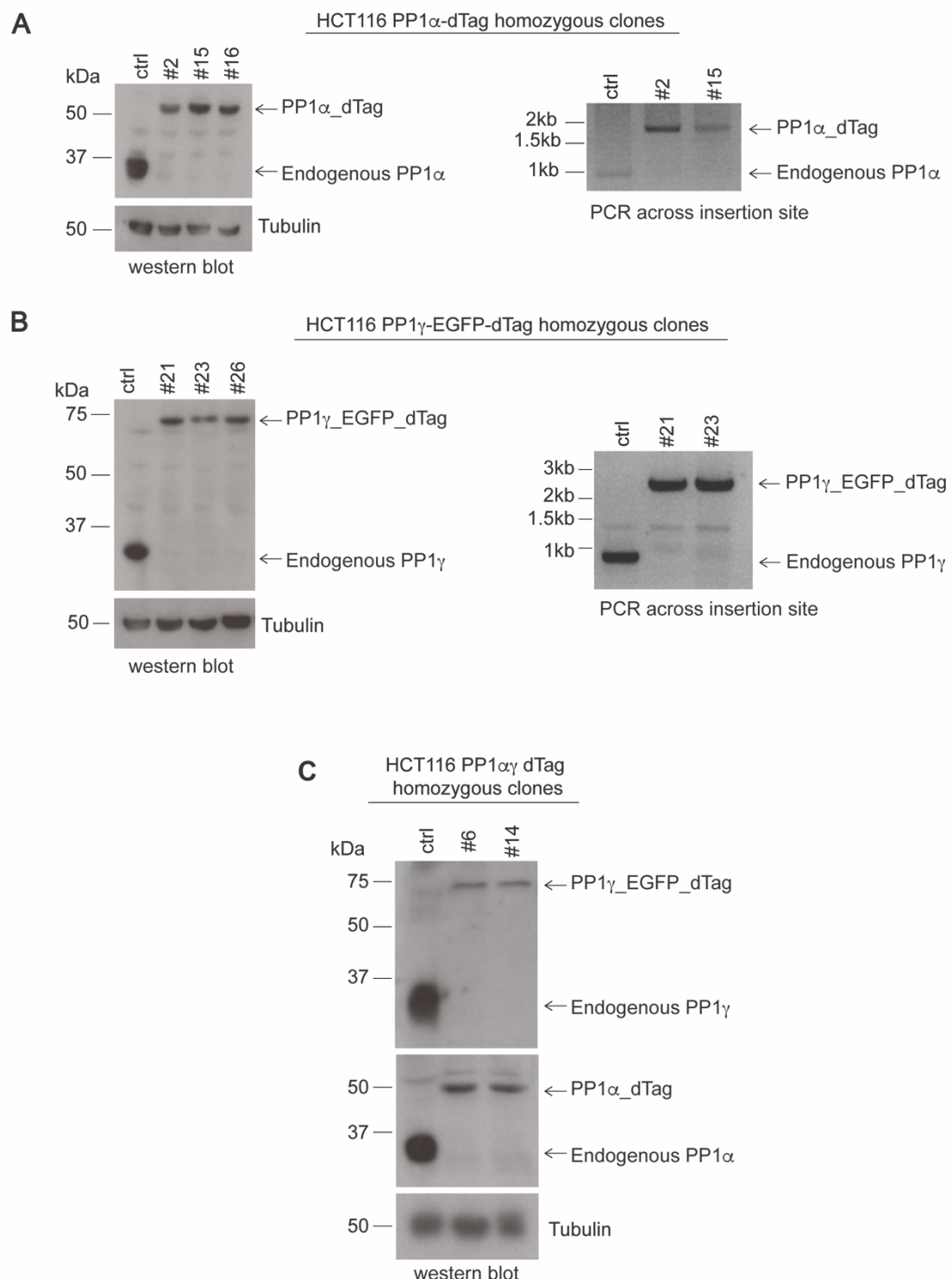


Figure 2.5: Generating HCT116 PP1 dTag cell lines.

(A) Western blot and PCR confirming successful homozygous tagging of PP1 α in HCT116 cells. (B) Western blot and PCR confirming successful homozygous tagging of PP1 γ in HCT116 cells. For PCRs in both (A) and (B), PCR primers anneal to genomic DNA either side of the integration locus for PP1 α or PP1 γ respectively, thereby amplifying across the insertion site. (C) Western blot confirming successful homozygous tagging of both PP1 α and PP1 γ in the same HCT116 cell lines. Expected sizes of protein bands in western blots: PP1 α ~ 35kDa. PP1 γ ~ 35kDa. PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

of band at the endogenous size for PP1 revealed successful generation of

homozygous clones. As before, to confirm that CRISPR tagging did not affect normal mitotic progression, the cells were imaged on the EVOS M7000 microscope imaging system and compared with parental HCT116 cells. No difference in mitotic timing was observed (data not shown).

2.6 dTag1 results in faster degradation of PP1 than dTag13 in HeLa cells

Next, dTag1 and dTag13 were both tested for their efficiency at degrading PP1 in the generated dTag systems. HeLa PP1 α dTag and PP1 γ dTag CRISPR tagged cells were incubated with either dTag1 (100nM) or dTag13 (100nM). The cells were harvested after either 60 or 120 minutes of treatment with dTag reagent, lysed in lysis buffer and run on an SDS-PAGE gel. The membrane was blotted for PP1 α or PP1 γ , dependent on which cell line was being tested (Figure 2.6A,B). Western blot analysis indicated that in the generated PP1 dTag cell lines, degradation with dTag1 was more substantial than dTag13, following a 2-hour incubation.

A dTag incubation time-course was carried out using the HeLa PP1 α dTag and PP1 γ dTag cell lines to more closely examine the rate at which degradation occurs (Figure 2.6C). Cells were incubated with dTag1 (100nM) for the indicated durations and loss of protein abundance in the cell was observed by western blot analysis. As before, a 2-hour incubation resulted in substantial loss of PP1. Quantification of this time-course (Figure 2.6D) illustrated that around 50% of detectable PP1 was lost after only 30 minutes. The majority was removed within the first 60 mins. There was little quantifiable change between 1 hour and 3-hour incubations.

dTag1 was more efficient at degrading PP1 in this system than dTag13. However, they were both used at the same concentration, and it is possible that dTag13

was not used at a saturating amount and a higher concentration could lead to faster degradation rate of tagged PP1. To test this hypothesis, PP1 γ dTag cells were incubated with 1mM dTag13 (a 10-fold increase in concentration to that used previously) and harvested at the indicated timepoints. Western blot analysis suggested that increasing the concentration of dTag13 to 1mM did not increase degradation rates and that the 100mM used previously was used at a saturating concentration (Figure 2.4E). It would be interesting to know what the limiting factor in this degradation pathway is. One possible explanation is that the limiting factor is the rate at which CRBN can induce degradation. Alternatively, it could be the endogenous proteasome itself, or the number or accessibility of lysines in the POI. If it were indeed the CRBN E3 ligase that was limiting the rate of PP1 degradation, it could be that incubating the cells with multiple dTag reagents that recruit different E3 ubiquitin ligases would lead to an even more rapid and acute degradation of tagged PP1.

Western blot analysis indicated that the 2-hour incubation with dTag1 (100nM) resulted in a successful degradation of CRISPR tagged PP1. To understand more fully how much degradation of PP1 had occurred, the detection limit of the PP1 antibodies used in this study was investigated. PP1 α/γ dTag cells were left untreated, lysed, and prepared for western blotting. Dilutions of the control (100%) sample to the indicated percentages were prepared by diluting in 1X sample buffer and analysed by western blot (Figure 2.6F,G). 10 μ L of sample was loaded per lane. The 100% sample corresponds to sample at 10ng/ μ L. PP1 signal is lost in the 12.5% sample, where signal intensity of the 100% sample is comparable to the control samples of Figure 2.6C, suggesting that $\geq 87.5\%$ of PP1

is degraded after a 2-hour incubation with dTag1. A comparison of the degradation efficiencies between asynchronous and mitotic cells is shown in Figure 2.8.

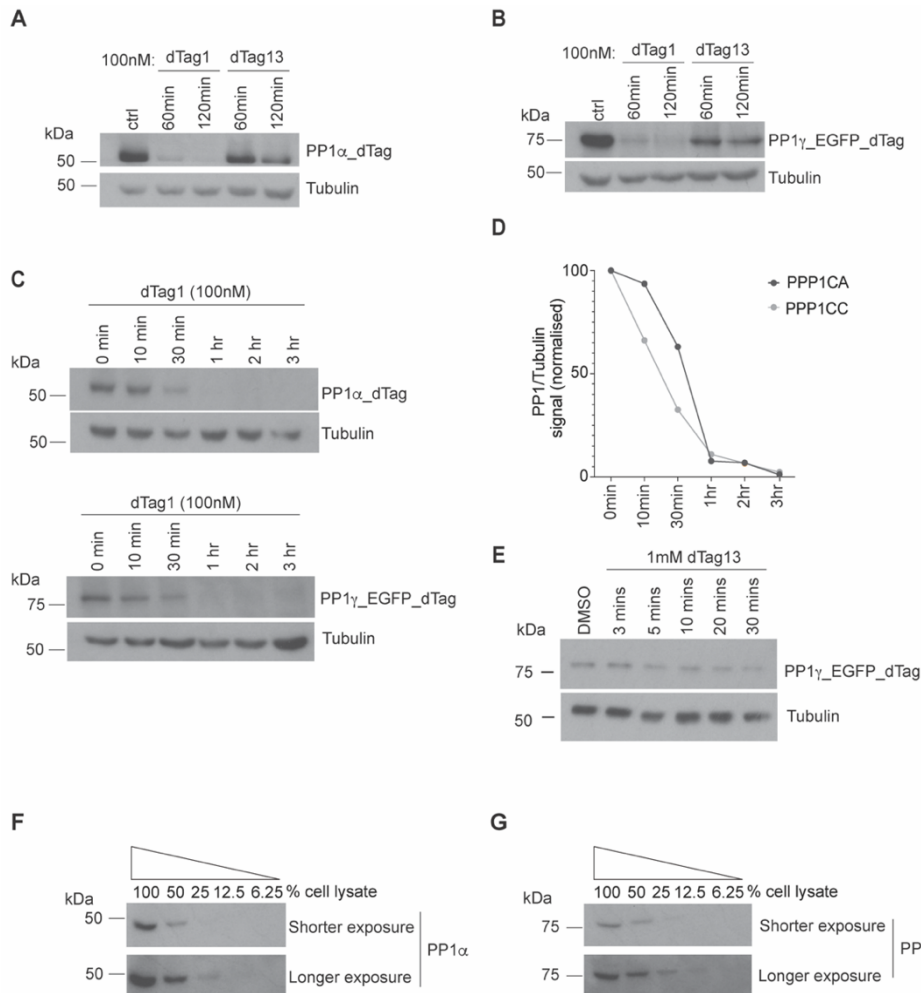


Figure 2.6: dTag1 results in faster degradation of target PP1 than dTag13 in HeLa cells

(A,B) Western blot of PP1 α dTag clone #A3 (A) or PP1 γ EGFP dTag clone #A5 (B) incubated with dTag1 (100nM) or dTag13 (100nM) for either 60 or 120 mins. (C) dTag1 incubation time-course using PP1 α dTag clone #A3 or PP1 γ EGFP dTag clone #A5. (D) Intensity of PP1 signal in western blots in (C) quantified in ImageJ. Tif file of western blot scan loaded into ImageJ and inverted using the Invert function, to view image as 'white-on-black'. Using the rectangle tool, a box (w=50, h=10) was drawn in the central point on the PP1 α or PP1 γ band, and the intensity of the signal was measured using the 'Measure' tool. A background measurement was taken for each timepoint and subtracted from the PP1 band intensity. The intensity of each corresponding tubulin band was measured in the same way. PP1 signal was normalised over tubulin and measurements plotted in Prism. (E) PP1 γ EGFP dTag cells incubated with dTag13 (1mM) for the indicated incubation periods and blotted for PP1 γ . (F,G) HeLa PP1 α / γ dTag cells (Clone #B5) were harvested and lysed. Dilutions of the lysate (100% sample) were created by dilution with Laemmli buffer to the indicated percentages. Samples were run on SDS-PAGE gels and blotted for PP1 α and PP1 γ . Expected sizes of protein bands in western blots: PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

2.7 dTag1 rapidly degrades dTag CRISPR tagged PP1 catalytic subunits

As outlined in the previous section, dTag1 is more efficient than dTag13 in degrading target PP1. To confirm that the FKBP12^{F36V} CRISPR tag on PP1 was functional in all of the generated PP1 dTag CRISPR cells, separate clones were incubated with dTag reagent (Figure 2.7A-F) for 2 hours. Western blot analysis confirmed that degradation of PP1 catalytic subunits was successful in all tested clones upon the addition of dTag reagent.

The CRISPR tag on PP1 γ contains a GFP tag in addition to the FKBP12^{F36V} protein. Therefore, the degradation of PP1 can be inferred by the lack, or reduction, of a GFP signal when visualised on a fluorescent microscope. PP1 α/γ dTag cells were incubated with dTag1 (100nM) for two hours before being fixed with PTEMF and stained with the kinetochore protein CENP-C and Hoechst (Figure 2.7G). As shown in the representative images presented, the addition of dTag1 (100nM) led to a drop in GFP signal, below the observable limit when contrasted the same in ImageJ as the control (-dTag) condition indicating that PP1 γ had been degraded. In future work, it would be interesting to carry out timelapse imaging to closely examine when and where the degradation begins in the cell, and whether the cytoplasmic or nuclear pool is degraded more rapidly.

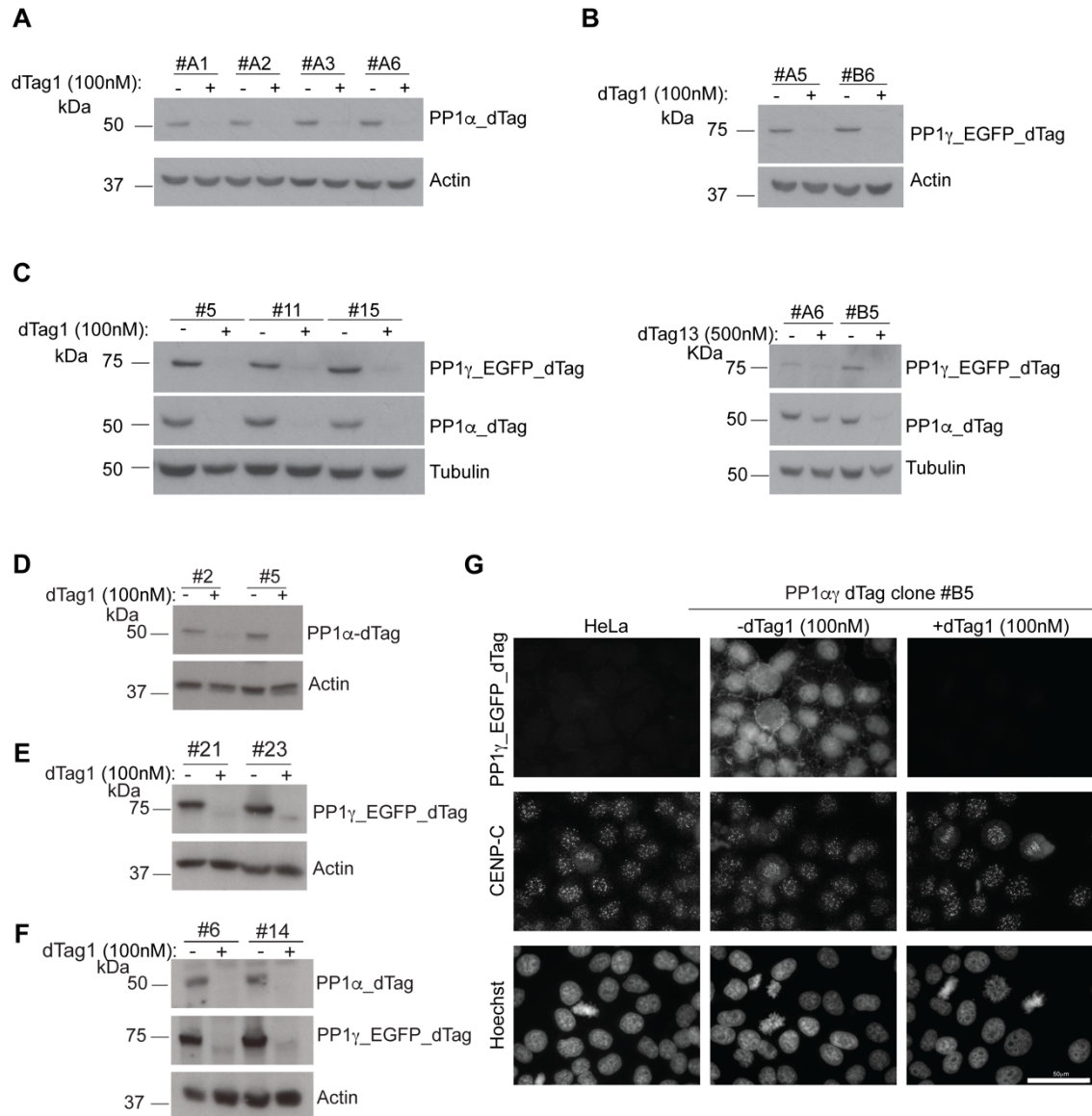


Figure 2.7: dTag1 rapidly degrades dTag CRISPR tagged PP1 catalytic subunits.

(A,B) PP1 α dTag HeLa CRISPR clones (A) and PP1 γ EGFP dTag HeLa CRISPR clones (B) were incubated with dTag1 (100nM) for 2 hours. Western blot shows successful degradation of PP1 catalytic subunits (C) PP1 α/γ dTag HeLa CRISPR clones were incubated with dTag1 (100nM) or dTag13 (500nM), as indicated, for 2 hours. Western blot shows successful degradation of PP1. (D, E, F) Functional test of the dTag efficiency in HCT116 cell lines. PP1 α (D), PP1 γ (E) and PP1 α/γ (F) cell lines were incubated with dTag1 (100nM) for 2 hours prior to harvesting and lysing the cells. Analysis on SDS-PAGE gels indicate successful degradation of PP1 catalytic subunits. (E) Immunofluorescence of HeLa cells and PP1 α/γ dTag clone #B5. CRISPR tagged PP1 γ contains EGFP. PP1 α/γ dTag cells were treated with either DMSO (-dTag1) or with dTag1 (100nM) for 2 hours before fixing with PTEMF. Cells were stained with CENP-C and Hoechst. Loss of GFP signal in the presence of dTag1 (100nM) represents successful degradation of PP1. Expected sizes of protein bands in western blots: PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

2.8 The rate of PP1 degradation using dTag1, but not dTag13, differs between different cell cycle stages

Various cell cycle synchronisation methods are widely used for increasing the number of cells in a population at a particular cell cycle stage. These include the microtubule polymerising poison, nocodazole, and the selective CDK1 inhibitor RO-3306 (Vassilev et al., 2006). To test the efficiency of the dTag system in different stages of the cell cycle, PP1 γ dTag cells were synchronised with nocodazole for 18 hours or left unperturbed as an asynchronous population. They were then incubated with 500nM dTag13 for different lengths of time, between 10 minutes and 2 hours. In both the asynchronous and nocodazole arrested cells, the rate of PP1 γ degradation using dTag13 appeared very similar (Figure 2.8A). In contrast, when dTag1 was added to nocodazole arrested cells, the amount of PP1 degraded was much lower than in the asynchronous population (Figure 2.8B). This might suggest that the VHL E3 ubiquitin ligase is less active in mitotic cells, but CRBN is not affected, possibly due to some mitosis-dependent post-translational modifications on VHL.

To generate a high proportion of mitotic cells, RO-3306 can be used instead of nocodazole. RO-3306 inhibits the mitotic kinase, CDK1, which reversibly arrests cells at the G₂/M border of the cell cycle (Vassilev et al., 2006). Cells enter mitosis soon after RO-3306 washout and can then be trapped in mitosis with a cell cycle synchronisation drug, such as nocodazole, MG132 (a proteasome inhibitor) or proTAME (an APC/C inhibitor) (Zeng et al., 2010b). The PP1 α/γ CRISPR cells were synchronised with nocodazole, either with or without an earlier RO-3306

arrest, and treated with different concentrations of dTag1 for 2.5 hours. As previously shown (Figure 2.8B), the addition of dTag1 in cells already arrested in mitosis by nocodazole led to a less efficient degradation of PP1 than that of an asynchronous population. Increasing the concentration of dTag1 reagent to 500nM slightly increased the amount of degradation in a 2.5-hour incubation. However, the degradation was more efficient when the cells were arrested with RO-3306 followed by nocodazole, and the dTag1 reagent was added upon RO-3306 wash out. Again, increasing the concentration of dTag1 to 500nM in this synchronisation method improved the degradation efficiency, but no effect was observed by increasing the concentration further to 1mM (Figure 2.8C).

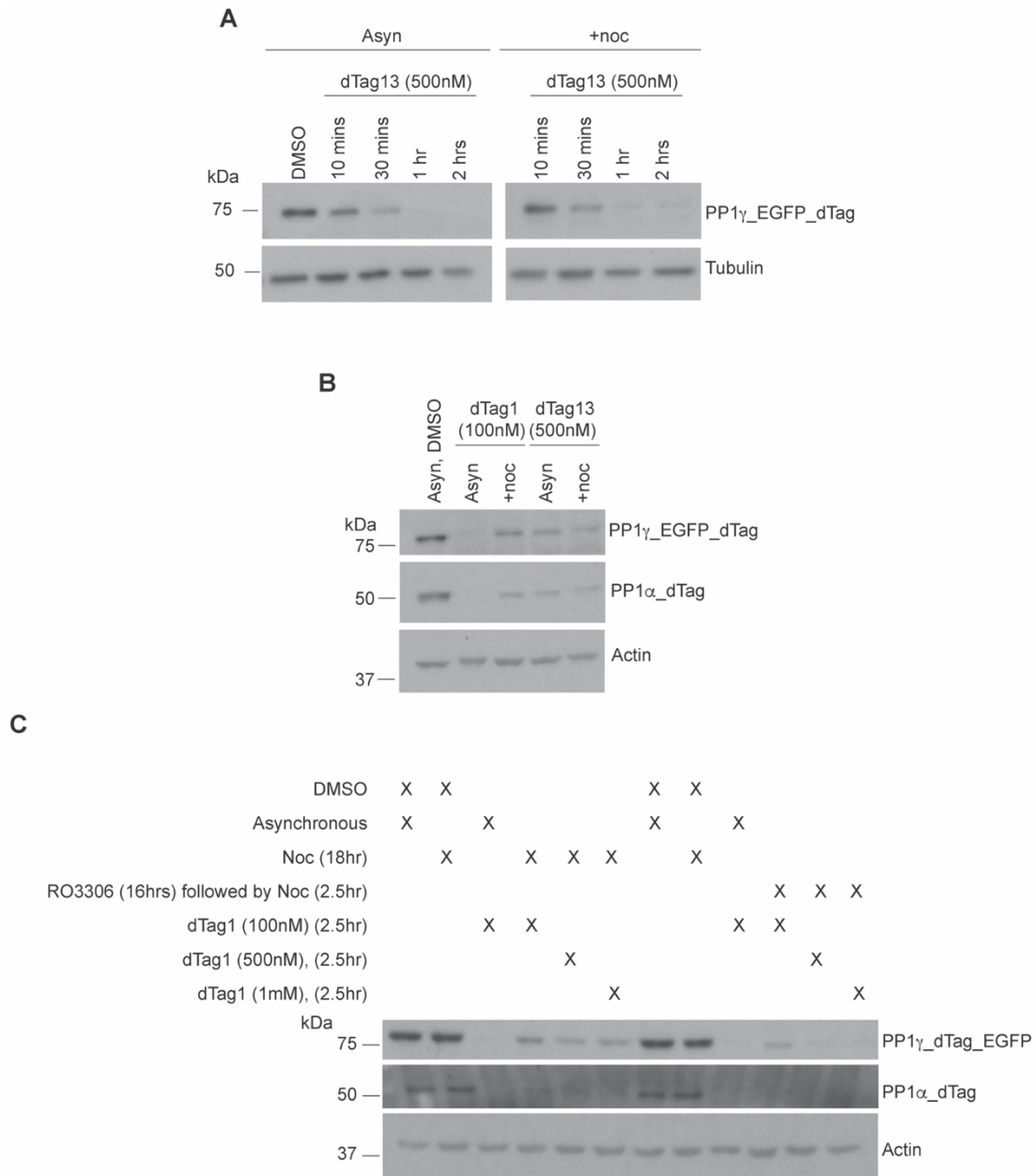


Figure 2.8: The rate of PP1 degradation using dTag1, but not dTag13, differs between different cell cycle stages.

(A) PP1 γ _EGFP_dTag cells were incubated with dTag13 (500nM) for the indicated durations, in asynchronous and nocodazole arrested populations. Western blot analysis shows the degradation efficiency of PP1 γ . (B) PP1 α / γ dTag cells, treated with or without nocodazole, were incubated with dTag1 (100nM) or dTag13 (500nM) for 2 hours. Degradation efficiency of PP1 was analysed by western blot. (C) PP1 α / γ dTag cells were synchronised with RO-3306 and/or nocodazole as indicated. Cells were treated with dTag1 at either 100nM, 500nM or 1mM. Western blot analysis shows the degradation efficiency of PP1 catalytic subunits. Expected sizes of protein bands in western blots: PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

2.9 Discussion

The precise roles of PP1 and its catalytic subunits in mitosis remain unclear, partly due to a lack of reagents in which endogenous PP1 can be precisely manipulated in mitosis. Protein degradation systems, including the dTag system, are powerful tools for rapidly degrading a target protein from cells offering a faster way of removing target protein compared to an RNAi depletion (Bekes et al., 2022). Additionally, degradation systems have fewer off-target effects compared to RNAi and small molecule inhibitors. Given that PP1 is a prolific phosphatase in mammalian cells and responsible for around half of all Ser/Thr dephosphorylation events (Bollen et al., 2010), such a system would provide a more precise way of studying the role of PP1 in mitosis, avoiding the cell stress caused by a long-term depletion and potential DNA replication issues.

The dTag system utilises a mutant of the FKBP12 protein in which one residue has been mutated (F36V) to create a binding interface for dTag reagents that bind exclusively to the mutant FKBP12 (FKBP12^{F36V}) and not to WT FKBP12. dTag reagents also bind to an E3 ligase, but the exact E3 ligase differs between the dTag reagents. dTag1 and dTag13 recruit VHL and CRBN, respectively (Figure 2.2A,B), to form ternary complexes between the tagged protein of interest and an E3 ligase and promote ubiquitination and subsequent proteasomal degradation of the target protein. Importantly, addition of either dTag1 or dTag13 to plain HeLa cells did not affect normal mitotic progression (Figure 2.2) indicating that any difference seen in later studies with the generated PP1 dTag cell lines in which the timing of mitosis is affected would be a result of PP1 degradation and side effects caused by the addition of dTag reagent.

In order to study the independent and combined roles of PP1 catalytic isoforms, CRISPR/Cas9 was used to tag PP1 α and PP1 γ in both HeLa and HCT116 cells with the FKBP12^{F36V} protein. Cell lines with only PP1 α or PP1 γ tagged were generated and screened for homozygosity, as well as cells in which both isoforms were fused to FKBP12^{F36V} (Figures 2.3, 2.4 and 2.5). Importantly, live cell imaging confirmed that the CRISPR tagging of PP1 did not affect normal mitotic timings (Figures 2.3D,G and 2.4C) suggesting that regular PP1 function had not been perturbed.

Once the cell lines had been generated, functional analysis of the dTag system in these cells could be undertaken. Both dTag1 and dTag13 led to a rapid loss of PP1 from all cell lines tested, with dTag1 being more efficient in asynchronous cells compared to dTag13 (Figure 2.6A,B). Interestingly, a difference in degradation efficiency was observed between an asynchronous population of PP1 dTag cells compared to a mitotically arrested population when they were incubated with dTag1, but this was not seen when the same analysis was carried out with dTag13 (Figure 2.8B). The reason for this is not known, although one hypothesis could be that there is a cell cycle-dependent reduction of VHL, but not CRBN, E3 ligase activity in mitosis. Through a dilution test for the PP1 α and PP1 γ antibodies used in western blot analysis, the amount of PP1 degraded via a 2-hour incubation with dTag1 is estimated to be $\geq 87.5\%$. This drastic degradation in such a short timeframe is improvement on what is typically a 48- or 72-hour RNAi depletion and will be beneficial in further studies of the mitosis specific role of PP1.

In summary, this chapter presents work in which CRISPR/Cas9 mediated locus-specific knock-in technology was used to generate homozygous CRISPR tagged dTag cell lines in both HeLa and HCT116 backgrounds. The timeframe in which degradation of PP1 was achieved after the addition of dTag to the cells was tested. These PP1 dTag cell lines will be valuable in further uncovering the roles of PP1 in mitotic events.

Chapter 3 - Functional Analysis of PP1 using the dTag system

3.1 PP1 is required for timely mitosis in HeLa cells

Mitosis is a carefully controlled process that requires coordinated events such as mitotic spindle formation, chromosome condensation, and organelle reorganization. Key mitotic kinases including CDK1:Cyclin B, Aurora B, and PLK1 regulate these events. Their actions are balanced by opposing phosphatases to coordinate dynamic phosphorylation reactions and ensure timely mitotic exit. PP1 plays a significant role in the regulation of mitosis, although its specific targets are not fully understood. Previous studies suggest that PP1 α and PP1 γ together are required for the regulation of histone modifications, the metaphase to anaphase transition and chromatin decondensation (Nijenhuis et al., 2014, Bancroft et al., 2020, Qian et al., 2011, Vagnarelli et al., 2011, Liu et al., 2010). Depletion of PP1 α and PP1 γ together disrupts the timing of mitotic events, prolonging the duration of mitosis (Bancroft et al., 2020). PP1 β has been implicated in myosin light chain and PLK1 dephosphorylation but is not thought to participate in mitotic timing control (Bancroft et al., 2020, Matsumura et al., 2011, Yamashiro et al., 2008, Kiss et al., 2019).

To investigate the role of PP1 in the timing of mitosis, analysis using the generated HeLa PP1 dTag cells introduced in Chapter 2 was performed. Using the HeLa PP1 α , PP1 γ and PP1 α/γ dTag cell lines, a comparison between the role of PP1 α and PP1 γ as well as their combined role can be made. Cells were pre-

synchronised with thymidine (2mM) and then imaged on the EVOS M7000 microscope at 3-minute intervals, using SiR-DNA to visualise the DNA (Figure 3.1A). Cells were either treated with DMSO (-dTag) or dTag1 (100nM, +dTag), which was added to the cells 6 hours after release from thymidine block. This allows the cells to progress through the majority of S-phase and replicate their DNA, a process that PP1 contributes to (Hiraga et al., 2017, Gnan et al., 2021, Jenkinson and Zegerman, 2022), largely unaffected (Figure 3.1A).

Quantification of mitotic timing from NEBD to META, META to ANA and NEBD to ANA highlights the need for PP1 in coordinating mitotic events. In all three cell lines, the degradation of PP1 prolonged overall time spent in mitosis (Figure 3.1D, G). Degradation of PP1 α or PP1 γ alone did not affect NEBD to META timings (Figure 3.1B,E), but resulted in an increase in time from META to ANA (Figure 3.1C,F). However, when both PP1 α and PP1 γ were degraded together in the same cell line, time between NEBD to META increased (Figure 3.1B,E). META to ANA also took longer in the PP1 α/γ cells when PP1 was degraded, similar to the individual PP1 α and PP1 γ dTag cell lines. These observations were consistent in multiple clones of each cell line, confirming that the phenotype was not clone specific. Additionally, the observation that removal of PP1 catalytic subunits from HeLa cells by RNAi-mediated depletion extends the META to ANA transition has been reported before (Bancroft et al., 2020) further supporting the data presented here using PP1 dTag cell lines. Representative images of the HeLa PP1 dTag cell lines during mitosis (Figure 3.1H) illustrate both the chromosome alignment delay and the anaphase onset delays.

The mitotic delay between NEBD and META was observed in the PP1 α/γ dTag cell lines, but this delay did not occur when only one of the catalytic isoforms was degraded. However, there was a META to ANA delay in all three cell lines. In summary, these data suggest that there may be some redundancy between PP1 catalytic isoforms in some mitotic events, but not all.

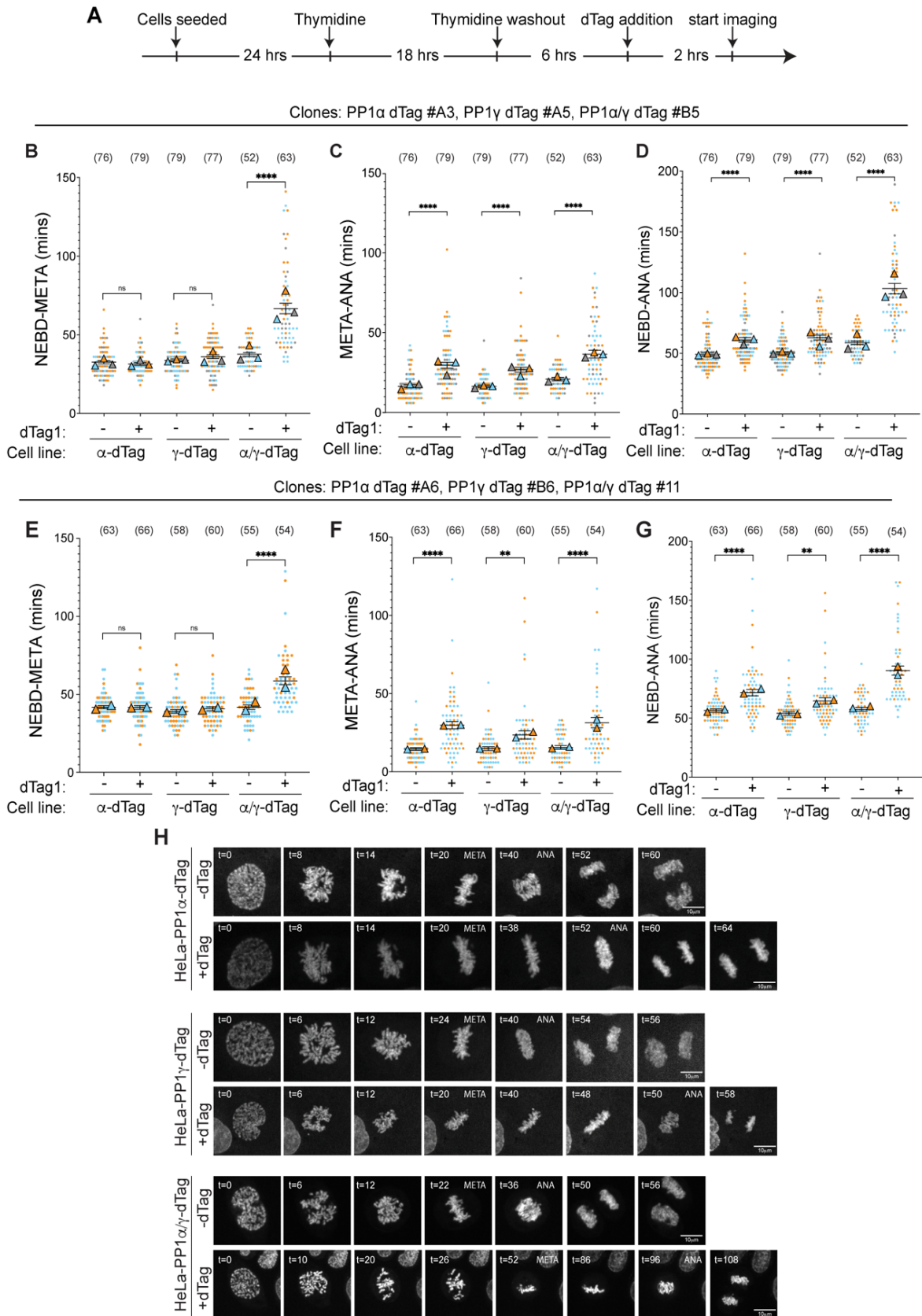


Figure 3.1: PP1 is required for timely mitosis in HeLa cells.

(A) Schematic of the drug treatments and synchronisation protocol for live cell imaging of PP1 dTag cell lines. (B-G) Quantification of nuclear envelope breakdown (NEBD) to

Figure 3.1 (continued). metaphase (META) (B and E), META to anaphase (ANA) (C and F) and combined NEBD to ANA (D and G) of HeLa PP1 dTag cell lines imaged on the EVOS M7000 microscope. Images taken every 3 mins for 12 hours. Quantification based on DNA morphology. Graphs plotted show mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. (B-D) is quantification of mitotic timings using the following dTag cell line clones: PP1 α dTag #A3, PP1 γ dTag #A5, PP1 α/γ dTag #B5. (E-G) is quantification of live cell imaging mitotic timings using the following dTag cell line clones: PP1 α dTag #A6, PP1 γ dTag #B6, PP1 α/γ dTag #11. (H) Representative stills of PP1 dTag cells (clone #B5) progressing through mitosis, imaged every 2 mins on the Olympus SoRa microscope. SiR-DNA (50nM) was added to the cells 1 hour before imaging was started to visualise DNA for imaging experiments on both EVOS and SoRa microscopes.

3.2 PP1 is required for timely mitosis in HCT116 cells

HeLa cell lines are aneuploid and have an extra copy of each PP1 isoform in comparison to a diploid cell line. This difference may alter the expression levels of the PP1 catalytic subunits compared to a normal somatic cell and therefore have an effect on the function of the enzyme. Therefore, to test the function of PP1 in both aneuploid and diploid cellular backgrounds, CRISPR/Cas9 tagging of PP1 isoforms was carried out in HCT116 cells, a diploid human colorectal carcinoma cell line in addition to the HeLa cell background. Generation of these cell lines is described in Chapter 2. HCT116 PP1 α , PP1 γ and PP1 α/γ dTag cell lines were imaged on the EVOS M7000 microscope in the same manner as the HeLa cell lines (Figure 3.1A) and mitotic timings were quantified. As with the HeLa cells, degradation of either PP1 α , PP1 γ or PP1 α/γ together resulted in a delay at the META to ANA transition (Figure 3.2B). There was a slight, but not statistically significant, increase in the NEBD to META timings in the individual PP1 α and PP1 γ cell lines. However, the NEBD to META time for PP1 α/γ dTag cell line was

almost twice as long when both PP1 isoforms are degraded, compared to the control cells (Figure 3.2A). Stills from live cell imaging of the HCT116 PP1 α/γ dTag cell lines (Figure 3.2D) show both the NEBD to META delay as well as the META to ANA delay.

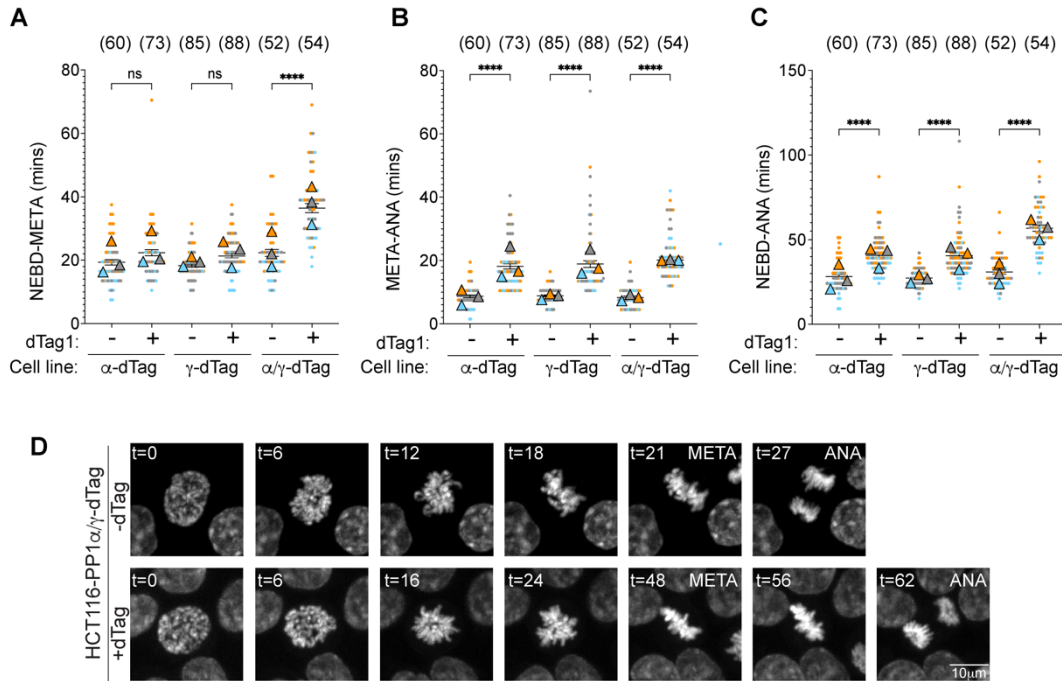


Figure 3.2: PP1 is required for timely mitosis in HCT116 cells.

(A-C) Quantification of nuclear envelope breakdown (NEBD) to metaphase (META) (A), META to anaphase (ANA) (B) and combined NEBD to ANA (C) of HCT116 PP1 dTag cell lines imaged on the EVOS M7000 microscope. Images taken every 3 mins for 12 hours. Quantification based on DNA morphology. Graphs plotted show mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. The following clones were used: PP1 α dTag #2, PP1 γ dTag #21, PP1 α/γ dTag #6. (D) Representative stills of HCT116 PP1 α/γ dTag cells (clone #14) progressing through mitosis, imaged every 3 mins on the Olympus SoRa microscope. SiR-DNA (50nM) was added to the cells to visualise DNA for imaging experiments on both EVOS and SoRa microscopes.

3.3 PP1 degradation does not increase chromosome segregation errors

The timing of chromosome segregation in mitosis is tightly controlled by the SAC. The SAC is active when there are unattached kinetochores and needs to be rapidly silenced at the correct time to promote a timely anaphase once stable kinetochore-microtubule attachments have formed. This promotes genomic fidelity so that progeny cells will contain the correct number of chromosomes. PP1 has been implicated in SAC silencing (Nijenhuis et al., 2014, Holder et al., 2020, Moura et al., 2017) and therefore it is plausible that its degradation may affect the occurrence of chromosome segregation errors. To test this, high resolution imaging of PP1 dTag cell lines, treated with either DMSO (ctrl) or dTag1 (100nM, +dTag1) was carried out on the Olympus SoRa microscope and the incidence of lagging chromosomes or DNA bridges was counted (Figure 3.3). Surprisingly, there was little difference between control cells and cells which had been degraded of PP1 in any of the cell lines tested. However, this quantification does not rule out the possibility of chromosome segregation errors such as ultrafine DNA bridges, which cannot be seen with conventional DNA staining methods (Liu et al., 2014).

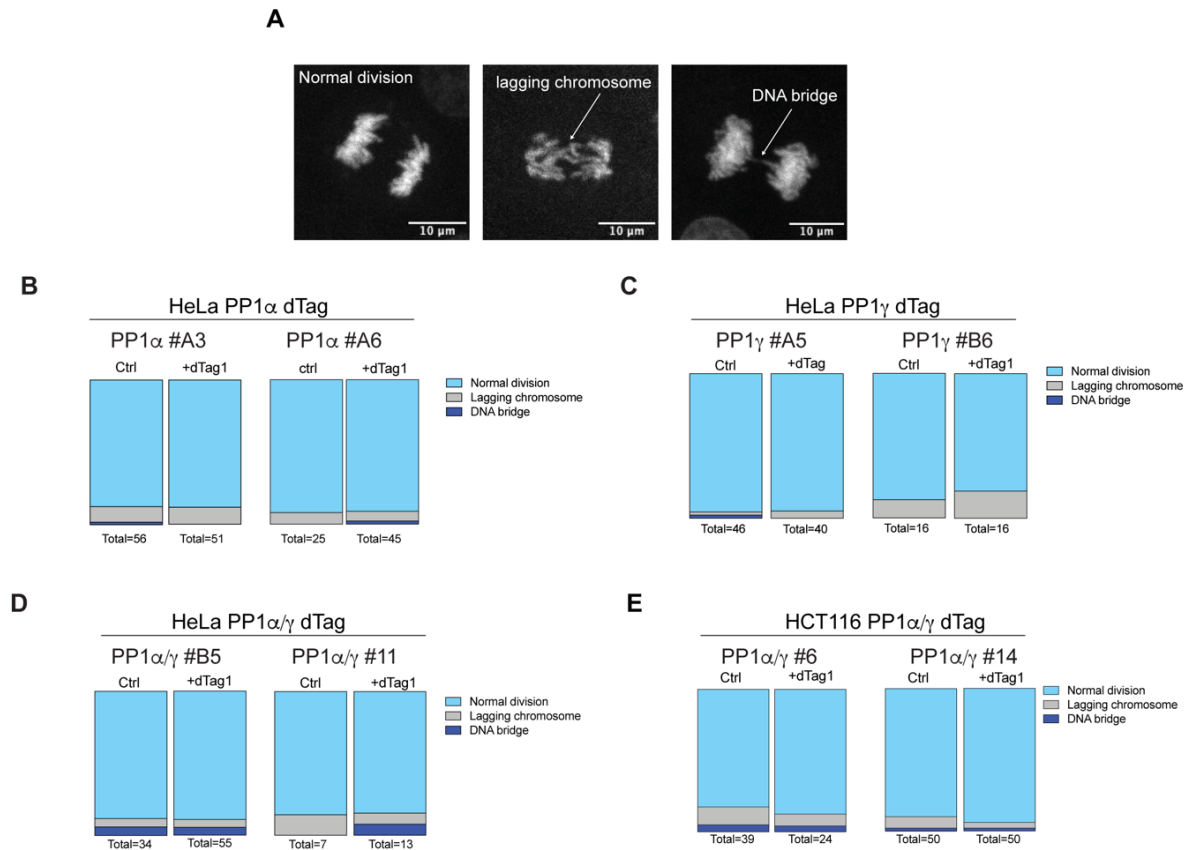


Figure 3.3: PP1 degradation does not increase chromosome segregation errors.

(A) Representative images of a normal division of sister chromatids, a lagging chromosome and a DNA bridge in anaphase of mitosis. (B-E) Quantification of chromosome segregation errors during anaphase in PP1 dTag cell lines, with or without dTag-induced degradation of PP1 catalytic subunits. Each panel is a different cell line (PP1 α , PP1 γ and PP1 α/γ). Two clones per cell line were tested. Quantification is based on live cell movies from the Olympus SoRa microscope, where cells were imaged every 2 mins (HeLa cell lines) or 3 mins (HCT116) cell lines. Total number of cells quantified per condition stated below each column.

3.4 PP1 γ , but not PP1 α , dephosphorylates Histone H3 phospho-Thr3

During mitosis, chromatin undergoes substantial structural changes, many of which are brought about through phospho-regulation of histone molecules. For example, phosphorylation of histone H3 threonine 3 (H3pThr3) by haspin kinase promotes chromatin condensation early in mitosis (Dai et al., 2005). During

mitotic exit, chromatin decondenses to reinstate its interphase structure compatible with DNA replication and transcription. PP1 γ is recruited to chromatin during anaphase by its targeting substrate Repo-Man, where it dephosphorylates H3pThr3 to facilitate chromatin decondensation (Qian et al., 2011, Trinkle-Mulcahy et al., 2006).

To confirm the previously reported observation that H3pThr3 is specifically a PP1 γ target and not a PP1 α target, HeLa PP1 α and HeLa PP1 γ dTag cells were incubated with dTag13 (500nM) for 2 hours to degrade PP1 catalytic subunits. 10 minutes before fixing the cells with PTEMF, cells were treated with a potent inhibitor of the haspin kinase 5-iodotubercidin (5-iTu, 5 μ M) (Wotring and Townsend, 1979, De Antoni et al., 2012) as indicated. PP1 dTag cells were stained for H3pThr3 and H3pThr3 intensity was quantified (Figure 3.4A,B). Treatment with 5-iTu resulted in a rapid loss of H3pThr3 staining due to loss of the phosphorylated residue. When cells were treated with 5-iTu while PP1 γ was degraded, H3pThr3 signal was partially retained. However, when PP1 α was degraded, there was no retention of H3pThr3 phospho-signal suggesting that H3pThr3 is specifically dephosphorylated by PP1 γ and not PP1 α .

Degradation of PP1 catalytic subunits using dTag13 was less efficient than with dTag1 (Figure 2.4). Therefore, to examine whether a more complete degradation of PP1 γ would make the retention of H3pThr3 signal more pronounced than shown in Figure 3.4A, the previous described assay was repeated with dTag1 (100nM). Indeed, dTag1 resulted in an improved retention of H3pThr3 signal to around 40% (Figure 3.4C,D), up from around 20% with dTag13 (500nM). Again,

there was no change when PP1 α was degraded and treated with 5-iTu compared to 5-iTu treatment alone.

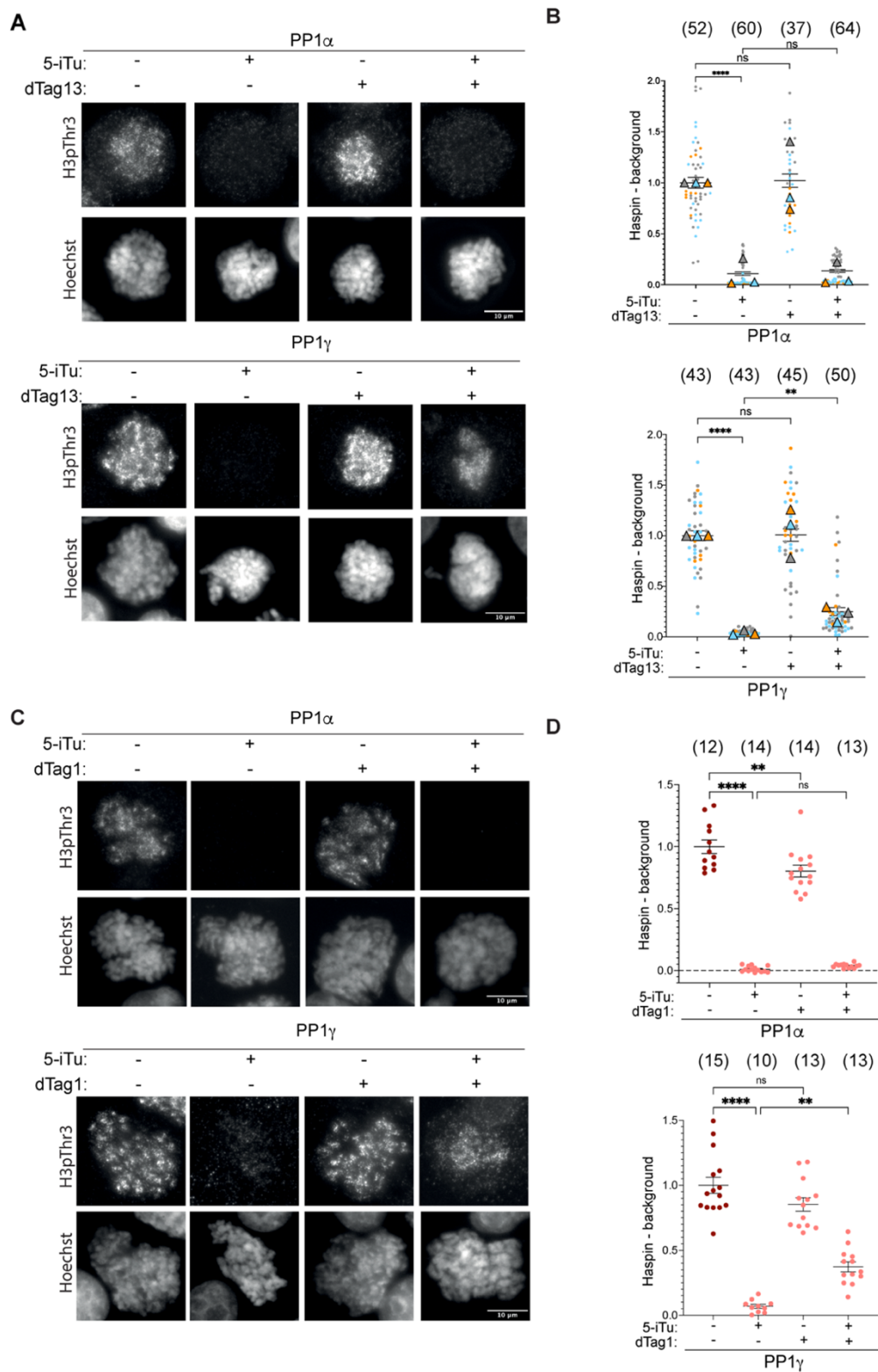


Figure 3.4: PP1 γ , but not PP1 α , dephosphorylates H3pThr3.

Figure 3.4 (continued). (A) HeLa PP1 α dTag and HeLa PP1 γ dTag cell lines were arrested in mitosis with nocodazole (0.3 μ M) for 4 hours. Cells were treated with dTag13 (500nM, 2 hours) and/or 5-iodotubercidin (5-iTu, 5 μ M, 10mins) as indicated. Cells were fixed with PTEMF for 12 mins and stained for histone H3 phospho-threonine 3 (H3pThr3). (B) Quantification of (A). Intensity of H3pThr3 signal on the chromatin was measured. Graphs plotted show mean $\pm$ SEM where each dot represents one cell measurement. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. Conditions were compared using a one-way ANOVA test. (C) Same experimental set up as (A), with dTag1 (100nM) used in place of dTag13 (500nM). All other parameters are not changed. (D) Quantification of (C), measured and analysed in the same manner as (B). One biological repeat only is presented.

3.5 PP1 is involved in chromatin decondensation during anaphase

Histone H3 is highly phosphorylated during mitosis to promote chromatin condensation, important for chromosome congression and alignment, on residue Thr3 as described above as well as residues Ser10, Thr11 and Ser28 (Van Hooser et al., 1998, Hendzel et al., 1997, Sauve et al., 1999). During anaphase, these phosphorylation modifications are removed, although interestingly with different kinetics; Thr3 and Ser28 are dephosphorylated before Ser10 and Thr11 (Qian et al., 2011). As mentioned above, PP1 γ was previously identified as a phosphatase that opposes haspin-mediated H3pThr3 phosphorylation (Qian et al., 2011, Trinkle-Mulcahy et al., 2006), and this phenotype was confirmed in the HeLa PP1 dTag cells (Figure 3.4). The dephosphorylation of histones is important for the next cell cycle, allowing for chromatin decondensation and reformation of interphase nuclei, which therefore facilitates transcription and DNA replication.

To examine the role of PP1 on chromatin decondensation during anaphase, HeLa PP1 α/γ dTag cell were imaged live on the Olympus SoRa microscope at 5-minute

intervals. Cells were either treated with dTag1 (100nM, +dTag) or DMSO (ctrl) for a minimum of 1 hour before imaging started. The chromatin area as cells exited mitosis was quantified manually in ImageJ using the 'measure' feature (Figures 3.5A,B). Initially, as cells exited mitosis, there was little difference between the cells with or without PP1 α/γ . However, later on during mitotic exit the difference in chromatin area increased such that at 90 minutes after anaphase onset the control nuclei were 1.25 times greater in size than the nuclei of the cells which had been treated with dTag1. This could be due to a delay in the dephosphorylation of H3pThr3 or other histone phosphorylation sites. Lagging chromosomes may also affect nuclear size. However, the acute loss of PP1 from cells did not increase the number of lagging chromosomes observed during live cell imaging experiments (Figure 3.3) and therefore this is unlikely to be the cause of the different nuclear sizes during and after anaphase.

To confirm that the reduction of nuclei size was a mitotic effect and not a phenotype that occurred solely by degrading PP1 before mitosis, the size of pre-mitotic nuclei was measured. Indeed, there was no difference in area of the chromatin in these interphase, pre-mitotic cells (Figure 3.5C), indicating that the reduction in nuclei size observed 90 mins after anaphase onset was caused by mitotic phospho-regulation. Representative images as used for the quantification of decondensation during anaphase are shown in Figure 3.5D.

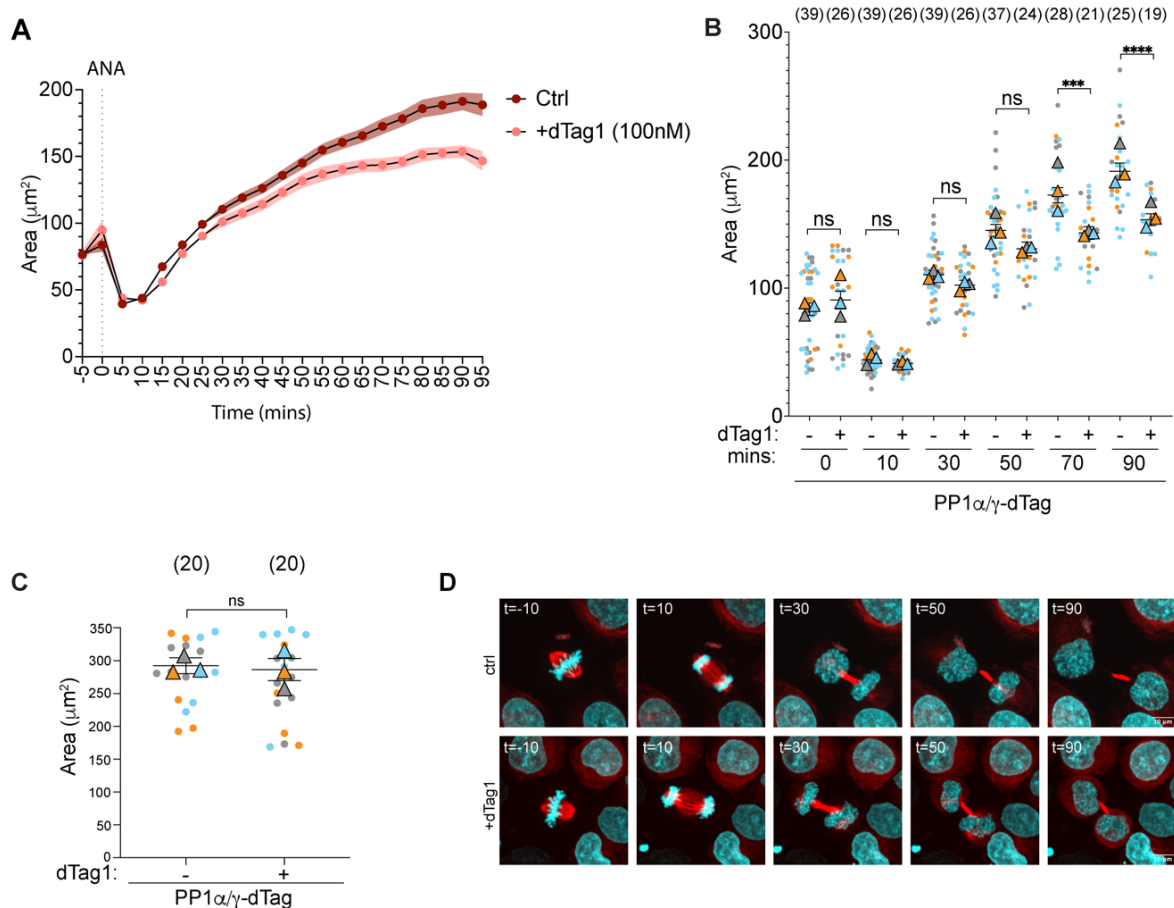


Figure 3.5: PP1 is involved in chromatin decondensation during anaphase.

(A) Quantification of chromatin area (μm^2) as cells progressed through anaphase (ANA) with or without a dTag-induced degradation of PP1 catalytic subunits. HeLa PP1 α/γ dTag (Clone #B5) was used. The -5 mins timepoint is the last metaphase timepoint before anaphase onset, the 0 min timepoint is the first anaphase timepoint. Live cell movies were imported into ImageJ and chromatin area was measured manually using the 'Measure' feature. (B) Same data as in (A) presented as a scatter plot of the indicated timepoints only. Graphs plotted show mean $\pm$ SEM where each dot represents one cell measurement. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. Within each timepoint, conditions were compared using Two-Tailed Unpaired T-test. (C) Quantification of chromatin area (μm^2) prior to mitotic entry. All measurements taken at the timepoint 5 mins before nuclear envelope breakdown. (D) Representative images of PP1 α/γ dTag (Clone #B5) used for the quantification presented in this figure. SiR-DNA (555, 50nM) and SiR-Tubulin (647, 50nM) was used to visualise the DNA and the tubulin, respectively

3.6 PP1 induces metaphase plate thinning

Chromosome congression is a very active process during which chromosomes oscillate and are being moved by microtubules to align them at the metaphase plate. During congression, the oscillation of chromosomes has been suggested to prevent chromosomes becoming intertwined with each other and components of the microtubules (Ke et al., 2009). However, as cells progress towards anaphase, the oscillatory movements decrease, and the metaphase plate thins to support chromosome segregation, a process involving the kinesin KIF18A. CDK1 and PP1 have been shown to have antagonising roles in the regulation of KIF18A, with expression of a PP1-binding mutant of KIF18A displaying an increased metaphase plate width (Hafner et al., 2014, De Wever et al., 2014).

To examine the effect of an acute PP1 degradation on metaphase plate width, PP1 α/γ dTag cells were arrested with proTAME (10 μ M, 3 hours), an inhibitor of the APC/C to trap cells in metaphase (Zeng et al., 2010b), with or without a dTag1 (100nM, 3 hours) treatment. Cells were fixed with PTEMF and stained for tubulin and the kinetochore marker CENP-C (Figure 3.6A). Measurement of the metaphase plates demonstrated that in the absence of PP1 α/γ , metaphase plates were slightly wider (Figure 3.6B). While this experiment did not directly measure KIF18A, the wider metaphase plate phenotype agrees with previous work that indicated that the dephosphorylation of KIF18A by PP1 is important for reducing oscillatory movements and thereby plate thinning (Hafner et al., 2014). Additionally, astrin has been implicated in the compaction of the metaphase plate, and expression of astrin mutated at the PP1 docking site has reduced inter-centromeric distances (Conti et al., 2019). Due to time constraints within this

project, astrin was not investigated, however it will be important to further characterise the relationship between astrin and PP1 in future work. It could also be interesting to investigate whether the metaphase plate widening phenotype in response to an acute PP1 loss, which could be affecting microtubule dynamics at the kinetochore, is linked to the mitotic delays observed above (Figure 3.1).

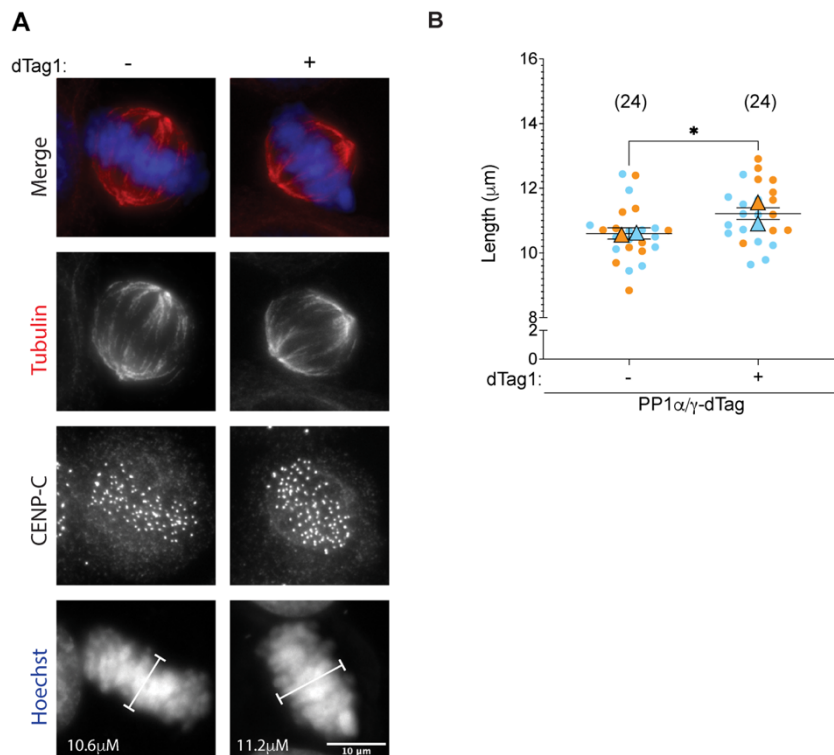


Figure 3.6: PP1 induces metaphase plate thinning.

(A) HeLa PP1 α/γ dTag cells were pre-synchronised with RO-3306 (9 μ M, 18hours). Cells were released from RO-3306 into proTAME (10 μ M, 3 hours) with or without dTag1 (100nM, 3hours) treatment, as indicated. Cells were fixed with PTEMF (12mins) and stained for tubulin and CENP-C. (B) Quantification of metaphase plate widths of cells from (A). Tiff files of cell images were loaded into ImageJ and the width across the metaphase plate was quantified using the 'Measure' tool on ImageJ. Graph plotted shows mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition.

3.7 Acute PP1 degradation does not influence the formation of stable kinetochore-microtubule attachments

As the delay at the metaphase to anaphase transition in the absence of PP1 has already been well described (Bancroft et al., 2020, Holder et al., 2019), whereas the chromosome congression phenotype observed is less well understood, attention was shifted to investigating this further.

The formation of stable kinetochore-microtubule attachments is an essential requirement for successful chromosome separation during anaphase. Astrin-bound PP1 at end-on attached kinetochores has previously been proposed to stabilise non-bioriented kinetochore-microtubule attachments to support proper segregation of chromosomes in mitosis (Song et al., 2021, Conti et al., 2019). Additionally, KNL1-recruited PP1 has been suggested to oppose Aurora B-mediated phosphorylations to stabilise attachments (Liu et al., 2010). To assess the ability of cells to form stable kinetochore-microtubule attachments in a PP1-degraded system, an established cold-treatment method was employed, originally designed to differentiate between stable end-on and transient microtubule-kinetochore attachments (Rieder, 1981) and still frequently used in more recent studies (Thein et al., 2007, Hayward et al., 2022, Conti et al., 2019, Hanisch et al., 2006). Under these conditions, any microtubules in the cell that are not stably bound to a kinetochore will depolymerise. HeLa PP1 α/γ dTag cells were treated and synchronised as illustrated (Figure 3.7A). When treated with the microtubule depolymerising agent nocodazole (noc, 0.3 μ M), a microtubule depolymerising agent, as expected there was no tubulin staining. In the control

RNAi situation (GL2, targeting firefly luciferase), cells were able to form a bipolar spindle with stable kinetochore-microtubule attachments. Interestingly, in this study, an acute degradation of PP1 via incubation with dTag1 (100nM, 2 hours) in the presence of proTAME (10 μ M), an APC/C inhibitor, did not affect the cell's ability to form stable kinetochore-microtubule attachments. RNAis against B56, important for error correction, (Kruse et al., 2013, Suijkerbuijk et al., 2012, Xu et al., 2013) and Nuf2, a component of the NDC80 complex and therefore essential for microtubule attachment formation (Nabetani et al., 2001, DeLuca et al., 2006, Cheeseman et al., 2006) were used as positive controls, in which the majority of stable kinetochore-microtubule attachments were abrogated (Figure 3.7B,C).

Very similar results were obtained in acutely PP1-degraded, diploid HCT116 cells, indicating that the result was not ploidy dependent (Figure 3.7D). These data are consistent with previous observations (Figure 3.1, 3.2 and 3.3) showing that the timing but not the fidelity of chromosome segregation was affected by acute PP1 degradation.

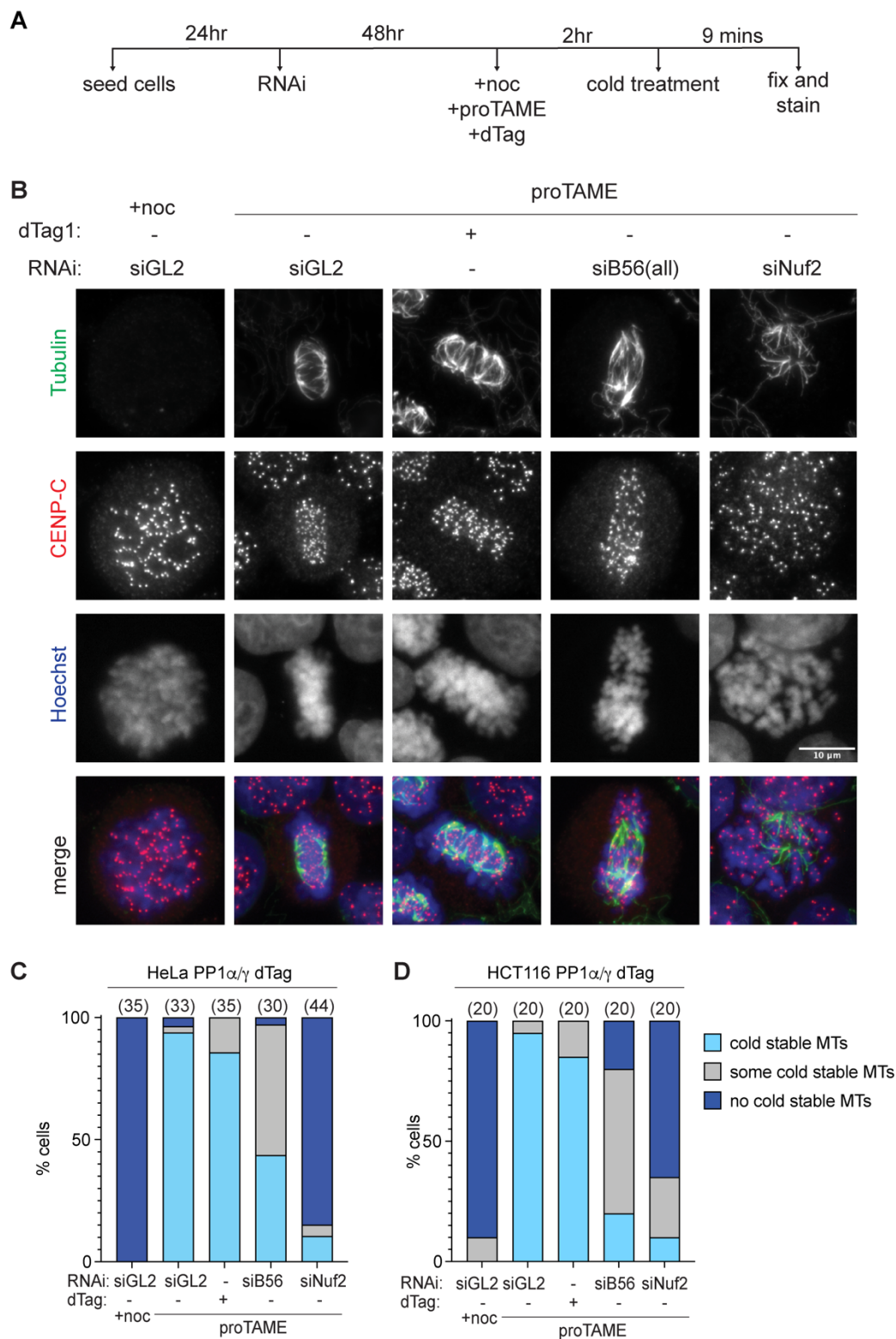


Figure 3.7: Acute PP1 degradation does not influence the formation of stable kinetochore-microtubule attachments.

(A) Schematic of the drug treatments and synchronisation protocol for a cold stability assay investigating the formation of stable kinetochore-microtubule attachments. Noc = nocodazole (0.33mM). proTAME (APC/C inhibitor, 10 μ M). dTag = dTag1 (100nM). (B) Representative images

Figure 3.7 (continued). of the experiment described in (A) using HeLa PP1 α/γ dTag cells. HeLa PP1 α/γ dTag cells were fixed with PTEMF (12mins) following a 9 min cold treatment and stained as indicated. (C, D) Quantification of HeLa (C) and HCT116 (D) PP1 α/γ dTag cells from experiment described in (A). Mitotic spindle was classified into 3 groups (cold stable spindle, some cold-stable MTs, no cold-stable MTs) based on visual impression. MTs = microtubules. Number of cells quantified per condition shown above each bar on the bar graph. Data merged from 2 independent biological repeats per cell line.

3.8 PP1 is not the principal phosphatase involved in error correction

During chromosome congression, a number of different attachment geometries aside from amphitelic attachments can be generated. These need to be corrected before anaphase onset to ensure equal segregation of chromosomes into the daughter cells, in a process known as ‘error correction’ (Nicklas and Ward, 1994, Nicklas, 1988, Nicklas et al., 1993). Error correction is regulated by the kinases Aurora B and MPS1, as well as the phosphatase PP2A-B56 (Hayward et al., 2019c). As PP1 and Aurora B have a suggested antagonistic relationship on certain substrates including KNL1 (Welburn et al., 2010, Nasa et al., 2018), it is conceivable that PP1 is also involved in error correction. To investigate this possibility, an established monastrol washout assay was used to test the HeLa PP1 dTag cells’ ability to correct erroneously attached kinetochores (Lampson et al., 2004, Kapoor et al., 2000, Khodjakov et al., 2003). Monastrol is a small-molecule inhibitor of kinesin-5 (Eg5) that induces a collapse of the mitotic spindle into a monopolar state containing a range of attachment states, including syntelic attachments (Kapoor et al., 2000). Removing monastrol from the cells through gentle washout with media allows the spindle to re-establish bi-polarity and continue mitotic progression. The continued presence of syntelic attachments

after monastrol washout suggests a failure to correct faulty kinetochore-microtubule connections (Lampson et al., 2004).

To investigate whether PP1 has a principal role in error correction along with PP2A-B56, HeLa PP1 α/γ dTag cells were treated with RNAi as indicated (Figure 3.8) and synchronised with monastrol (100 μ M, 14 hours) before being released into proTAME (10 μ M, 2 hours). Cells were subsequently fixed with PTEMF following a cold treatment (Figure 3.8A). The ability of the cell to re-establish bipolarity was assessed and the mitotic spindle was classified as either perfectly aligned, suggesting error correction was not perturbed, partially aligned or totally disorganised, indicative of a faulty error correction pathway. Cells treated with an Aurora B inhibitor (ZM447439, 10min, 10 μ M) or depleted of B56 through RNAi were not able to re-establish a bi-polar spindle following monastrol washout, however PP1-degraded cells were able to (Figure 3.8B,C). This suggests that, in contrast to PP2A-B56, PP1 is not a major regulator of the error correction process during mitosis.

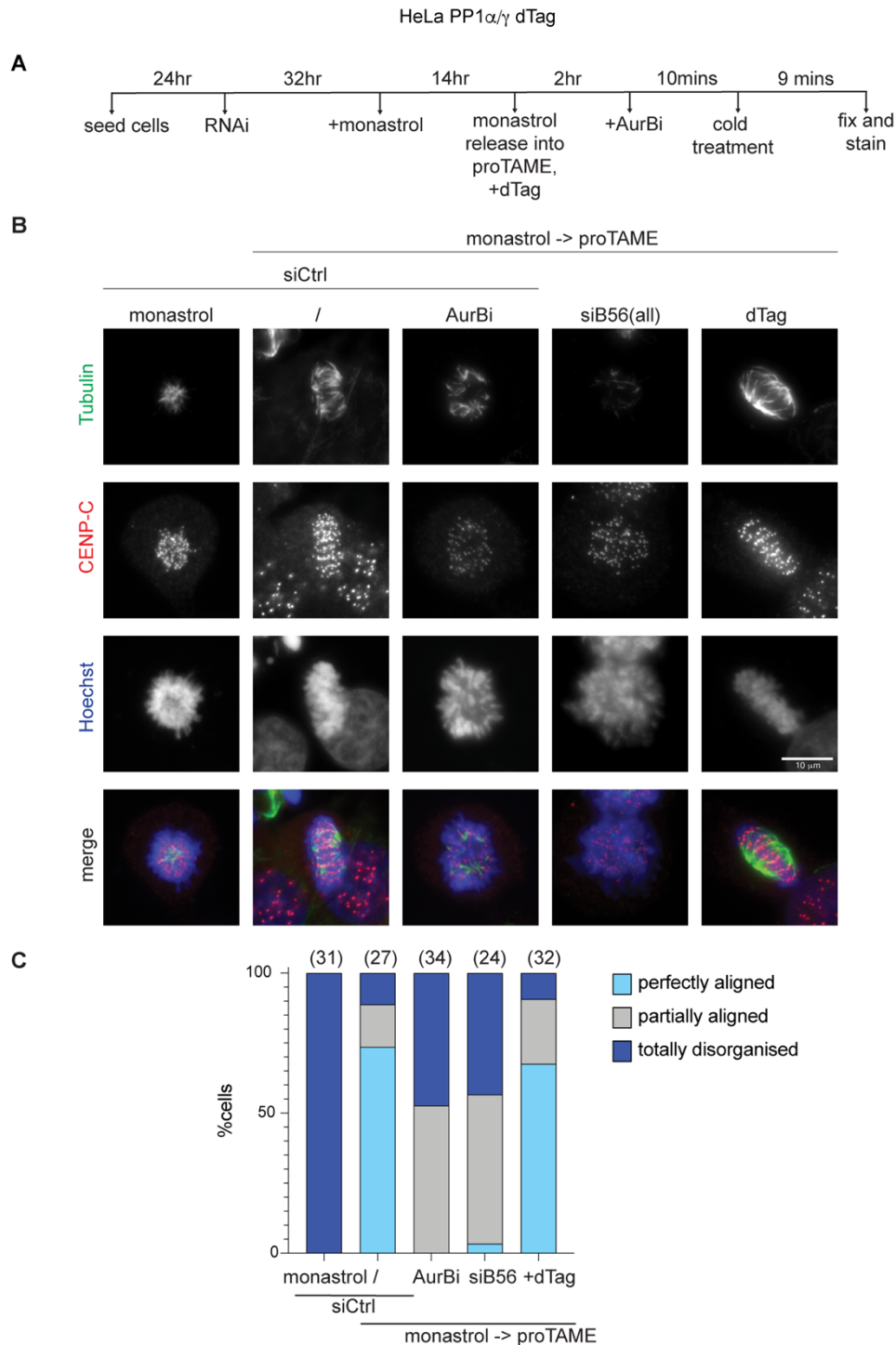


Figure 3.8: PP1 is not the principal phosphatase involved in error correction.

(A) Schematic of the drug treatments and synchronisation protocol for an error correction assay investigating the formation of stable kinetochore-microtubule attachments following monastrol (Eg5 inhibitor) treatment. Monastrol (100 μ M, 14 hours). proTAME (APC/C inhibitor, 10 μ M, 2 hours). dTag = dTag1 (100nM, 2 hours). AurBi = Aurora B inhibitor (ZM447439, 10min, 10 μ M) (B) Representative images of the experiment described in (A). HeLa PP1 dTag cells were fixed with PTEMF (12mins) following a 9min cold treatment and stained as indicated. (C) Quantification of cells from experiment described in (A). Alignment of the metaphase plate was classified into 3 groups (perfectly aligned, partially aligned, totally disorganised) based on visual impression. Number of cells quantified per condition shown above each bar on the bar graph. Data merged from 2 independent biological

3.9 PP1 regulates mitosis by spindle checkpoint dependent and independent pathways

Live cell imaging of PP1 dTag cell lines demonstrated the need for PP1 in regulating mitotic timings as degradation led to prolonged mitosis (Figures 3.1 and 3.2), in line with previously published data from our group (Bancroft et al., 2020). In this work, it was shown that PP1 had roles in coordinating mitotic timings beyond the SAC. To confirm this previous report using the generated PP1 dTag system, HeLa PP1 α/γ dTag cells were imaged in the presence of AZ3146, an inhibitor of the principal checkpoint kinase MPS1. Inhibition of MPS1 artificially silences the SAC as downstream checkpoint signalling is prevented due to a loss of phosphorylation activity on the MELT motifs of KNL1, and cells proceed into anaphase regardless of kinetochore attachment status, leading to chromosome segregation errors (Hewitt et al., 2010). As expected, when treated with AZ3146 (2 μ M), PP1 α/γ dTag cells progressed through mitosis significantly faster than when left untreated and failed to form an aligned metaphase plate before progressing into anaphase. However, when these cells were treated with both AZ3146 (2 μ M) and dTag1 (100nM), although they similarly failed to align their chromosomes and enter anaphase with segregation errors, they did not enter anaphase as rapidly after NEBD as when treated with AZ3146 alone (Figure 3.9A-C). To confirm the efficiency of the dTag-induced degradation of PP1 catalytic isoforms, cells were harvested after imaging and analysed by western blot (Figure 3.9D). Taken together, these data suggest that PP1 has mitotic roles independent of checkpoint signalling that are important for proper mitotic timings. This observation agrees with previously published work from our group that

shows that PP1 is important for activation of the APC/C at the metaphase to anaphase transition, but it is not required for SAC silencing itself (Bancroft et al., 2020) (Figure 3.9E).

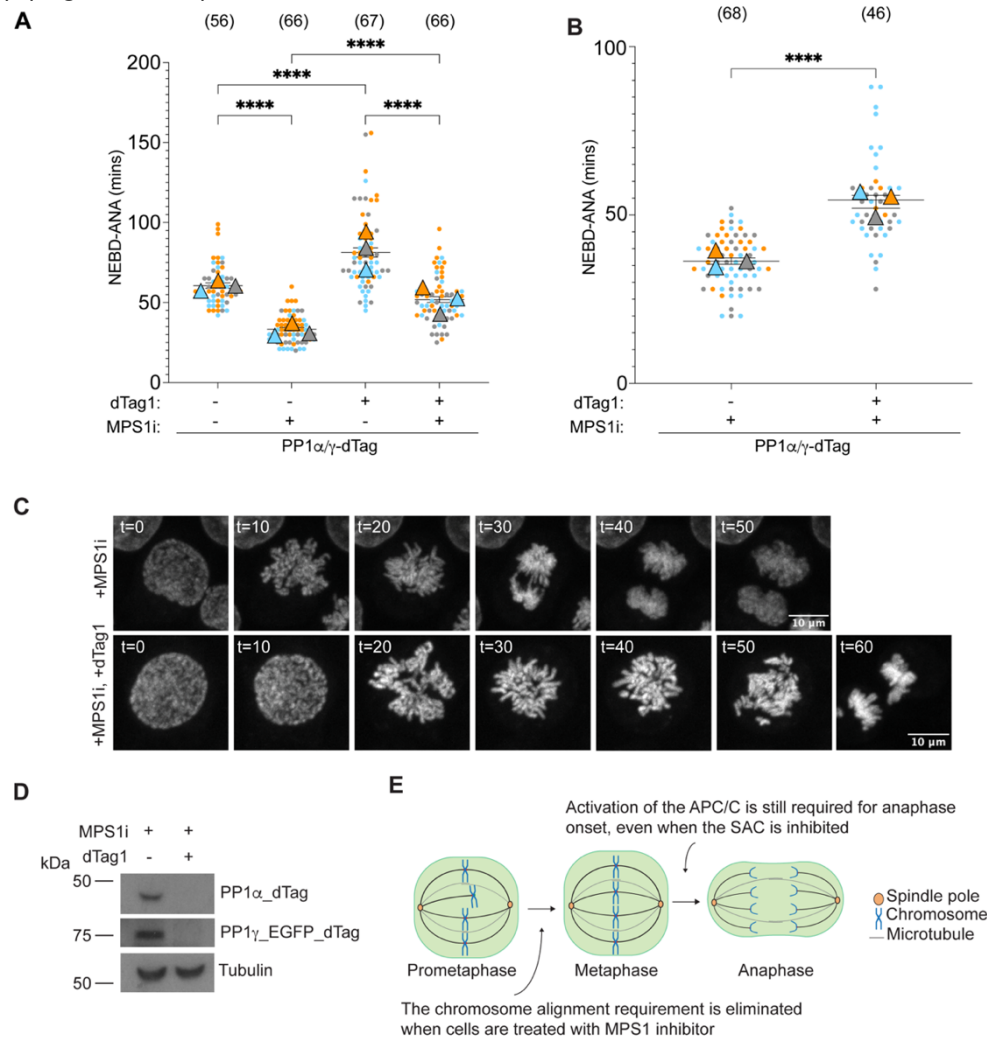


Figure 3.9: PP1 regulates mitosis by spindle checkpoint dependent and independent pathways.

(A) HeLa PP1α/γ dTag cells were pre-synchronised with thymidine (2mM, 18hours). 6 hours following release from thymidine block, cells were treated with dTag1 (100nM) as indicated. 2 hours later, the MPS1 inhibitor AZ3146 (MPS1i, 2μM) was added to indicated samples (+), and imaging was started. Cells were imaged at 3 min intervals on the EVOS M7000 microscope. Quantification based on DNA morphology of time taken from nuclear envelope breakdown (NEBD) to anaphase (ANA) is presented. Graphs plotted show mean ± SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. (B) Same experimental set up as (A), with only the +MPS1i conditions, with or without dTag1 (100nM). Cells imaged on the Olympus SoRa at 2 min intervals. (C) Representative stills from the imaging in (B). (D) Western blot analysis of cell lysates, harvested after imaging from (B) to test efficient degradation of PP1 catalytic subunits. (E) Illustration showing a model to explain the mitotic delays observed when both MPS1 is inhibited and PP1 is degraded.

3.10 PP1 depletion induces a metaphase to anaphase delay beyond checkpoint silencing

A hallmark of SAC silencing is the loss of SAC proteins, such as MAD1 and MAD2, from kinetochores, at which point the APC/C is rapidly activated to promote the degradation of Cyclin B and securin. Previous work from our group showed that when PP1 was inhibited, there was a delay in ANA onset following SAC silencing due to a delay in the activation of the APC/C caused by a delay in the dephosphorylation of CDC20 (Bancroft et al., 2020). To confirm this using the dTag system for acute PP1 degradation, HeLa PP1 dTag cells expressing mScarlet tagged MAD1 were generated (PP1 α/γ dTag + MAD1). MAD1 is a checkpoint protein that localises to unattached kinetochores as part of the MAD1:MAD2 dimers required for catalysing MAD2 activation and MCC assembly (Faesen et al., 2017). Upon end-on kinetochore-microtubule attachment formation, MAD1 is lost from kinetochores (Kuhn and Dumont, 2017). While MPS1 remains at the kinetochore to monitor tension and reactivate the checkpoint if required, MAD1 loss from kinetochores is widely recognised as a key aspect for SAC silencing (Lara-Gonzalez et al., 2021). CRISPR/Cas9 technology was used to tag MAD1 with an mScarlet fluorescent tag in the PP1 α/γ dTag cells. Successful tagging was confirmed by western blot (Figure 3.10A). These cell lines were subsequently screened by live cell imaging to check that CRISPR tagging MAD1 did not affect normal mitotic behaviour (Figure 3.10B) and fixed cell imaging to confirm that MAD1 localisation was not perturbed (Figure 3.10C).

PP1 α/γ dTag + MAD1 cells were imaged on the Olympus SoRa microscope at 2-minute intervals, with or without treatment with dTag1 (100nM). Quantification of NEBD to ANA indicated that PP1 degradation led to a mitotic delay (Figure 3.10D), as seen previously with the PP1 α/γ dTag cells (Figure 3.1D,G). The time from the loss of MAD1 signal to ANA was also quantified (Figure 3.10E), indicating that the acute degradation of PP1 led to a metaphase arrest after loss of the last MAD1-positive kinetochore (Figure 3.10G) and hence checkpoint silencing. The kinetics of the MAD1 signal decrease during early mitosis corresponded to the delay in chromosome congression seen when both PP1 α/γ catalytic subunits are degraded together (Figure 3.10F). This data therefore confirms that degradation of PP1 does not affect SAC silencing but leads to a metaphase arrest, most likely due to a delay in the activation of the APC/C, after spindle checkpoint silencing has been completed.

Given the time limitations with this project, it has not been possible to directly confirm the delay in Cyclin B and securin degradation. However, future work will explore this by carrying out biochemical timecourse assays (similar to those presented in Chapter 4 (Figure 4.6)) and western blotting for Cyclin B, securin and CDC20.

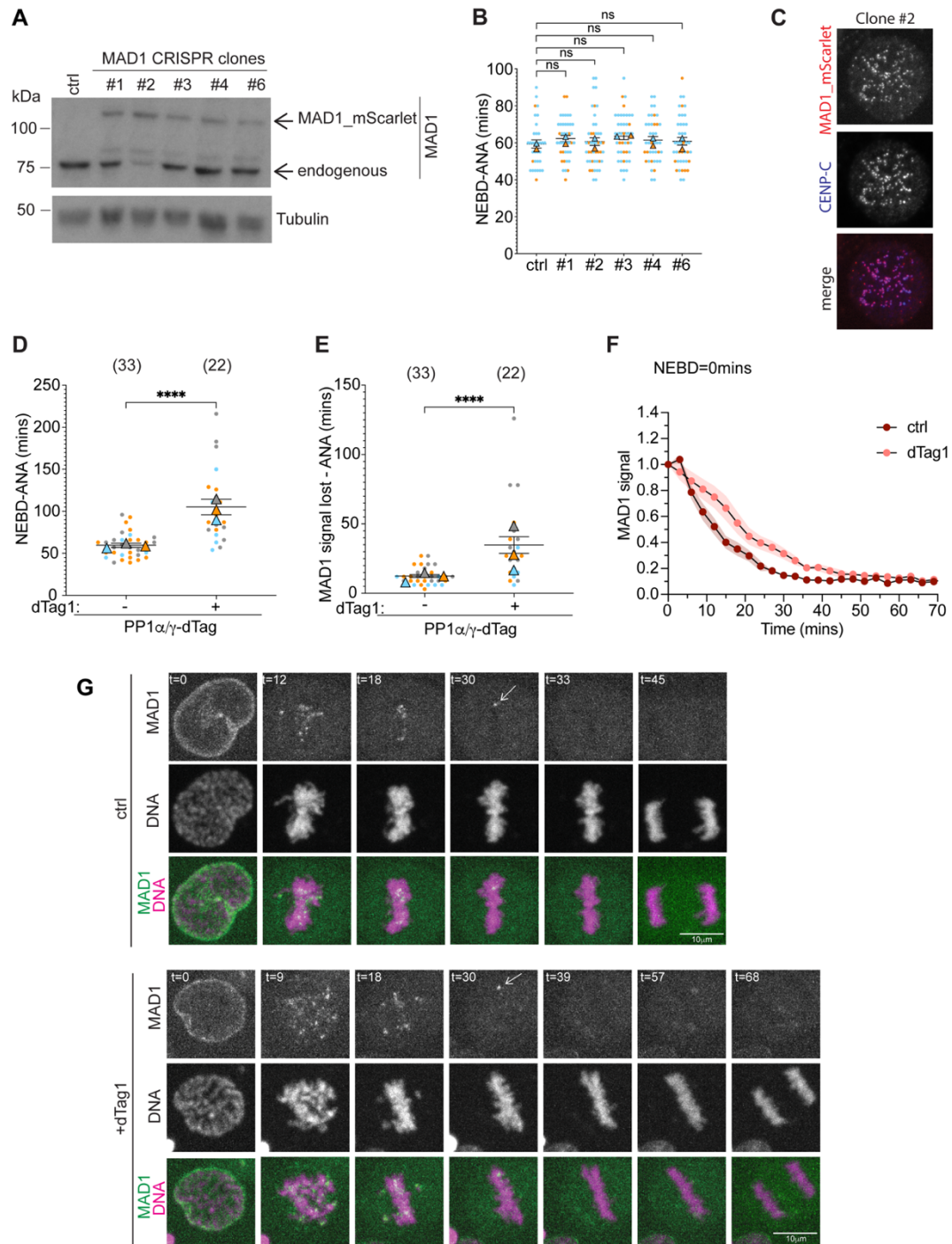


Figure 3.10: PP1 degradation induces a metaphase to anaphase delay beyond checkpoint silencing.

Figure 3.10 (continued). (A) MAD1-mScarlet CRISPR clones were generated in the HeLa PP1 α/γ dTag cell line (PP1 α/γ dTag + MAD1). PP1 α/γ dTag + MAD1 CRISPR clones were harvested, lysed and samples run on an SDS-PAGE gel. Membrane was blotted for MAD1 to confirm successful tagging of endogenous MAD1. (B) PP1 α/γ dTag + MAD1 CRISPR clones were imaged on the EVOS M7000 microscope, without drug treatments. Nuclear envelope breakdown (NEBD) to anaphase (ANA) was quantified. Ctrl = HeLa PP1 α/γ dTag cells (C) PP1 α/γ dTag + MAD1 CRISPR clones were fixed with PTEMF (12mins) and stained for CENP-C. Representative images of clone #2 shown. (D and E) (D) NEBD-ANA timings. (E) Time from when the MAD1 signal is lost to ANA. Graphs plotted show mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. (F) Quantification of MAD1 signal in single cells during mitosis was measured. n=9 per condition. MAD1 intensity normalised within each cell. (G) PP1 α/γ dTag + MAD1 CRISPR clone #2 was pre-synchronised with thymidine (2mM, 18hours) and then imaged on the Olympus SoRa microscope with or without dTag1 (100nM) treatment. Images were taken at 3 min intervals. Hoechst was used to visualise the DNA. Expected sizes of protein bands in western blots: MAD1 ~ 75kDa. MAD1_mScarlet ~ 100kDa.

3.11 PP1 influences tubulin dynamics during mitosis

Chromosome congression and alignment in mitosis relies on the dynamic nature of microtubules of the mitotic spindle. The physical forces applied through the microtubules onto chromosomes are essential for cells to reach an aligned metaphase plate. Perturbation of microtubules, for example by nocodazole treatment, which induces microtubule depolymerisation, or taxol, which stabilises microtubule bundles causes mitotic arrests (Horwitz et al., 1993). Live cell imaging of PP1 α/γ dTag cell lines demonstrated that when both catalytic subunits were degraded the cells were delayed in reaching META after NEBD. The live cell imaging movies showed that during this time, in the absence of PP1, the chromosomes appeared more 'twitchy', possibly as a result of irregular tubulin dynamics. Indeed, PP1 has been shown to interact with a number of MAPs, such as KIF18A.

To investigate the tubulin phenotype in the PP1 α/γ dTag cells, cells were incubated with SiR-DNA and SiR-tubulin dyes for one hour to facilitate visualisation of DNA and tubulin simultaneously, and then imaged on the Olympus SoRa microscope at 5-minute intervals with or without dTag1 (100nM) treatment. When treated with dTag1, cells displayed irregular tubulin morphology and did not form a symmetrical bipolar spindle, whereas control cells were able to (Figure 3.11A). Cells were harvested after imaging for western blot analysis (Figure 3.11B) to confirm that the degradation of PP1 was successful. Quantification of mitotic timings also demonstrate that PP1 degradation was efficient as mitosis was prolonged in these cells (Figure 3.11C).

Specific tubulin phenotypes were assessed to examine the difference between the mitotic spindle with or without PP1. All measurements were taken at the final metaphase timepoint before anaphase onset. Firstly, the length of the mitotic spindle increased in the absence of PP1 from an average of 13.4 μ M to 15.2 μ M (Figure 3.11D,E). Furthermore, the metaphase plate was less evenly centred between the two spindle poles when PP1 was degraded than in a control cell (Figure 3.11F,G). Finally, the loss of PP1 resulted in the angle between the two spindles deviating from 180° (Figure 3.11H,I). Together, this suggests that PP1 may be regulating tubulin dynamics and structure, either directly or indirectly via modification of one or more microtubule motor proteins.

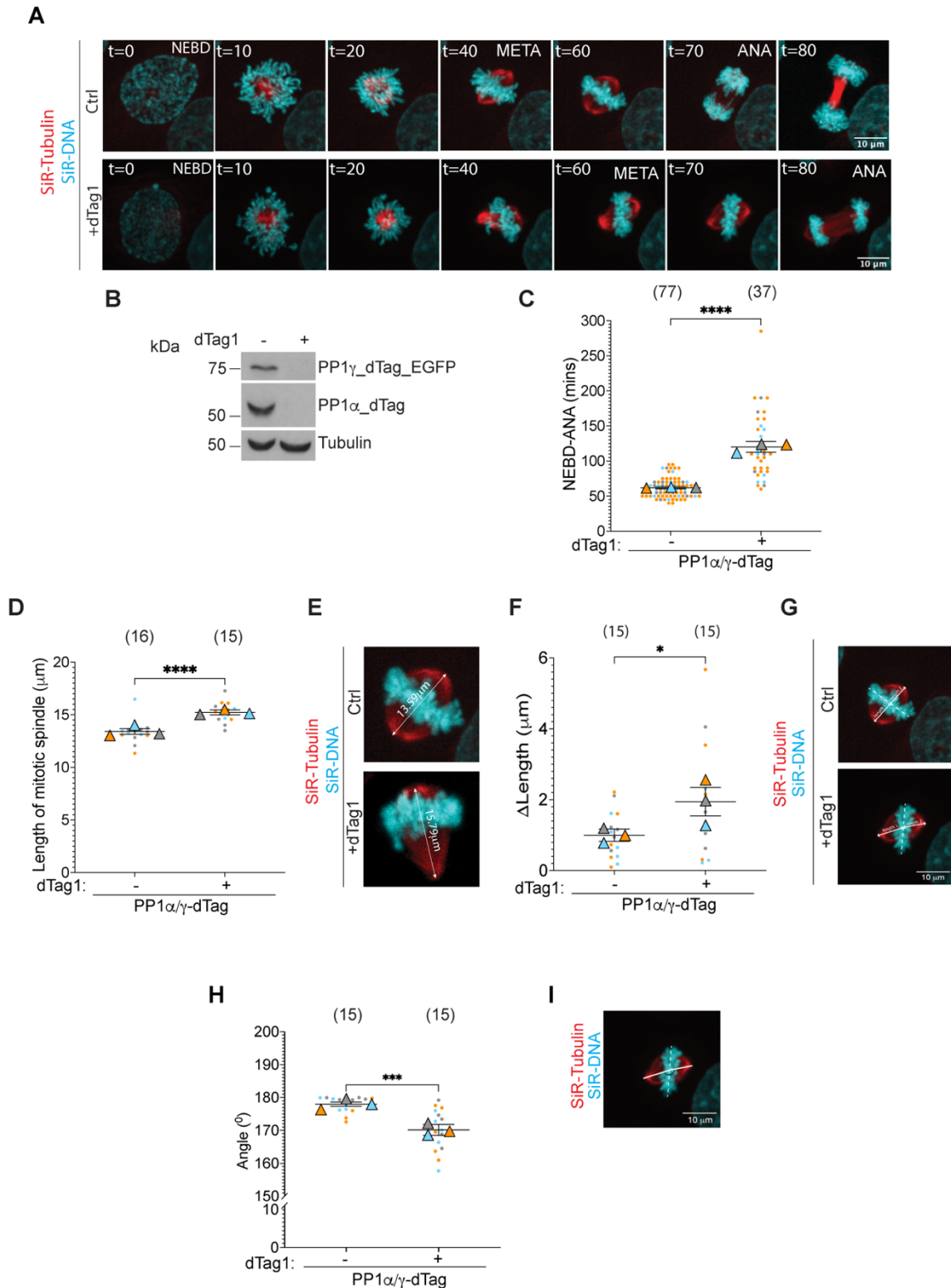


Figure 3.11: PP1 influences tubulin dynamics during mitosis.

(A) PP1 α/γ dTag cells were pre-synchronised with thymidine (2mM, 18hours) and then imaged on the Olympus SoRa microscope with or without dTag1 (100nM) treatment, 8 hours after release from thymidine block. Images were taken at 5min intervals. SiR-DNA (555, 50nM) and SiR-Tubulin (647, 50nM), added one hour before imaging started, were used simultaneously to visualise both the DNA and the tubulin during mitosis. (B) Western blot analysis of cell lysates, harvested after imaging from (A) to test efficient degradation of PP1 catalytic subunits. Expected sizes of protein bands in western blots: PP1 α _dTag ~ 50kDa. PP1 γ _EGFP_dTag ~ 75kDa.

Figure 3.11 (continued). (C) Quantification of mitotic timings from (A). NEBD=nuclear envelope breakdown. ANA=anaphase. Graphs plotted show mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. (D) Measurement of the length of the mitotic spindle (μm) from one spindle pole to the other across the metaphase plate. (E) Representative images from (D). (F) The difference in length (Δlength) (μm) between the centre of the mitotic plate and one spindle pole, and the centre of the mitotic plate to the other spindle pole. (G) Representative images from (F). (H) Angle ($^\circ$) across the metaphase plate. (I) Image showing how the angles ($^\circ$) were measured in (H). In ImageJ, a line was drawn from each spindle pole to the centre of the metaphase plate. The angle at this juncture was measured. All measurements in (D), (F) and (H) were carried out from cells at their last metaphase timepoint before anaphase onset.

3.12 PP1 degradation increases the number of polar chromosomes during chromosome congression

CENP-E (kinesin-7) has been suggested to directly interact with PP1 (Kim et al., 2010). CENP-E is a large plus-end-directed kinesin motor protein that plays an important role in congressing polar localised chromosomes during prometaphase by converting laterally attached kinetochores to end-on attached kinetochores, as well as maintaining chromosome alignment (Wood et al., 1997, Barisic et al., 2014, Shrestha and Draviam, 2013, Sikirzhytski et al., 2018, Gudimchuk et al., 2013). Interfering with CENP-E function by inhibiting its motor activity increases the number of polar localised chromosomes (Kapoor et al., 2006, Barisic et al., 2014). CENP-E is also phospho-regulated; phosphorylation of CENP-E at residue Thr422 has been reported to be important for CENP-E motor activity and mutating this residue to alanine increases the incidence of polar localised chromosomes. PP1 is proposed to dephosphorylate this phospho-site to promote stable biorientation (Kim et al., 2010).

Live cell imaging of PP1 dTag cell lines showed that degradation of both PP1 α and PP1 γ together resulted in a delay in chromosome congression (Figures 3.1 and 3.2). Therefore, the PP1 α/γ dTag cells were imaged in live with both SiR-DNA and SiR-Tubulin, to simultaneously visualise DNA and tubulin, to investigate whether acute degradation of PP1 increases the frequency of polar localised chromosomes during chromosome congression. Indeed, analysis of these cells revealed that the incidence of cells with polar chromosomes became more frequent (Figure 3.12A), suggesting that CENP-E activity may be perturbed. Additionally, polar localised chromosomes took longer to be aligned on the metaphase plate in the absence of PP1 α and PP1 γ (Figure 3.12B). This phenotype was reproducible in two independent HeLa PP1 α/γ clones.

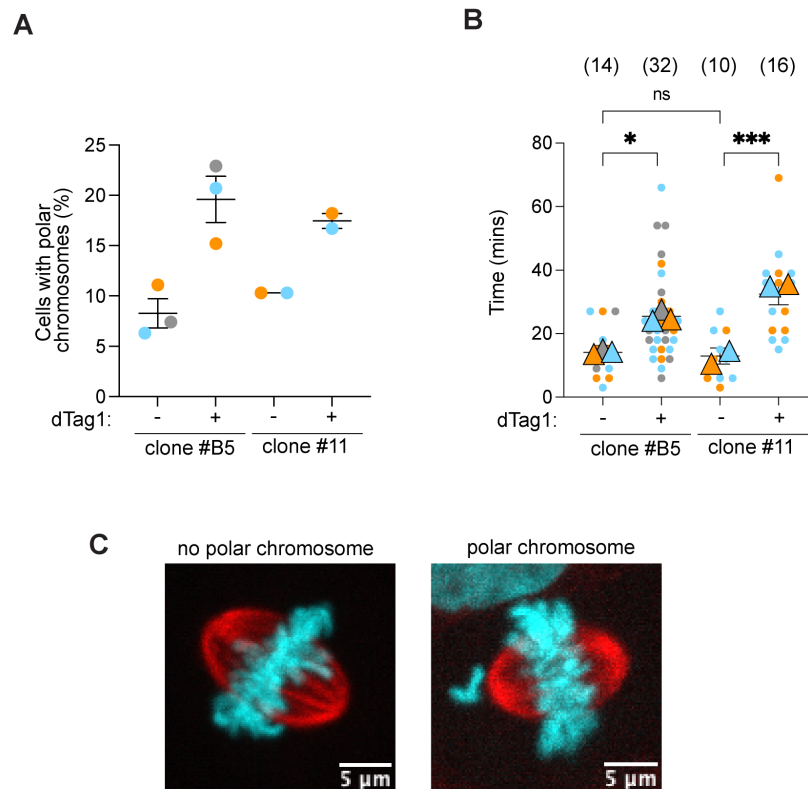


Figure 3.12: PP1 degradation increases the number of polar chromosomes during chromosome congression.

Figure 3.12 (continued). (A) Percentage of mitotic cells with polar chromosomes during chromosome congression. HeLa PP1 α/γ dTag cells were either left untreated or incubated with dTag1 (100nM) and imaged on the EVOS M7000 microscope at 3min intervals. SiR-DNA was used to visualise the DNA. The number of cells that had visible polar localised chromosomes during congression was counted. 3 independent biological repeats were counted for PP1 α/γ dTag clone #B5 and 2 repeats for PP1 α/γ dTag clone #11. Each dot on the graph is the percentage of cells of each biological repeat that has polar chromosomes. (B) Of the cells that have polar chromosomes, the time these chromosomes spent at, or near, the pole before being aligned at the metaphase plate, was quantified. Graph plotted shows mean $\pm$ SEM where each dot represents one cell. Different colours represent independent biological repeats. Triangles represent the mean value within each biological repeat. Numbers above each condition indicate the number of cells quantified in the corresponding condition. (C) Images to illustrate an aligned metaphase plate with no polar chromosomes compared to a (near) metaphase plate with a polar localised chromosome. DNA is shown in light blue, tubulin is shown in red.

3.13 CENP-E as a candidate PP1 target during chromosome congression

Live cell analysis implicates CENP-E as a potential PP1 target during mitosis as PP1 α/γ degradation increased the frequency of polar localised chromosomes during congression, in line with previously published data (Kim et al., 2010) which identified CENP-E as an interaction partner of PP1. To more closely examine the proposed misregulation of CENP-E, asynchronous HeLa PP1 α/γ dTag and HCT116 PP1 α/γ dTag cells were examined in fixed cell analysis using a CENP-E antibody to visualise CENP-E localisation. Cells were treated with either a CENP-E inhibitor (CENPEi, GSK923295, 50nM) or dTag1 (100nM) for 2 hours before fixing with PTEMF. CENPEi locks CENP-E in an inactive state, with the motor domain unable to use its ATPase activity (Qian et al., 2010) and thus CENP-E localised strongly at the spindle poles because it is unable to move along the microtubules towards the plus ends. Agreeing with data from live cell analysis, the degradation of PP1 increased the percentage of cells with polar

chromosomes (Figure 3.13C). When cells were not perturbed, CENP-E localised to the aligned kinetochores of a metaphase plate. However, when PP1 was degraded CENP-E presented a range of localisation phenotypes. When there were no polar chromosomes, CENP-E behaved in the same way as the control and localised to the aligned kinetochores (fourth panel in Figure 3.13A (HeLa cells) and 3.13B (HCT116 cells)). When there were polar localised chromosomes, CENP-E usually mislocalised away from the aligned kinetochores, but localised to the polar chromosomes to varying degrees.

Quantification of CENP-E localisation in the cells which displayed polar localised chromosomes is represented in Figure 3.13D. Importantly, even in the presence of polar chromosomes in the control cells (4 for HeLa out of 300 cells counted, and 3 for HCT116 out of 250 cells counted), CENP-E localised to aligned kinetochores as normal. It is possible that the range of phenotypes observed in the PP1-degraded condition was a result of varying levels of degradation efficiency; some cells in the population analysed may have an almost complete removal of PP1 while others may have a low level remaining. Additionally, it should be noted that this investigation was carried out in fixed cell analysis with an asynchronous population of cells. From live cell imaging data presented above (Chapter 3.12), it is known that the polar localised chromosomes persisted for around 30 minutes when PP1 was degraded before becoming aligned at the equator. Therefore, in an asynchronous population of cells there will be cells at a range of mitotic stages and few of them will be within this roughly 30-minute timeframe, so it will be useful to repeat this experiment with synchronised cells in the future. To further the investigation into the role of PP1 on CENP-E localisation

and function, visualisation of CENP-E in the PP1 α/γ dTag cells without the need for an antibody with either an endogenous fluorescent CRISPR tag or transient transfection would be beneficial to allow for live cell imaging experiments.

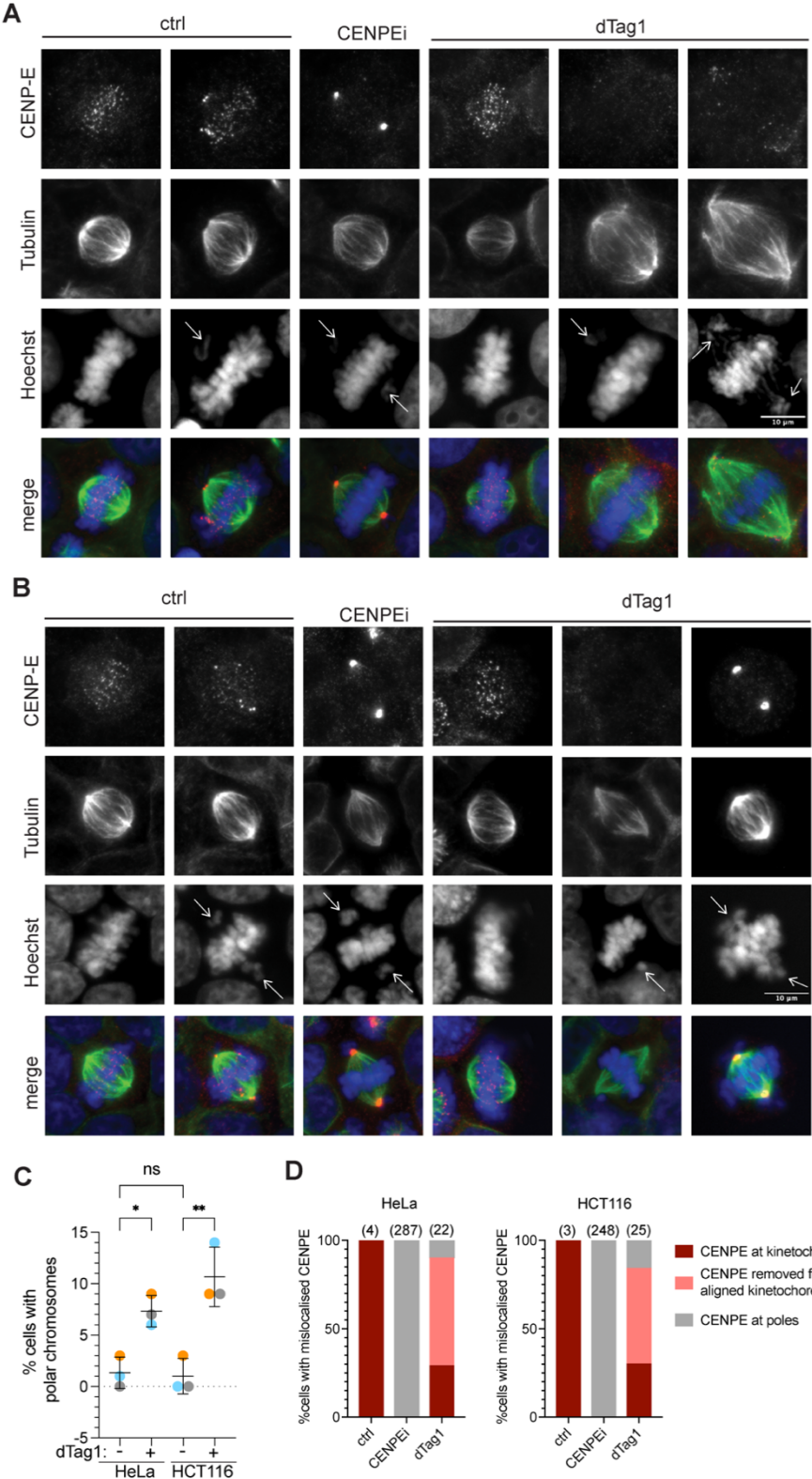


Figure 3.13: CENP-E as a candidate PP1 target during chromosome congression.

Figure 3.13 (continued). (A) Asynchronous HeLa PP1 α/γ dTag cells and (B) asynchronous HCT116 PP1 α/γ dTag cells were treated with either DMSO (ctrl), a CENP-E inhibitor GSK923295 (CENPEi, 50nM) or dTag1 (100nM). Cells were fixed with PTEMF (12mins) and stained as indicated. Representative images of the range of CENP-E localisation phenotypes are shown. See main text for detailed discussion. (C) Percentage of cells that had polar localised chromosomes in the ctrl and +dTag conditions for both HeLa (A) and HCT116 (B) PP1 α/γ dTag cells. (D) CENP-E localisation was categorised into the phenotypes as follows: CENPE at kinetochores, CENPE removed from aligned kinetochores or CENPE at poles. CENPE at poles is a subcategory of CENPE removed from aligned kinetochores, as the strong localisation of CENPE at spindle poles was only observed when CENPE was also removed from the aligned kinetochores. Number of cells per condition is displayed above the corresponding column in the graphs. Data merged from 3 biological repeats. 300 cells (HeLa) and 250 cells (HCT116) counted in total.

Discussion

It has long been known that PP1 is an important player in the regulation of mitosis. PP1 has been implicated in many mitotic processes, including SAC silencing (Vanoosthuyse and Hardwick, 2009, Pinsky et al., 2009, Meadows et al., 2011, Nijenhuis et al., 2014), activation of the APC/C at the metaphase to anaphase transition (Bancroft et al., 2020) and stabilisation of end-on kinetochore-microtubule attachments (Conti et al., 2019, Song et al., 2021, Liu et al., 2010). However, PP1's mitotic specific roles are not fully understood. Generation of PP1 dTag cell lines (Chapter 2) allows for the precise and rapid degradation of PP1 catalytic isoforms PP1 α and PP1 γ and provides a novel way of studying the relative contributions of the PP1 catalytic subunits.

Initial characterisation of the generated HeLa and HCT116 PP1 dTag cell lines was carried out by live cell imaging to closely analyse the mitotic delay phenotype upon acute degradation of PP1 catalytic isoforms (Figures 3.1 and 3.2). Previous reports in which PP1 α and PP1 γ were depleted together through RNAi

demonstrated that overall timing of mitosis increased (Bancroft et al., 2020). However, due to PP1's roles in both DNA replication and controlling the transcription machinery (Hiraga et al., 2017, Gnan et al., 2021, Jenkinson and Zegerman, 2022, Estell et al., 2023, Estell and West, 2024, Cortazar et al., 2019), this observation did not exclude the possibility that this mitotic delay was due to knock-on effects of DNA damage that had occurred during S-phase. Using the PP1 dTag cell lines, PP1 can be acutely degraded just before entry into mitosis, limiting the risk of DNA damage. Live cell imaging analysis presented in this thesis confirms the importance of PP1 for the overall timing of mitosis, while also providing further insight into isoform-specific function (Figure 3.1 and 3.2). At the META to ANA transition, PP1 has been implicated in both SAC silencing and APC/C activation (Bancroft et al., 2020, Moura et al., 2017, Holder et al., 2020, Nijenhuis et al., 2014), however the potential isoform specific roles are not yet known. It may be the case that despite initial phenotypic observation revealing a similar META to ANA delay in the individual PP1 α and PP1 γ dTag cell lines, at a molecular level the events causing this delay are different, which could explain the more prominent delay when both isoforms are degraded. Investigating this possibility will be an important avenue for further research. Furthermore, the imaging experiments presented in Figures 3.1 and 3.2 were carried out after a thymidine arrest and release. It would be insightful to repeat these imaging experiments with a prior RO3306, which reversibly arrests cells at the G₂/M border of the cell cycle (Vassilev et al., 2006), and examine whether degradation of PP1 during a prolonged G₂/M arrest results in different mitotic phenotypes compared to those observed when cells were pre-synchronised with thymidine before dTag reagent was added.

While there was a META to ANA delay in all cell lines tested, only cells with both PP1 α and PP1 γ degraded together resulted in an NEBD to META delay. This could indicate that the roles of PP1 during chromosomes congression do not require both catalytic isoforms, and therefore only removing one isoform does not affect mitotic timing for this part of the cell cycle. An alternative intriguing possibility is that certain functions may require a 1X amount of PP1 activity, whereas others may require a 2X amount, such that degradation of only one subunit does not decrease the overall level of PP1 activity for there to be a phenotypic effect on the cells' behaviour.

The formation of stable kinetochore-microtubule attachments is critical for safeguarding chromosome segregation. Two independent pools of PP1 at kinetochores, an astrin-bound and a KNL1-bound pool, have been reported to be important for the stabilisation of kinetochore-microtubule attachments (Liu et al., 2010, Song et al., 2021, Conti et al., 2019). These studies opted for a replacement system in which the endogenous copies of astrin and KNL1 were replaced by mutant versions in which the RVxF motifs of astrin and KNL1 were mutated to AAAA (four alanine residues) to prevent PP1 binding while preserving PP1 abundance so as not to interfere with other cellular processes. In contrast, in this study, an acute PP1 degradation and subsequent cold treatment did not reveal any errors in stable kinetochore-microtubule attachment formation (Figures 3.7). The error correction pathway was also not affected when PP1 was degraded (Figure 3.8). The dTag system permits rapid degradation of target proteins so that the phenotype of whole cell protein loss can be examined, however the study of

possible intricate feedback loops in this system is more challenging. Therefore, the differing observations of PP1's role in kinetochore-microtubule attachment stability could be due to the different systems used to probe them. It could perhaps point towards a more complicated system of stabilising attachments than originally thought. Further work will be needed to fully uncover the role of PP1 in stable attachment formation. It will also be important in future work to determine whether the cell lines generated and used in this study have the same kinase and phosphatase (in the absence of dTag addition) activity levels as the parental HeLa and HCT116 cells by examining phospho-substrate levels.

When chromosomes have aligned at the metaphase plate and stable kinetochore-microtubule attachments have formed, the SAC needs to be silenced to cease production of the MCC and allow APC/C activation. Interestingly, despite the proposed role of PP1 in checkpoint silencing, acute degradation of PP1 did not increase the frequency of lagging chromosomes or DNA bridges during anaphase (Figure 3.3) suggesting that the SAC was silenced prior to anaphase onset and that these cells were able to form stable kinetochore-microtubule attachments to separate their chromosomes equally. This was confirmed using PP1 α/γ dTag + MAD1 cells (Figure 3.10), which was also reported in previously published work from our group (Bancroft et al., 2020). This publication identified CDC20 as a PP1 target at the metaphase to anaphase transition. Confirming this data in the dTag system and identifying other PP1 substrates at the metaphase to anaphase transition is an exciting avenue for further study.

In order to investigate the SAC-independent mitotic delay, PP1 α/γ dTag cells were imaged in the presence of an MPS1 inhibitor, AZ3146, which overrides the checkpoint (Figure 3.9). It is well known that when MPS1 is inhibited, cells enter anaphase regardless of kinetochore attachment status, resulting in a large number of erroneous chromosome segregations (Hewitt et al., 2010). When PP1 α/γ dTag cells were incubated with both dTag reagent, to degrade PP1, and MPS1 inhibitor, to override the SAC, the time from NEBD to ANA was around 50 minutes on average, which is similar to the control cells that have not been treated with either reagent (Figure 3.9A). Despite overall similar mitotic timings, an important distinction between these conditions is shown in that the dTag and MPS1 inhibitor treated cells proceed into anaphase before reaching an aligned metaphase plate and separate their chromosomes with many errors (Figure 3.9C). This further supports the idea that PP1 has mitotic roles independent of checkpoint signalling that are important for proper mitotic timings, most likely the activation of the APC/C.

The delay in chromosome congression observed when PP1 α/γ were both degraded points towards a role of PP1 in regulating the mitotic spindle. Previous research has presented evidence for the interaction of PP1 with a number of proteins important for mitosis, including KIF18A (De Wever et al., 2014, Hafner et al., 2014), astrin (Conti et al., 2019, Song et al., 2021) and CENP-E (Kim et al., 2010). PP1 has been proposed to negatively regulate CENP-E motor activity by opposing the activating Aurora phosphorylation (Kim et al., 2010). In agreement with previous reports, PP1 degradation led to an increase in the frequency of polar localised chromosomes during congression. Interestingly, polar localised

chromosomes took longer to be aligned at the metaphase plate in the absence of PP1 supporting the idea that CENP-E function is perturbed (Figure 3.12).

Subsequent analysis of CENP-E, specifically its localisation in fixed cell experiments, revealed that in the presence of polar localised chromosomes when PP1 had been degraded, CENP-E often localised away from the aligned kinetochores and occasionally localised strongly at the poles (Figure 3.13), suggesting a degree of inhibition of the motor activity of CENP-E. It is possible that the range of phenotypes seen represents a dynamic localisation pattern of CENP-E during chromosome congression in the absence of PP1. Additionally, degradation of PP1 resulted in a delay at the metaphase to anaphase transition after chromosome alignment and SAC silencing (Figure 3.10). This means that in an asynchronous population of cells it is to be expected that there would be a higher number of cells arrested with an aligned metaphase plate when PP1 is degraded compared to control cells. In this quantification, this fact would sway the cell count as the mitotic index of PP1 degraded cells would be higher. Despite this, there is still a substantial increase in the number of cells with polar chromosomes compared to the control cells (Figure 3.13C). However, this explains the discrepancy between the higher percentage of polar localised chromosomes counted in live cell imaging (Figure 3.12A) compared to fixed cell imaging (Figure 3.13C).

Aurora phosphorylation of CENP-E Thr422 promotes CENP-E motor activity (Kim et al., 2010). If phosphorylation of this residue in CENP-E is prevented, it becomes trapped at the spindle poles, unable to move towards the plus ends of

microtubules and fulfil its function. This phosphorylation site, Thr422, is located within the RVxF motif of CENP-E, phosphorylation of which prevents PP1 from binding. At kinetochores, this residue is dephosphorylated and PP1 binding is permitted (Kim et al., 2010). CENP-E is then removed from kinetochores in a dynein-dependent manner (Howell et al., 2001, Weber et al., 2024, Auckland and McAinsh, 2019). In addition to Thr422, CENP-E has two canonical CDK1 phosphorylation sites (S/T-P) located at the C-terminus, Ser2639 and Ser2654. The relevance of these phospho-sites in regulating CENP-E function during prometaphase and the potential involvement of PP1 in dynamically regulating these is not well understood. Furthermore, it could be hypothesised that the CENP-E at kinetochores in the absence of PP1 is hyperphosphorylated as PP1 cannot dephosphorylate it. How and why this potentially hyperphosphorylated CENP-E is removed from aligned kinetochores remains an open area of investigation. Due to the nature of the transient CENP-E phenotype, studying CENP-E localisation in fixed cells is challenging and the mechanism(s) by which CENP-E is mislocalised away from aligned kinetochores remains to be elucidated. To further investigate the dynamic localisation of CENP-E during chromosome congression and the potential inhibitory effect of PP1 degradation on CENP-E function, a cell line in which CENP-E localisation could be tracked, either with an endogenously fluorescent tag or by transient transfection, would be most insightful.

Chapter 4: p31^{comet} as a potential PP1 target during mitotic exit

4.1 The known role of p31^{comet} in silencing the spindle assembly checkpoint

The metaphase to anaphase transition of mitosis is tightly regulated to ensure that cells only enter anaphase when all kinetochores are correctly attached to microtubules. The spindle assembly checkpoint (SAC) and the anaphase promoting complex/cyclosome (APC/C) both contribute to the regulation of this transition (Musacchio, 2015, Lara-Gonzalez et al., 2021). In response to unattached kinetochores, the SAC generates the mitotic checkpoint complex (MCC) which inhibits the APC/C, together denoted as APC/C^{MCC}, preventing the degradation of securin and Cyclin B and delaying anaphase until all kinetochores are stably bound to microtubules (Alfieri et al., 2016). Failures in the regulation of the SAC or the APC/C can result in cells entering anaphase prematurely before stable kinetochore-microtubule attachments have formed and increase the risk of chromosome segregation errors and aneuploidy (Thompson et al., 2010). Therefore, it is essential that SAC activation and silencing, and timely activation of the APC/C, is tightly controlled to maintain genomic stability.

The SAC and the APC/C are both regulated by reversible phosphorylation. At unattached kinetochores, the kinase monopolar spindle 1 (MPS1) initiates a phosphorylation signalling cascade that recruits several checkpoint proteins (Ji et al., 2017, Abrieu et al., 2001). Key to this signalling pathway is the recruitment

and subsequent activation of inactive cytosolic O-MAD2 to active C-MAD2 at MAD1:C-MAD2 dimers located on unattached kinetochores (Fang et al., 1998, Simonetta et al., 2009, Faesen et al., 2017). The product of the SAC pathway is the MCC which consists of the SAC proteins BUB3, BUBR1, C-MAD2 and the protein CDC20, which together inhibit the activity of the APC/C (McAinsh and Kops, 2023). Once all kinetochores are correctly and stably bound to microtubules, SAC signalling is terminated and MCC formation ceases. Additionally, in order to rapidly activate the APC/C to promote timely anaphase onset, the MCC already formed and present in the cytosol needs to be disassembled (Lara-Gonzalez et al., 2021). In vitro studies suggest that MCC disassembly is an active process as the metaphase to anaphase transition occurs more rapidly than would be expected if MCC were to be spontaneously disassembled to allow for APC/C activation (Braunstein et al., 2007, Simonetta et al., 2009, Kulukian et al., 2009, Eytan et al., 2014). The protein p31^{comet} (also known as MAD2L1 binding protein or MAD2L1BP) has been implicated in both SAC silencing and MCC disassembly to reduce MCC in the cell (Figure 1A) (Habu and Matsumoto, 2013, Yang et al., 2007, Teichner et al., 2011, Westhorpe et al., 2011, Hagan et al., 2011, Date et al., 2014). Human p31^{comet} has a canonical CDK1 site that has been shown to be phosphorylated in mitosis (Date et al., 2014). The biological relevance of this phosphorylation site, and its potential dynamic control during mitosis, requires further investigation.

p31^{comet} was first identified by the yeast two-hybrid system as a MAD2 binding protein (Habu et al., 2002). Studies show that RNAi-mediated depletion of p31^{comet} does not affect the time taken between nuclear envelope breakdown and

metaphase, but it does result in delayed anaphase entry of around 30 minutes on average and a delay in MCC disassociation from the APC/C^{MCC} (Varetti et al., 2011). A delay in mitotic exit was also observed in a p31^{comet} knockout cell line (Lok et al., 2020). Overexpression of p31^{comet} has been shown to override the SAC, suggesting that it is an important protein involved in SAC silencing (Habu et al., 2002, Westhorpe et al., 2011, Hagan et al., 2011). p31^{comet} can bind to both MAD1-bound and CDC20-bound C-MAD2 (Xia et al., 2004), giving rise to two proposed models for how p31^{comet} functions to silence the checkpoint.

The first model, referred to as the 'capping model', arose due to the observation that p31^{comet} binds specifically to C-MAD2 and localises to unattached kinetochores in a MAD2-dependent manner (Westhorpe et al., 2011, Yang et al., 2007). p31^{comet}, like MAD2, is a HORMA domain protein. Despite having little sequence homology, p31^{comet} and C-MAD2 adopt similar folds owing to similarity in regions defining their secondary structural elements (Yang et al., 2007). p31^{comet} interacts extensively with the C-terminal tail of C-MAD2. When O-MAD2 is activated at the MAD1:C-MAD2 dimers located at unattached kinetochores, the C-terminal region entirely changes conformation and becomes trapped between two beta-strands within the MAD2 structure (Sironi et al., 2002) which may explain why p31^{comet} specifically interacts with C-MAD2 and not O-MAD2. Whereas CDC20 and MAD1 bind to C-MAD2 on different surfaces to p31^{comet}, p31^{comet} and O-MAD2 have significantly overlapping binding interfaces on C-MAD2, preventing dimerization (Yang et al., 2007, Mapelli et al., 2006). Based on these observations, the 'capping model' suggests that p31^{comet} binds to MAD1-

bound C-MAD2 at kinetochores to stop activation of MAD2 and attenuates SAC signalling (Mapelli et al., 2006, De Antoni et al., 2005, Luo et al., 2004, Yu, 2006).

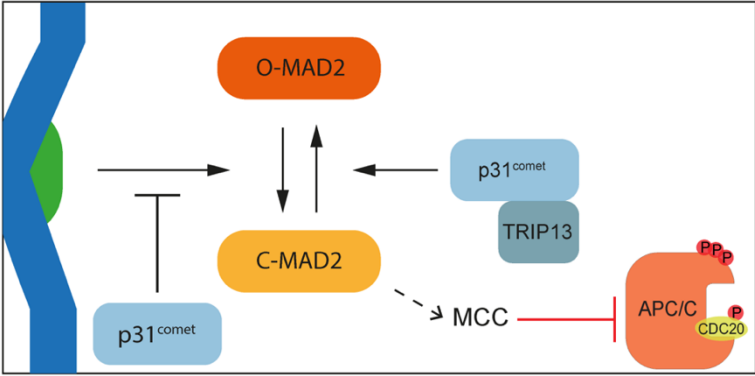
However, modulation of p31^{comet} levels does not affect O-MAD2 recruitment to kinetochores (Westhorpe et al., 2011). If the current capping model was indeed correct, and O-MAD2 was competing with p31^{comet} for C-MAD2 binding, modulation of p31^{comet} abundance in the cell would be expected to affect O-MAD2 recruitment. Additionally, p31^{comet} interacts with MAD2 at all stages of the cell cycle (Westhorpe et al., 2011, Varetto et al., 2011). In mitotic cells treated with nocodazole, a microtubule depolymerising agent, where the checkpoint is fully active, p31^{comet} localises with MAD2 to unattached kinetochores (Date et al., 2014). How p31^{comet} does not silence the checkpoint under these conditions requires further investigation.

The second proposed model of p31^{comet} activity in silencing the SAC is referred to as the 'disassembly model'. p31^{comet}, along with its partner TRIP13, is implicated in disassembling already formed MCC (Westhorpe et al., 2011). TRIP13 is an AAA+ protein that uses the energy of ATP hydrolysis to drive conformational changes of HORMA domain proteins (Ye et al., 2015). p31^{comet} acts as a TRIP13 "adaptor protein" so that TRIP13 can catalyse the inactivation of MAD2 that forms a part of the soluble MCC (Teichner et al., 2011, Ye et al., 2015, Alfieri et al., 2018). This ATP-dependent conformational change of MAD2 stimulates the disassembly of the MCC by promoting the dissociation of CDC20 from BUBR1 (Teichner et al., 2011). Additionally, p31^{comet} promotes the

ubiquitination of CDC20 which drives the reactivation of APC/C (Reddy et al., 2007).

Despite recent progress in understanding the function of p31^{comet} in the SAC, little is known about its regulation. A recent study used mass spectrometry to identify residue Ser102 as a phospho-site in human p31^{comet} (Date et al., 2014). Although this residue is not widely conserved across p31^{comet} orthologs (Figure 1B), it is a canonical CDK1 phosphorylation site in human p31^{comet}. Therefore, we

A



B

HUMAN	FPGPVSOEGCCQFTCELLKHIMYQRQQLPLPYEQLKHFYRKPSQAEEMLKKKPRATTEVSSR	122
MOUSE	FPGPVSOEDCCQFTCELLKHILYQRHQLPLPYEQLKHFYRKVPQAEEDTARKKAWLATEARNR	124
COW	FPGPVSOEGCCQFTCELLKHIMYQRQQLPLPYEQLKHFYRKFPQAEEDVVKKKPRATAEVSSR	122
RAT	FPGPVSOEGCCQFTCELLKHIMYQRQQLPLPYEQLKHFYQKSSQAEEDTARKKPRLTTEASNR	125
DROSOPHILA	NIEVLTSGTTAEFVNGILDFFLYQRRI PFVYKTYKYYVDKWSDAESGESKDQESFAHYQR	75
■		
HUMAN	-----KCQQALAELESVLSHLEDF FARTLVPRVLI LLGGNALS PKEFYELDLSLLAPY-----	175
MOUSE	-----KCQQALAELESVLSHLEDF FARTLVPRVLI LLGGNALS PKEFYELDLSRLAPF-----	177
COW	-----KCQQTAELESVLSHLEGL FARTLVPRVLI LLGGNALS PKEFYELDLSRLAPN-----	175
RAT	-----KCQQALAELESVLSHLEDF FARTLVPRVLI LLGGNALS PKEFYELDLSRLAPF-----	178
DROSOPHILA	NQGRS KAKATKESISDMREIRQAFRSSEVKSLRFLFGNNMFMPSEAYTLHIPHDSISRHYC	138
■		
HUMAN	-----SVDQSL S-----TAACLRRRLFRAI-FLADAFSELQA-----PPLMGTV	212
MOUSE	-----GVDQGLN-----TAACLRRRLFRAI-FLADPFSELQT-----PPLMGTI	214
COW	-----SMDPSLS-----TAACLRRRLFRAI-FLADAFSELQV-----PPLMGTI	212
RAT	-----SVDQGLN-----TAACLRRRLFRAI-FLADAFSELQA-----PPLMGTI	215
DROSOPHILA	EHHALPEGRINDALLRLTCEEIYRLFSTELKVTNVLEMELLTDSDRPQGSHCDSEFNLI PKH	201

Figure 4.1: The known role of p31^{comet} in silencing the spindle assembly checkpoint

(A) p31^{comet} has been implicated in spindle assembly checkpoint silencing in two distinct pathways. Firstly, through binding to C-MAD2 localised at kinetochores, p31^{comet} prevents activation of MAD2 from O- to C-MAD2 to silence the SAC signalling cascade. This slows down the rate of MCC formation. Secondly, p31^{comet} binds to C-MAD2 that has been incorporated into the MCC and promotes its structural interconversion back to inactive O-MAD2 thus promoting MCC disassembly. (B) Sequence homology of p31^{comet} across species shows that the protein is not highly conserved. Black square indicates the location of the phospho-site in human p31^{comet}.

hypothesised that dephosphorylation of Ser102 in p31^{comet} at the metaphase to anaphase transition, possibly by PP1, may regulate p31^{comet} activity at this cell cycle transition.

4.2 p31^{comet} localisation resembles that of other known SAC proteins

It has been shown that p31^{comet} is a binding partner of MAD2 and has similar localisation pattern during the cell cycle (Westhorpe et al., 2011, Hagan et al., 2011). In order to visualise the localisation of endogenous p31^{comet} and confirm this localisation, CRISPR/Cas9 knock-in system was used to generate cell lines with GFP-tagged p31^{comet}. The C-terminus contains regions that interact with the MAD2 binding interface, important for p31^{comet}'s role in SAC silencing, whereas the N-terminus is dispensable for MAD2 binding (Yang et al., 2007, Date et al., 2014). Therefore, the GFP tag was added to the N-terminus (Figure 4.2A) to minimise potential of interfering with p31^{comet} function. CRISPR cell lines were generated in HeLa cells and successful homozygous tagging of p31^{comet} was confirmed by western blot analysis (Figure 4.2B).

In order to visualise the dynamic localisation of p31^{comet} during mitosis, the GFP-p31^{comet} CRISPR cells (clone #12) were imaged on an Olympus SoRa microscope at 5-minute intervals (Figure 4.2C). At the onset of mitosis, p31^{comet} signal rapidly accumulates at punctate foci on the chromatin. As cells progress through mitosis and the chromosomes align, the p31^{comet} signal starts to drop. There is no punctate p31^{comet} signal as the cells enter anaphase. This supports the p31^{comet} localisation phenotypes previously observed using antibodies

against p31^{comet} (Westhorpe et al., 2011, Hagan et al., 2011) in which p31^{comet} dynamically localises in a similar manner to other checkpoint proteins, including MAD2, p31^{comet}'s well known binding partner.

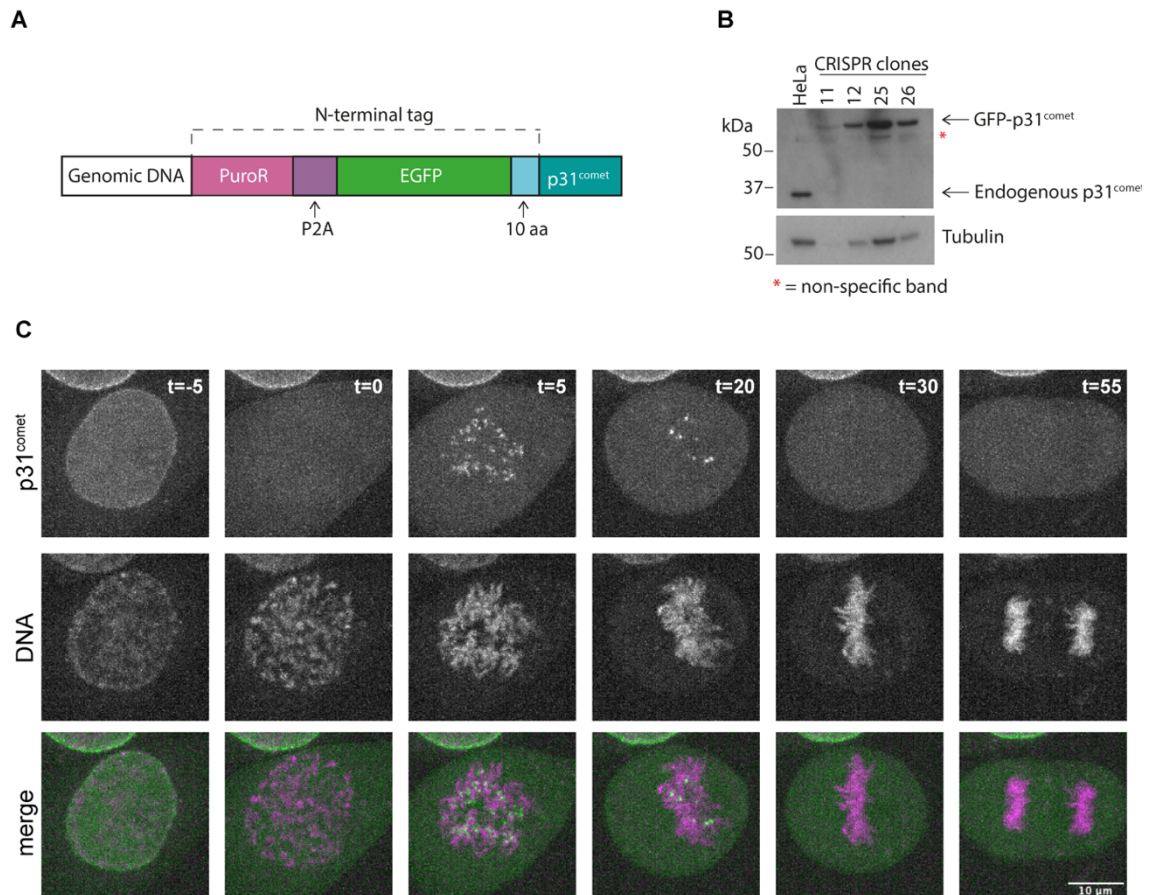


Figure 4.2: Visualising endogenous p31^{comet} in HeLa cells using CRISPR/Cas9 fluorescent tagging.

(A) Schematic of the CRISPR/Cas9 insert used to tag endogenous p31^{comet} at the N-terminus with a fluorescent GFP tag. A 10 amino acid (aa) linker is included between the GFP tag and endogenous p31^{comet}. PuroR = puromycin resistance gene. (B) Western blot screen confirms successful CRISPR tagging of p31^{comet}. (C) GFP-p31^{comet} CRISPR cells were imaged on the SoRa microscope, using siR-DNA (50nM) to visualise the DNA. Images were taken at 5 min intervals. Expected sizes of protein bands in western blots: p31^{comet} ~ 31kDa. GFP p31^{comet} ~ 60kDa.

4.3 p31^{comet} is mitotically phosphorylated on Ser102

Work from a decade ago suggested that p31^{comet} is mitotically phosphorylated on Ser102 (Figure 4.3A) and that this phosphorylation attenuates its activity during early mitosis (Date et al., 2014). Ser102 is located within a flexible loop between two alpha helices (Figure 4.3B), not directly at the MAD2 binding interface, but nearby (Yang et al., 2007), so its phosphorylation could be regulating the p31^{comet}-C-MAD2 interaction.

To study the potential phospho-regulation of p31^{comet}, the HeLa Flp-In/T-Rex system (Invitrogen, (Tighe et al., 2008)) was utilised to generate cell lines with inducible p31^{comet} harbouring mutations at the proposed Ser102 site. These cell lines contain a single integrated FRT site where the desired transgene is inserted into the genome. In these cells, the expression of a GFP-p31^{comet}, siRNA resistant, transgene could be induced upon the addition of small amounts of doxycycline (2µM). The cell lines generated contained an inducible transgene either with the Ser102 site mutated to alanine (S102A) as a phospho-null p31^{comet} mutant or to aspartate (S102D) as a phospho-mimetic p31^{comet} mutant. A cell line with inducible WT p31^{comet} was also generated as a control. Biochemical analysis of the p31^{comet} Flp-In cell lines show that endogenous p31^{comet} can be knocked down by 48-hour treatment with siRNA oligos and transgene expression can be simultaneously induced through the addition of doxycycline (2µM) for 24 hours (Figure 4.3C).

To first confirm that p31^{comet} is phosphorylated in vivo during mitosis, HeLa cells were harvested either in an asynchronous state or in a mitotic arrest. These cells

were lysed in lysis buffer containing phosphatase inhibitors, to ensure preservation of any phosphorylated residues. A portion of the mitotic sample was subsequently treated with λ -phosphatase to dephosphorylate phosphorylated residues in the cell lysate. The samples were analysed on a PhosTag SDS-PAGE gel (Figure 4.3D). PhosTag reagent binds to phosphate groups of proteins and slows the mobility of these proteins on the gel allowing for better resolution of phosphorylated protein from the unphosphorylated counterparts (Kinoshita et al., 2006). The results confirmed that p31^{comet} is phosphorylated in mitosis as shown by the enriched upshifted band on the PhosTag SDS-PAGE gel, and that the upshift was indeed caused by the phosphorylated state of the protein as a 2-hour treatment with λ -phosphatase resulted in the loss of this band in the PhosTag SDS-PAGE gel (Figure 4.3D).

To determine whether it was phosphorylation of Ser102 that was causing this observed enriched upshift in the PhosTag SDS-PAGE, the novel WT p31^{comet} and S102A p31^{comet} Flp-In inducible cell lines were examined on PhosTag SDS-PAGE. The expression of the GFP-p31^{comet} transgenes was induced with doxycycline (2 μ M) for 24 hours before harvesting asynchronous and mitotically arrested samples. Indeed, when these cells were examined on a PhosTag SDS-PAGE gel, the mitotic S102A GFP-p31^{comet} sample did not have an upshifted band at the size expected of the transgene however it was present in the mitotic WT Flp-In cells (Figure 4.3E). Therefore, Ser102 of p31^{comet} is phosphorylated in mitosis and this modification causes a visible upshift when run on PhosTag SDS-PAGE.

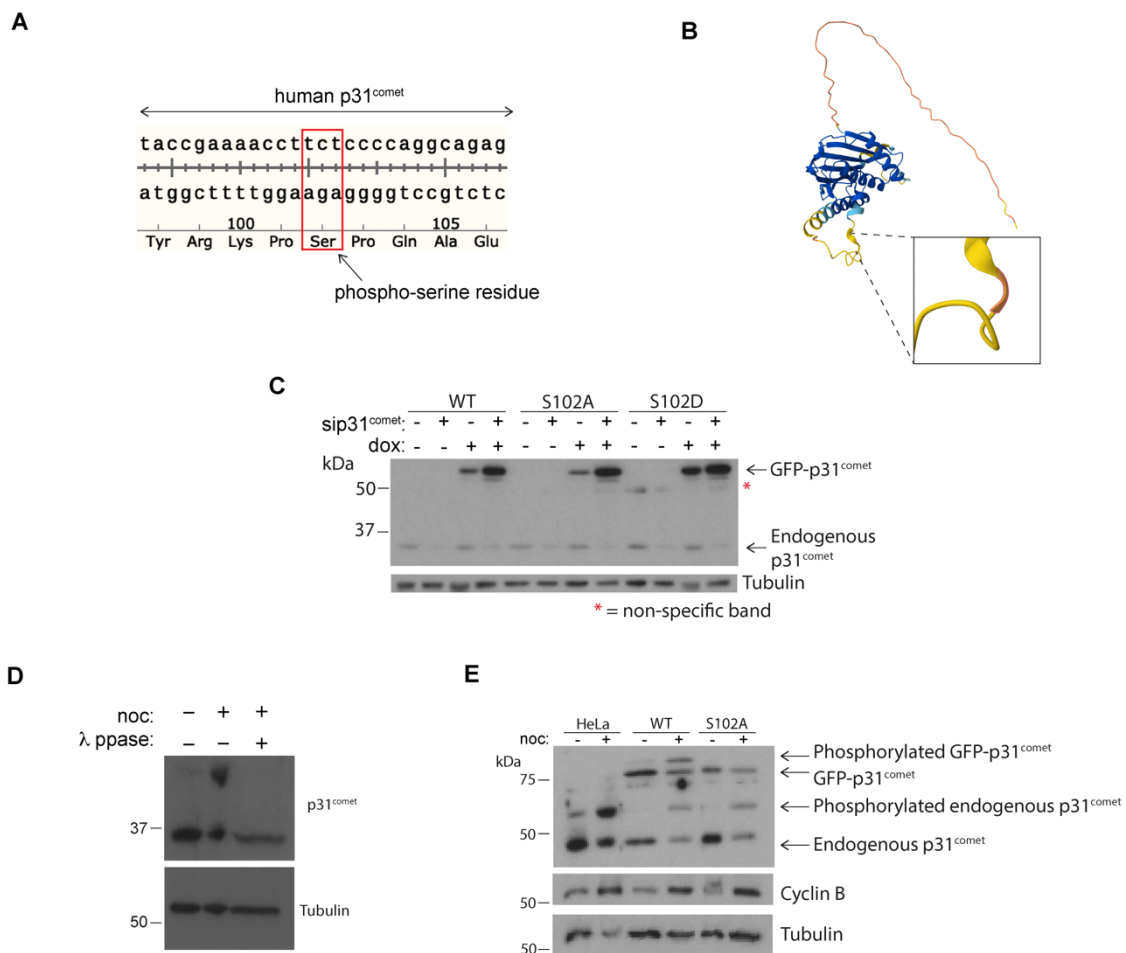


Figure 4.3: p31^{comet} is mitotically phosphorylated on Ser102.

(A) Location of Ser102 of p31^{comet} in gene sequence is shown. (B) Structure of p31^{comet} (alpha-fold). Pink highlighted region shows location of Ser102 within the structure, based on alpha fold prediction. (C) Western blot confirming successful RNAi rescue with GFP-p31^{comet} transgene protein. HeLa Flp-In/T-REx GFP-p31^{comet} cells were depleted of endogenous p31^{comet} for 48 hours and GFP- p31^{comet} transgenes were induced by addition of doxycycline (dox) as indicated for 24 hours. (D) Asynchronous and mitotically arrested HeLa cells were harvested. Some of the mitotic sample was treated with λ-phosphatase, as indicated. (E) HeLa, WT p31^{comet} Flp-In and S102A p31^{comet} Flp-In cells were harvested, in either an asynchronous state or in a mitotic arrest (noc). These cells were lysed, and samples analysed on SDS-PAGE gels. Expected sizes of protein bands in western blots: p31^{comet} ~ 31kDa. GFP p31^{comet} ~ 60kDa.

4.4 Phosphorylation of Ser102 promotes p31^{comet} localisation to unattached kinetochores

It has been suggested that phosphorylation of p31^{comet} Ser102 weakens the binding of p31^{comet} to MAD2 and that this phosphorylation event is therefore an important aspect in the regulation of p31^{comet} during mitosis (Date et al., 2014). The Ser102 residue does not lie directly in the p31^{comet}-MAD2 binding interface; instead, it is found within a nearby flexible loop region next to residue Gln83, an important residue for MAD2 binding (Yang et al., 2007, Date et al., 2014). To test whether the phosphorylation state of p31^{comet} interferes with its localisation, the generated GFP-p31^{comet} Flp-In cell lines were used. Expression of the endogenous protein was knocked down by RNAi and expression of the GFP-tagged transgene was induced with doxycycline (2 μ M) for 24 hours. The localisation of WT, S102A and S102D GFP-p31^{comet} was examined via fixed cell imaging. An S-Trityl-L-cysteine (STLC) arrest was carried out for two hours before fixing the cells with PTEMF (12 mins). STLC is an inhibitor of the kinesin motor protein Eg5 which is responsible for the formation of bipolar spindles in mitosis. Addition of STLC (10 μ M) collapses the spindle and results in a cell that has a mixture of attached and unattached kinetochores (Figure 4.4A) (DeBonis et al., 2004). Using astrin as a marker for attached kinetochores or MAD1, a binding partner of MAD2, as a marker for unattached kinetochores, it was shown that p31^{comet} specifically localises to unattached kinetochores (Figure 4.4B-E). WT p31^{comet} did not co-localise with astrin at the attached kinetochores and there was no change in this localisation when Ser102 was mutated to alanine. MAD1 is a SAC protein involved in the SAC signalling cascade and localises specifically to

unattached kinetochores in a MAD1:C-MAD2 dimer. WT, S102A and S102D p31^{comet} transgenes all co-localised with MAD1 to unattached kinetochores, albeit to different extents. The phospho-null S102A p31^{comet} localised around 50% less strongly to unattached kinetochores than WT p31^{comet} or the phospho-mimetic S102D mutant. There was no observable difference between WT and S102D p31^{comet} localisation. This observation does not fit with the proposed model based on *in vitro* competition assays that phosphorylation of p31^{comet} weakens its binding to MAD2 (Date et al., 2014). However, *in vivo*, it is possible that other post-translational modifications, such as phosphorylation of MAD2, are also involved in regulating the p31^{comet}-C-MAD2 interaction. Understanding the interaction between p31^{comet} and C-MAD2 will require further study.

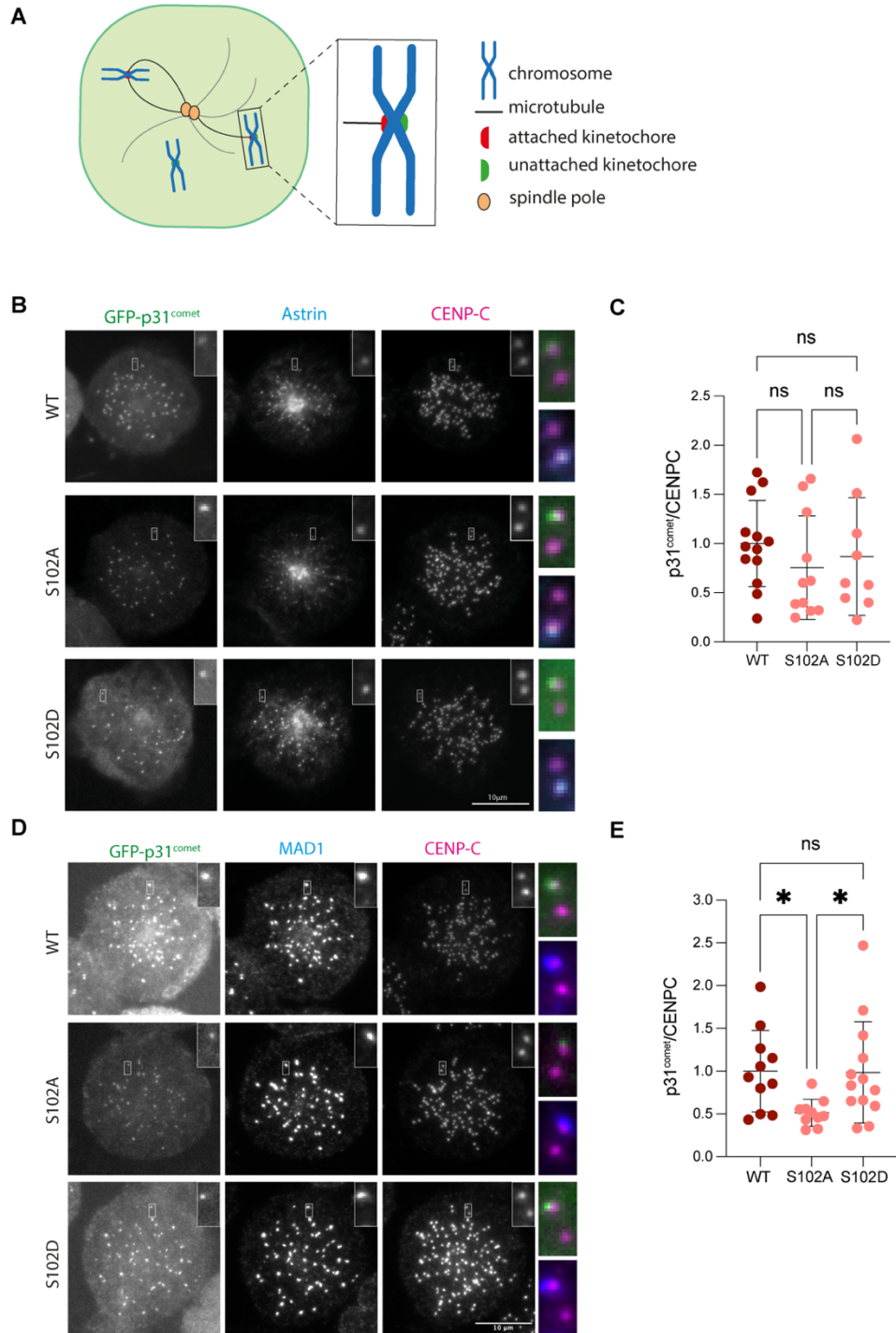


Figure 4.4: Phosphorylation of p31^{comet} promotes its localisation to unattached kinetochores.

(A) Illustration of a cell during an STLC arrest, showing a mix of kinetochore attachment statuses. (B) p31^{comet} does not localise to attached kinetochores. Astrin is used as a marker for attachment. (C) Quantification of (B). (D) Phosphorylation of p31^{comet} promotes localisation to unattached kinetochores. (E) Quantification of (D). In the colour merges in (B) and (D), GFP-p31^{comet} is shown in green, astrin or MAD1 in blue and CENP-C in magenta.

4.5 Phosphorylation of p31^{comet} Ser102 affects mitotic timings

p31^{comet} has been implicated in SAC silencing (Mo et al., 2015, Hagan et al., 2011, Varetti et al., 2011, Habu and Matsumoto, 2013, Yang et al., 2007, Teichner et al., 2011) but the biological relevance of the mitotic phosphorylation of p31^{comet} is not fully understood. To investigate the effect of p31^{comet} phosphorylation on mitotic progression, WT GFP-p31^{comet} and S102A GFP-p31^{comet} Flp-In cells were synchronised with a single thymidine (2 μ M) block, and transgene expression was induced by doxycycline (2 μ M) (Figure 4.5A). The cells were imaged live on an EVOS M7000 microscope (Thermo Fisher Scientific) going through mitosis. Images were taken at 5-minute intervals for a period of 12 hours. The time taken for the cells to proceed from nuclear envelope breakdown (NEBD) to anaphase (ANA) was measured (Figure 4.5B). Mitotic timing of WT p31^{comet} was also compared to the phospho-mimetic S102D p31^{comet} Flp-In cell line (Figure 4.5C). A control RNAi against GL2 (luciferase) was used for the WT p31^{comet} conditions in all experiments. Knockdown of the endogenous p31^{comet} by RNAi increased the time taken between NEBD and ANA (Figure 5B,C,E) which agrees with previous reports and supports the idea that p31^{comet} plays a role in checkpoint silencing (Habu et al., 2002, Hagan et al., 2011, Westhorpe et al., 2011).

S102A GFP-p31^{comet} Flp-In cells progressed through mitosis on average slightly faster than the WT counterparts (Figure 4.5B). There was no significant difference between the mitotic timings of WT and S102D p31^{comet} cells. Therefore, this data may suggest that failure to phosphorylate p31^{comet} at Ser102 improves its capability of silencing the SAC. However, it would be insightful to repeat this experiment to confirm this observation. It would also be beneficial to optimise the

expression level of the p31^{comet} transgene to more closely match the endogenous protein levels. While the expression levels of the induced p31^{comet} transgenes were well matched in the cells harvested after imaging (Figure 4.5D), they were significantly higher than that of the endogenous protein. The NEBD to ANA timing of the WT rescue conditions, in both Figure 5B and Figure 5C, was on average faster than the control uninduced conditions of the same cell line. This could be due to the fact that upon induction there was more p31^{comet} in the cells, so the SAC is more efficiently silenced, and cells progress through mitosis faster. Therefore, optimising the p31^{comet} expression levels either by a shorter incubation with doxycycline or a lower concentration could be insightful for future work.

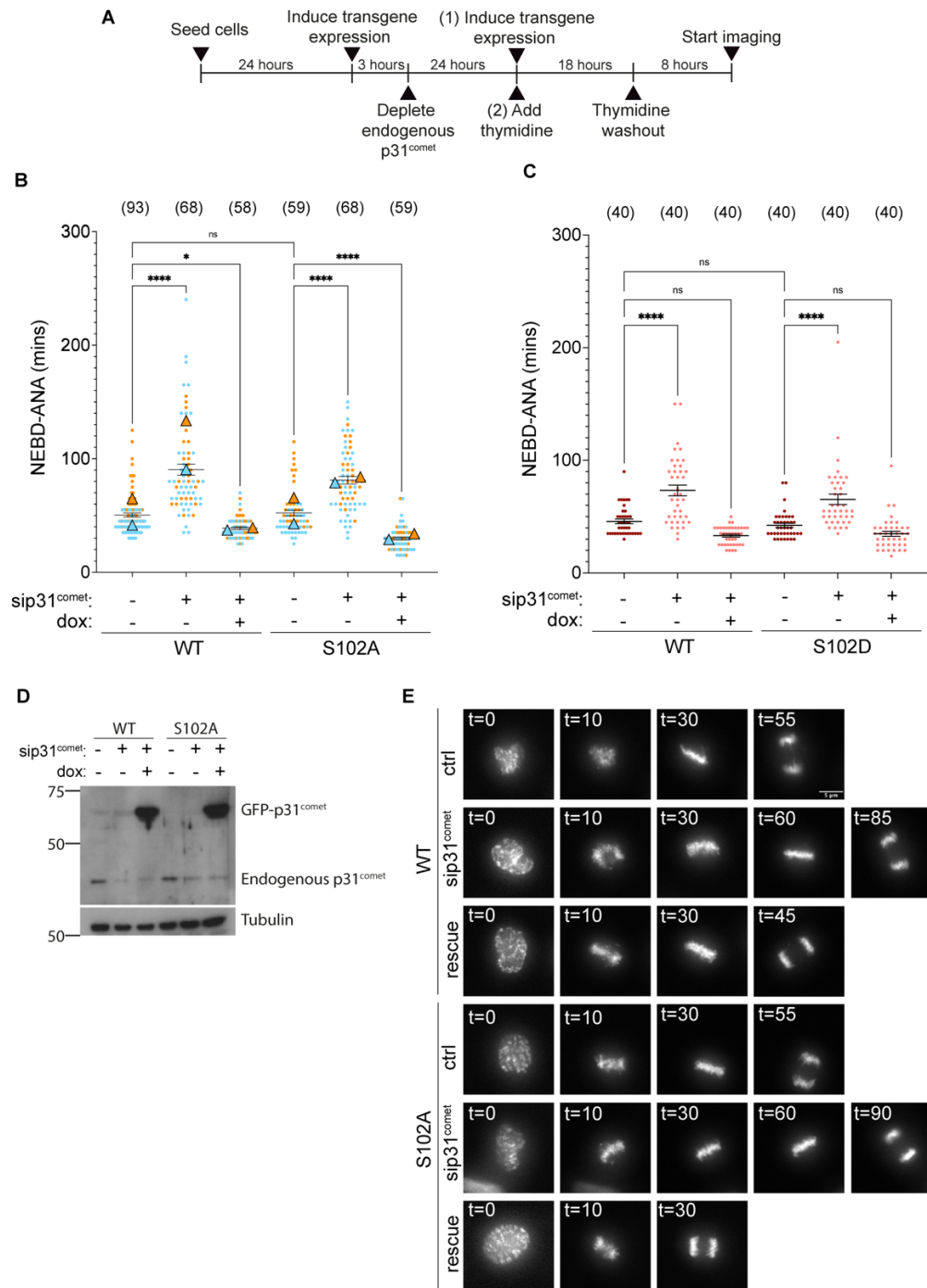


Figure 4.5: Phosphorylation of Ser102 of p31^{comet} affects mitotic timings.

(A) Illustration of the experimental set up for live cell imaging of p31^{comet} HeLa Flp-In/T-Rex cell lines. (B) Quantification of NEBD-ANA (mins) for WT vs. S102A p31^{comet} HeLa Flp-In/T-Rex cell lines. (C) Quantification of NEBD-ANA (mins) for WT vs. S102D p31^{comet} HeLa Flp-In/T-Rex cell lines. (D) Western blot against p31^{comet} using cell lysates harvested immediately after the 12-hour imaging experiment was completed. (E) Representative stills from live cell imaging using the EVOS microscope showing the metaphase-to-anaphase delay observed when endogenous p31^{comet} is knocked down through RNAi. This delay is rescued when GFP-p31^{comet} transgene expression is induced. Expected sizes of protein bands in western blots: p31^{comet} ~ 31kDa. GFP p31^{comet} ~ 60kDa.

4.6 p31^{comet} is not dephosphorylated during mitotic exit

The SAC is widely regulated by reversible phosphorylation reactions. Many checkpoint proteins are phosphorylated at the onset of mitosis by mitotic kinases (Stucke et al., 2002, Musacchio, 2015, Alfonso-Perez et al., 2019, D'Angiolella et al., 2003). These phosphorylations are then removed systematically during mitotic exit by PP1 and PP2A phosphatases (Rosenberg et al., 2011, Visintin et al., 1998). Given that p31^{comet} is phosphorylated at Ser102 during mitosis and knowing that many phosphorylations are removed upon SAC silencing to support mitotic exit, the hypothesis that the phosphorylation of Ser102 is removed as the cells exit mitosis and enter the next cell cycle was tested.

Cells can be synchronised in mitosis and biochemically induced to enter anaphase by chemically inhibiting CDK1 or MPS1 (Figure 4.6A) (Cundell et al., 2013). To test whether p31^{comet} is dephosphorylated during mitotic exit, biochemical analysis of mitotic exit was performed. HeLa cells were synchronised in mitosis using nocodazole for 18 hours and then released into media containing flavopiridol (10 μ M) (Figure 4.6B) or AZ3146 (2 μ M) (Figure 4.6C) to biochemically induce anaphase. Flavopiridol inhibits CDK1 (Losiewicz et al., 1994), while AZ3146 inhibits MPS1 (Hewitt et al., 2010). Samples were taken at 10-minute intervals over the course of 90 minutes and washed once with PBS before adding lysis buffer. These samples were run on PhosTag SDS-PAGE gels to analyse the phosphorylation state of p31^{comet}. Cyclin B degradation is used as an indicator for APC/C activation and synchronous mitotic progression. APC3/CDC27 is phosphorylated by CDK1 during mitosis and is a component of the APC/C (Zhang et al., 2016). Using Cyclin B degradation and the dephosphorylation of

APC3/CDC27 as indicators for synchronous APC/C activation and mitotic progression, the results show that p31^{comet} was not dephosphorylated under these conditions as the SAC was disassembled and cells exited mitosis. This surprising result suggests that the phosphorylation of p31^{comet} is not dynamically regulated and dephosphorylation of this residue is not essential for p31^{comet} function at the metaphase to anaphase transition of mitosis.

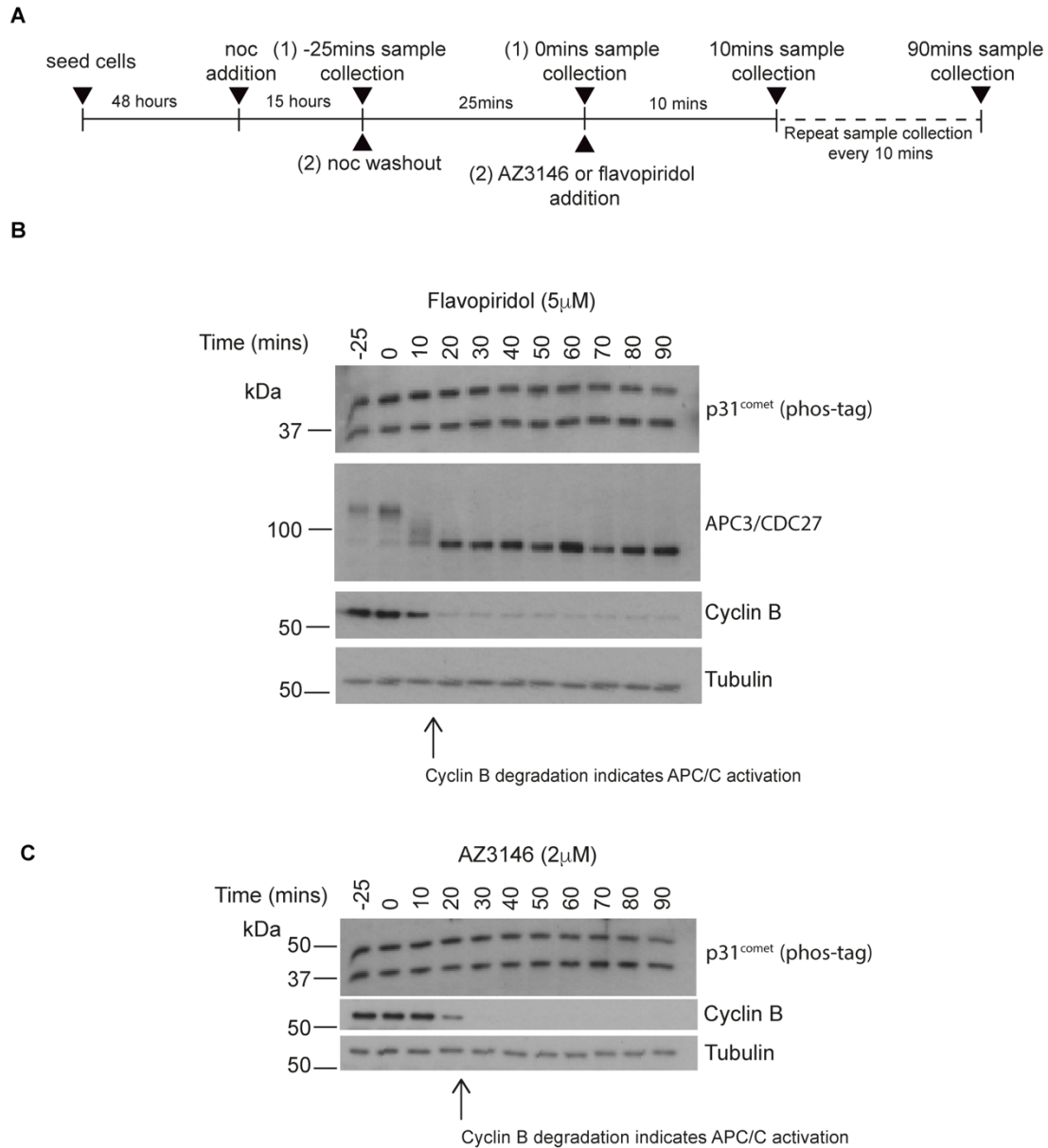


Figure 4.6: p31^{comet} is not dephosphorylated upon mitotic exit.

(A) Illustration of experimental set up for biochemical mitotic exit timecourse. (B,C) SDS-PAGE gels of mitotic exit timecourse samples. CDK1 was inhibited with Flavopiridol (5 μ M) (B) or MPS1 was inhibited with AZ3146 (2 μ M) (C). Samples of the culture were taken at the times indicated and western blotted for the indicated proteins. p31^{comet} blots are PhosTag SDS-PAGE gels. The other blots are regular SDS-PAGE gels. Expected sizes of protein bands in western blots: p31^{comet} ~ 31kDa. APC3/CDC27 ~ 90kDa. Cyclin B ~ 50kDa. Tubulin ~ 50kDa.

4.7 Discussion

The spindle assembly checkpoint (SAC) is a dynamic cell surveillance mechanism that is crucial for the correct segregation of chromosomes in mitosis. Reversible phosphorylation reactions of checkpoint proteins play an important regulatory role in both SAC activation and silencing, as well as the activation of the anaphase promoting complex/cyclosome (APC/C). The balance of CDK1-Cyclin B1 mediated phosphorylation and the dephosphorylation by protein phosphatases such as PP1, PP2A-B55 and PP2A-B56 is important to coordinate timely APC/C activation.

Recent work showed that PP1 depletion results in a delay in the metaphase to anaphase transition and that this was due to delayed activation of the APC/C (Bancroft et al., 2020). CDC20 was identified as one important substrate of PP1 involved in the activation of the APC/C. However, it is likely that PP1 has other substrates at this point in the cell cycle. p31^{comet}, shown to be mitotically phosphorylated on a consensus CDK1 site (Date et al., 2014), was a candidate PP1 substrate at the metaphase to anaphase transition as it is involved in SAC silencing and promotes MCC disassembly and hence indirectly regulates APC/C activity (Hagan et al., 2011, Habu and Matsumoto, 2013, Yang et al., 2007, Teichner et al., 2011, Westhorpe et al., 2011).

In this chapter, inducible Flp-In/T-Rex cell lines with p31^{comet} mutants were generated and it was confirmed that p31^{comet} is phosphorylated at Ser102 specifically during mitosis using PhosTag SDS-PAGE gels (Figure 4.3D). Lambda phosphatase treatment was used as a control to confirm that the upshift was due

to phosphorylation. Carrying out 2D-gel electrophoresis could also be useful for future work to test whether there are phosphorylated residues within p31^{comet} that are not captured by PhosTag gels. Preliminary live cell imaging data suggests that S102A GFP-p31^{comet} Flp-In cells proceed faster through mitosis than WT GFP-p31^{comet} Flp-In cells (Figure 4.5B). However, surprisingly, it appears that this phosphorylation is not dynamically regulated and removed during mitotic exit (Figure 6B,C) suggesting that it is unlikely to be a PP1 substrate at the metaphase to anaphase transition. Taken together, the data presented here suggests that the phosphorylation of the Ser102 residue affects mitotic timings to a small extent, but removal of the phosphorylation is not essential for anaphase entry.

In this chapter, and also previously reported (Hagan et al., 2011, Westhorpe et al., 2011), it is shown that p31^{comet} co-localises with MAD2 at unattached kinetochores. This itself is an interesting observation as MAD2 is promoting the recruitment of a protein involved in its own inactivation. It is interesting to note that p31^{comet} localises to unattached kinetochores, where the SAC is expected to be active, and not to attached kinetochores where the SAC is silenced. Given that p31^{comet} activity is presumably inhibited while at unattached kinetochores until kinetochore-microtubule attachments have formed, so as to not prematurely silence the SAC, it is likely that its behaviour is regulated in a separate way to its localisation. The identification that p31^{comet} is mitotically phosphorylated (Date et al., 2014) introduced phosphorylation as a potential method for p31^{comet} regulation. *In vitro* studies demonstrated that the p31^{comet}-MAD2 interaction is weakened by phosphorylation of p31^{comet} (Date et al., 2014). However, *in vivo* work using the inducible Flp-In/T-Rex cell lines in this chapter suggests that the

phosphorylation has the opposite effect as the non-phosphorylatable S102A mutant localises less efficiently to unattached kinetochores. PhosTag analysis of mitotic vs asynchronous cell populations show that only around half of the p31^{comet} in the cell is phosphorylated in mitosis (Figure 4.3D). It could be possible that the phosphorylation affects the binding of p31^{comet} to one, but not the other, pool of C-MAD2. Understanding the binding of p31^{comet} to C-MAD2, both the MAD1-bound pool at unattached kinetochores and the CDC20-bound pool within the MCC, and potential differences between p31^{comet} binding to these two pools will be important to examine further.

Chapter 5 - B56 recruitment to the kinetochore

5.1 Regulation of kinetochore-microtubule attachments

The formation of a bipolar mitotic spindle is an essential requirement for accurate chromosome segregation. The mitotic spindle is a complex cytoskeletal structure made up of dynamic microtubules, which undergo rapid cycles of polymerisation and depolymerisation (Kollman et al., 2011, Mitchison and Kirschner, 1984). They are asymmetric with faster growing plus ends projecting outwards from the spindle poles and slower growing minus ends. During mitosis, microtubules become attached to kinetochores in a bipolar arrangement with each kinetochore of every chromatid pair stably bound to microtubules emanating from opposing spindle poles which promotes equal sister chromatid separation in anaphase (Monda and Cheeseman, 2018, Prosser and Pelletier, 2017) (Figure 5.1A)

Association of microtubules with kinetochores is a stochastic process that functions through a 'search and capture' method (Kirschner and Mitchison, 1986). In the process, many incorrect and unstable attachments will form that need to be corrected in a process known as 'error correction' (Nicklas and Ward, 1994). Aurora B is implicated in this process. It has been suggested that Aurora B phosphorylates kinetochore proteins of the KMN network which reduces their affinity for microtubules and destabilises them so that they disassociate from the kinetochore (Welburn et al., 2010, Cheeseman et al., 2006, DeLuca et al., 2006, Wei et al., 2007). When correct attachments form, processes exist to recognise and stabilise these attachments to facilitate chromosome segregation in the later

stages of mitosis (Geraghty et al., 2021, Foley et al., 2011, Conti et al., 2019, Suijkerbuijk et al., 2012, Elowe et al., 2007, Lenart et al., 2007, Matsumura et al., 2007).

In addition to Aurora B, Polo-like kinase 1 (PLK1) as well as the PP2A-B56 are associated with the formation of stable microtubule-kinetochore attachments (Foley et al., 2011, Suijkerbuijk et al., 2012, Elowe et al., 2007, Lenart et al., 2007, Matsumura et al., 2007). Inhibition of PLK1 results in the pronounced loss of stable kinetochore fibres (Lenart et al., 2007), indicating its importance for bipolar attachment formation. Additionally, PLK1 has a role in centrosome maturation (Lane and Nigg, 1996), mitotic entry (Lobjois et al., 2009), sister chromatid cohesin during the 'prophase pathway' (Hauf et al., 2005, Sumara et al., 2002, Haarhuis et al., 2014), and cytokinesis (Petronczki et al., 2007).

Structurally, PLK1 consists of an N-terminal kinase domain that contains a T-loop residue phosphorylated by Aurora A promoting the activation of PLK1 (Macurek et al., 2008, Bruinsma et al., 2014, Seki et al., 2008). PLK1 has two polo-box domains (PBDs) that bind to phosphorylated phospho-Ser and phospho-Thr residues on target substrates which helps to recruit PLK1 to specific locations in the cell (Elia et al., 2003) (Figure 5.1C). PLK1 is only recruited once these residues on its binding partners have been phosphorylated. One such example is that of the PLK1 binding partner BUBR1, which is phosphorylated by CDK1 on Thr620 for the recruitment of PLK1 (Elowe et al., 2007). During prometaphase, PLK1 localises to kinetochores following a priming CDK1-dependent phosphorylation event on PLK1's binding proteins CENP-U and BUB1 (Chen et

al., 2021, Singh et al., 2021, Kang et al., 2006). PLK1 also associates with the SAC protein BUBR1, as mentioned above. If the interaction between PLK1 and BUBR1 is prevented, cells lose the ability to form stable K fibres (Lenart et al., 2007, Elowe et al., 2007).

BUBR1 is a SAC protein that is recruited to unattached kinetochores and incorporated into the MCC which inhibits APC/C until all kinetochores are stably bound to microtubules. BUBR1 has multiple domains, each with an important function (Figure 5.1B). The N-terminal region contains two lysine (K) – glutamate (E) – asparagine (N) (KEN) boxes which supports BUBR1 binding to CDC20 and thus interaction with and inhibition of the APC/C to prevent premature anaphase (Lara-Gonzalez et al., 2011). The N-terminus of BUBR1 also has tetratricopeptide repeats (TPRs) which are important for binding both to kinetochores and the APC/C. The Gle2-binding sequence (GLEBS) binds to BUB3 to further promote BUBR1 recruitment to kinetochores (Overlack et al., 2015). The C-terminal region of BUBR1 contains the kinetochore attachment regulatory domain (KARD). The KARD is essential for the formation of stable attachments (Suijkerbuijk et al., 2012). Deletion of this region does not affect the ability of BUBR1 to localise to kinetochores but causes the cells to be defective in forming stable kinetochore-microtubule attachments (Suijkerbuijk et al., 2012). Additionally, the phosphorylation of residues Ser670, Ser676 and Thr680 within the KARD have been suggested to be important for stable attachment formation and chromosome alignment (Elowe et al., 2007, Kruse et al., 2013, Matsumura et al., 2007, Suijkerbuijk et al., 2012). In fact, the expression of a BUBR1 phospho-null mutant or a BUBR1 truncation that lacks the KARD domain in place of the

endogenous BUBR1 gives rise to the same phenotype as a BUBR1 knock-down (Suijkerbuijk et al., 2012, Lampson and Kapoor, 2005). Phosphorylation of BUBR1 on Thr620, although not in the KARD but located adjacent to it, also plays an important role in mitosis. Phosphorylation of this residue creates a PBD docking site for PLK1 which then is primed to phosphorylate Ser670, Ser676 and Thr680 within the KARD (Elowe et al., 2007, Kruse et al., 2013).

As mentioned above, Aurora B activity has a destabilising effect on kinetochore-microtubule attachments. Importantly, the loss of BUBR1 is able to partially rescue the Aurora B inhibition phenotype, suggesting that BUBR1 functions against Aurora B to stabilise attachments (Lampson and Kapoor, 2005). The phosphatase PP2A-B56, which is recruited to kinetochores via BUBR1, has been suggested to be the phosphatase that opposes Aurora B activity (Kruse et al., 2013, Suijkerbuijk et al., 2012, Xu et al., 2013).

PP2A is involved in many aspects of the cell cycle including sister chromatid cohesion (Riedel et al., 2006, Kitajima et al., 2006), formation of kinetochore-microtubule attachments (Kruse et al., 2013, Suijkerbuijk et al., 2012) and the SAC (Espert et al., 2014, Nijenhuis et al., 2014). The phosphatase PP2A is made up of a catalytic (C) subunit, a scaffold (A) subunit and a regulatory (B) subunit (Figure 5.1D) (Janssens and Goris, 2001). Specificity of PP2A to its substrates is defined by the regulatory subunits, of which there are four groups: B (B55), B' (B56), B'' and B'''. There are five isoforms of B56 (B56 α - ϵ) which display overlapping but distinct localisations. B56 α and B56 ϵ localise predominantly at centromeres, whereas B56 γ and B56 δ are found at kinetochores and B56 β can

be seen at both (Vallardi et al., 2019). In the context of the formation of kinetochore-microtubule attachments, B56 γ and B56 δ are of most relevance. PP2A-B56 is localised to the kinetochore by interaction with BUBR1. BUBR1 has a specific short linear motif (SLiM) defined as LxxIxE which is found within the KARD of BUBR1. It has four residues that are important for the binding of B56. These are K668, L669, I672 and E674 (Wang et al., 2016b).

It is known that PLK1 is required for the formation of stable microtubule-kinetochore attachments (Lenart et al., 2007). Based on observations described above, the proposed model suggests that PLK1 phosphorylates the BUBR1 KARD which recruits PP2A-B56 that opposes Aurora B-dependent phosphorylation of the KMN (Figure 5.1E) thus stabilising attachments. However, whether the phosphorylation of BUBR1 by PLK1 promoting PP2A-B56 recruitment is the essential role of PLK1 in stabilising kinetochore-microtubule attachments has not been formally tested and it is not known whether PP2A-B56 recruitment independent mechanisms are also essential for PLK1-mediated attachment formation. PLK1 also interacts with and regulates proteins which specifically localise to attached kinetochores, such as astrin (Geraghty et al., 2021) and Clasp2 (Maia et al., 2012). The interaction between PLK1 and these proteins may be the more important contribution to kinetochore-microtubule attachment stabilisation. This chapter aims to generate novel reagents to investigate how important the role of PLK1 in recruiting PP2A-B56 is for the formation of K-fibres.

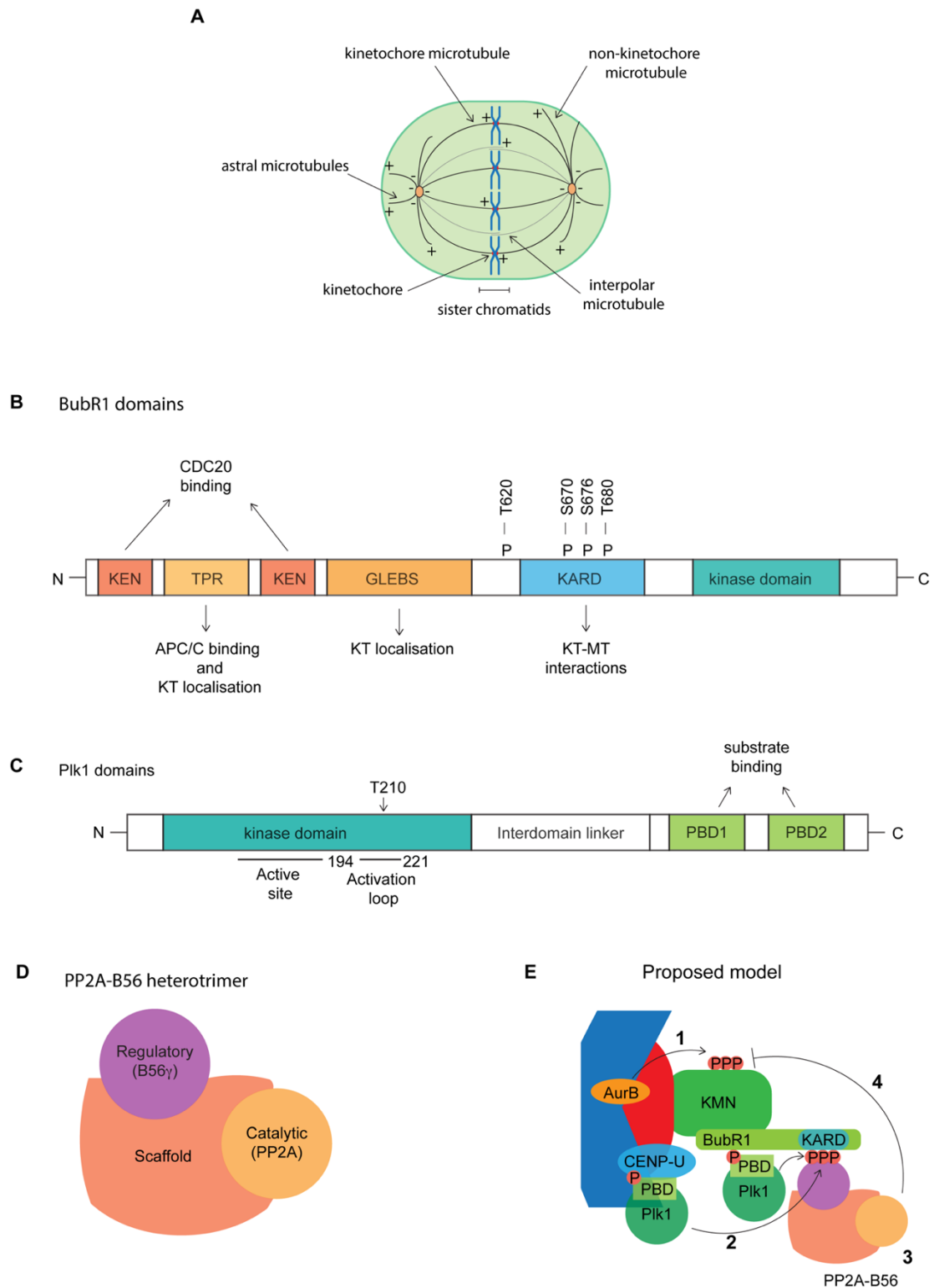


Figure 5.1: Key players in the formation of stable kinetochore-microtubule (KT-MT) attachments.

(A) The mitotic spindle and types of microtubules characterised based on their interaction with kinetochores. + indicates the microtubule plus ends, - indicates the microtubule minus ends. (B) BUBR1 domain organisation. (C) PLK1 domain organisation. (D) Heterotrimeric structure of PP2A-B56, consisting of the scaffold subunit, the catalytic PP2A subunit and the regulatory B56 γ subunit.

Figure 5.1 (Continued). (E) Proposed model of PLK1-mediated stabilisation of kinetochore-microtubule attachments. (1) Aurora B (AurB)-mediated phosphorylation of components of the KMN network destabilise KT-MT attachments. (2) PLK1 is recruited to the kinetochore via its PBD. PLK1 phosphorylates residues within the KARD domain of BUBR1. (3) PP2A-B56 is recruited to the KARD (4) PP2A-B56 opposes AurB activity on KMN components to stabilise the kinetochore-microtubule attachments.

5.2 Generating an endogenous GFP B56 CRISPR cell line

In order to investigate whether PLK1 acts to stabilise kinetochore-microtubule attachments primarily through PP2A-B56 localisation to the kinetochore via BUBR1, it is useful to be able to visualise B56 localisation within the cell. To this end, CRISPR/Cas9 technology was used to endogenously tag B56 γ in HeLa cells at the N-terminus with a fluorescent GFP tag. B56 γ is one of the isoforms of B56 that localises to unattached kinetochores and is thought to be more involved in the formation of stable kinetochore-microtubule attachments than the isoforms that localise to the centromeres. Western blot analysis (Figure 5.2A) confirmed successful integration of the GFP tag onto endogenous B56 γ as shown by the upshift in protein size. The cell lines were also screened by fixed cell immunofluorescence (Figure 5.2B). The cells were fixed with PTEMF for 12 minutes and then stained as indicated. This showed that GFP-B56 γ co-localised with its binding partner BUBR1 to kinetochores, suggesting that addition of the GFP tag via CRISPR/Cas9 technology did not interfere with the ability of B56 γ to localise normally to unattached kinetochores (Figure 5.2B).

To further confirm that CRISPR tagging B56 γ did not affect its mitotic function, GFP-B56 γ clone #D3, which was shown to be homozygous (Figure 5.2A), was imaged in live on the SoRa microscope. Images were taken at 3-minute intervals.

In line with previous reports (Vallardi et al., 2019, Suijkerbuijk et al., 2012, Kruse et al., 2013), B56 γ rapidly localised to kinetochores following NEBD as shown by the punctate GFP-B56 γ signal (Figure 5.2C). The punctate signal remained throughout prometaphase, gradually diminishing in intensity as chromosomes moved towards alignment. As the cells entered ANA, GFP-B56 γ was not detectable. The cells presented normal mitotic behaviour. Importantly, chromosome segregation occurred with no visible errors suggesting that the GFP CRISPR tag did not interfere with B56 γ 's role in formation of stable KT-MT attachments.

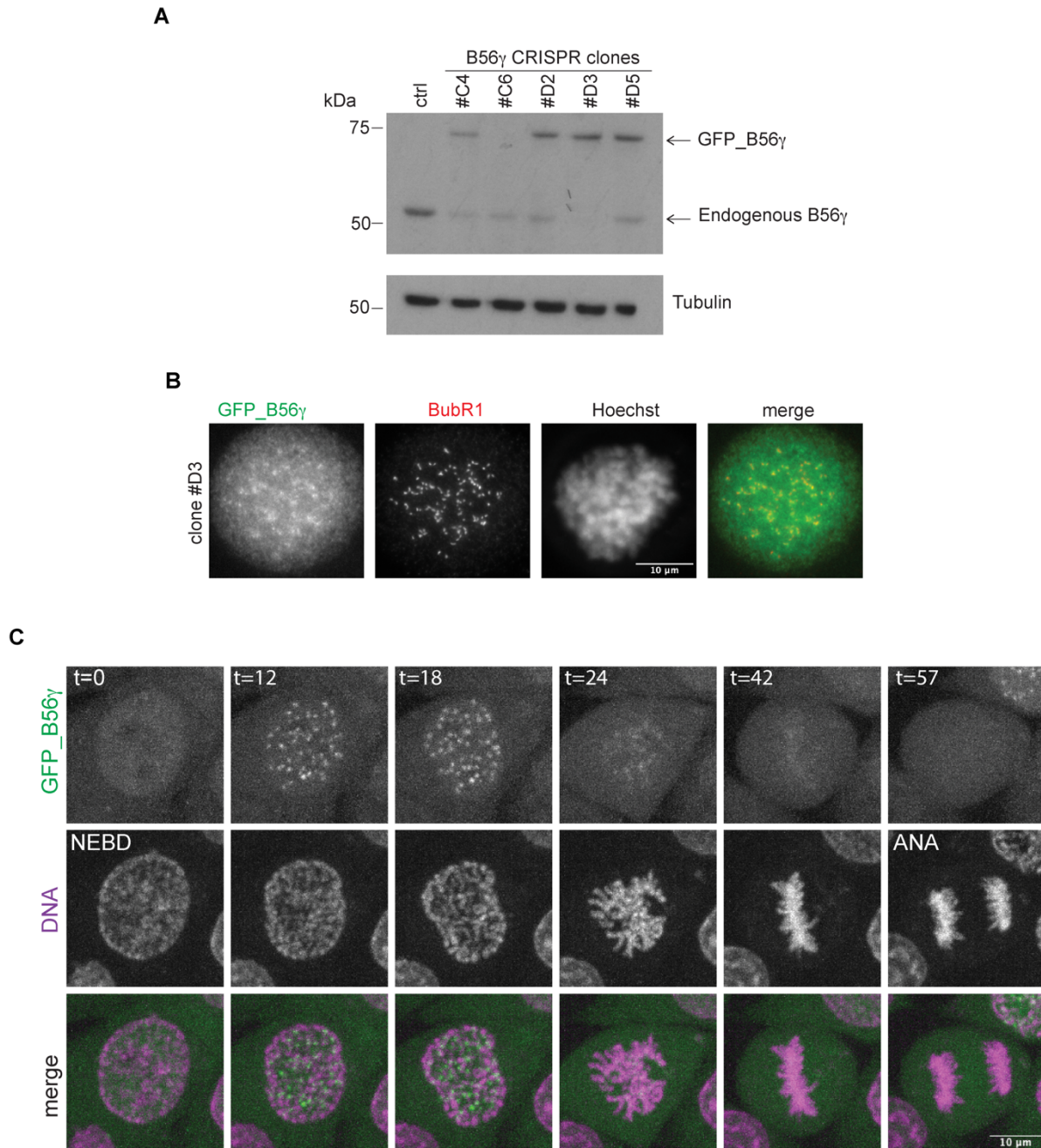


Figure 5.2: Generation of HeLa GFP-B56 γ CRISPR cell lines.

(A) Western blot screen confirms successful CRISPR tagging of B56 γ . (B) GFP-B56 γ HeLa CRISPR cells were fixed with PTEMF for 12 minutes and stained for BUBR1 and Hoechst. (C) GFP-B56 γ CRISPR cells (clone #D3) were imaged on the SoRa microscope, using siR-DNA (50nM) to visualise the DNA. Images were taken at 3 min intervals. Expected sizes of protein bands in western blots: B56 γ ~ 50kDa. GFP_B56 γ ~ 75kDa. Tubulin ~ 50kDa.

5.3 PLK1 activity at the G₂/M transition is required for PP2A-B56 localisation to unattached kinetochores

PP2A-B56 localisation to kinetochores in a PLK1-mediated manner has been implicated in the stabilisation of microtubule-kinetochore attachments (Suijkerbuijk et al., 2012, Foley et al., 2011). Using the B56γ CRISPR/Cas9 HeLa cells, the role of PLK1 activity for B56γ localisation was investigated.

To facilitate PLK1 inhibition in early mitosis, before stable kinetochore-microtubule attachments have had the time to form, cells were first pre-synchronised using the CDK1 inhibitor, RO-3306. RO-3306 is a cell-permeable and reversible potent CDK1 inhibitor that arrests cells at the G₂/M transition (Vassilev et al., 2006). Following an 18-hour incubation with RO-3306 (9μM), the RO-3306 was washed out and cells were released into media containing nocodazole (noc, 0.3μM), with or without the PLK1 inhibitor BI2536 (1μM) (Steegmaier et al., 2007) for 2 hours (Figure 5.3A). The addition of nocodazole, a microtubule depolymerising agent, results in a mitotic arrest with no microtubule-kinetochore attachments as all microtubules had depolymerised due to nocodazole treatment. This means that the role of PLK1 in localising B56γ specifically to unattached kinetochores at the start of mitosis can be tested. Following a 2-hour incubation with BI2536, after the RO-3306 washout, cells were fixed with PTEMF for 12 minutes and stained as indicated (Figure 5.3B). In the control condition where the cells were synchronised with RO-3306 but not treated with BI2536, B56γ co-localised with BUBR1 to kinetochores. In contrast, in the presence of BI2536, B56γ was not able to localise to unattached kinetochores as

efficiently (Figure 5.3B,C). This suggests that PLK1 kinase activity in early mitosis is essential for B56 γ localisation to unattached kinetochores.

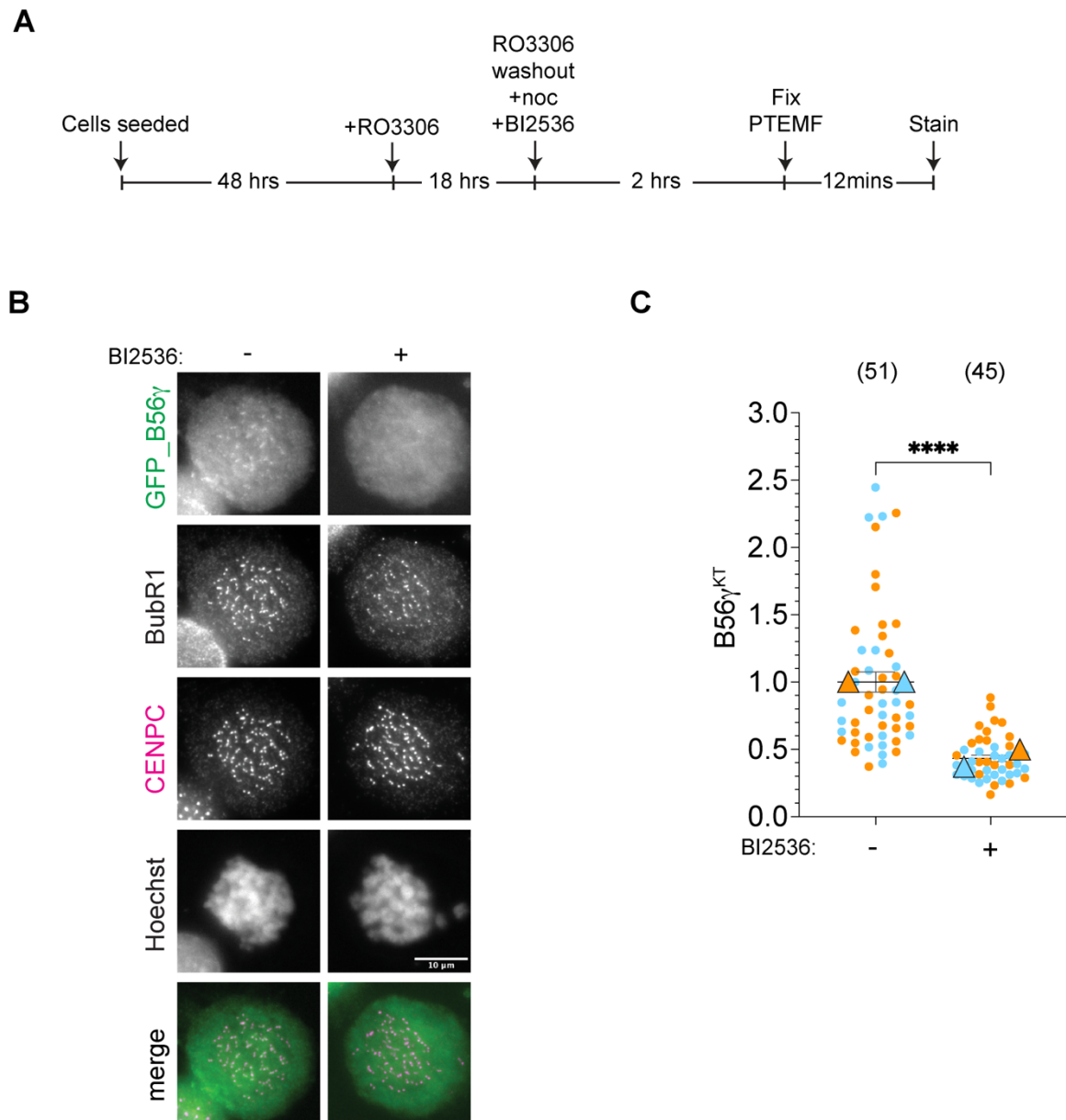


Figure 5.3: Inhibition of PLK1 at G₂ reduces B56 γ levels at unattached kinetochores.

(A) Schematic depicting the cell synchronisation protocol and experimental timeline. noc = nocodazole, 0.3 μ M. RO-3306 = CDK1 inhibitor, 9 μ M. BI2536 = PLK1 inhibitor, 1 μ M. (B) Representative immunofluorescent images of GFP-B56 γ CRISPR cells treated with either DMSO (-) or BI2536 (+, 1 μ M) upon washout of RO-3306. Cells were fixed with PTEMF and stained for BUBR1 and CENP-C. (C) Quantification of (B). 10 kinetochores per cell, normalised to CENP-C. Bars indicate the SEM (each point represents a single cell average intensity of 10 kinetochores, data merged from 2 independent experiments presented as different colours).

The maintenance of B56γ at unattached kinetochores during mitosis was also tested. B56γ CRISPR/Cas9 cells were not pre-synchronised as they were previously; instead, they were subjected to a 2-hour nocodazole arrest before fixing with PTEMF (Figure 5.4A). At 15, 30 or 60 minutes prior to fixing, the cells were treated with BI2536 (1μM), and then stained as indicated (Figure 5.4B). Interestingly, there is only a subtle decrease in B56γ signal at unattached kinetochores (Figure 5.4C), suggesting that once at kinetochores B56γ localisation becomes largely independent of PLK1 activity.

Taken together, the analysis presented in Figures 5.3 and 5.4 suggests that initial B56γ recruitment to unattached kinetochores requires PLK1 activity. However, once B56γ has localised to kinetochores, PLK1 activity is not essential for it to remain there.

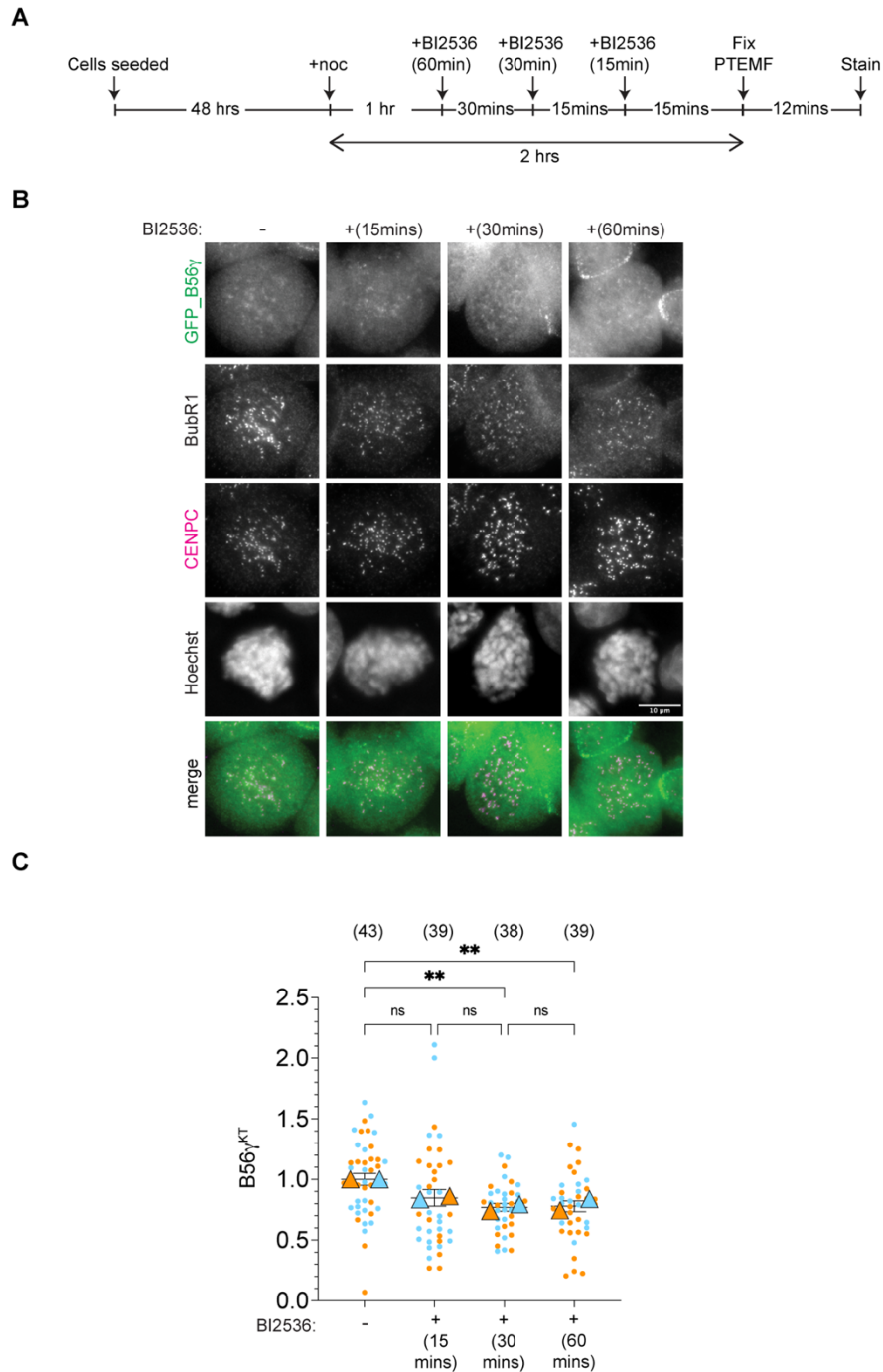


Figure 5.4: Inhibition of PLK1 during mitosis does not have a drastic effect on B56γ localisation to kinetochores.

(A) Schematic depicting the cell synchronisation protocol and experimental timeline. (B) Representative immunofluorescent images of GFP-B56γ CRISPR cells treated with either DMSO (-) or BI2536 (+, 1μM) for 15, 30 or 60 mins. Cells were fixed with PTEMF and stained for BUBR1 and CENP-C. (C) Quantification of (B). 10 kinetochores per cell, normalised to CENP-C. Bars indicate the SEM (each point represents a single cell average intensity of 10 kinetochores, data merged from 2 independent experiments presented as different colours).

5.4 Co-localisation of PP2A-B56 and BUBR1 throughout mitosis

PLK1 phosphorylation of BUBR1 has been suggested as a crucial step in the recruitment of PP2A-B56 to unattached kinetochores, which in turn promotes the stabilisation of kinetochore-microtubule attachments. To investigate the relationship between PP2A-B56 and BUBR1, the HeLa T-Rex/Flp-In system (Invitrogen (Tighe et al., 2008)) was utilised to generate cell lines with inducible expression of BUBR1.

First, CRISPR/Cas9 mediated knock-in technology was utilised to tag endogenous B56 γ in parental HeLa T-Rex/Flp-In cells. Successful tagging was confirmed by western blot (Figure 5.5A). Following this, integration of an siRNA resistant mScarlet-BUBR1 transgene was carried out in the B56 γ CRISPR HeLa T-Rex Flp-In cell line. In these cells, the expression of mScarlet-BUBR transgene could be induced upon the addition of small amounts of doxycycline (2 μ M, 48hours) (Figure 5.5B).

High resolution live cell imaging was carried out with the generated inducible WT BUBR1 cell line that had endogenous B56 γ CRISPR tagged with GFP. Cells were pre-synchronised with thymidine (2mM, 18 hours). 7 hours after a thymidine washout, cells were transferred to the SoRa microscope and imaged every 5 minutes. SiR-DNA (50nM) was added to the cells to facilitate visualisation of the DNA. It has previously been reported that B56 γ recruitment to kinetochores is promoted by the interaction of B56 γ and BUBR1. Interestingly, in this imaging

experiment, punctate B56γ localisation is visible before that of WT BUBR1 in early mitosis soon after the nuclear envelope begins to break down. This may suggest that initial B56γ is not dependent on BUBR1 at the kinetochore to bring about its localisation. However, as endogenous BUBR1 was not depleted in this imaging experiment, another possible explanation is that endogenous BUBR1 is localised prior to being able to see a punctate mScarlet-BUBR1 signal, and therefore is recruiting B56γ. It will be important to confirm that depletion of BUBR1 results in a loss of B56 levels at kinetochores in future experiments.

To assess whether the induction of the WT mScarlet-BUBR1 transgene altered the mitotic timings of the cells compared to an uninduced condition, time spent in mitosis was quantified (Figures 5.5D-F). There was no significant difference in the timings between NEBD to META or from META to ANA, suggesting that the expression of the WT BUBR1 transgene does not affect normal mitosis, at least in the situation where the endogenous has not been depleted.

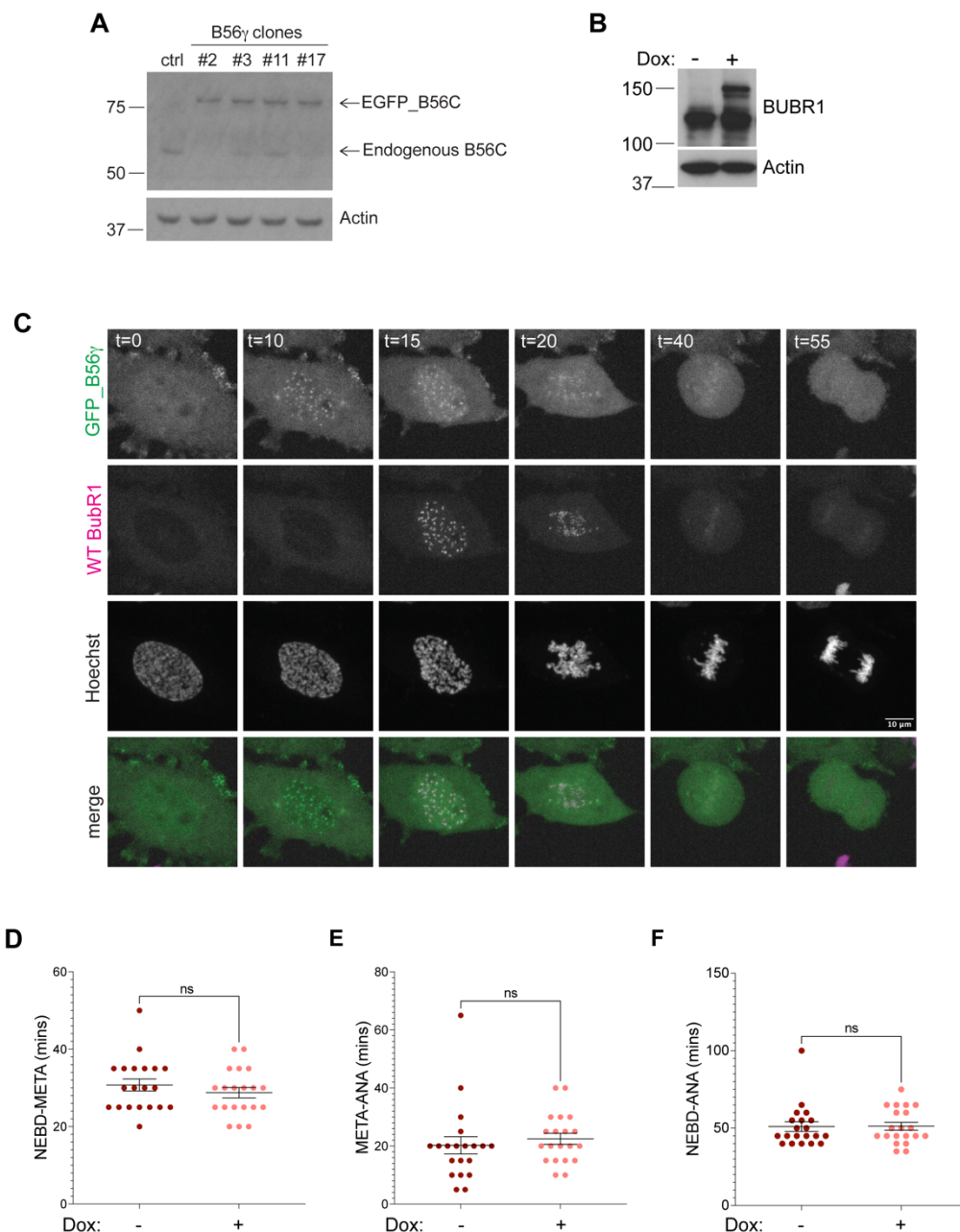


Figure 5.5: Co-localisation of PP2A-B56 γ and BUBR1 throughout mitosis in inducible BUBR1 Flp-In cells.

(A) Western blot screen confirms successful CRISPR tagging of B56 γ in the parental HeLa T-Rex Flp-In cell line. (B) Western blot to show the expression of BUBR1 transgene upon the addition of doxycycline (dox, 48 hours, 2 μ M). (C) GFP- B56 γ CRISPR cells with inducible WT BUBR1 were imaged, using siR-DNA to visualise the DNA on the SoRa microscope. Images were taken at 3min intervals. Expression of WT BUBR1 was induced by the addition of doxycycline (dox, 2 μ M). Representative images of the induced (+) condition shown. (D,E,F) Quantification of mitotic timings for uninduced (-) and induced (+). NEBD=nuclear envelope breakdown. META=metaphase. ANA=anaphase. (D) NEBD-META (E) META-ANA (F) NEBD-ANA. Expected sizes of protein bands in western blots: B56 γ ~ 50kDa. GFP_B56 γ ~ 75kDa. BUBR1 ~ 120kDa..

5.5 Discussion

PLK1 is known to be important for the formation of stable kinetochore-microtubule attachments. Previous work has led to a potential model in which PLK1 phosphorylates the BUBR1 KARD to recruit PP2A-B56 (Elowe et al., 2007, Suijkerbuijk et al., 2012). Once at kinetochores, PP2A-B56 antagonises Aurora B-mediated destabilising events at the kinetochore-microtubule interface which promotes the stabilisation of a bipolar mitotic spindle in preparation for anaphase. Indeed, studies in which B56 is depleted or prevented from localising to kinetochores demonstrate that loss of B56 from kinetochores leads to a loss of K-fibres and defects in chromosome alignment, but can be rescued by Aurora B inhibition (Foley et al., 2011, Suijkerbuijk et al., 2012, Xu et al., 2013, Kruse et al., 2013).

An outstanding question, however, is whether PLK1 regulates kinetochore-microtubule attachments through PP2A-B56 independent pathways that make a greater contribution to their stabilisation. The experiments presented in this chapter focus on the generation of critical reagents and preliminary experiments. to further address this question. Using HeLa GFP-B56 γ CRISPR cell lines, the localisation of B56 γ to unattached kinetochores was examined in the presence of a PLK1 inhibitor. As expected, initial recruitment of B56 γ to unattached kinetochores at the onset of mitosis requires PLK1 kinase activity (Kruse et al., 2013, Suijkerbuijk et al., 2012, Xu et al., 2013) (Figure 5.3). However, surprisingly, once B56 γ had localised, PLK1 activity became dispensable to the maintenance of B56 γ at kinetochores (Figure 5.4).

Live cell imaging of the HeLa GFP-B56 γ CRISPR cell lines (Figure 5.2C) as well as WT mScarlet-BUBR1 HeLa T-Rex/Flp-In cells (Figure 5.5C) demonstrated that B56 γ localised rapidly to kinetochores after NEBD and co-localised with BUBR1. Due to time constraints with this project, functional experiments with the WT BUBR1 Flp-In cell line were not possible. In addition to WT BUBR1, Flp-In cell lines in which the BUBR1 transgenes contain mutations that affect the level of B56 binding affinity, termed 'LIAA' and 'superKARD' will be valuable for the future steps in this project. BUBR1 LIAA has two residues of its SLiM within the KARD, L669 and I672, mutated to alanine. This mutation has been shown to eliminate B56 (Kruse et al., 2013, Espert et al., 2014). In contrast, the SuperKARD peptide, identified as the PP2A-B56 binding region of nucleoprotein in the Ebola virus, has a high B56 binding affinity that does not rely on phosphorylation (Kruse et al., 2018). Therefore, BUBR1 superKARD should be able to constitutively recruit B56 regardless of PLK1 activity. Using RNAi rescue assays and through mutations in the inducible BUBR1 transgenes influencing B56 binding affinity (SuperKARD (high affinity) and LIAA (low affinity)) as well as a T620A mutation that disrupts the PLK1-BUBR1 interaction, the formation of kinetochore-microtubule attachments can be investigated. For example, it will be interesting to examine whether or not constitutive B56 recruitment to the kinetochore using the BUBR1 SuperKARD peptide can rescue stable kinetochore-microtubule attachment formation, independent of PLK1 activity or BUBR1-bound PLK1. The ability to visualise B56 γ in these LIAA and SuperKARD BUBR1 Flp-In cell lines will be important to use as a readout for how well they are able to recruit B56 γ . If the SuperKARD is not able to rescue stable kinetochore-microtubule attachments when PLK1 function at the kinetochore is disrupted, this may point towards there

being additional PLK-mediated mechanisms required for generating stable kinetochore-microtubule attachments other than recruitment of B56. Other PLK1 targets such as astrin (Geraghty et al., 2021) and Clasp2 (Maia et al., 2012) are also thought to contribute to the stable kinetochore-microtubule attachment formation. Further insight into which of these PLK1 interactions are most critical for attachment stabilisation may shed light on the link between PLK1 misregulation and cancer development (Lee et al., 2014).

Chapter 6: Conclusions

Mitosis is the part of the cell cycle when the cell's replicated DNA is equally segregated into two new daughter cells. In order to preserve genomic integrity and maintain the correct number of chromosomes in the progeny cells, mitosis is tightly controlled by ordered phosphorylation, dephosphorylation and proteolysis events. The Ser/Thr-directed phosphatases PP1 and PP2A are implicated in several aspects of mitotic regulation including the spindle assembly checkpoint (SAC), activation of the anaphase promoting complex/cyclosome (APC/C) and stabilisation of kinetochore-microtubule attachments. However, their roles are not fully understood.

Many advancements in our understanding of the cell cycle have come from work carried out in yeast. Indeed, the discovery of CDK genes in budding and fission yeast was a landmark discovery that has laid the foundation of subsequent research into cell cycle regulation (Hartwell et al., 1973, Nurse et al., 1976, Hartwell et al., 1974). Furthermore, the existence of cell cycle checkpoints was first shown in yeast in which cells arrested in response to faulty DNA replication (Hartwell and Weinert, 1989, Weinert and Hartwell, 1988). However, it has become apparent since these groundbreaking discoveries that the wiring of phospho-regulation is very different between yeast and mammalian cells. For example, fission yeast only contains one CDK whereas mammalian cells contain several. Additionally, it has been shown that the dual-specificity phosphatase Cdc14 is critical for mitotic exit in budding yeast but not for human cells (Berdougo et al., 2008). Therefore, it is not possible to directly extrapolate data from yeast

to human studies and experiments should also be conducted in mammalian systems to examine the roles of kinases and phosphatases in higher eukaryotes.

In this thesis, PP1 degron tag (dTag) systems were established in both HeLa and HCT116 cells to facilitate the rapid degradation of target PP1 catalytic subunits. In this way, possible side effects of DNA damage in the absence of PP1 can be minimised and the mitotic-specific role of PP1 can be investigated. Characterisation of the dTag cell lines (Chapter 2) demonstrated that the degradation efficiency of PP1 was high. However, it is a possibility that some phosphatase activity may persist if the degradation of all target PP1 in the cell was incomplete. In addition to the dTag system, there are other targeted protein degradation systems such as the HaloTag. While also recruiting the VHL E3 ligase, it could perhaps provide a more complete removal of all of the PP1 catalytic subunits and lead to an even greater phenotype. HaloTag technology also provides additional applications as well as targeted proteasomal degradation, such as the study of protein-protein interactions and protein localisation (England et al., 2015) which could be useful for further study of PP1.

The dTag system was shown to be efficient at acutely degrading the target protein. In line with previous data (Bancroft et al., 2020), loss of PP1 resulted in metaphase to anaphase delays, with the delay being more prolonged in the absence of both PP1 α and PP1 γ simultaneously. Strikingly, a chromosome congression defect with increased frequency of polar chromosomes was only observed when both PP1 α and PP1 γ were degraded (Chapter 3.12). CENP-E was shown to be mislocalised in these cells (Chapter 3.13). Looking beyond this

thesis, it will be important to further investigate the role of phospho-regulation on CENP-E to build a complete picture of its PP1-mediated regulation and provide insight into chromosome congression mechanisms. The ability to visualise CENP-E in live cell experiments will provide an avenue for precisely analysing its dynamic localisation pattern to better understand how PP1 influences its localisation during mitosis and how this functions alongside the Aurora-mediated activation of CENP-E (Kim et al., 2010).

Using the cell lines generated in this thesis, it will be important to further investigate the isoform specific functions of PP1 in mitosis. Generating a PP1 β dTag cell line could prove useful to confirm that, in an acute degradation system, PP1 β does indeed not affect mitotic timing, as has previously been reported using RNAi studies (Bancroft et al., 2020). Further comparison between HeLa and HCT116 cells could also prove insightful. As many solid tumours in cancer are aneuploid (Replogle et al., 2020, Lukow et al., 2021), identifying differences in mitotic regulation could support the development of cancer treatments. In the future, it would also be informative to investigate PP1's role in mitosis in a stable diploid non-transformed cell line, such as hTERT-RPE1 cells, and compare this with the role of PP1 in HeLa and HCT116 cells which are both transformed.

Previous data from our lab identified CDC20 as a PP1 target at the metaphase to anaphase transition, but it is likely that there are other yet unidentified targets (Bancroft et al., 2020). In this thesis, the SAC protein p31^{comet}, which has a canonical CDK1 site, was examined as a possible PP1 target during mitotic exit. However, surprisingly, although it is a mitotically phosphorylated protein (Chapter

4.3 and also reported previously (Date et al., 2014)) it was not dephosphorylated upon mitotic exit (Chapter 4.6). It would be interesting to understand when this phosphorylation event is turned over. As p31^{comet} is seemingly not phosphorylated significantly in an asynchronous population of cells and does not seem to be turned over at the protein level, the phosphorylation would be expected to be reversed at some point. Other proteins that are known to be involved in regulating SAC silencing, such as TRIP13, and APC/C activity, such as the ubiquitin conjugating enzyme UBE2S, also contain canonical CDK1 sites that may be subject to dynamic regulation by PP1 during mitosis and worth examining. Identifying additional PP1 targets at this stage of the cell cycle that contribute to mitotic regulation will be an important advancement in understanding the phospho-regulation of mitosis.

It has been suggested that PLK1 stabilises kinetochore-microtubule attachments by phosphorylating BUBR1, thereby facilitating the recruitment of PP2A-B56 (Elowe et al., 2007, Kruse et al., 2013, Foley et al., 2011, Suijkerbuijk et al., 2012). However, whether the phosphorylation of BUBR1 by PLK1 promoting PP2A-B56 recruitment is the essential role of PLK1 in stabilising these attachments has not been formally tested. Additionally, it remains unclear if mechanisms independent of PP2A-B56 recruitment also play a crucial role in PLK1-mediated attachment formation. Work presented in this thesis focused on the generation of reagents that will be valuable for investigating this further. Using the generated WT BUBR1 Flp-In cell line (Chapter 5) along with mutant BUBR1 transgenes with altered B56 binding affinities, a deeper understanding of the PLK1 interactions most critical for stabilizing kinetochore-microtubule attachments can be achieved.

In conclusion, this thesis has explored the role of phosphatases in the regulation of mitosis using the dTag system. This work and the tools generated has provided the foundation for subsequent studies exploring the dynamic regulation of chromosome congression mechanisms, stable kinetochore-microtubule attachments and a timely metaphase to anaphase transition.

Chapter 7: Materials and Methods

7.1 General Reagents

General laboratory chemicals and reagents were purchased from Sigma-Aldrich and Thermo Fisher Scientific, unless otherwise stated. All drugs were resuspended in Dimethyl sulfoxide (DMSO) purchased from Merck (Cat#276855) unless otherwise stated. All drugs used in this study are listed in the table below.

Table 1: List of drugs and inhibitors

Drug	Use	Supplier	Cat #	Working conc.
AZ3146	MPS1 kinase inhibitor	Tocris Bioscience	3994	2 μ M
5-iodotubercidin	Haspin inhibitor	Tocris Bioscience	1745	5M
BI2536	PLK1 inhibitor	Tocris Bioscience	17351	1 μ M
Calyculin A	Protein phosphatase inhibitor	Tocris Bioscience	1336	50nM
Doxycycline	Tetracycline class antibiotic	Insight	SC-204743	2 μ M
dTag1	FKBP12 ^{F36V} degrader	Tocris Bioscience	6914	Variable (usually 100nM)
dTag13	FKBP12 ^{F36V} degrader	Tocris Bioscience	6605	Variable (usually 500nM)
Flavopiridol	CDK1 kinase inhibitor	Tocris Bioscience	3094	5 μ M
GSK923295	CENP-E inhibitor	Cambridge bioscience	CAY18389	50nM
Hoechst 33342	Blue light emitting DNA dye	ThermoFisher	62249	16.2 μ M
MG132	Proteasome inhibitor	Insight	Sc201270	20 μ M

Monastrol	Kinesin Eg5 inhibitor	Tocris Bioscience	1305	100µM
Nocodazole	Microtubule depolymerising agent	Merck Chemicals Ltd	484728	330nM
Okadaic acid	S/T protein phosphatase inhibitor	Tocris Bioscience	1136	100nM
proTAME	APC/C inhibitor	MedChemExpress	HY-124955	10µM
RO-3306	CDK inhibitor	Insight	SC358700	9µM
S-trityl-L-cysteine (STLC)	Eg5 inhibitor	Sigma-Aldrich	164739	10µM
SiR-DNA 555	DNA dye for live cell imaging	Spirochrome	CY-SC201	50nM
SiR-DNA 652	DNA dye for live cell imaging	Spirochrome	CY-SC007	50nM
SiR-Tubulin 652	Tubulin dye for live cell imaging	Spirochrome	CY-SC002	50nM
Thymidine	For S-phase arrest	Millipore	6060	2mM (stock resuspended in water)
ZM447439	Aurora B inhibitor	Millipore	189410	10µM

For transfection of siRNA oligos, oligofectamine (Invitrogen) was used according to manufacturer's instructions. TransIT-LT1 (Mirus-Bio) or TransIT-X2 (Mirus-Bio) were used following manufacturer's instructions for transfection of plasmids. TransIT-LT1 was used for HeLa and HCT116 cells. Opti-MEM (Gibco) was used for making up the transfection mix. siRNA oligos used in this study are listed in Table 2.

Table 2: siRNA oligos.

siRNA target	Oligonucleotide sequence (5'-3')	Part of transcript targeted	RNAi duration	Source
GL2 (luciferase)	CGUACGCGGAUACUUCGAUU	ORF	variable	Dharmacon, (#D-001100-01-20)
PPP2R5A	On-target plus Smartpool siRNA oligos against PPP2R5A (5'-GCUCAAAGAUGCCACUUCA-3', 5'-CAAUACAAGUGCCGAAUAA-3', 5'-UGAAUGAACUGGUUGAGUA-3', 5'-GGAAAUGAAUGGCAAGCUU-3')	UTRs and ORF	48hr	Dharmacon , Cat#L-009352
PPP2R5B	On-target plus Smartpool siRNA oligos against PPP2R5B (5'-CGCAUGAUCUCAGUGAAUA-3', 5'-UCAAGUCGCUGUCUGUCUU-3', 5'-CAAACCAUCGUAUCACUGA-3', 5'-GAACAAUGAGUAUAUCCUA-3')	UTRs and ORF	48hr	Dharmacon, Cat#L-009366
PPP2R5C	On-target plus Smartpool siRNA oligos against PPP2R5C (5'-GGAUUUGCCUUACCACUAA-3', 5'-GGAAGAUGAACCAACGUUA-3', 5'-CAUCAGAAUUUGUGAAGAU-3', 5'-CAGAAGUAGUCCAUAUGUU-3')	UTRs and ORF	48hr	Dharmacon, Cat#L-009433
PPP2R5D	On-target plus Smartpool siRNA oligos against PPP2R5D (5'-GUACAUCGACCAGAAGUUU-3', 5'-UCCAUGGACUGAUCUAUAA-3', 5'-UGACUGAGCCGGUAAUUGU-3', 5'-GUAGGCAGAUAACCAU-3')	UTRs and ORF	48hr	Dharmacon, Cat#L-009799

PPP2R5E	On-target plus Smartpool siRNA oligos against PPP2R5E (5'-UUA AUGAACUGGUGGACUA-3', 5'-GCACAGCUGGCAUAUUGUA-3', 5'-GACACGCUAUCUGAUCUUA-3', 5'-GGAUAAAGUAGACGGAUUU-3')	UTRs and ORF	48hr	Dharmacon, Cat#L-008531
p31^{comet} (MAD2L1BP)	CUGUAAUCAUCGCUGAACAUU	3'-UTR	48hr	Dharmacon, custom oligo
Nuf2	GCAUGCCGUGAAACGUAUAUU	ORF	48hr	Dharmacon, custom oligo

PPP2R5 = B56

7.2 Antibodies

Primary antibodies used in this thesis are detailed in the Table 3.

IF = immunofluorescence. WB = western blotting.

Table 3: Primary antibody details.

Target	Species	Stock concentration	Dilution of stock		Source
			IF	WB	
p31 ^{comet} (MAD2L1BP)	Mouse	1mg/mL	1:2000	1:2000	Sigma, E29.19.14, MABE451
Cyclin B	Mouse	1mg/mL	/	1:2000	Sigma, 8A5D12, 05-373
Tubulin (DM1A)	Mouse	1mg/mL	/	1:1000	Sigma, T6199
Tubulin	Rabbit	8µg/mL	1:2000	/	Cell signalling, 2125S
CENP-C	Guinea Pig	1mg/mL	1:2000	/	MBL, PD030
MAD1	Rabbit	1mg/mL	1:2000	/	GeneTex, GTX105079
PPP1CA	Rabbit	1mg/mL	/	1:1000	Bethyl, A300- 904A

PPP1CC	Goat	0.2µg/mL	/	1:1000	Santa Cruz Biotechnology, sc- 6108
β-Actin (conjugated to HRP)	Mouse	0.2µg/ml	/	1:2000	Santa Cruz, sc- 47778
Histone H3 pThr3	Rabbit	/	1:40,000	/	Cell signalling, 9714
CENP-E	Mouse	1mg/mL	1:1000	/	Abcam, ab5093
BUBR1	Rabbit	1mg/mL	1:5000	/	Bethyl, A300- 386A
CDC27/APC3	Mouse	0.2mg/mL	/	1:1000	Santa Cruz, sc13154
PPP2R5C	Mouse	0.2mg/mL	/	1:1000	Santa cruz, E6, sc- 374380

7.3 Molecular Biology

All DNA primers were obtained from Invitrogen. Mammalian expression constructs for T-REx doxycycline-inducible Flp-In cells were made in pcDNA5/FRT/TO vectors modified to encode EGFP or mScarlet. Mutagenesis of pcDNA/FRT/TO vectors was carried out using the mutagenesis primers listed in Table 4 and the QuickChange method (Agilent Technologies).

Table 4: Mutagenesis primers.

TARGET	FW OR RV	DNA SEQUENCE (5'-3')
p31^{COMET} S102A	FW	TTTTACCGAAAACCTGCTCCCCAGGCAGAGGA
p31^{COMET} S102A	RV	TCCTCTGCCTGGGGAGCAGGTTTTCGGTAAAA
p31^{COMET} S102D	FW	TTTTACCGAAAACCTGATCCCCAGGCAGAGGA
p31^{COMET} S102D	RV	TCCTCTGCCTGGGGATCAGGTTTTCGGTAAAA
PPP1CA	FW (PAM SITE)	AACCCCCGCAGCTTACCCTGTGCCCCAGATGA
PPP1CA	RV (PAM SITE)	TCATCTGGGGCACAGGGTAATGTGCGGGGGTT
PPP1CC	FW (PAM SITE)	GTTTTGACACTGCTTAGTCGGGACTTGTAAC
PPP1CC	RV (PAM SITE)	AAGTCCCGACTAAGCAGTGTCAAAACGAC
MAD1	FW (PAM SITE)	TGTGGGAGGGCTAACCTGCAGGCTCGGGGGCATAGC CGGAGCCAC
MAD1	RV (PAM SITE)	GTGGCTCCGGCTATGCCCCGAGCCTGCAGGTTAGC CCTCCCACA

PPP1CA = PP1 α . PPP1CC = PP1 γ .

7.4 Cell Culture

7.4.1 Tissue Culture

HeLa cells were cultured in DMEM with 1% (vol/vol) GlutaMAX (Life Technologies) containing 10% (vol/vol) bovine calf serum and 1% (v/v) penicillin-streptomycin (Gibco, 10,000 U/ml) at 37°C and 5% CO₂.

HCT116 cells were cultured in McCoy's 5a media (Life Technologies) containing 10% (vol/vol) bovine calf serum and 1% (vol/vol) penicillin-streptomycin (Gibco, 10,000 U/ml) at 37°C and 5% CO₂.

7.4.2 Production of Flp-In Stable Cell Lines

HeLa cell lines with single integrated copies of the desired transgene under an inducible promoter were created using the T-Rex doxycycline-inducible Flp-In system (Invitrogen), using pcDNA5/FRT/TO vectors as follows. Parental HeLa Flp-In T-REx™ cells were seeded at 80,000 cells/well in DMEM with 1% (vol/vol) GlutaMAX (Life Technologies) containing 10% (vol/vol) bovine calf serum and 1% (v/v) penicillin-streptomycin (Gibco, 10,000 U/ml). 24 hours after seeding, 100ng of pcDNA5/FRT/TO/EGFP vectors were co-transfected into Flp-In TRex doxycycline HeLa cells with 900ng of the Flp recombinase encoding plasmid pOG44. Positive clonal cells for the integration of the desired transgene were selected for, and subsequently cultured, in DMEM with 1% (vol/vol) GlutaMAX (Life Technologies) containing 10% (vol/vol) bovine calf serum supplemented with 4µg/ul blasticidin (InvivoGen) and 200 µg/ul hygromycin (InvivoGen). Successful clone formation was confirmed by immunofluorescence and western blotting.

Transgene expression was induced by addition of 2 μ M doxycycline for a minimum of 24 hours.

7.4.3 Production of CRISPR edited cell lines

CRISPR/Cas9-edited knock-in HeLa and HCT116 cell lines were generated. Gene sequences were downloaded from NCBI, and suitable guide RNA sequences were identified. Homology recombination cassettes containing the desired knock-in DNA with flanking regions of homology to the target locus were generated and PAM sites were mutated. The guide RNA was ligated into pSpCas9 (Addgene, 48139). The homology recombination cassettes were co-transfected with pSpCas9 (Addgene, 48139) containing the corresponding guide RNA (Table 5). The homology arms varied between 150bp (for MAD1) and 1830bp (for PPP1CA (PP1 α)). The knock-in sequence contained the relevant desired insert (fluorescent protein and/or FKBP12^{F36V}), a glycine-rich flexible linker, a P2A ribosomal skipping site and an antibiotic resistance marker (Table 6). Homology arm cassettes for PP1 α and PP1 γ were made by a previous member of the Gruneberg lab. The homology arm cassette for MAD1 was a gift from the Barr lab. Antibiotic resistance markers were also swapped as required. The p31^{comet} and PPP2R5C (B56 γ) homology arms were amplified from genomic DNA and cloned into pSCA vectors. The primers used for amplifying the homology arms for p31^{comet} and PPP2R5C (B56 γ) are shown in Table 7. All homology arm pSCA vectors were edited via Gibson Assembly (NEB) to include the desired insertion.

For HeLa and HCT116 CRISPR cell line generation, 30,000 cell/well were plated in a 6-well dish 24 hours prior to transfection. After transfection, cells were incubated for 48 hours before transferring to a 15cm dish and selection antibiotic-containing media added. Clonal populations were expanded and screened for successful homozygous knock-in.

For all CRISPR cell lines, the knock-in cassette contained the desired fluorescent and/or FKBP12^{F36V} protein sequence, a glycine-serine rich flexible linker between this sequence and the homology arm, a P2A ribosome-skipping sequence and a blasticidin, puromycin or geneticin (G418) resistance marker. Antibiotic resistant clones were selected, and successful modification confirmed by PCR screening, immunofluorescence and western blotting as required. For individual PP1 CRISPR cells, a blasticidin resistance marker was used. For cell lines that were subjected to multiple rounds of CRISPR editing, different antibiotics were used for selection at each stage. For each of the double PP1 α/γ dTag CRISPR cells, the PP1 γ dTag blasticidin resistant cell line was used as the parental cell line to then add in the PP1 α dTag puromycin-containing tag. For the HeLa PP1 α/γ dTag cell line with MAD1-mScarlet, the MAD1 CRISPR homologous recombination repair construct confers resistance to geneticin (G418). A summary of the cell lines generated for this thesis is shown in Table 6 below.

For PCR screening, genomic DNA was extracted from cells using squishing buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 25 mM NaCl, 200 μ g/ml Proteinase K) and amplified using primers either side of the inserted region and Q5 polymerase (NEB). Homozygous clones were then screened again by PCR using genomic

DNA extracted from cell pellets using the QIAgen DNeasy Blood and Tissue kit (following manufacturer's instructions).

Table 5: CRISPR/Cas9 guide RNAs.

Target gene	Guide RNA (5'-3')
PPP1CA	CACCGCCATCATCTGGGGCACAGGGTGG
PPP1CC	CTATGTTACAAGTCCCGACT
p31^{comet}	CTAACCGCAAGGAGTAGCGG
PPP2R5C	CGTCAACATCTAGACTTCAGC (Vallardi et al., 2019)
MAD1	GGCGTAGCCTGCAGGCTCGG

PPP1CA = PP1 α . PPP1CC = PP1 γ . PPP2R5C = B56 γ .

Table 6: CRISPR knock-in cell lines generated.

Target gene	N- or C-terminal	Cell background	Antibiotic resistance
PPP1CA	C	HeLa	BSD, 4 μ g/mL
PPP1CC	C	HeLa	BSD, 4 μ g/mL, or Puro, 0.4 μ g/mL
PPP1CA	C	HCT116	BSD, 4 μ g/mL
PPP1CC	C	HCT116	BSD, 4 μ g/mL, or Puro, 0.4 μ g/mL
p31^{comet}	N	HeLa	Puro, 0.4 μ g/mL
PPP2R5C	N	HeLa	Puro, 0.4 μ g/mL
PPP2R5C	N	HeLa Flp-In T-REx TM	Puro, 0.2 μ g/mL
MAD1	C	HeLa	Geneticin (G418), 500 μ g/mL

PPP1CA = PP1 α . PPP1CC = PP1 γ . PPP2R5C = B56 γ .

Table 7: Primers for amplifying homology arms for CRISPR cell lines.

Target gene	Primer (5'-3')
p31^{comet} (Forward primer)	TCCCCATCTGCCTACAGGAT
p31^{comet} (Reverse primer)	GTGGGGAGAAAGTGACCGAA
PPP2R5C (Forward primer)	CACGAACCAGCCAGTTCC
PPP2R5C (Reverse primer)	AATAGCCATTTATTAACCTACCCACGGAAG

PPP2R5C = B56 γ .

7.5 Western Blotting and PhosTags SDS-PAGE Gels

For western blotting and PhosTag SDS-PAGE gels, cell pellets were lysed at 4°C for 30 mins, in lysis buffer supplemented with phosphatase and protease inhibitors (20 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 % IGEPAL [vol/vol]; Phosphatase inhibitor cocktail 1:100 [Sigma-Aldrich]; Protease inhibitor cocktail 1:250 [Sigma-Aldrich]; 0.5 M β -glycerol phosphate; 10 mM NaF; 100 nM Okadaic acid; 50 nM Calyculin A; PMSF 1 mM). Protein concentrations were measured by Bradford assay using Protein Assay Dye Reagent Concentrate (Bio-Rad).

For western blotting, proteins were separated by SDS-PAGE gels and transferred from the gel to nitrocellulose membranes using a Trans-blot Turbo system (Bio-Rad): 2.5A, 25V, 7 min for two gels. For high molecular weight proteins over 100kDa, the duration of the transfer protocol was extended to 10 min for two gels. Membranes were blocked in 5% cow's milk in phosphatase buffered saline (PBS), 0.1% Tween-20 (PBST). Antibodies were incubated in milk/PBST for 2-18hours. Membranes were washed with PBST between primary and secondary antibody incubations and prior to developing. Western blots were revealed using ECL (GE Healthcare) on films.

For analysis of protein phosphorylation state, SDS-PAGE gels were prepared and supplemented with 100mM MnCl₂ and 25mM PhosTag reagent (Wako Chemicals, AAL-107S1). Prior to transfer, gels were equilibrated with 3x7 min washes in transfer buffer (20 mM Tris; 150 mM Glycine; 0.1% [wt/vol] SDS; 20% [vol/vol] MeOH; 20 mM EDTA) and subsequently 3x7 min washes in transfer

buffer without EDTA (20 mM Tris; 150 mM Glycine; 0.1% [wt/vol] SDS; 20% [vol/vol] MeOH).

7.6 Imaging Techniques

7.6.1 Immunofluorescent Staining

For fixed cell analysis, cells were seeded on #1.5 thickness coverslips 48-72 hours prior to fixing. Cells were fixed with PTEMF (20 mM Pipes-KOH, pH 6.8, 0.2% [vol/vol] Triton X-100, 1 mM MgCl₂, 10 mM EGTA, and 4% [wt/vol] formaldehyde) for 12 minutes at room temperature. Coverslips were incubated in blocking buffer (3% w/v bovine serum albumin, PBS) for 1 hour. Coverslips were incubated face-down on 70µl droplets of primary antibodies in a humidified chamber for 1 hour. Following primary antibody incubation, coverslips were washed 3 times in 1X PBS. Secondary donkey antibodies against mouse, rabbit, guinea pig, or sheep, labelled with Alexa Fluor 405, Alexa Fluor 555, or Alexa Fluor 647 (Molecular Probes) were used at 1:1000. Coverslips were incubated face-down on 70µl droplets of diluted antibodies in a humidified chamber for 45 minutes. Coverslips were washed 3 time in 1X PBS and once in distilled deionised water. Coverslips were left to dry completely before being mounted with 7µl of Moviol 4-88 (Sigma) according to manufacturer's instructions.

7.6.2 Cold Treatment

Cold treatment was performed by placing cell dishes prior to fixing on ice for 9 minutes and replacing the existing media with pre-chilled media. After 9 mins, the

dish was removed from the ice and media was replaced with PTEMF at room temperature. Cells were fixed and stained as above (7.6.1 Immunofluorescent Staining).

7.6.3 Fixed Cell Image Acquisition

For fixed cell microscopy, imaging was carried out using a 60X/1.35 NA oil immersion objective on a BX61 Olympus microscope fitted with filter sets for DAPI, EGFP/Alexa Fluor 488, Alexa Fluor 555, Alexa Fluor 647 (Chroma Technology Corp.) to sequentially excite and collect fluorescence images on a CoolSNAP HQ2 camera (Roper Scientific) using MetaMorph 7.5 imaging software (GE Healthcare). Cell stacks were 2.4mm total, taken at 0.2 mm intervals.

7.6.4 Fixed Cell Image Processing and Analysis

Image processing and analysis was carried out using the Fiji distribution of ImageJ. Image stacks were imported into ImageJ (Schindelin et al., 2012). Maximum- and Sum-projected 8-bit TIFF files were generated from the raw image. MAX-projection TIFF files were imported into Illustrator CS6 (Adobe) for figure production.

To determine intensity of kinetochore localised signal, background-corrected kinetochore intensities were determined by placing an 8-pixel circular region of interest (ROI) over individual kinetochores, measuring the mean grey value,

normalizing to mean gray value of the relevant channel (as stated) within the same ROI, and calculating single cell averages.

To measure the overall signal on chromatin, the area of chromatin based on Hoechst staining was measured, as well as the mean intensity of the signal and a background measurement. The intensity of the signal in the channel of interest over the same area was measured.

7.6.5 Live Cell Image Acquisition

For live cell imaging to determine mitotic timings, an EVOS M7000 fluorescent microscope (Thermo Fisher Scientific) was used. Cells were seeded at 50,000 cells/well on 6-well glass bottomed dishes (34.94mm, 0.170 ± 0.005 mm, IBL) 3 days before imaging. For live cell imaging, cells were pre-synchronised with a single thymidine (2mM) block for 18 hours. After an 18-hour incubation, cells were released from the thymidine arrest by carefully washing four times with DMEM. Cells were released into Flurobrite media (Thermo Fisher Scientific) supplemented with 10% FBS and 1X GlutaMAX (Thermo Fisher Scientific) with siR-DNA (Spirochrome; 50nM).

For live cell imaging of p31^{comet} Flp-In cells, cells were seeded at 50,000 cells/well. After 24 hours, cells were transfected with either an siRNA against the 3'-UTR of p31^{comet} or a control siRNA against GL2 (luciferase) (Table 2). After 24 hours, a single thymidine block was performed as described above. Doxycycline (2 μ M) was also added to induce the expression of the transgene at this time. 6 hours after the thymidine release, imaging was started using the EVOS M7000

fluorescent microscope. Cells were placed in a 37°C and 5% CO₂ environmental chamber. Imaging was performed using a 20X/0.75 objective lens. Images were captured of each FOV at 5-minute intervals for 12 hours.

For live cell imaging of HeLa PP1 dTag cell lines on the EVOS M7000 fluorescent microscope, dTag reagent was added to cells 6 hours after release from thymidine and 2 hours before imaging was started. Images were captured of each FOV at 3-minute intervals for 12 hours.

For high resolution live cell imaging of p31^{comet} CRISPR cells, 60,000 cells were seeded into 35-mm dishes with 1.5-thickness cover glass bottom (Fluorodishes; Applied Precision), 48 hours before imaging. 7 hours before imaging was started, DMEM was removed from the dish and carefully replaced with Flurobrite with siR-DNA (Spirochrome; 50nM) was added. For high resolution of all other cells lines, cells were seeded at 50,000 cells/well on μ -Slide 2 Well #1.5H Glass Bottom chamber slide (Ibidi) 3 days prior to imaging. Imaging was carried out on an Olympus SoRa super-resolution spinning disc confocal microscope. Cells were placed in a 37°C and 5% CO₂ environmental chamber. Imaging was performed using a 60X/1.5 oil HR immersion objective lens. 4 FOVs per well were selected. Cell stacks were 12mm total, taken at 1mm intervals.

7.6.6 Live Cell Image Processing and Analysis

For quantification of mitotic timings, MAX projections of the movies were used. Nuclear envelope breakdown (NEBD), metaphase (META) and anaphase (ANA) timepoints were determined based on DNA morphology.

For quantification of DNA decondensation during anaphase, areas of interest were drawn manually around the chromatin at each timepoint and the area measured using the measurement tool in ImageJ.

7.7 Biochemical Assays

7.7.1 Mitotic Exit Timecourses

HeLa cells were seeded at 1,200,000 cells/dish onto 15cm² dishes. Nocodazole (330nM) was added 16 hours before the timecourse was carried out to arrest the cells in mitosis. Mitotic HeLa cells were harvested by mitotic shake off and separated into two falcon tubes in such a way that one contained 1/10th the final volume of the mitotic cell sample (for a pre-nocodazole washout sample) and the other contained the other 9/10th. Both falcon tubes were centrifuged for 4 minutes at 400g. The pre-nocodazole washout sample was washed with 1mL 1X phosphate-buffered saline (PBS) pre-warmed to 37°C and centrifuged for 1 min at 400g. Cells were resuspended in 100mL ice-cold lysis buffer and snap frozen using liquid nitrogen. Lysis buffer was made as described for Chapter 7.5. To release the cells from the nocodazole-induced mitotic arrest, the remaining cells were washed twice as follows: cells resuspended with 20mL DMEM with 1% (vol/vol) GlutaMAX (Life Technologies) containing 10% (vol/vol) bovine calf serum pre-warmed to 37°C and centrifuged for 4 minutes at 400g. The cells were resuspended in 990mL DMEM with 1% (vol/vol) GlutaMAX (Life Technologies) containing 10% (vol/vol) bovine calf serum to give a final concentration of 15,000,000 cells/mL. The cells were incubated at 37°C and 5% CO₂ for 25 mins

for the cells to re-establish a mitotic spindle, mixing gently every 5 minutes. After 25 minutes, Flavopiridol (5 μ M) or AZ3146 (2 μ M) diluted in warm media were added to the cells. Samples were collected every 10 minutes for a total of 90 minutes. Upon each sample collection, the collected cells were treated with calyculin A (50nM) to quench the reaction and prevent any further dephosphorylation events during the centrifugation and wash steps. Cells were resuspended in 100 μ L lysis buffer as described above and snap frozen.

7.7.2 Lambda Phosphatase treatment

HeLa cells were arrested in nocodazole (330nM) for 18 h and harvested by mitotic shake-off. Cell pellets were lysed (as described Chapter 7.5 Western blotting) and subsequently incubated with lambda (λ)-protein phosphatase (NEB) for 2 hours at 30°C according to the manufacturer's instructions. 1x Laemmli buffer was added, and samples denatured at 100°C for 5 minutes. Samples were run on PhosTag or SDS-PAGE gels and analysed by western blotting.

7.8 Statistical Analysis

Production of graphs and statistical analysis was performed in GraphPad Prism (GraphPad Software, Inc.). Error bars represent the standard error of the mean (SEM) unless otherwise stated. P-values are shown on graphs as follows: $p > 0.05$ = not significant (ns), $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****. If only two conditions were compared, they were compared using a Two-Tailed Unpaired T-test. If more than two groups were compared, a one-way

ANOVA was used. Where multiple colours are used for the same condition in graphs, each colour represents an independent biological repeat. Cells are represented as individual dots. The mean value of each condition within each repeat is represented as a triangle in the corresponding colour. Numbers above each condition indicate the number of cells quantified in the corresponding condition.

Chapter 8: Bibliography

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