A New Chemical Genetics Toolbox to Analyse Genes Essential for the Mitotic Entry

by

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This is to certify that I have examined the above Dual PhD thesis
and have found that it is complete and satisfactory in all respects,
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Abbreviations

Abbreviation	Full name
A.U.	arbitrary unit
Ab	antibody
AML	acute myeloid leukaemia
AID	auxin-inducible degron
Apcin	APC/C inhibitor
Asv	Asunaprevir
BCA	bicinchoninic acid
BFP	vlue fluorescent protein
bp	base pair
BrdU	Bromodeoxyuridine
BSA	bovine serum albumine
CaP	calcium phosphate
CLS	centrosomal localisation signal
CRISPR	clustered regularly interspaced short palindromic repeats
CRS	cytoplasmic-retention signal
D-box	destruction box
DAPI	4',6-diamidino-2-phenylindole
dd	double degron
degron	degradation tag
DIA	doxycycline, IAA, and Asunaprevir
DIAA	doxycyline and IAA
DIC	differential interference contrast
DSB	double strand break
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOX	doxycycline
DSB	double strand break
EdU	5-ethynyl-2'-deoxyuridine
ER	endoplasmatic reticulum
EtOH	Ethanol

FCS	foetal calf serum
FACS	fluorescence-activated cell sorting
FRET	Förster resonance energy transfer
FSC	forward scatter
G_1	first growth stage
G_2	second growth stage
GOI	gene of interest
gDNA	genomic DNA
gRNA	guide RNA
HBS	Hepes buffered saline
HeLa	Henrietta Lacks, cervical cancer cells
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	homologous recombination
HtTA1	HeLa cells expressing tTA1
IAA	indole-3-acetic acid
IP	immunoprecipitation
k-MT	kinetochore-microtubule
kDa	kilo-dalton
ko	knock-out
LCI	live-cell imaging
M phase	mitosis
mAID	mini AID
NCI	National Cancer Institute
MEF	mouse embryonic fibroblast
MEM	Dulbecco's minimum essential medium
miRNA	micro RNA
mRNA	messenger RNA
NEBD	nuclear envelope breakdown
NES	nuclear export signal
NHEJ	non-homologous end joining
NLS	nuclear-localisation sequence
NOC	nocodazole

O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
POI	protein of interest
ProTAME	pro-N-4-tosyl-L-arginine methyl ester
RING	Really Interesting New Gene domain
RNA	ribonuclein acid
RNAi	RNA interference
RPE-1	Retinal pigment epithelial cells
RT	room temperature
S phase	synthesis phase
SB	sleeping beauty
SDS	sodium dodecyl sulphate
siRNA	small interference RNA
SiR	silicon rhodamine
SMASh	small-molecule-assisted shut-off
SSC	side scatter
TEMED	N,N,N',N'-tetramethyl- ethylenediamine
TET	tetracycline
TC	tissue culture
thy	thymidine
TPR	tetratricopeptide
tTA	tetracycline-controlled transcriptional activator
YFP	yellow fluorescent protein

Gene and protein abbreviations

Abbreviation	Full name
β-TrCP	β-transducin repeat containing E3 ubiquitin protein ligase
53BP1	p53-binding protein 1
A / E	ARPP19 / ENSA
APC/C	anaphase-promoting complex
ARPP19	cAMP Regulated Phosphoprotein 19
ATR	Ataxia Telangiectasia and RAD3 related protein
ATM	Ataxia Telangiectasia Mutated protein
AURK	aurora kinase
BORA	protein aurora borealis
BUB	budding uninhibited by benzimidazole
BY	cyclin B1-YFP
BYN	cyclin B1-YFP-NLS
C-TAK	CDC25C-associated kinase
CAK	CDK-activating kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine 3',5'-monophosphate
CCNA2	cyclin A2
CCNB1	cyclin B1
CCNB2	cyclin B2
CDC	Cell Division Cycle
CDK	cyclin-dependent kinase
CDH1	CDC20 homolog 1
CHK	checkpoint kinase
CKS	CDK subunit protein
DP-1	dimerization partner 1
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
EMI1	early mitotic inhibitor 1
ENSA	α-endosulfine

ER	estrogen receptor
ERK	extracellular signal-regulated kinase
GFP	green fluorescent protein
HPIP	hematopoietic PBX-interacting protein
KIF	kinesin family member
MAD	mitotic arrest-deficient
MAPK	mitogen-activated protein kinase
MASTL	Microtubule-associated S/T kinase-like
MCC	mitotic checkpoint complex
MIG16	monokine induced by IFN-γ 16
MPF	mitotic-promoting factor
MPS1	monopolar spindle protein 1
MRE11	meiotic recombination 11
MRN	MRE11-RAD50-NBS1 complex
mRFP	monomeric red fluorescent protein
MYT1	membrane-associated Y/T kinase
NEK2A	NIMA-related expressed kinase 2A
NF	nuclear transcription factor
NFAT	nuclear factor of activated T cells
NIMA	never in mitosis A
ORC	origin recognition complex
ORF	origin of firing
p-CDK sub	phosphorylated CDK substrates
p21 / p27	Cyclin-dependent kinase inhibitor
p53	tumour protein 53
p107	retinoblastoma-like protein 1
PCNA	proliferating cell nuclear antigen
PD-1	programmed cell death protein 1
PIN	Prolyl isomerase
PLK	polo-like kinase
PLX	Xenopus polo-like kinase
PP	protein phosphatase

PRC1	protein regulator of cytokinesis 1
RAD	radiation sensitive
Rb	Retinoblastoma
RhoA	Ras homolog family member A
SAC	spindle assembly checkpoint
SCAPER	S phase cyclin A associated protein in the ER
SCF	SKP1-Cullin-F-box protein complex
SKP1	S-Phase Kinase Associated Protein 1
TF	transcription factor
TIR1	transport inhibitor response 1
TOR	target of rapamycin
TrCP	transducin repeats-containing protein
Ub	Ubiquitin
USF	upstream transcription factor
USP37	ubiquitin specific peptidase 37
YB-1	Y box binding protein 1

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Abstract

Two mitotic cyclins, cyclin A and cyclin B, are believed to promote mitotic events. However, previous studies have implicated both specific requirement and redundancy of these cyclins in human cells in mitosis. Using degron-tagging approaches, the roles of cyclin B were analysed in RPE-1 and in HeLa cells. Striking differences were uncovered between the two cell lines. RPE-1 cells require cyclin B for specific substrate phosphorylations only in metaphase, but HeLa cells without cyclin B stall in an intermediate prophase-like stage which was characterized by a specific morphology and a unique pattern of CDK-substrate phosphorylation. In the absence of cyclin B, endogenous levels of cyclin A alone are responsible for establishing this prophase-like stage found in HeLa cells; but overexpressing cyclin A promoted separate mitotic events in a dose-dependent manner. On the other hand, RPE-1 cells rely on a combination of cyclin A-mediated CDK activity, as well as phosphatase inactivation, to promote separate mitotic events. These results implied that HeLa cells depend on the quantity of, rather than the specific, CDK-cyclin activity during mitosis, whereas RPE-1 cells require specific CDK phosphorylations conferred by different cyclins. As the expression of mitotic cyclins is altered in many cancer cells, a comprehensive understanding of the regulation of these cyclins in healthy and cancer cells will allow the design of targeted treatments and a better prediction of patient outcome after therapies.

Chapter 1

Thesis summary and objective

1.1 Hypothesis

There are two types of CDK-binding cyclins that control mitotic progression in human cells, cyclin A and cyclin B. Both are present during mitotic entry, but cyclin A is degraded in prometaphase [1], slightly earlier than cyclin B which is destroyed during the metaphase-to-anaphase transition [2, 3]. The difference in the timing of cyclin degradation are believed to aid in the temporal control of mitotic progression and exit [4].

Cyclin A is already expressed in S and G_2 phases of the cell cycle, during which it has well-established roles in cell cycle progression by activating CDK2 and CDK1 [5–8]. Owing to the cyclin's oscillating expression pattern (Figure 2.2), conventional protein depletion methods have not been able to pinpoint cyclin A's functions during mitotic entry, although it has been implicated to trigger the activation of the CDK1-cyclin B complex [9]. Separating cellular phenotypes that were caused by the lack of cyclin A in the current or preceding stages of the cell cycle was made difficult due to the inefficiency of protein depletion methods.

Similarly to cyclin A, the roles of cyclin B are not entirely clear. One study showed that cyclin B promotes early stages of mitosis, specifically aiding with nuclear envelope breakdown (NEBD) in mouse embryos [10]. This is contradicted by other work demonstrating that the main activity of cyclin B-mediated CDK phosphorylations is carried out after NEBD [11, 12], although the latter study by Gong et al. implied a level of redundancy between cyclin A and cyclin B prior to NEBD [12]. These implications are supported by the observation that CDK1-cyclin B is translocated into the nucleus during prophase [13–16]. The above studies, together with others that defined the importance of CDK1-cyclin B in mitosis after NEBD [13, 17–21], show the importance of cyclin B throughout the cell division stage. Nevertheless, the specifics of cyclin B's functions prior to NEBD remain to be clarified.

Novel degron tagging techniques can achieve an abrupt protein depletion within a few hours in cultured cells [22–24], making them an attractive tool for cell cycle studies. Furthermore, the degron tags can be established in different cell line systems to address and compare the functions of essential cell cycle proteins in untransformed and cancer cells.

1.2 Objective

The objective of this thesis is to define the specific roles of human cyclins A and B in mitosis and to additionally separate between the functions in early and late stages of mitosis. Next, I aim to compare the roles of cyclins A and B during the cell cycle in healthy and cancerous cells. I plan to define the importance of each cyclin in two different cell lines and address their level of redundancy and specificity. Moreover, I plan to investigate the key players required to establish a prophase stage in human cells.

1.3 Summary

Human hTERT immortalised non-cancerous retinal pigment epithelial cells (RPE-1) require cyclin A2 for mitotic entry, and either cyclin B1 or B2 (hereafter cyclin B unless specifically stated) for a faithful metaphase and mitotic exit. Cyclin A2's mitotic, but not S phase, roles can be partially fulfilled by an excess of nuclear cyclin B1, whereas stabilising cyclin A2 in mitosis cannot compensate for the loss of cyclin B. Furthermore, RPE-1 cells lacking cyclin B with a highly active mitotic phosphatase can establish a stable prophase-steady state during which they maintain medium levels of cyclin-depdendent kinase (CDK) mitotic substrate phosphorylations. The prophase-steady state was further characterised by chromosome condensation, centrosome separation, and mitotic cell rounding.

In contrast, cyclin A2 is not essential in human cervical cancer cells (HeLa) whereas cyclin B is crucial for mitotic progression. HeLa cells lacking cyclin B are unable to progress past nuclear envelope breakdown, but have promoted some mitotic events including cell rounding and a significant amount of mitotic CDK substrate phosphorylations. Further analyses demonstrated that exogenous cyclin A2 dose-dependently compensates for mitotic events, with high levels of overexpression achieving a faithful mitotic exit. HeLa cells with insufficient cyclin activity enter mitosis but then revert to late G_2 prior to NEBD. Interestingly, cells with an intermediate CDK activity, controlled by the level of cyclin A2 overexpression, can progress past NEBD but fail to segregate their chromosomes into two cells. Instead, these cells continue their cell cycle, as judged by an appearance of an 8N population, but eventually become apoptotic.

The data in this thesis show that HeLa cells progress through separate stages of mitosis

depending on the quantity of cyclins that can activate the mitotic CDKs. These findings underlined the importance of controlling cyclin expression levels in HeLa cells to ensure their proliferation. RPE-1 cells, on the flip side, largely base their decision to progress through mitosis according to specific CDK substrate phosphorylations, conferred by either cyclin A in early mitosis, or by cyclin B in later stages of mitosis. Notably, phosphatases also aid with the regulation of mitotic progression in these cells. Additional information is required to assess the level of cyclin redundancy in these cells.

Chapter 2

Introduction

2.1 The cell cycle

Human proliferating cells must replicate their genome exactly once per cell cycle, and divide each copy into two cells during mitosis. Several mechanisms are in place to ensure the proper sequence of these events is intercepted with growth periods. Cells commit to entering the cell cycle in the first growth stage G_1, replicate their DNA during S phase, enter another growth stage G_2 and finally complete the cell cycle by dividing their contents into two cells during mitosis. Newly established cells will either repeat the cycle, or exit into G_0 in the absence of nutrients or growth signalling.

Mitosis is further divided into stages starting with prophase, prometaphase, metaphase, anaphase, and telophase. Each of this stages has its own characteristics that are also visually summarised in Figure 2.1 [25]. In prophase, cells round up, condense their DNA into chromosomes, begin organising the spindle and separate the centrosomes into two opposing poles. Afterwards, nuclear envelope breakdown (NEBD) marks the onset of prometaphase and the cells begin to transition into metaphase by assembling their spindle and attaching the newly formed microtubules to chromosomes. In metaphase, chromosomes are aligned to the equatorial plane of the cell, while the mitotic spindle is organised and sister-chromatids are eventually attached to opposite poles. Once the metaphase events are completed, and cells have securely attached all sister-chromatids to microtubules, mitotic substrate dephosphorylation occurs, thereby triggering a rapid separation of the sister chromatids into two daughter cells during anaphase and telophase. Next, cells undergo cytokinesis and re-form the nuclear envelope around their DNA in telophase to then decondense the chromosomes and enter G_1.

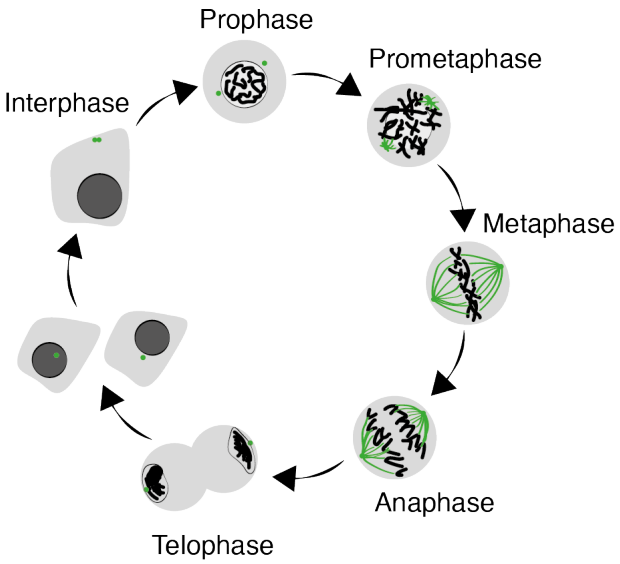

Figure 2.1: A schematic view of the cell cycle. Stages of mitosis are as noted on the Figure, starting with prophase. Chromosomes are marked in black, centrosomes and microtubules, observed emanating from the centrosomes in prometaphase, are shown in green. Interphase includes the whole non-mitotic cell cycle, including G_1 , S, and G_2 stages, and is not entirely pictured here.

CDKs and cyclins drive cell cycle progression A controlled transition from the second growth phase into mitosis (G_2/M) is imperative as any errors could lead to catastrophe during mitosis and give rise to genomic instability, eventually either leading to death or promoting cancer development. Checkpoints and restriction points can pause the cycling cell in case of DNA damage or stress (i.e. poor conditions, lack of nutrients or growth hormones) until it meets the requirements for progression into the next stage. Once a cell is ready to divide, a bistable switch is flipped to trigger mitotic events. This decision has been studied immensely, but there are still many unknowns surrounding this point of the cell cycle. At the centre of this switch is a mitotic cyclin-dependent kinase (CDK1) that is responsible for over a thousand mitotic phosphorylations [26] and as its name suggests, it requires a cyclin partner for its activity. These complexes are highly evolutionarily conserved drivers of the cell cycle and were initially described in *Rana* oocytes [27], *Xenopus* egg extracts [28], and yeast [29]. Interestingly, both fission and budding yeast have several CDK-cyclin homologues but fission yeast are able to proliferate with just one CDK and cyclin partner, whereas two cyclin partners are necessary in budding yeast [30–34]. Fission yeast research indicates that the quantitative amount of

CDK activity, capable of executing mitotic phosphorylations, is the core requirement for a successful cell division. However, budding yeast studies implied that cyclin specificity may play a more important role in some organisms.

Research in other model systems, such as in mouse embryonic fibroblasts (MEFs), showed that the cells are tolerant for the loss of several interphase CDK proteins such as the G_1 specific CDK4/6 [35] and the G_1/S driver CDK2 [36, 37], as mitotic CDK1 is able to compensate for them [38]. CDK1 has the highest affinity for binding with the mitotic cyclin B, but it is also able to form a functional complex with D-, E-, and A-type cyclins [32, 38]. Cyclin partners can confer substrate specificity in yeast [39], (reviewed in [40]), mammals [38, 41], and in vitro [42, 43]. Thus, the ability of CDK1 to partner up with other cyclins is likely the underlying reason for its ability to drive the whole cell cycle.

Cyclins control cell cycle progression by activating specific CDK proteins Cyclins are famous for their oscillating expression pattern that depends on the stage of the cell cycle. They are only expressed when required to activate their partner CDK, and degraded by the proteasome as soon as the CDK has fulfilled its role. There are three cyclin types driving the cell cycle – D, E, and A – that normally bind with interphase CDK4 / 6 or CDK2, but only two cyclin sub-families – A and B – are expressed during G_2/M in higher eukaryotes (Figure 2.2). Cyclins A and B both form functional complexes with CDK1, however it is unclear what their designated roles in mitotic entry and progression are.

Cyclin A is present in the cells as early as in late G_1 and early S phase, and its levels remain high until early mitosis. Cyclin B however, is a well-established mitotic cyclin which is only detectable in late G_2 and destroyed in mitosis, slightly later than cyclin A (Figure 2.2). These two cyclins each have a preferential partner to bind with and drive the cell cycle; cyclin A largely associates with CDK2 and cyclin B normally binds with CDK1. Nevertheless, CDK1-cyclin A complex is also present in a normal cell cycle, and there is evidence for a specific role of this pair in late S phase in chicken DT-40 [44] and mammalian cells [8]. However, not much more is known about the function of CDK1-cyclin A in later stages of the cell cycle.

Despite the fact that many studies have been conducted on mitotic entry, specific roles for each cyclin remain elusive. Cyclins cannot be inhibited with drugs as they do not have enzymatic activity. Thus, researchers have often resorted to model systems

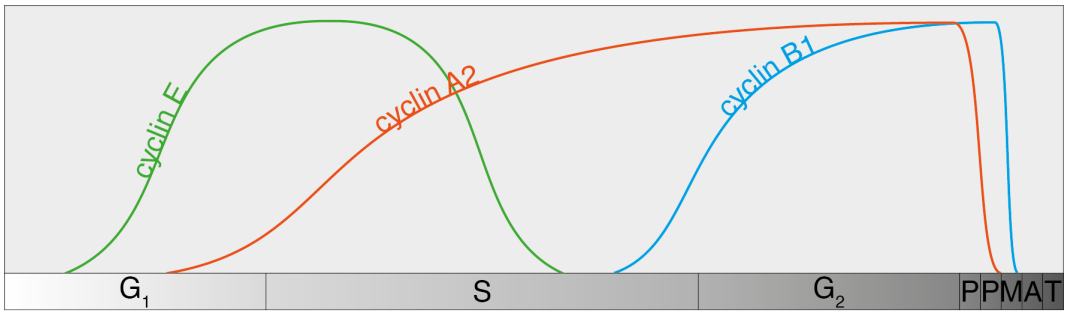

Figure 2.2: Protein levels of three cyclins types responsible for cell cycle progression. Cyclin E is involved in G_1/S transition and early S phase. Cyclin A2 promotes S and G_2/M events, and cyclin B1 is largely responsible for G_2/M and progression through early and mid M phase. Cyclin A2 is degraded in prometaphase and cyclin B1 in late metaphase / early anaphase [1]. Cyclin D is omitted from this graph as it is expressed from G_1 to M. The stages of mitosis are as follows from left to right: prophase, prometaphase, metaphase, anaphase, and telophase.

or RNA interference (RNAi) to deplete protein levels as much as possible. However, cyclins are very strongly expressed each cell cycle and that has added to the inefficiency of conventional depletion methods. While contradictory, the research carried out so far has still provided us with many insights on when each cyclin is required, and these will be reviewed next.

2.2 Mitotic cyclins and their functions

The cyclin family includes many proteins based on sequence similarity, but not all are involved in cell cycle progression. In the past, suggestions to redefine cyclins based on their structure and not just their sequence arose, but to date these have not been separated into 'cell cycle' and 'non-cell cycle' cyclins. For the purposes of this thesis I will concentrate on the ones relevant for the cell cycle, especially the two CDK1-binding types that are believed to have roles in mitotic progression, cyclins A and B.

There are other cyclins present in mitosis, for example the functionally distinct cyclin F that contains an F-box motif and forms SCF ubiquitin ligase complexes [45]. Even though its expression pattern matches cyclin A's, the regulation of cyclin F is completely different to the control of cyclins A and B [46]. Thus, cyclin F will not be discussed in this thesis. Any future references to mitotic cyclins exclude cyclin F, unless stated differently.

There are non-somatic and somatic-specific sub-types of cyclins A and B in human cells Cyclin A sub-family encompasses two proteins in higher eukaryotes, meiotic A1 and somatic A2, encoded by *CCNA1* and *CCNA2*, respectively. B-type cyclins have three homologues; embryonic and human testis-specific cyclin B3, and two somatic cyclins B1 and B2 (their gene names are *CCNB3*, *CCNB1*, and *CCNB2*, respectively).

The specifics of CDK-cyclin association are not entirely clear As noted previously, the two types of cyclins are different in their expression pattern, and also primarily form complexes with separate CDK proteins: cyclin A2 binds with CDK2, and cyclin B with CDK1. However, both cyclins can actually form a functional complex with either CDK1 or CDK2, and the affinity for CDK2-cyclin A2 and CDK1-cyclin B association is regulated by differences in the respective CDK's pattern of activation [47, 48].

CDK-activating kinases (CAK) play a role in this selective binding [47] as CDK2 can bind with cyclin A2 prior to being activated by a phosphorylation on its T-loop which is mediated by CAK. This step is necessary for CDK1 to bind any cyclin protein [48]. In contrast, yeast CDC2 (cell division cycle 2, homologous to mammalian CDK1) was previously shown to bind with cyclin B1 prior to it's T-loop phosphorylation in vitro [49].

The specific roles of each CDK-cyclin complex are not yet fully established, owing to

their essential roles in cell cycle progression, as well as their overlapping activities.

Cyclin A2 is most known for its interphase roles, but it has been shown to also contribute to mitotic progression. Specifically, it has been implicated in the regulation of kinetochore-microtubule (k-MT) attachments in prometaphase [50, 51]. Whether that is cyclin A2's only mitotic function is unclear and further research is needed to clarify its roles.

The next Section focuses on the cyclin A sub-family with more emphasis on the somatic cyclin A2 and its structure, localisation, functions, and control of expression, respectively. Lastly, as cyclins A1 and A2 have been indicated to play a role in cancer development, reports on this topic will also be discussed. Then, cyclin B sub-family will be analysed in a similar manner, followed by reviews on other mitotic players (CDK1, PLK1, PP2A and MASTL).

2.2.1 Cyclin A

The two homologues of A-type cyclins, embryonic A1 and somatic A2 are highly conserved in *Xenopus* [52], mouse [53, 54], and human [55], but interestingly not in *Drosophila* where only one cyclin A protein exists [56]. Furthermore, as shown in *Xenopus* embryos, cyclin A1 is only present until the midblastula, when cyclin A2 takes over [57].

Both cyclins A1 and A2 normally associate with CDK2 to drive the cell cycle through G_1/S, S and G_2 phases [5, 12, 16, 58]. They have well-established roles during replication, but their G_2 functions are not entirely clear as not much is known about this phase of the cell cycle in general. It is believed that cyclin A2 promotes some early mitotic events [59] as well as aids in triggering CDK1-cyclin B activation [9, 12]. As noted above, there are still many unknowns surrounding the mitotic activity of cyclin A2.

2.2.1.1 Cyclin A1

This meiotic cyclin was initially discovered in marine invertebrates [60] but its homologues were also described in *Xenopus* [53, 61] and mice model systems [54], and humans [55]. Its function in mammals seems to be restricted to embryos, and in adults to germ cells in testis where it is essential for spermatogenesis [54, 55, 62].

Interestingly, cyclin A1 has roles in the development of some types of leukaemia [55, 63]. Its role has also been implicated in the proliferation of a human osteosarcoma cell line

– MG63 [64]. Overexpression of cyclin A1 promotes tumour development and indicates a poorer outcome in patients diagnosed with acute myeloid leukaemia (AML) [65]. This was linked to a change in protein localisation which enabled it to form a complex with CDK1, thereby promoting cell proliferation [66].

2.2.1.2 Cyclin A2

Here, cyclin A2 will be described in detail, starting with a short overview and a description of its structure, followed by a summary of studies on its interphase, G_2/M, and mitotic functions, respectively. Afterwards, transcriptional control and degradation mechanics will be reviewed, with an emphasis on the mitotic proteasome, the Anaphase Promoting Complex/Cyclosome (APC/C). In the end, implications for cyclin A2 in some cancer tumours will also be described.

Overview Cyclin A2 is essential for mouse development [67] and has roles in S, G_2 and M phases in healthy mammalian cells. In mammals, cyclin A2 promotes a faithful replication when complexed with CDK2 [5–7], aids late origin firing by activating CDK1 [8], and helps with the G_2/M transition, but the specific CDK partner during this stage is unknown [9, 12, 16, 52, 68]. The many functions of this protein are likely due to its complex structure, which will be described below.

Protein structure Human cyclin A2 is a 48 kDa (kilodalton) protein consisting of an N-terminal α-helix domain, a destruction box motif (D-box), a highly conserved cyclin box and a cyclin box fold. The N-terminus contains sites that contribute to its timely degradation but are actually redundant [69]. However, the N-terminal and cyclin box α-helix domains are together crucial for the activation of its partner CDK [70, 71]. The cyclin box is a motif which distinguishes all cyclin proteins based on sequence similarity. It encodes an MRAIL motif that is required for the binding of proteins containing an RXL domain (p27, p107 and p21) [71, 72]. Residues downstream of the MRAIL aid with kinase activation, and different CDKs require specific sites that help with their binding and activation [71].

In contrast to the above, Schulman et al. did not find any interference with CDK2-cyclin A2 binding following MRAIL mutations in a human osteosarcoma cell line U2OS [72], however retinoblastoma-like protein p107 and transcription factor E2F1 binding was

severely disrupted in those. These data indicated that there are additional sites in cyclin A2 that are responsible for CDK2 binding, but to date they have not been defined.

Cyclin A2 may have specific roles at centrosomes A centrosomal localisation signal (CLS) has been identified near the MRAIL motif that is different from a cyclin E CLS [72–74]. Modification of cyclin A2's CLS resulted in the CDK inhibitor p27-binding defects, displacement of cyclin A2 from centrosomes, and replication inhibition [74]. The halt in replication may be caused by defects in the centrosomal cycle as this link has previously been established [75–77]. CDK2-cyclin E has also been shown to play a similar role as CDK2-cyclin A2 at the centrosomes [78, 79] and since cyclin E was not disrupted in the above studies, it is not clear whether the centrosome defects, if any, could indeed halt replication. However, it is not entirely clear whether cyclins A and E have distinct functions at the centrosomes. Unlike cyclin E, cyclin A2 is able to localise to the centrosomes regardless of CDK binding [74], thus showing that there may be inherent differences between the two cyclins' activities at centrosomes. Furthermore, other roles of cyclin A2 could be affected by the CLS mutation, promoting the several phenotypes mentioned above.

Cyclin A2's crystal structure has been solved Cyclin A2 is a complex protein, and there are still many unknowns surrounding its structure. Brown et al. have solved a crystal structure of cyclin A2 and found that it does not undergo a significant conformational change upon binding with CDK [80]. Instead, cyclin-binding induces conformational changes in its CDK partner, as demonstrated by crystal structure analyses of the CDK2-cyclin A2 complex [81, 82]. It is unclear whether CDK1 undergoes similar changes as CDK2 when binding with cyclin A2, as only CDK1-cyclin B1 crystal structure has been solved to date [83]. Overlapping, but distinct, cyclin A2 residues are involved with binding and activation of CDK1 as compared with CDK2 [71]. More research is needed to further elucidate how cyclin A2's structure affects its CDK partner. Cyclin A2 is also regulated by its localisation pattern, and there are many unknowns surrounding the control of its localisation. Both the structure and its binding partners have been implicated to aid in its subcellular regulation.

Localisation Cyclin A2's localisation is dependent on the cell cycle as it is found in the nucleus during S phase, and also exported to the cytoplasm during G_2/M [84, 85]. Interestingly, cyclin A2's sequence does not encode a nuclear localisation signal (NLS) [70] and it is not entirely clear how its localisation is controlled.

The role of cyclin A2's binding partners in its localisation Cyclin A2's nuclear presence has previously been attributed to its partner CDK, especially CDK2 [70, 86]. However, Bendris et al. have found that neither binding of CDKs or p21 and p107 conferred nuclear localisation, instead this is apparently dependent on the whole cyclin A2 protein and not a single region [71].

On the flip side, one other report implicated that SCAPER (S phase cyclin A associated protein in the ER), a poorly characterised protein, controls CDK2-cyclin A2's export to the cytoplasm by binding with cyclin A2 [87]. Owing to a lack of research on SCAPER, it's involvement in this process is not yet fully established.

Control of cyclin A2's localisation is important for its function Lindqvist lab have recently found that the cytoplasmic localisation is entirely abolished as a response to DNA damage by p21, but were unable to pinpoint what promotes this translocation under normal conditions [88], albeit they did not look into SCAPER-mediated control. Instead, they highlighted the importance of cyclin A2's cytoplasmic localisation and showed that it is responsible for PLK1 activation via BORA and Aurora A (AURKA). More on PLK1 function will be described in later parts of this thesis. Interestingly, they show that exogenously expressed cytoplasmic cyclin A2 promotes mitosis more than exogenous cyclin A2 fused with an NLS [88], hinting that mitotic events need to be triggered from outside of the nucleus.

Not much more is known about the complex control of cyclin A2's localisation and further research is still needed to address some important questions in this field. As this is not within the scope of this thesis, I will instead focus on defining cyclin A2's function, regardless of its localisation.

Functions of cyclin A2 Cyclin A2 is essential in *Drosophila* [56, 89, 90], but dispensable for proliferation in mouse embryonic fibroblasts (MEFs) [91]. On the other hand, mouse embryos are not viable without cyclin A2, regardless of cyclin A1's presence [53,

67]. Cyclin A2 was found to be essential for early mitotic events in human cancer HeLa cells [9, 59]. But according to Gong and colleagues, it is not crucial for mitosis as HeLa cells depleted of cyclin A2 using RNAi were still able to divide after a delay in mitosis [12, 16].

As noted previously, some contradictions on cyclin functions may have occurred due to RNAi methods used to achieve protein depletion which do not fully diminish the protein in cells, and could also have some off-target effects. These and other contrasting reports provided the grounds for setting up novel methods with higher depletion efficiency that were used to address some fundamental questions in this thesis. Nevertheless, the following Section will provide an overview of known functions for cyclin A2, starting with S phase, G_2/M transition, and lastly, its roles in mitosis.

S phase Mammalian cyclin A has important roles throughout replication [5–7] and it seems that these are at least partially affected by its binding partner (or the lack of one). It has recently been found that zebrafish embryos lacking CDK1 and cyclin A2 are unable to enter S phase [92], but this has not yet been demonstrated in mammalian cells.

CDK2-cyclin A2 complex promotes E2F transcription factor (TF) release from the Retinoblastoma protein (Rb), thereby promoting G_1/S progression along with other G_1 CDK-cyclin complexes [68, 93, 94]. Research in human S phase cell extracts showed that depletion of cyclin A2, but not cyclin E – another CDK2 activating protein – negatively influenced replication mechanics [6], implying that cyclin A2 has more important roles during S phase. It is not entirely clear what the specific differences between CDK2-cyclin E and CDK2-cyclin A2 in S phase are, and more research is needed to clarify any differences between the two complexes. Furthermore, cyclin A2 has additional roles in S/G_2 transition where it binds with an E2F dimerization partner, DP-1, to decrease its DNA-binding activity – thus reducing E2F-mediated initiation of gene transcription by interfering with its DNA association mechanics [95].

While the majority of interphase cyclin A2 functions are attributed to its binding with CDK2, the CDK1-cyclin A2 complex has been shown to regulate late origin firing, supported by the lack of CDK2 at these sites [8]. Katsuno et al. elegantly implicated a specific late S phase role for this complex via the ATR-CHK1 pathway, whereby CHK1 regulates CDC25A turnover, a well-established CDK1-activating phosphatase [8]. In contrast, CDK1 inhibition by Hochegger et al. did not result in significant defects during

S phase in mouse and chicken DT-40 cells, implying a more overlapping function for CDK2-cyclin A2 and CDK1-cyclin A2 to fulfill this role [44].

Addressing the importance of a specific kinase partner for cyclin A2 is challenging due to the overlapping activity of these highly similar kinases and the cyclin's peculiar expression and destruction pattern. In zebrafish, the CDK1-cyclin A2 complex has been implicated to play roles in promoting S phase [92]. But in humans, the same complex becomes more abundant only as cells progress further through the cell cycle [47], indicating that this secondary kinase partner may have additional roles later on. There are still many unknowns surrounding the difference in function of cyclin A2 with either of the two CDK partners, and more research is required to further clarify the necessity for both of these complexes in human cells.

G_2/M transition Cyclin A2 is required for mitotic entry in *Xenopus* egg extracts [96] and *Drosophila* [56, 89, 90], but its depletion caused only a delay in mitotic events in HeLa, and no other obvious defects [9, 16]. This indicated that cyclin A2 has functions in G_2 to control the timing of mitotic entry.

Data from *Xenopus* egg extracts showed that cyclin A2 triggers PLX1 (PLK1) activation by phosphorylating aurora borealis (BORA), which promotes mitotic entry [96]. This was recently also confirmed by Cascales et al. in human cells [88]. On top of this, cyclin A2 has been shown to cooperate with the CDC25 family of phosphatases to help CDK1-cyclin B activation [59]. Thus, cyclin A2 may have several roles to trigger mitosis, and more research is needed to elucidate its specific requirements during this transition.

Another function of cyclin A2 in mitosis is keeping the Anaphase Promoting Complex/Cyclosome (APC/C) machinery inactive by phosphorylating one of its activatory components, CDC20, thereby contributing to the control of entry into and progression through mitosis [97]. APC/C is essential for a faithful proliferation, but it must be inactive during mitotic entry to avoid premature degradation of key mitotic proteins, including cyclins, and thus avoiding the premature loss of mitotic kinase activity [98]. APC/C will be described in greater detail after this Section.

During the G_2/M progression a key mitotic phosphatase must be inactivated by a separate pathway via a microtubule-associated serine/threonine kinase-like (MASTL, also known as Greatwall) [99], which will be introduced in more detail in Section 2.3.3. CDK1 has been implied in the activation of MASTL [100], however given the inactivity of CDK1-

cyclin B until prophase onset, a CDK1-cyclin A2 complex may instead contribute to these events. Further studies are necessary to determine the timing of MASTL activation to separate between the roles of cyclin A and cyclin B during mitotic entry.

Mitosis Cyclin A2 is degraded in early mitosis, just after Nuclear Envelope Breakdown (NEBD) [1], indicating that it may have roles in the preceding stages.

Apart from its implied necessity in mitotic entry, cyclin A2 seems to also be required for the regulation of kinetochore-microtubule (k-MT) attachments in prometaphase [50, 51]. Kinetochores are large protein structures that serve as an anchor to attach microtubules to the centromeres of sister-chromatids in prometaphase. Incorrect links are detected by a highly sensitive checkpoint and corrected before cells progress into the next stage of mitosis [25].

The spindle-assembly checkpoint (SAC) SAC maintains the genomic integrity of cells by inhibiting sister-chromatid separation until all kinetochores are attached to microtubules emanating from opposite poles (reviewed in [101–103]). It is a highly complex and sensitive checkpoint that blocks the binding of a co-activator of the Anaphase Promoting Complex / Cyclosome (APC/C) [104]. The APC/C is an E3 ubiquitin ligase responsible for cyclin A and B degradation in mitosis, and will be described in more detail in the next Section on Transcriptional control and degradation mechanisms. Briefly, the APC/C controls mitotic progression by temporally controlled degradation of key mitotic players. Apart from its other targets, the APC/C triggers chromosome segregation by degrading securin, a protein that binds to separase, to unlock the protease activity of separase. Separase is then released and promotes anaphase by cleaving cohesive structures between sister-chromatids to allow their separation (reviewed in [105]). If the APC/C degrades securin before all the chromatids have been attached to their respective opposite poles, it can result in mitotic catastrophe, characterised by aneuploidy, thus promoting genomic instability.

CDC20 is required to trigger initial APC/C activity, but it is specifically bound by the SAC to delay mitotic progression. MAD1, MAD2, BUBR1 and BUB1 were identified as SAC components using yeast screens [106, 107], and the former three bound with CDC20 are now known as the SAC effector, the Mitotic Checkpoint Complex (MCC).

During prometaphase, kinetochores recruit the MCC components [108] and three ad-

ditional kinases aiding to the APC/C's inhibition. These are aurora B (AURKB), BUB1 [109], and MPS1 [110] (the reader is pointed to a review on the SAC by Mussachio and Salmon, 2007 for more information [101]). AURKB is a part of the Chromosome Passenger Complex (CPC) which has roles throughout mitosis in chromosome structure, SAC, mitotic spindle organisation and additional functions in cell division (reviewed in [111]). AURKB is involved in the SAC where it destabilises k-MT connections to allow repair of incorrect attachments [112, 113]. MPS1 is found on the surface of kinetochores and promotes the assembly of the MCC, thereby maintaining SAC activity at unattached kinetochores. Once the k-MT attachments are stable, MPS1 is removed and the SAC signal disappears [114, 115].

The main job of the SAC is keeping CDC20 from activating the APC/C, and the kinases simply aid with the assembly of MCC. MAD2 and BUBR1 components of the MCC either bind CDC20 or block its substrate recognition, respectively [116, 117] (reviewed by Alfieri et al., 2017 [118]). The recruitment of proteins involved in kinetochore assembly and SAC function is highly complex and temporally controlled [119]. These details will not be reviewed here as they are not the focus of this study.

Continued: The roles of cyclin A2 in mitosis If the k-MT mismatches are not corrected, then the spindle-assembly checkpoint (SAC) will not be satisfied and either arrest cells in mitosis, or lead to aneuploidy due to defective chromosome segregation as the chromatid cohesion has not been removed. The reader is directed to these excellent reviews for more details on the effect of defective k-MT repair as it is beyond the scope of this thesis: Tanaka, 2010 [120], and Monda and Cheeseman, 2018 [121].

Elegant experiments in Duane Compton's lab using photoactivatable GFP-tubulin in three different cell lines showed that cyclin A2 – mediated k-MT stability shifts from prometaphase to metaphase [50]. Cells lacking cyclin A2 were shown to have premature k-MT stabilisation whereas its overexpression lead to unstable k-MT attachments in metaphase. The authors proposed that cyclin A2 is required to maintain unstable k-MT links during prometaphase to allow any erroneous attachments to be repaired, followed by APC/C proteasomal degradation of cyclin A2 that then allows these attachments to stabilise once cells enter metaphase [50]. The same group later on showed that this control is carried out by cyclin A2-regulated PLK1 activity in prometaphase [51], supporting the fact that cyclin A2 controls PLK1 in late G_2 and throughout mitosis. However, it is

unclear whether cyclin A2 achieves this function while bound with CDK1 or CDK2.

Additionally, CDK1-cyclin A2 abolishes Origin Recognition Complex (ORC) binding to chromatin during mitosis by hyperphosphorylating ORC1, the largest subunit of ORC [122], thereby suppressing replication.

Interestingly, a role for cyclin A2 after metaphase has also been demonstrated. Residual levels persisting after proteasomal degradation have been implicated to play an important role in the remodelling machinery [123]. Rho GTPases are essential for metaphase and cytokinesis-related signalling. RhoA is specifically relevant for actin reorganisation and helps with mitotic cell rounding and stiffening of the cortex (reviewed by Chircop, 2104 [124]). Low levels of cyclin A2 seem to activate RhoA after metaphase as well as interact with actin directly to promote cytokinesis in a human breast cancer cell line (MCF-7) and in murine mammary gland cells (NMuMG) [123]. These experiments were conducted with small-hairpin RNA to deplete cyclin A levels, and FRET analysis to check for RhoA activity in telophase. Interestingly, the cells entered and progressed through mitosis fairly normally, albeit they needed more time to complete this stage. Not a lot of work has been done on cyclin A2's function in telophase, so further investigations are needed to determine its relevance and function during cytokinesis.

There is more and more convincing data that cyclin A2 functions extend into control of mitotic entry and its progression. This is an interesting area of research as many tumours modify cyclin A2's expression and knowing its distinct functions will help in future research and may pave a road to targeted cancer therapy. Control of cyclin expression is a particularly interesting area as they are strongly expressed when required and degraded during each cell cycle. The next Section will address how cyclin A2 levels fluctuate and why.

Transcriptional control and degradation mechanisms Cyclin A2 expression is tightly controlled throughout the cell cycle transcriptionally, as well as post-translationally. Normally, cyclin A2 becomes detectable in cells in late G_1 and its levels remain high during S and G_2 phases, followed by degradation in prometaphase.

CCNA2 promoter consists of CRE and CAAT boxes that can be occupied by several

families of transcription factors (TF) which can either promote or inhibit transcription in response to growth factors and extracellular stimuli. E2-F is one such transcription factor that responds to cell cycle progression [125]. Cyclin A2 promoter has also been shown to be activated by YB-1 [126], HMGA2 [127], and others.

TGFII-β is a well known growth factor that can trigger several signalling pathways such as ERK, p38 cascade, and MAPK signalling, depending on the cell type [128–132]. Activating ERK promotes a CCAAT-specific Nuclear transcription factor Y (NF-Y) [133] by translocating its NF-YA subunit into the nucleus [134]. Once active, the TF ubiquitously initiates transcription of CCAAT-containing promoters, such as cyclin A2, cyclin B, CDC25C, CDK1, PCNA, DNA polymerase α and others [135, 136]. However, this pathway may have an additional layer of complexity as fibroblasts and epithelial cells have been shown to have distinct responses to the growth factor. The cells trigger NF-YA nuclear accumulation at varying times, depending on their basal p38, ERK and MAPK activities [134]. This confers a higher degree of transcriptional control over the core cell cycle proteins, which is essential in the case of cyclin A2 transcription, as its overexpression can induce DSBs in mammalian cells [137]. Moreover, NF-Y activation is dependent on phosphorylation by CDK2 and can trigger arrests at either G_1/S or G_2/M in human colorectal carcinoma cells if it can not be activated [138]. Concomitant with this, NF-Y inhibition was linked to a G_2 arrest in several mammalian cell lines [139, 140]. On the other hand, CDK2 is not essential in mice [36, 141] or human cells (unpublished, Poon lab). Thus, the control of NF-Y activation can be achieved by other kinases as well.

Chae et al. have also shown that CDK2-cyclin A2 is involved in a positive feedback loop, further activating the NF-Y and promoting the transcription of other cell cycle genes such as cyclin B and CDK1 [136].

On the flip side, the NFAT (Nuclear Factor of Activated T cells) negatively controls cyclin A2 expression by silencing its promoter in lymphocytes. This was supported by mouse models with defective NFAT1 presenting with high levels of cyclin A2 [142], however it is unclear what its role in healthy somatic cells is.

Cyclin A2 has additional levels of transcriptional control, however these are beyond the scope of this thesis, but the reader is directed to an excellent review by Bendris et al. for more information [143]. Apart from transcriptional regulation, cyclin A2's levels are also dictated by a complex degradation mechanism. Timely destruction of cyclins is

crucial for a successful cell cycle, and cells have employed several systems to ensure a swift destruction of these proteins when their partner kinase activity is no longer required. Both A- and B-type cyclins are destroyed in mitosis once all phosphorylations necessary for cell division are achieved. While they share some similar mechanisms of destruction, there are some stark differences between them as cyclin A2 is degraded first during prometaphase and cyclins B1/B2 are destroyed later, in metaphase [1, 144]. This implies that they are controlled differently even though they both contain a highly conserved destruction box (D-box) motif that enables the ubiquitination by the APC/C in mitosis as shown in the *Xenopus* model organism [61, 145, 146]. The RING-type E3 ubiquitin ligase is required to target both cyclin A and B sub-families for destruction, so it will be shortly summarised below.

The Anaphase Promoting Complex / Cyclosome (APC/C) The mitotic E3 ubiquitin ligase APC/C is a large complex consisting of 15 proteins (Figure 2.3) that can be divided into three groups: (I) the scaffolding platform, (II) the catalytic and substrate identification group, and (III) a tetratricopeptide repeat (TPR) arm [147]. The arm is required for co-activator binding, initially CDC20, and CDH1 in later stages of mitosis and during G_1 [147, 148]. The two co-activators are regulated in a different manner and confer substrate specificity as well as inflict a temporal control on APC/C activation [148]. While CDC20 activates the complex in prometaphase and targets the D-box motif (RxxL), CDH1 is also able to bind the KEN-box KENxxxN motif and is active in metaphase [149, 150].

CDK1 and PLK1 both activate APC/C^{CDC20} in prometaphase [151–153]. Importantly, the MCC inhibits CDC20 activity towards D-box containing cyclin B1 and securin until the SAC is satisfied as mentioned above. Once active, APC/C^{CDC20} deregulates CDK1 activity by ubiquitylating cyclin A2 [154, 155]. Furthermore, APC/C^{CDC20} also promotes the subsequent activation of CDH1, which functions in a negative feedback loop that inhibits and aids in degradation of CDC20 [156]. During the metaphase-to-anaphase transition, APC/C^{CDC20} followed by APC/C^{CDH1} target cyclin B1 and securin for degradation, allowing the release of separase and eventual chromosome segregation. The complex is kept active during mitotic exit and G_1 to block premature CDK1 activation and cell cycle entry (reviewed in [148, 157]).

In G_1, increasing CDK2 activity and accumulation of EMI1 then lead to APC/C

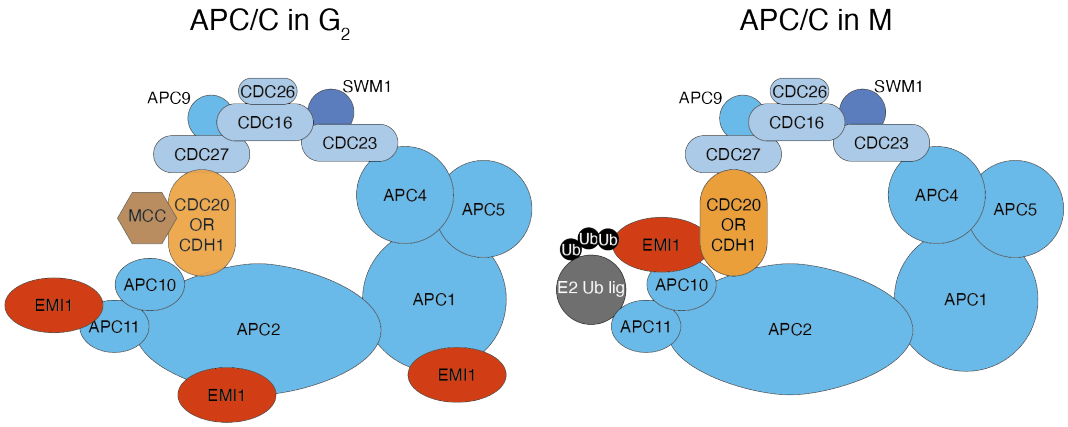

Figure 2.3: A schematic view of the APC/C and EMI1 interplay in G_2 (left), and in M phase (right). EMI1 binds APC1, APC2 and APC11 subunits as an inhibitor in G_2, and as a substrate with its D-box motif to CDC20 / CDH1 in M. The MCC is the mitotic checkpoint complex, SAC effector that maintains CDC20 inactive until SAC is satisfied. The timing of MCC disassembly and EMI1 degradation is not yet fully clear. Model adapted from [162].

inhibition and commitment to the cell cycle [158]. EMI1 is especially interesting as it is capable of being an APC/C^{CDH1} substrate as well as its inhibitor. This appears to be regulated by its concentration: while low amounts of EMI1 are targeted for degradation, as soon as its concentration surpasses APC/C^{CDH1} levels, it becomes very efficient as an APC/C inhibitor [159]. This is achieved by two different binding sites – as a substrate, EMI1 binds to the APC/C with its D-box as other substrates (Figure 2.3, right), but as an inhibitor it binds to APC1, APC2, and APC11 subunits, thus blocking the docking of E2 (Figure 2.3, left). This is possible because EMI1 must be poly-ubiquitinated and if there is more EMI1 than APC/C, even single-ubiquitinated EMI1 is still able to achieve inhibitory binding to APC/C. Mutating the site required for inhibitory binding confirmed that if EMI1 is not able to bind as an inhibitor, it will be degraded [158, 160, 161].

APC/C specific degradation of cyclin A2 Even though both types of cyclins are targets of APC/C, they are degraded at different times of mitosis and as mentioned above, this is largely conferred by CDC20. The co-activator has two roles: (i) it directly binds to cyclin A2 via a CDK subunit (CKS), thereby promoting destruction independently of the SAC [154, 155, 163–165], and (ii) it activates APC/C^{CDH1} as a response to a satisfied SAC, which also targets cyclin B, securin, and others in metaphase [166].

This dual role of CDC20 has been attributed to a change in the requirement for its

interaction with APC/C subunits. It seems that when the SAC is active, CDC20 needs to only bind with APC8 to trigger substrate recognition and ubiquitylation. However, when SAC is satisfied, it must bind with APC8 and APC3 as well as cooperate with APC10 to allow SAC-sensitive substrate binding [164, 166]. Other studies have also indicated that cyclin A2 is able to outcompete SAC components to trigger its own degradation prior to metaphase [154, 164, 165].

Cyclin A2 is not the only early substrate of APC/C^{CDC20}. A centrosomal kinase NEK2A is also destroyed prior to SAC inactivation, and initially it was believed this is owing to its similarity with the cyclin A2 D-box and its surrounding residues [167]. However, it was later demonstrated that the mechanism of NEK2A targeting is vastly different from cyclin A2 ubiquitylation. NEK2A is recruited to the APC/C in prophase independently of CDC20 and destroyed as soon as CDC20 activates APC/C, regardless of its inhibition by the MCC [168]. This is possible because NEK2A is a very sensitive substrate for APC/C^{CDC20} and it is initially recruited to APC/C via its APC/C-binding tail and not by its destruction motifs. Then, as soon as CDC20 is localised to APC/C, regardless of MCC binding, it is able to promote the destruction of substrates containing the D-box motif by associating with the APC/C [168]. These data further highlight the complexity of APC/C-mediated protein degradation during mitosis and have provided us with an unique insight into these complex mechanics.

The question that remains is – why does cyclin A2 need to be degraded earlier than cyclin B? Many researchers have attempted to address this, and they showed that stable cyclin A2 interferes with mitotic progression demonstrated by delays in chromosome alignment and anaphase onset [1, 4]. This, in combination with its role in destabilising k-MT attachments [50], support a model where a prometaphase destruction of cyclin A2 is essential for a stable metaphase plate alignment.

Other mechanisms of cyclin A2 degradation Abrupt cyclin A2 depletion is also aided by the SCF complex. The F-box SKP2 has been found to interact with cyclin A2 (reviewed in [169]), however not much is known about this aspect of cyclin A2 degradation to date.

Next, cyclin A2 can bind to acetyl transferases that aid ubiquitination, but it is not yet clear to what extent they aid protein turnover due to inconclusive mutagenesis data [143, 170].

Additionally, activated by CDK2-cyclin E / A, USP37 has been linked to promoting cyclin A2 accumulation while also deubiquitinating its APC/C site [171]. This promotes the re-accumulation of cyclins and S phase entry independently of APC/C activity and contributes to cell cycle commitment.

A tight control of expression and degradation is necessary for a controlled cell proliferation. Several researchers have looked into the role of augmented cyclin A2 expression in cancer tumours, and some of these findings are outlined below.

Cyclin A2 in cancer Online databases provide information gathered with CRISPR screens which can be found on the Depmap Portal (URL: `https://depmap.org/portal/gene/CCNA2?tab=overview` accessed on 9/6/2021) and Project Score analyses that use the same database (URL: `https://score.depmap.sanger.ac.uk/gene/SIDG03827` accessed on 9/6/2021) show that over 60% of cancer types are negatively affected by the loss of cyclin A2. These data imply that more than half of the analysed cancer types rely on cyclin A2 for their proliferation, but also show that a subset is unaffected by its loss.

Concomitant with the above, cancers that overexpress or downregulate cyclin A2 have been identified, but interestingly, studies have linked both genotypes with a poorer prognosis in patients [137, 172, 173]. High expression levels can induce more DNA double strand breaks (DSBs) and trigger premature replication [137]. The abundance of DNA breaks would normally activate a cell cycle checkpoint, but these are disrupted in a large proportion of cancers, aiding to genomic instability.

A separate investigation into ER positive/negative breast cancers linked cyclin A2 overexpression to a poor prognosis for distant metastasis free survival in ER positive but not in ER negative cancers [174]. They also linked cyclin A2 overexpression with a poorer prognosis for patients with ER positive cancers. Moreover, they indicated that the widely used tamoxifen treatment for ER positive tumours is less efficient in cancers with elevated cyclin A2 levels.

On the other hand, knock-down of cyclin A2 has been linked with a lower MRE11 mRNA translation [173, 175], which is essential for the recognition and repair of DSBs [176, 177]. In agreement with this, Gu and colleagues have found that breast cancer cells utilise cyclin A2 to promote DNA repair by controlling protein levels of two DNA damage

recognition proteins, MRE11 and RAD51 [173].

Another interesting study demonstrated that cyclin A2 becomes downregulated in colorectal cancers in order to promote tumour invasiveness by regulating a cytoskeleton regulator RhoA [178]. Low RhoA activity was in that case directly linked to an increase in membrane motility and cell invasiveness, shown by an altered actin structure. Further investigation carried out in the same lab confirmed the role of cyclin A2 in Rho modulation but also unveiled that cyclin A2 is actually present until metaphase and might have additional functions during mitosis [179].

It is essential to mention that cyclin A2 may have roles in poly- and aneuploidy of cancerous cells. Endoreduplication is a process that is normally carried out in proliferating human cells during cell differentiation of blood, liver and placenta cells, to name a few [180]. These processes are tightly regulated with the TOR and MAPK singalling pathways and it is thought that cyclin E oscillations are the main driver of the endocycle. Cancerous cells sometimes utilise endoreduplication to achieve and maintain aneuploidy, and also to acquire resistance to chemotherapy drugs [180]. Interestingly, it has been shown that cyclin A2 deficiency promotes endoreduplication in Arabidopsis [181]. It is intriguing to imagine that some cancer tumours downregulate cyclin A2 to aid in poly- and aneuploidy.

There has been a substantial amount of research done on CDK2 inhibitors as anti-cancer treatments, regardless of its cyclin partner. Designing specific CDK2 inhibtors is challenging as they are likely to also target other CDK proteins (reviewed by Chohan et al., 2015 [182]). Nevertheless, CDK2 inhibitors have recently been patented by Pfizer [183], however more information on their potency is not yet available. On the other hand, aspirin and salicylic acid have been implicated to hinder cancer cell proliferation by directly downregulating both CDK2 and cyclin A2 [184]. Moreover, Kim et al. have recently described novel inhibitors of the CDK2-cyclin A2 complex that could have potential clinical significance [185]. Specific CDK2-cyclin A2 inhibitors may prove to have great clinical significance in CDK2-dependent cancer types, and more studies on these promising compounds are required to achieve their full potential.

To really understand the pathways in which cyclin A2 promotes cancer, it is essential to clearly define its role in healthy human cells and compare it with cancer cells. This

thesis aims to provide some insight by analysing its function in human cell line systems.

The next Section will provide on a similar summary of cyclin B, following the same format as for cyclin A. While the two cyclins share some similarities, there are many distinctions between them and the reader will be familiarised with these by the end of the next part.

2.2.2 Cyclin B

The B-type cyclins are highly evolutionarily conserved and essential in the majority of studied organisms as they activate CDK1 to drive mitotic progression [11, 90, 186, 187]. Notably, cyclin B binding is not sufficient to activate CDK1. The mitotic kinase is controlled with two inhibitors [188] that allows the inactive CDK1-cyclin B complex to accumulate during G_2 and activate rapidly at mitotic entry to ensure a faithful progression into mitosis. CDK1 is at the basis of a bistable switch system [189, 190] that drives the cells through mitosis and maintains thousands of phosphorylations that promote mitotic events [26]. When the cells are ready to divide, CDK1 is inactivated as its regulatory subunits are degraded by the proteasome [1]. Cells then dephosphorylate CDK1 substrates and split their chromatids into two cells during anaphase and cytokinesis, respectively. More on CDK1, and the speculated trigger that induces its activity at the start of mitosis, will be discussed after this Section.

Cyclin B sub-family There are three homologues belonging to the cyclin B sub-family in vertebrate model systems: cyclins B1, B2 and B3. Cyclin B3 is expressed exclusively in embryos and adult testes [191] and it will not be mentioned in detail here as it is beyond the scope of this thesis. Cyclins B1 and B2 are both somatic but their functions during an unperturbed cell cycle are not yet entirely clear – they can compensate for each other in mammalian cells [192], but cyclin B1 is essential for mouse development, whereas cyclin B2 is not [193].

According to Gong et al., cyclins A2 and B1 cooperate during mitotic entry and progression in human cancer cells [12, 16]. To date it is not fully clear whether the cyclins are also redundant in non-transformed cells and further studies are needed to provide more insight into these interplays.

There are two somatic B-type cyclins in mammals Cyclins B1 and B2 have distinct localisation patterns where cyclin B1 is translocated into the nucleus in prophase [13, 14] and associates with specific cellular compartments such as microtubules and kinetochores after nuclear envelope breakdown (NEBD) [19]. On the other hand, cyclin B2 is restricted to the Golgi apparatus, presumably aiding with the organelle's reorganisation at the onset of mitosis [17]. After mitotic entry, cyclin B2 is found to be distributed

equally in the cells, and not particularly linked with any compartment, unlike cyclin B1 [17]. These distinct localisation patterns are due to specific motifs in the N-terminus of each protein and swapping the sequences also lead to an exchange in their localisation [194].

It is difficult to separate the functions between cyclins B1 and B2 as there are a few contradictions in the field. However, overexpression of either protein is a marker for poor prognosis in cancer patients [195]. Following is what is known about each of these proteins to familiarise the reader with their roles in human cells. While the differences between cyclins B1 and B2 could be a separate investigation, this was not addressed in this study. Thus, because cyclin B2 is not essential and less relevant to the research conducted, it will be reviewed first and in less detail than cyclin B1.

2.2.2.1 Cyclin B2

It is not yet clear why cyclin B2 cannot compensate for the roles of cyclin B1 during mouse development. They are structurally very similar proteins, with some crucial differences in their N-termini as noted above [194]. Not much is known about specific functions of cyclin B2, and more striking phenotypes are shown in cells when it is depleted along with cyclin B1 [187]. Indeed, depleting both proteins in HeLa cells resulted in a delay in mitotic entry and more apparent defects in later stages of mitosis [12, 16].

Interestingly, elevated cyclin B2 expression has been linked to a poorer prognosis for some cancer types, including non small cell lung cancer [196] and breast cancer [197]. Furthermore, down-regulating its high levels in mouse bladder cancer lead to a decrease in its invasiveness and metastasis [198]. It is essential to clarify the functions of cyclin B2 in human cells and what roles this dispensable protein acquires to promote the development and proliferation of some tumours.

Implications in cancer A study on mouse cancer cells overexpressing cyclin B2 showed that cyclin B2 influences centrosome separation mechanics [195] (reviewed by [199]). Nam et al. show that high levels of cyclin B2 during mitosis cause defects such as lagging chromosomes and anaphase bridges, while cells have an intact SAC. Mitotic defects were attributed to issues in k-MT mis-attachment repair. Furthermore, the authors linked cyclin B2 overexpression with non-linear poles in metaphase cells, owing to impaired

centrosome positioning. They demonstrated that the overexpression caused premature centrosome separation via aurora A (AURKA) and PLK1 hyperactivation [195].

Investigating the levels of cyclin B2 expression in specific cancer types may help to predict patient outcome and narrow down therapeutic targets.

2.2.2.2 Cyclin B1

Overview Strongly expressed during G_2 and M, cyclin B1 is required for a faithful mitosis in mammalian cells [12, 193]. RNAi data in human cancer cells HeLa implicated its importance in the proper timing of NEBD, but it seems to cooperate with cyclin A2 in early mitotic events [12, 16]. A crystal structure for cyclin B1 has been solved [200] and it will be summarised below. The description of cyclin B1 will follow a similar format as for cyclin A2 in the previous Section – starting with protein structure and localisation, followed by a review of its functions in separate stages of mitosis. In the end, a short summary of some studies that analysed cyclin B1's role in cancer development will be provided.

Protein structure and localisation Petri et al. have solved the crystal structure of cyclin B1 and showed that its N-terminus is very similar to two CDK2-binding cyclins, cyclin A2 and E [200]. This part contains the cyclin box domain that is shared among the cyclin family of proteins, so the similarity with other cyclin proteins was not surprising. Additionally, like cyclins A2 and E, cyclin B1 also contains a second cyclin box in its C-terminus, and both the N- and C-terminal cyclin boxes seem to be dependent on each other for stability [200]. Unlike cyclins A and E, the N-terminus of cyclin B includes a cytoplasmic retention signal (CRS) which will be further described below in the paragraphs discussing localisation.

Despite the fact that both cyclins A and B are able to form a functional complex with CDK1, they are fundamentally distinct in other evolutionarily conserved sequences that encode the amino acids building their surface [200]. These are likely the source of previously established cyclin specificity towards a selection of substrates [38, 39, 41–43], (reviewed in [40]). Furthermore, the sequence surrounding cyclin B1's RXL motif that is involved in substrate recognition is markedly dissimilar to cyclin A2 [200], implying that there are fundamental differences in substrate recognition between A- and B-type cyclins.

The CDK1-cyclin B1 complex was previously shown to be recruited to a variety of cellular compartments during mitosis (including microtubules, centrosomes, kinetochores and chromosomes) where it phosphorylates crucial substrates required for a faithful mitotic progression [17, 19, 201, 202]. Specific segments of cyclin B1 are apparently responsible for the control of CDK1-cyclin B1 sub-cellular localisation. For example, a short motif, in combination with some specific residues in the N-terminus, have been linked with the recruitment of CDK1-cyclin B1 to mitotic chromosomes [202]. Moreover, cyclin B1's N-terminal cyclin box is required for CDK1-cyclin B1 association with kinetochores during prometaphase [19].

The protein structure of cyclin B is highly complex and the specific roles of distinct sequences are not yet entirely clear. There is an additional level of complexity owing to a specific regulation of cyclin B1's sub-cellular localisation.

Localisation Cyclin B1's localisation is tightly controlled during G_2 and M phases. The protein is kept in the cytoplasm in G_2 due to a CRS motif in its N-terminus [203]. Following mitotic entry, cyclin B1 is translocated from the cytoplasm into the nucleus during prophase [13], and this is one of the most remarkable events in mitosis. The importance of retaining CDK1-cyclin B1 in the cytoplasm until prophase has been the focus of many studies. Researchers have found that the sudden translocation contributes to the control of mitotic progression [13, 204] and marks a restriction point as cells can still arrest their cell cycle if CDK1-cyclin B1 has not yet been accumulated in the nucleus [205]. In MEFs, nuclear localisation and cytoplasmic retention of cyclin B1 triggers and delays mitotic entry, respectively [10]. It is unclear whether human proliferating cells control mitotic entry in a similar manner.

Studies have shown that CDK1 phosphorylates components of nuclear lamina in vitro and in vivo on sites that are required for their destruction [206–208]. Hagting et al. have shown that this is achieved by CDK1-cyclin B1 [14]. This activity could be the reason why CDK1-cyclin B1 is actively exported from the nucleus until prophase. Notably, CDK1-cyclin A2 that is already inside the nucleus could also aid to NEBD, although it is unclear how a premature disassembly of the nuclear envelope is avoided in that case.

The translocation of CDK1-cyclin B1 occurs during prophase once cells have already triggered some mitotic events [13, 14], but it is not yet entirely clear how it is achieved.

Neither CDK1 or cyclin B1 have conventional NLS signals, and the timely translocation is apparently controlled in at least two different ways: by importin β-mediated translocation, and by blocking cyclin B1's CRS [14, 209]. Importin β is a Ran-dependent GTP-ase [210], but it can aid in the nuclear accumulation of cyclin B1 regardless of Ran presence [211]. On the other hand, mutating only the CRS to hinder cyclin B1's translocation signal resulted in a halted cell cycle in *Xenopus* [212, 213], thereby highlighting the importance of this motif.

The CRS sequence is located in cyclin B1's N-terminus, which encodes additional nuclear export signal (NES) motifs [214]. CDK1-cyclin B1 actually actively shuttles between the nucleus and cytoplasm in G_2, but it is constantly exported until prophase at which point the import signals prevail over the export ones, thus resulting in a nuclear accumulation of the complex [214–216]. The inactivation of CRS by phosphorylation has been implied to tip this balance in favour of nuclear import [14].

Cyclin B1's nuclear accumulation is not yet entirely understood, and there is some uncertainty surrounding the role of PLK1 in this event. While there is evidence that PLK1 promotes nuclear transport by phopshorylating cyclin B1 at the NES [217], contrasting reports demonstrate that PLK1 inhibiton does not abolish cyclin B1's nuclear localisation [218]. Furthermore, others have disagreed on the site where cyclin B1 is phosphorylated by PLK1 [15, 219], but they support that PLK1 does play a role in CDK1-cyclin B1 translocation by phosphorylating sites at or near the CRS. Moreover, Yuan et al. also noted the importance of other kinases such as MAPK and ERK2 in promoting nuclear import of cyclin B1 [219].

Contrasting the above reports is a study from Gavet and Pines that redefined our understanding of CDK1-cyclin B1 spatio-temporal control. Using a novel biosensor for CDK1-cyclin B1 activity, they demonstrated that the translocation is actually dependent on the activity of CDK1-cyclin B1 itself, and not on PLK1 or a formation of a nuclear import signal on cyclin B1 [220]. This has not been investigated further to date, thus the true mechanism of CDK1-cyclin B1 localisation remains elusive.

G_2 / M transition Several studies have attempted to address whether cyclin B1 is necessary for mitotic entry [11, 12, 16, 187]. They show that cyclin B is not required to trigger mitosis, but it becomes essential in later stages. This is in agreement with

the above information showing that CDK1-cyclin B1 complexes become activated and translocated into the nucleus during prophase [13–15]. These data indicate that there is a separate trigger to initiate mitotic events, and CDK2-cyclin A2 has been speculated as the upstream activator of CDK1-cyclin B1 [9].

In contrast, Pines lab have shown that mouse embryos are unable to progress past NEBD after depleting their maternal pool of cyclin B [10]. However, based on the observable condensation of DNA into chromosomes, it is clear that these cells have triggered some mitotic events. Strauss et al. have attributed this to a separate mechanism controlling chromosome condensation in mouse embryos described previously [221, 222]. It is unclear whether a separate CDK activity could also lead to this condensation, e.g. CDK2-cyclin A or CDK1-cyclin A.

Mitosis Due to the above mentioned contradictory reports, it is difficult to attribute specific roles for cyclin B1 during mitotic entry. More research is needed to clarify its specific or redundant functions during this stage in mammalian cells.

Prophase CDK1-cyclin B1 has been implicated to aid NEBD by phosphorylating nuclear membrane components, lamins A, B, and C [14]. To date, this role is unclear as CDK1 could also carry out some phosphorylations as part of a CDK1-cyclin A2 complex. This is supported by cyclin A2 localising inside the nucleus at the onset of mitosis [71], and previous data showing that the depletion of cyclin B did not significantly interfere with the onset of NEBD until cyclin A2 was also knocked down in HeLa cells [16]. Furthermore, the CDK1-cyclin A2 complex has a specific function during mitotic entry to promote PLK1 activation via BORA [88, 96], thus showing that the complex is indeed active during this stage. Further research is needed to determine whether CDK1-cyclin B1 is truly involved and required for NEBD.

Prometaphase CDK1-cyclin B1 is recruited to kinetochores [19] by a SAC component MAD1 [223–225]. The three studies by Alfonso et al., Jackman et al., and Allan et al., demonstrated an interaction between cyclin B1 and MAD1, but they had different views and models to explain its function. First, the Barr lab indicated that CDK1-cyclin B1 is recruited by MAD1 to promote MPS1 localisation to unattached kinetochores and aid the spindle checkpoint activity [223]. Second, Pines lab showed that CDK1-cyclin

B1 translocates MAD1 from nuclear pores to kinetochores to ensure the SAC activation prior to NEBD [224]. Third, Saurin and Musacchio labs implied that CDK1-cyclin B1 is a scaffold for MAD1 at the kinetochores, thereby aiding SAC activation [225]. Their differing views have been summarised in a commentary by Houston et al. where the authors highlighted a common finding between the above studies: MAD1-mediated recruitment of cyclin B1 to kinetochores contributes to the SAC [226]. Future research is crucial to clarify the exact mechanism of the interaction between MAD1, cyclin B1 and the kinetochores.

Once the k-MT attachments are stable, CDK1-cyclin B1 is relocated in a dynein / dynactin-dependent manner to the spindle poles [19, 227]. The CDK1-cyclin B1 complex is known to interact with microtubules [13, 17, 18] and promote spindle pole separation in budding yeast by phosphorylating kinesin motor proteins [228, 229]. Kinesins transport various types of cargo along microtubules [230] and CDK1 phosphorylates the kinesin5 family protein EG5 to regulate its localisation to the mitotic spindle [20, 231]. EG5 is an essential protein that helps in the formation of the spindle as well as with centrosome separation in mitosis and meiosis [20, 232]. However, there are also other pathways aiding to the organisation of microtubule bundles into a spindle such as AURKA-mediated phosphorylation of another kinesin protein, KIF15 [233].

In parallel, CDK1-cyclin B1 also aids to the spindle assembly by activating KIFC1 and TPX2 [21].

Metaphase Cyclin B1 promotes cytokinesis by activating PRC1, a microtubule bundling protein [234]. Mitotic substrates must be dephosphorylated for a faithful mitotic exit. To contribute to this, cells inactivate CDK1 by degrading cyclin B1 *Drosophila* [4] and re-activate mitotic phosphatases (reviewed in [235, 236]).

Controlling levels of cyclin B1 protein in cells is crucial for a successful cell cycle. This is achieved by activating its transcription and degradation depending on the stage of the cell cycle.

Control of expression and degradation in mitosis Firstly I will summarize the transcriptional control of cyclin B1, followed by the mechanism and importance of its degradation during mitosis.

Transcription Cyclin B1's transcription is activated at the end of S phase and it is expressed levels increase throughout G_2 [237]. Cyclin B1's promoter is complex and allows several transcription factors to bind, likely as an additional control mechanism. Transcription factors involved include USF [238], cyclin A2-activated NF-Y [134, 140, 239], YB-1 [126]. Additionally, E2F, previously shown to promote the expression of cyclin A2, has also been implicated to aid cyclin B1's transcription [240]. Interestingly, cyclin B1 continues to be transcribed by transcription factor NF-Y even during mitosis in HeLa cells [241].

Tumour suppressor p53 halts the expression of cyclin B1 in response to DNA damage in two ways: (i) via NF-Y [139] and (ii) via another transcription factor Sp1 [242]. Depleting cyclin B1 and other cell cycle promoters allows the cells to arrest in G_2.

Transcriptional control of cyclin B1 is complicated due to the interplay of various transcription factors and inhibitors involved. More is known about how cyclin B1 is degraded during mitosis, as it is an essential step for a successful mitotic exit [4, 243].

Degradation Cyclin B1 is targeted for destruction in late metaphase, which is mainly driven by the APC/C [144, 244] that was described previously (see Section 2.2.1.2). Briefly, the APC/C recognises substrates with the help of a co-activator, either CDC20 or CDH1 (reviewed in [148, 157]). While the SAC is active, cyclin B1 and securin are not targeted by the APC/C^{CDC20}, whereas cyclin A2 is able to outcompete APC/C^{CDC20} inhibitors to trigger its own degradation [155, 164], as noted previously (see Section 2.2.1.2).

Cyclin B1 and securin are recognised by APC/C^{CDC20}, because of their D-box motifs (RXXL) after the SAC is satisfied, but their destruction mechanics are different [245]. Furthermore, the precise mechanism of cyclin B1 targeting depends on the stage of mitosis, regardless of which APC/C co-activator is bound. The D-box motif is recognised during metaphase and anaphase, but an additional upstream sequence of cyclin B1 is apparently important to increase the affinity for its destruction in anaphase [246].

The ubiquitin signal placed on cyclin B1 by the APC/C has itself been examined. A study has shown that cyclin B1 is targeted by ubiquitin chains linked in multiple different ways [247]. It is supposedly recognised by the 26S proteasome due to a common K48 ubiquitin chain [248, 249]. However, Kirkpatrick et al. have found that is is also marked by non-conventional poly-ubiquitin links on other lysine residues such as K63, which was

thought to play a role separate to the proteasome 26S degradation [250, 251], and K11 [247]. Another study by Dimova et al. showed that K11 poly-ubiquitylation is required, but only in the absence of a number of mono-ubiquitylation sites [252].

Ubiquitin chains are likely comprised of several branches, especially knowing that one ubiquitin molecule can be further ubiquitylated on several different sites. Recently, the branched ubiquitin structures were looked into and implied to be playing an important role in protein targeting (reviewed in [253–255]). However, this will not be further discussed here as it is not relevant to the findings in this thesis.

A study conducted in the Wolthuis group demonstrated that CDK1-cyclin B1 is recruited to APC/C^{CDC20} even in prometaphase with the help of a CDK subunit protein (CKS) [256], similar to cyclin A2 [155]. However, the authors also showed that cyclin B1 is not degraded until SAC is satisfied [256]. Furthermore, cyclin B1 also interacts with APC/C^{CDC20} via a different mitotic kinase: microtubule-associated serine/threonine kinase-like (MASTL, also known as Greatwall) [257, 258]. MASTL coordinates the timing of cyclin B1 degradation with the subsequent separase activation [258], but it is not yet clear whether the defects in mitotic exit of MASTL-depleted cells are only due to impaired timing of cyclin B1 depletion. MASTL will be reviewed separately as its function in mitotic entry is important for the findings in this thesis, thus only its role in the degradation of cyclin B1 is noted here.

Since cyclin B1 is actively recruited to the APC/C^{CDC20} in at least two ways described above, cells must ensure that it does not become degraded before the SAC is inactivated. To this end, cyclin B1 seems to be at least partially dependent on a microtubule-associated protein, hematopoietic PBX-interacting protein (HPIP) [259]. HPIP stabilizes cyclin B1 by interfering with the activity of APC/C^{CDC20}. HPIP is an inhibitor and a substrate of APC/C^{CDC20} [259] and its role is regulated by a post-translational modification.

Failure to fully deplete cyclin B1 can either block mitotic progression in HeLa [3] or result in a re-activation of the SAC in anaphase when kinetochores lose tension, leading to an arrest which blocks cytokinesis as observed in U2OS and HeLa cells [260]. It is unclear whether persisting levels of cyclin B1 have different effects in other cancer cell lines and tumours, but its levels are often dysregulated simply by the intrinsic genomic instability of cancer cells and aneuploidy. Moreover, cancer cells have also been reported to modify

the expression levels of cyclin B1 to aid their survival, and some of these studies will be discussed next.

Implications in cancer There are various reports of cyclin B1 overexpression in several cancer types, including breast, human papillomavirus, cervical, colorectal and non-small cell lung cancer [261–265]. In many cases, cyclin B1 overexpression was linked to a poorer prognosis for the patients [264, 266–268]. A meta-analysis found that high levels of cyclin B1 lead to a poor outcome in most of the lung and oesophageal cancer types, but indicate a better survival of patients with colorectal cancer [269].

Work in mouse model systems demonstrated that promoting *CCNB1* transcription influences separase activity. Specifically, it seems that higher levels of cyclin B1 present on chromosomes interfere with timely separase activation by maintaining its inhibitory phosphorylation, leading to anaphase bridges and mitotic failure [195]. Furthermore, Huang et al. have investigated how altered expression levels of cyclin B1 by miRNA contribute to tumour development in mice [270].

Cyclin B1 levels were found to be high even during G_1 phase of several cancer types, and it was speculated that the synthesis of cyclin B1 during G_1 could contribute to tumorigenesis [271].

A large number of research articles have implicated further roles for cyclin B1 in cancer development and proliferation, but they will not be described here as they are beyond the scope of this thesis. Establishing cyclin B1's function and how this is altered during the development of some cancer types will provide further insights for patient therapy.

Summary Cyclins A2 and B1 are both involved in numerous pathways during mitotic entry, and there are many uncertainties surrounding their specific roles. While studies have indicated distinct roles between the two, these were conducted using conventional depletion methods that could cause some functions to be overlooked or misunderstood.

Next, I will provide summaries of other mitotic players that are relevant in this thesis starting with CDK1, followed by PLK1, MASTL and PP2A.

2.3 Key players in mitosis

CDK-cyclin complexes are responsible for the majority of phosphorylation events that drive the cell cycle. The main cyclin-dependent kinase active during mitosis is CDK1. It is responsible for over a thousand mitotic phosphorylations that ensure a faithful cellular division [26]. I discussed the properties and control of both of CDK1's regulatory subunits (cyclin A and cyclin B) in the previous Sections. However, there is an additional layer of control imposed on CDK1 directly that will be described in the following paragraphs. Furthermore, CDK1 is a part of a complex network that drives mitosis, and some key mitotic kinases and phosphatases that are relevant for the work in this thesis will also be described. Firstly, the functions of PLK1 will be summarised, followed by a short overview of the anti-mitotic phosphatases. Next, I will describe MASTL and its role as the link to inactivating one of the phosphatases. In the end, the key events mentioned throughout this Section will be summarised by focusing on the sequence of events that trigger mitosis.

2.3.1 CDK1

2.3.1.1 Overview

CDK1 is a mitotic kinase that partners with cyclins B1, B2, and A2 during an unperturbed cell cycle and the specific roles for these activities are as described in the preceding Sections of this thesis. As noted previously, it can also compensate for the roles of other CDK proteins by binding with their respective cyclins to drive the whole cell cycle [30–34, 38]. Normally however, CDK1-cyclin B complexes ensure a faithful mitotic progression while the CDK1-cyclin A2 activity is restricted to a specific role during late S phase [8, 44]. To date it is unclear whether CDK1-cyclin A2 has additional roles during G_2 and M stages, although it's increasing abundance as the cell cycle progresses has been noted [47].

Cyclin binding is not sufficient for CDK1 activation. An additional phosphorylation at T161 by a CDK-activating kinase (CAK) CDK7 needs to be present for its kinase functions [272, 273]. Nonetheless, this is not the limiting factor for CDK1 activity because it is actually kept inactive by two inhibitors until cells are ready to enter mitosis [258]. Inhibitory kinases WEE1 and MYT1 maintain T14 and Y15 phosphorylations on CDK1 to suppress its activity [188, 274]. This allows the inactive CDK1-cyclin B1 complex

to accumulate during G_2 and not trigger mitotic events. When cells are ready to enter mitosis, the kinase / phosphatase balance of CDK1 regulators tips in favour of the CDC25 family, resulting in the removal of inhibitory phosphorylations and the beginning of CDK1 activation (reviewed in [275]).

The initial pool of active CDK1-cyclin B1 starts to suppress WEE1 and MYT1 kinases and promotes CDC25 phosphatases, thereby triggering an auto-amplification loop. CDK1-cyclin B1 then phosphorylates mitotic substrates and also aids in the inactivation of protein phosphatase 2A (PP2A), one of the main mitotic antagonists. PP2A must be inhibited because it directly dephosphorylates CDK1-cyclin B1 substrates in G_2 to delay mitotic entry [189]. To achieve this, CDK1 promotes MASTL activation which indirectly inhibits PP2A [100]. Furthermore, MASTL also additionally promotes CDC25 activation, resulting in a yet another CDK1 activatory feedback loop [276]. There is a sharp increase in activated nuclear CDK1-cyclin B1 during prophase and this sudden activity is required to drive the cells through the early stages of mitosis, allowing NEBD [220].

2.3.1.2 Control of CDK1-cyclin B1 activity

As noted above, CDK1 activity is governed by inhibitory kinases WEE1 and MYT1, and activatory phosphatases belonging to the CDC25 family. In fission yeast these antagonistic proteins share similar mechanisms of control that are linked to PP2A [277]. However, here I will focus on their activity in mammalian cells.

WEE1 and CDC25 families have been extensively reviewed by Perry and Kornbluth, 2007 [275], so here I will summarize their findings along with some recent developments. The reader is advised to follow up on the review for more details and specific references if interested.

The CDC25 family CDC25 phosphatases promote CDK activation by removing its inhibitory phosphorylations. Yeast model systems only contain one CDC25 phosphatase [278], but there are three members of the CDC25 family in higher eukaryotes; CDC25A, CDC25B and CDC25C. All three have roles in G_1 / S transition by activating CDK2 [279–282] as well as in G_2 / M progression where they promote CDK1 activity [283–288] (reviewed in [289, 290]). Moreover, all three phosphatases have so far been shown to be involved with the DNA-damage response as CHK1/2 can control them to induce a G_2

arrest [291–294] (reviewed in [295]). To date it is not entirely clear which sub-families of CDC25 are actually essential in mammals owing to contrasting information in the field.

Karlsson et al. found that CDC25B has an essential role as the initial trigger of CDK1 activation [285], supported by the fact that CDC25B is found in the cytoplasm in G_2, whereas CDC25C is nuclear [296, 297]. CDC25C is also essential [283], but it has roles after CDC25B has already triggered CDK1-cyclin B1 activation [296]. Furthermore, CDC25B was shown to be crucial for meiosis in mice [298] and for a faithful DNA damage response in mammalian cells [299]. In contrast to this, a study found that CDC25A and B can compensate for each other during mitotic entry [300]. In agreement with the latter, a different group has found that CDC25A alone is sufficient for proliferation and a faithful DNA damage response in mice [301].

It is currently believed that CDC25A is more important for the G_1 / S transition [279, 280], but it is clear that there is a level of compensation between all three sub-families. This is in agreement with yeast studies as the single CDC25 phosphatase capable of executing all cell cycle roles shares similarities with the three mammalian homologues [278]. More research is needed with today's rapid protein depletion tools to clarify and distinguish between their roles.

Even though the CDC25 sub-families share some similarities, the cells have developed distinct control mechanisms for each of them. They are spatiotemporally controlled throughout the cell cycle thanks to their NLS and NES motifs, postranslational modifications, as well as interactions with 14-3-3 proteins. As their regulation is quite complex, here I will summarize only CDC25C, as it is required for both mitotic entry and progression. See Figure 2.4 left hand side for a visual summary of the following steps.

CDC25C is kept inactive in G_2 by (i) PP2A:B56-mediated dephosphorylation of T130, promoted by CHK1 during replication, and by (ii) p-S216 maintained by CDC25C-associated kinases (C-TAK) kinases, which is additionally shielded by 14-3-3 proteins [275, 291, 302]. The latter phosphorylation is additionally aided by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (reviewed in [303]).

Mitosis At the start of mitosis, T130 is phosphorylated and thus transforms into a docking site for PLK1, which promotes CDC25C's nuclear localisation [275] and its nuclear retention by additionally phosphorylating S198 [302, 304]. T130 phosphorylation

in combination with CDK2 activity triggers the release of the 14-3-3 proteins and exposes the inhibitory p-S216 site [305]. Next, p-S214 promotes PP1-mediated dephosphorylation of p-S216, and that finally activates CDC25C [305, 306]. An initial pool of activated CDK1-cyclin B complexes then in turn further phosphorylate the S214 site which leads to an increase in CDC25C activation resulting in a positive feedback loop ([305]).

Furthermore, p-T48 and p-T67 aid in the maintenance of CDC25C activity [305]. These become phosphorylated by ERK and MAPK kinases to act as a docking site for prolyl isomerase 1 (PIN1), which increases the phosphatase activity of CDC25C [307]. However, in contrast to the above, CDK1 has also been implicated to phosphorylate these sites to aid CDC25C's activation and nuclear localisation [308].

On top of this, CDC25C activity appears to be regulated at least partially by CDK2-cyclin A2 complexes [302, 309], thus supporting a role for CDK2-cyclin A2 as a mitotic trigger. Further backing these claims is another study describing the importance of CDK2 in M entry but not in its progression [310].

On the other hand, a model describing two distinct populations of CDC25C differing in their phosphorylations exists. A population characterised by p-T67 that localises with chromosomes, and another population with p-T130 that is found at centrosomes and interacts with PLK1 [311]. In the model proposed by the authors these two groups are different and do not share the same roles in mitosis. It was further shown that only the p-T130 pool of CDC25C interacts with PLK1 [311]. Regulation of CDC25C is highly complex, and as this is not the focus of this thesis, it will not be explained in more detail.

WEE1 and MYT1 are both able to phosphorylate CDK1 Y15 [274, 312], but only MYT1 can also modify T14 [313, 314]. WEE1 is found in the nucleus [315, 316], while MYT1 is constricted to cellular membranes [313, 314] and also capable of binding to CDK1 [317]. Thus, MYT1 aids to cytoplasmic localisation of CDK1-cyclin B1, as well as maintains its inactive state. Furthermore, MYT1 also aids in checkpoint recovery following DNA damage. Despite its many functions, MYT1 is not essential for cell survival [318], whereas WEE1 is crucial for proliferation in *Drosophila* and mammals [319, 320].

WEE1 is active during G_2 and must be abruptly switched off and degraded at mitotic entry to allow CDK1 activation [321, 322]. To this end, cells have employed a number of

mechanisms that are not yet entirely understood. Here I will summarise what is known to date. The following steps are also summarised in Figure 2.4, right hand side.

G_2 WEE1 is stabilised and protected from proteasomal degradation by MIG16 tumour suppressor, which interferes with the binding of β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) to WEE1 [323]. Additionally, CDC14A shields WEE1 from degradation by dephosphorylating S123 and S139, two sites that can otherwise bind pro-mitotic PLK1 [324]. WEE1 is also positively regulated by 14-3-3 proteins that are bound to its C-terminus (aa639-646) [325], which is promoted by phosphorylation of WEE1 at S549 [326, 327].

G_2 / M CDC14A no longer dephosphorylates WEE1 on S123 and S139, thus allowing them to be phosphorylated. These promote PLK1's recruitment, while p-S123 also promotes the generation of two additional phospho-sites that enable β-TrCP binding - S121 and S53 [324]. However, it is not entirely clear which kinase is responsible for the majority of these phosphorylation events.

Since PLK1 is not required for mitotic entry [328] it is unclear how important this role is in an unperturbed cell cycle. On the other hand, PLK1 seems to have a bigger role in WEE1 inactivation following a DNA-damage checkpoint arrest [299]. Furthermore, CDK1-dependent phosphorylations are also responsible for WEE1 inactivation [329]. In *Xenopus*, PIN1 isomerase aids to WEE1 inactivation at mitosis [330], but this has not yet been demonstrated in human cells.

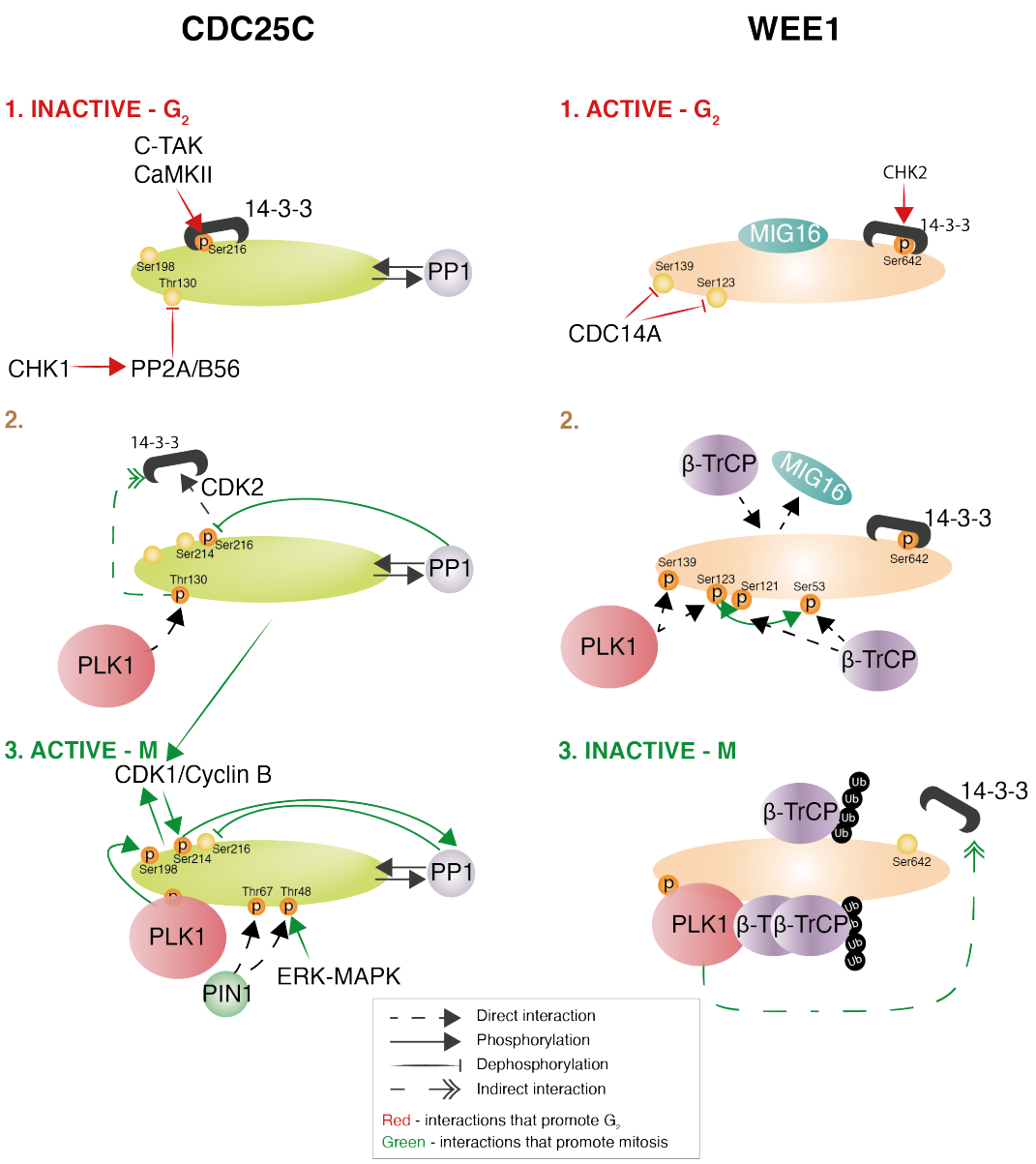

Figure 2.4: A schematic view of CDC25C and WEE1 regulation. Left: 1. CDC25C is inactive in G_2, 2. steps to activate CDC25C, 3. Activated CDC25C at the onset of mitosis. Right: 1. Active WEE1 in G_2 , 2. PLK1 and β-TrCP-mediated inactivation of WEE1, 3. Inactive WEE1 is targeted for degradation. Green arrows indicate events that mitosis and red arrows promote G_2. C-TAK is CDC25C-associated kinase, CaMKII is Ca^{2+}/calmodulin-dependent protein kinase II, PP2A/B55 is protein phosphatase 2A, subunit B55, CHK1 is checkpoint kinase 1. PLK1 is polo-like kinase 1, CDK are cyclin-dependent kinases, PP1 is protein phosphatase 1, PIN1 is prolyl isomerase 1, MAPK is mitogen-activated protein kinase. CDC14A is cell division cycle protein 14A, MIG16 is monokine induced by IFN-γ 16, β-TrCP is β-transducin repeat containing E3 ubiquitin protein ligase, Ub is ubiquitin.

2.3.1.3 CDK1 thresholds and hysteresis

CDK1 was initially thought to be an ON / OFF type kinase [331] to avoid losing mitotic phosphorylations before the cells are ready to divide. However, it is now known that cells ensure their commitment to mitosis by implementing a bistable switch-like system for mitotic entry. Cells require a high level of CDK1 activity to enter mitosis, but a lower amount is needed to maintain this stage [189, 190, 332].

To allow mitotic entry, there must be an increase in CDK1 activity sufficient to reach the entry activity threshold. The feedback loops that are described above aid cells in reaching the threshold in a timely manner. Once the balance is tipped in favour of mitotic protagonists and some initial CDK1-cyclin B1 complexes become active, they trigger several positive feedback loops, as well as negatively affecting the mitotic antagonist PP2A via MASTL [333–335].

Interestingly, it seems that the CDC25 / WEE1 pathway can be completely bypassed without losing mitotic entry controls [336]. Indeed, our lab in collaboration with others have also shown that WEE1 inhibition alone does not deplete CDK1 hysteresis. However, constitutively active PP2A on top of WEE1 inhibition abolishes CDK1 hysteresis. This confirmed that there are indeed two interlinked bistable switches that control mitotic entry [190].

On top of this, in the same study, an intermediate 'prophase-steady' state was defined where cells arrest in mitosis when lacking sufficient CDK1 activity to progress through NEBD [190]. This state was characterised by CDK1-cyclin B1 localising in the nucleus, and not triggering NEBD due to inhibited activity of CDK1 [190]. Thus, cells regulate CDK1 activity not only to enter mitosis but also to progress through different stages of cell division, adding to the complexity of mitotic progression.

2.3.1.4 CDK1 in S phase

An S phase specific role for CDK1 has been demonstrated in *Drosophila* syncytial blastoderm where it aids to synchronise the cell cycle of embryos [337]. In chicken DT-40 cells, CDK1 can initiate DNA replication and aids with centrosome duplication. However, this function is redundant with CDK2 and is only exerted in CDK2 knock-out cells [44].

In somatic mammalian cells, CDK1-cyclin A2 complex starts forming in mid S phase [47] and regulates late origin firing [8]. Katsuno et al. used several ways to demonstrate

that the role is confined specifically to CDK1-cyclin A2. Moreover, they also found that an increase of CDC25A activity promoted CDK1 activation sooner than normally and caused aberrant origin firing in early S phase [8].

Not much is known about the S phase specific CDK1-cyclin A2 activation and more research is needed to elucidate the control of this complex. This information supports a model where CDK1 is controlled in a different manner in S phase, either due to the stage of the cell cycle, or because of its binding partner cyclin A2. In this case, if CDK1-cyclin A2 is already activated in S phase, it is also possible that it can act as a mitotic trigger later on, depending on its mode of regulation.

2.3.2 PLK1

Overview PLK1 is a serine / threonine protein kinase that was discovered in *Drosophila* and has since been shown to be conserved across a variety of species from yeast to human (reviewed in [338]). Overexpression of PLK1 in cancer tumours is a marker for poor prognosis [339, 340], thus it is an attractive target for drug therapy. PLK1 inhibitors have been developed and are in use for some cancer treatments [341] (reviewed in [342]), but in spite of this progress there are still many unknowns surrounding PLK1's numerous functions in the cell cycle. Here, I will review what is known about its role in mitotic entry and progression

Functions PLK1 is involved in DNA replication, mitotic entry, and likely aids with cytokinesis (reviewed in [343, 344]). Additionally, it plays an important role in centrosome maturation at the G_2 / M transition [345, 346]

PLK1 activation PLK1's activity is dependent on BORA and AURKA which act together to phosphorylate the kinase activatory site in PLK1 T-loop T210 [347, 348]. Moreover, it has previously been shown that BORA is also required to activate AURKA [349], thus BORA promotes PLK1 activation both directly and indirectly. BORA is apparently activated by CDK1 [348], but it is unclear whether this is achieved by CDK1-cyclin A2 or CDK1-cyclin B1. As BORA must be activated prior to CDK1-cyclin B1, CDK1-cyclin A2 seems like a potent candidate for this role. Moreover, cyclin A2 directly interacts with PLK1 in late G_2 [350], possibly aiding its activation as well.

Interestingly, similar to CDK1-cyclin B1, PLK1 also translocates into the nucleus prior to NEBD [38, 347].

Mitotic entry Depleting or inhibiting PLK1 hindered mitotic entry [351], and even resulted in a G_2 arrest in some studies [350, 352]. On the other hand, depleting PLK1 to less than 10% in non-cancerous human cells resulted in a normal but delayed entry into mitosis [328]. These data underline its potency as a drug target for cancer patients.

PLX1 – a *Xenopus* homologue of PLK1- has been shown to interact with and activate CDC25C, thereby aiding with the activation of CDK1 [353] (reviewed in [354]). This has also been confirmed in mammalian cells (reviewed in [343]), supporting a role for PLK1 in mitotic entry, although it does not seem to be essential for it [328]. PLK1 seems to be more important for mitotic entry following a checkpoint arrest, as mentioned above [299].

Mitotic progression During mitotsis, PLK1 is involved with spindle and centromere assembly, kinetochores, and cytokinesis (reviewed in [338, 355]). Due to its interaction with a variety of proteins during mitosis such as AURKB, MPS1, the APC/C and the SAC, PLK1 is an indispensable protein required for the faithful progression into the next cell cycle (reviewed in [344, 355]).

Specific roles of PLK1 are less relevant for this thesis and will thus not be explained in more detail here. The reader is advised to follow up on these reviews [338, 344, 355].

2.3.3 Phosphatases and MASTL

Phosphatases are the main anti-mitotic activity since they directly dephosphorylate mitotic substrates phosphorylated by CDK1. There are a few protein phosphatases implicated in mitotic entry, progression and exit: PP1, PP2A, PP4 and PP6 (reviewed in [100]). Though some of their functions are defined, there are still many questions surrounding their activation, inhibition and overall function. Here I will focus on two that are most relevant for this thesis - PP1 and PP2A.

These two phosphatase families have stark differences in the structure of their catalytic sites, which suggests that they are specific towards distinct phospho-sites. Indeed, studies have shown that PP1 is required in later stages of mitosis, while PP2A has important functions during mitotic entry as well as exit (reviewed in [235, 236]).

During the G_2 / M transition both PP1 and PP2A must be inactivated to allow a successful entry and progression through mitosis. Later, they are re-activated to ensure a faithful chromosome segregation and mitotic exit [235]. These events are coordinated with kinase activity to achieve a timely mitotic substrate phosphorylation at entry and dephosphorylation at exit. The phosphatases are excellent targets for cancer therapy due to their involvement with cell cycle control [356].

2.3.3.1 PP1

There are a number of isoforms of PP1 with distinct localisation patterns and over 200 regulatory proteins that control their activity. PP1 can be inactivated by CDK1 during late G_2 and this site becomes auto-dephosphorylated when CDK1 activity is decreased in anaphase, thereby allowing PP1 activation, mitotic substrate dephosphorylation, and a faithful mitotic exit (reviewed in [357]).

The major role of PP1 seems to be in anaphase where it regulates chromosome architecture by interacting with KI67 and AURKB [358–360]. PP1 has not been attributed essential roles during mitotic entry as it is apparently more specific for late mitotic substrates (reviewed in [235, 236]).

2.3.3.2 PP2A

PP2A is required for the correct timing of mitotic entry and exit by dephosphorylating CDK1 substrates. It is strongly activated in interphase and inhibited prior to mitotic entry. The PP2A:B55 subunit is inactivated by the binding of MASTL-phopshorylated ENSA / ARPP19 [99, 333, 335]. It is possible that the trigger that leads to CDK1-cyclin B1 activation also helps to inactivate PP2A via MASTL.

On the other hand, CDK1-cyclin B1 itself has been indicated to promote mitotic entry by directly phosphorylating ARPP19, independently of MASTL . Nevertheless, this activity is insufficient for a faithful cyokinesis [361].

In *Xenopus* egg extracts, depletion or inactivation of PP2A:B55 during G_2 causes them to enter mitosis more readily but they suffer defects during the exit. However, if PP2A is inhibited specifically in mitotic extracts, they are surprisingly capable of exiting mitosis normally [362]. This indicated that the main function of the phosphatase in

Xenopus is delaying mitotic entry until cells are ready to commence cell division and that there are likely other phosphatases that can take over the role of substrate dephosphorylation [362]. However, this has not yet been shown in mammalian cells where PP2A has a known role during mitotic exit in inactivating the CDK1 activator CDC25C [363].

PP2A is an attractive target for cancer therapy and recently NCI has announced three clinical trials using LB-100. The small-molecule inhibitor is being promoted for its low toxicity and shown to enhance effects of other chemotherapeutics like PD-1, according to the information provided by NCI and LB-100 developer Lixte Biotechnology Holdings (unpublished).

2.3.3.3 MASTL

MASTL was first described in *Drosophila* as Greatwall, an essential mitotic kinase required for chromosome condensation and faithful mitotic progression [364]. Soon after, its role as a regulator of PP2A in the control of mitotic entry and exit was discovered [333–335, 365]. As noted above, MASTL inhibits PP2A by phosphorylating two small proteins, ENSA and ARPP19 [99, 333, 335]. The two paralog inhibitors are highly similar, but have distinct roles during mouse development [366].

Additionally, MASTL has roles in the reorganisation of cell architecture that are independent of its kinase activity [367] (reviewed in [368]).

Overexpression of MASTL in several cancer types is a marker for poor prognosis (reviewed in [368]) and it has also been linked with head and neck squamous cell cancer recurrence [369]. Researchers are investigating the potency of MASTL as a drug target in several cancer types, including breast, thyroid, head and neck squamous cell cancer [369–373].

Mitotic functions MASTL needs to be phosphorylated on several residues to become activated, including T193 or T206 which play important roles in its kinase activity, followed by autophosphorylation at S883 to further promote its own activation [374]. To date the initial trigger is unclear, but CDK1 has been implicated as it can activate MASTL in vitro [375]. CDK2 is also a possible candidate as it is active prior to mitotic entry.

G_2 / M MASTL is thought to promote mitotic entry as a nuclear component of the Mitosis Promoting Factor (MPF) [376]. Nevertheless, cells lacking MASTL can still enter mitosis, but they are unable to achieve a faithful cytokinesis [335, 377].

MASTL is also involved in restarting the cell cycle after a G_2 / M checkpoint arrest induced by DNA damage [378].

In addition to acting in the PP2A inhibitory pathway, MASTL acts as a CDC25 activator to promote CDK1-cyclin B1 activation and mitotic entry [276]. It is thought that MASTL contributes to the switch-like mechanism controlling mitotic entry [189].

Mitosis It is not entirely clear what roles MASTL plays in early mitosis. Its main role is likely to maintain PP2A in an inactive state to ensure mitotic substrates are not dephosphorylated too soon [379]. Apart from this temporal control, studies have not yet shown other specific functions of MASTL.

To promote mitotic exit, MASTL catalyses cyclin B1 binding with the APC/C, irrespective of CDC20 [258]. Furthermore, it becomes inactivated by PP1 on its auto-activatory site to allow PP2A re-activation and a faithful mitotic exit [380–382].

2.3.4 Events that lead to mitotic entry

As the most important players were described in the previous paragraphs, here I will briefly summarize their role in promoting mitotic events (Figure 2.5). While CDK1 is at the centre of these pathways, it is actually influenced by separate actors. Most importantly, CDK-cyclin A2 has been indicated as the trigger by kick-starting both pathways that promote CDK1 activation at mitotic entry. However, it is unclear whether this is carried out by CDK1-cyclin A2 or CDK2-cyclin A2 and further research is needed to separate between the roles of these two complexes.

After the change in the balance between CDK1 inhibitors and activators, negative and positive feedback loops allow the formation of an auto-amplification loop of CDK1-cyclin B1, which is required to drive the cells through mitosis [275, 361, 383, 384]. The initial pool of active CDK1-cyclin B1 suppresses WEE1 and MYT1 kinases and promotes the CDC25 family of phosphatases, thereby triggering a self-promoting loop.

CDK1-cyclin B1 complexes then phosphorylate mitotic substrates and also aid in the

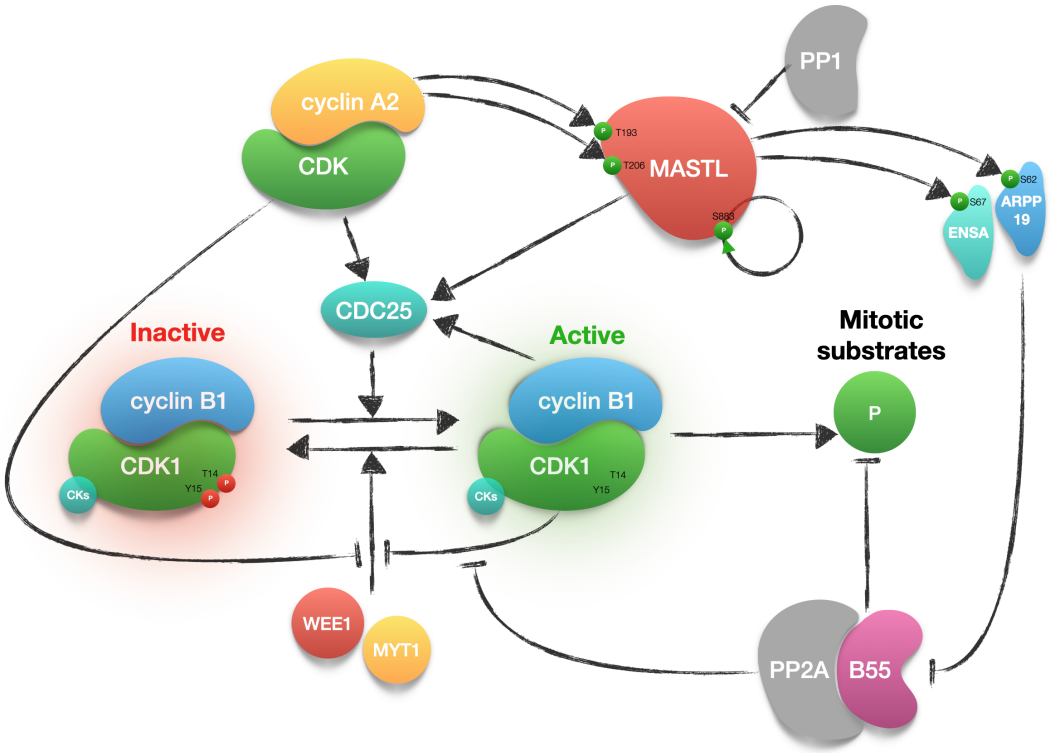

Figure 2.5: A schematic view of the interplay of key mitotic players in mitosis. The schematic shows proteins that promote CDK1-cyclin B1 activation and the subsequent mitotic substrate phosphorylations (marked by P in a green circle). Protein names are as noted in the main text. Promoting interactions are marked with arrows and inhibitory interactions are marked with flat arrows. Phosphorylation that promotes protein activity is marked in green, and inhibitory phosphorylations are marked in red.

inactivation of PP2A. The phosphatase must be inhibited because it directly dephosphorylates CDK1-cyclin B1 substrates in G_2 to delay mitotic entry [189]. To achieve this, CDK1 promotes MASTL activation which indirectly inhibits PP2A [100]. Furthermore, MASTL additionally promotes CDC25 activation, resulting in a yet another CDK1 activatory feedback loop [276].

Chapter 3

Materials and Methods

3.1 Hochegger lab methods

Due to the format of my dual-degree PhD, methods will be described separately depending on the lab the work was conducted in as there are some differences between them.

3.1.1 List of solutions

Reagent	**Components**
BSA	2 mg/ml BSA
$CaCl_2$	2M $CaCl_2$
DAPI	Dilute 5 mg/ml stock 10000 fold in PBS
DMEM	Supplied by the GDSC TC Facility
DMEM / F12	Supplied by the GDSC TC Facility
HBS (2X)	50 mM HEPES (pH 7.1), 280 mM NaCl, 1.5 mM, Na_2HPO_4
KCl	75 mM KCl
Lysis buffer	50 mM Tris-Cl pH 7.5, 2 mM EDTA, 1 mM DTT
MiliQ	Supplied by the GDSC TC Facility
PBS	Supplied by the GDSC TC Facility
PBS/NP-40	PBS / NP-40 (0.1%)
Sample buffer (6X)	0.01% v/v Bromphenol blue, 62.5 mM Tris-HCL pH 6.8, 7% w/v SDS, 20% w/v sucrose, 5% v/v β-mercaptoethanol
SDS-PAGE stacking gel	5% w/v acrylamide, 0.13% w/v bisacrylamide, 125 mM Tris-HCl (pH 6.8), 1% v/v APS, 1% v/v TEMED
12% SDS-PAGE resolving gel	12% w/v acrylamide, 0.086% w/v bisacrylamide, 375 mM Tris-HCl (pH 8.8), 1% v/v APS, 1% v/v TEMED
SDS-PAGE running buffer	2.88% w/v glycine, 0.6% w/v Tris base, 0.1% w/v SDS
Transfer Buffer Anode 1	300 mM Tris, 20% methanol, pH 10.4
Transfer Buffer Anode 2	25 mM Tris, 20% methanol, pH 10.4
Transfer Buffer Cathode	25 mM Tris, 4 mM 6-aminohexanoic acid, 20% methanol, pH 10.4

Tris-HCL, various pH	Supplied by the GDSC TC Facility

Table 3.1.1: List of solutions used in the Hochegger lab. A table of solutions that were used in the Hochegger lab for work conducted in Chapters 4, 5, and 6. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise, and diluted in MiliQ, unless stated otherwise.

3.1.2 List of DNA plasmids

Name	**Vector**
H2B-mRFP-P2A-mEmerald-NES	pFusionRed
Sleeping beauty transposase	pCMV (CAT) T7-SB100 [385]
Cyclin B1-YFP-NLS	pSBtet-BP (pUC19 backbone) [385]
Cyclin B1-YFP	pSBtet-BP (pUC19 backbone) [385]
YFP	pSBtet-BP (pUC19 backbone) [385]
YFP-NLS	pSBtet-BP (pUC19 backbone) [385]

Table 3.1.2: List of DNA plasmids used in the Poon lab. A table of DNA plasmids that were used in the Poon lab for work conducted in Chapters 4, 5, and 6.

3.1.3 List of antibodies

Name	**Host**	**Code**	**Manufacturer**
53BP1	Mu	N/A	Gift from O'Driscoll lab
α-tubulin	Mu	7291	Abcam
γ-tubulin	Rb	ab84355	Abcam
Alexa647 anti-rabbit	Donkey	A31573	Invitrogen (ThermoFisher)
Alexa555 anti-goat	Donkey	A21432	Invitrogen (ThermoFisher)
Alexa488 anti-mouse	Donkey	A21202	Invitrogen (ThermoFisher)
HRP anti-mouse	Gt	P0447	DAKO
HRP anti-rabbit	Gt	A120-201P	Bethyl Laboratories
p-APC1 (S355)	Rb	ab10923	Abcam
Aurora B	Mu	ab2254	Abcam
CDK1	Mu	A17	ThermoFisher

CDK2	Mu	ab6433	Abcam
p-CDK substrates [TPXK]	Rb	9477S	Cell Signaling Technology
CDC27	Mu	610455	BD Transduction Biolab.
p-CDC27 (T244)	Rb	ab12281	Abcam
CREST	Hs	HCT-0100	Immunovision
Cyclin A2	Mu	ab38	Abcam
Cyclin B1	Mu	ab72	Abcam
Cyclin B1	Rb	ab32053	Abcam
Cyclin B2	Mu	sc-28303	Santa Cruz
Myc	Mu	ab32	Abcam
GAPDH	Mu	GTX627408	Genetex
GFP	Mu	2555	Cell Signaling Technology
MASTL	Mu	HPA02717	Sigma-Aldrich
p-MASTL (T198)	Rb	N/A	Produced in Hochegger lab
ENSA	Mu	ab180513	Abcam
p-ENSA (S67)	Rb	5240	Cell Signaling Technology
KI67	Mu	ab16667	Abcam
Lamin A/C	Gt	sc-6215	Santa Cruz
p-Lamin A/C S22	Rb	2026S	Cell Signaling Technology
Pericentrin	Rb	ab4448	Abcam
PP1	Mu	sc-7482	Santa Cruz
p-PP1 (T320)	Rb	EP1512Y	Abcam
PP2A:B55	Gt	sc-18330	Santa Cruz
PP2A:B55α	Mu	sc-81606	Santa Cruz

Table 3.1.3: List of antibodies used in the Hochegger lab. A table of antibodies that were used in the Hochegger lab for work conducted in Chapters 4, 5, and 6. Manufacturer details are as follows: Abcam (Cambridge, UK), BD Transduction Laboratories (Franklin Lakes, New Jersey , USA), Bethyl Laboratories (Montgomery, TX, USA), Cell Signaling Technology, (Danvers, MA, USA), DAKO (Agilent, Santa Clara, CA), Genetex (Irvine, CA, USA), Immunovision (Springdale, AR USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma-Aldrich (St. Louis , MO, USA), ThermoFisher (Waltham, MA, USA).

3.1.4 Cell culture and transfections

RPE-1 cells were grown in DMEM/F12 (Sigma) supplemented with 10% of TET-FREE Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin antibiotic (Sigma). MCF7 cells were grown in DMEM (Sigma) supplemented with 10% of FCS and 1% Penicillin/Streptomycin antibiotic (Sigma). MCF10A cells were grown in DMEM supplemented with 100 ng/ml of Cholera toxin and 0.5 mg/ml of Hydrocortisone (all obtained from Sigma). All cells were grown in 37°C and 5% CO_2 unless stated otherwise.

RPE-1 cell lines were transfected either with Neon Electroporation Transfection kit (ThermoFisher) using 1.5 μg DNA, according to the manufacturer's protocol, optimised by Dr. Nadia Hegarat, or with FuGene (Promega) using 2 μg DNA and different concentrations of Fugene reagent, ranging from 2 : 1 to 4 : 1, transfected according to the manufacturer.

3.1.4.1 Drugs used in the Hochegger lab

Drugs used for this study and working concentrations were as noted here:

Abbreviation	**Drug name**	**Concentration**
PD	PD-166285	0.5 μM
N/A	Apcin	26 μM
N/A	ProTAME	6 μM
Asv	Asunaprevir	3 μM
IAA	Indole-3-acetic acid	500 μM
DOX	Doxycycline	1 μg/ml
thy	Thymidine	4 mM
N/A	SiR-DNA	50–100 nM
N/A	SiR-Tubulin	50–100 nM

Table 3.1.4: Drug names and concentrations. A table of drugs and their working concentrations as used in the Hochegger lab for work in Chapters 4, 5, and 6.

3.1.4.2 Sleeping Beauty gene incorporation method

Sleeping beauty plasmids were obtained from Addgene (plasmid nr. 60496 pSB-tet-BP) and using NEB HiFi Assembly according to their protocol, BspDI and NcoI sites were targeted to cut out the luciferase present in the received plasmid and incorporate the gene of interest (GOI). The plasmid includes a TET-ON promotor for the GOI and a stably expressed Blue Fluorescent Protein (BFP). To transfect cells, 1.9 μg of this plasmid along with 100 ng SB-100X (Addgene plasmid nr. 34879) was transfected into RPE-1 degron cells using electroporation. Cells were grown for 10 days and BFP-positive cells were then sorted into 96-well plates (excitation approx. 456 nm) using FACS Melody live cell sorting, with the help of GDSC staff. Cells were then grown and checked for protein expression after 24 h of DOX addition using western blotting. Afterwards, a few promising clones were chosen to work with.

3.1.4.3 siRNA transfections

All siRNA were diluted to 20 μM of stock solution using RNAse free water upon receival and stored in – 20°C. For one well in a 6-well plate transfection, 500 μl of Dulbecco's Minimum Essential Medium (MEM) was used to incubate 5 μl siRNA-MAX. Afterwards, 160 nM of siRNA was added to the mix and incubated for 10 – 15 min at room temperature (RT). In the mean time, cells were trypsinised and counted to 10 x 10^4 cells/ml. Next, 1.5 ml cells and the prepared mixture with siRNA were mixed and seeded into a well. After 7 – 8 h the media was aspirated and changed to the normal growth media.

In the case of siENSA/ARPP19 and siMASTL, cells were synchronised with 4 mM thymidine 12 h after siRNA transfection for a minimum of 22 h. They were released the same as described in Section 3.1.5.

For siRNA transfections, "ontarget plus" smart-pool siRNAs were used (Dharmacon or Horizon) for ARPP19, ENSA, CCNA2, CDC27, B55α, B55δ. As a negative control, All Stars negative control siRNA (Qiagen 1027280) was used.

3.1.4.4 Double degron tag method (^{dd})

The generation of degron tagged cells was not the topic of this thesis, more information on the technique and gene targeting is available in previous publications from the Hochegger lab [24, 386]. Briefly, a combination of two protein degradation tags was used: mAID

that is a mini-Auxin-Inducible Degron tag and SMASh (Small-Molecule Assisted Shutoff) that normally cleaves itself off. The SMASh tag triggers degradation of a protein when it remains attached to the protein of interest (POI) by addition of a small molecule inhibitor Asunaprevir (Asv). The mAID tag requires plant TIR1 protein to be expressed as it forms an E3 ubiquitin ligase with other endogenous proteins that trigger protein degradation and recognise the mAID tag after the addition of Auxin (IAA, Indole-3-acetic acid). Plant TIR1 derived from *Oryza sativa* (osTIR1) was incorporated into the commonly used Rosa26 locus with a tetracycline inducible promotor (TET-ON) (see Figure 3.1 for a schematic view of the degradation induced by the degron tags).

RPE-1 cells tagged with the double degron tags were treated with 1 μg/ml of doxycycline (DOX) for 2 h prior to adding 3 μM of Asv and 500 μM of IAA for 4 h (hereafter referred to as the DIA cocktail or DIA treatment) to activate both degron tags (Figure 3.1). Cells were incubated at 37°C during these treatments.

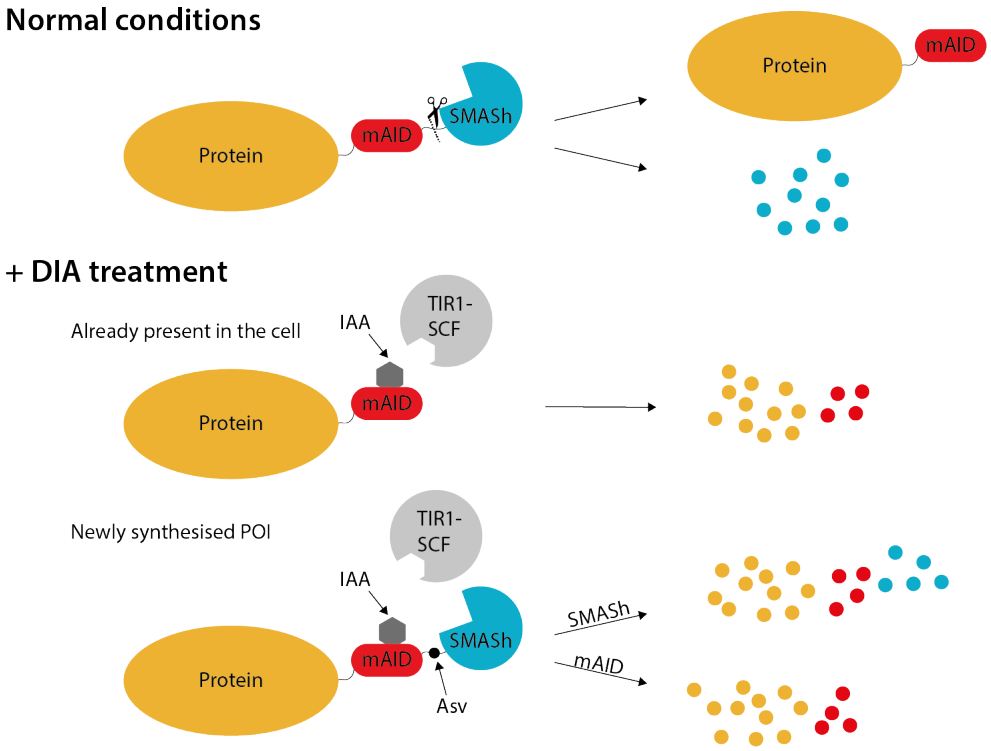

Figure 3.1: A double degron tag system used to study the roles of cyclins in RPE-1 cells. Under normal conditions, the cells express the protein of interest (POI) with the mAID tag attached, while the SMASh tag cleaves itself off in the absence of a small molecule inhibitor. Upon the addition of the DIA cocktail (1 μg/ml DOX, 3 μM of Asv and 500 μM of IAA), DOX promotes osTIR1 expression, which targets the mAID for degradation in the presence of IAA. Asv inhibits SMASh detachment from the protein, thereby targeting the newly translated tagged protein for degradation.

3.1.5 Single thymidine arrest

Cells were seeded into a P75 or P150 flask to approximately 75% confluency. On the same day, 4 mM of thymidine (thy) was added and the cells were then incubated for a minimum of 22 h. Next, cells were washed 5 – 10 times with warm PBS and either re-seeded for immunofluorescence analyses, or re-incubated with DIA-free or DIA-containing media for 9 h, then 25 μM of Apcin and 6 μM of ProTame were added for and additional 2 h prior to harvesting. If the cells were to be analysed by immunofluorescence they were diluted to $20x10^4$ cells/ml and 2 ml were seeded into a 6-well plate containing autoclaved coverslips provided by the GDSC TC facility with 17 – 19 μm thickness, or 200 μl were added into a 96-well PerkinElmer Cell Carrier plate. They were released the same as above and fixed with ice-cold or -20°C Methanol.

3.1.6 Single thymidine arrest in combination with an siRNA transfection

Cells were seeded in a 6-well plate on top of 22 x 22 mm and 17 – 19 μm thick coverslips that were previously autoclaved and provided by the Tissue Culture (TC) facility at the Genome Damage and Stability Centre (GDSC), or into a 96-well PerkinElmer Cell Carrier plate on day 1 to a total of 30 – 40 x 10^4, or 20 – 30 x 10^4 cells, respectively. On the same day, 4 mM of thymidine (thy) was added and the cells were then incubated for a minimum of 24 h. On day 2, cells were washed three times with 37°C PBS and incubated for further 14 h. They were then treated with siRNA and 4 mM of thy on day 3. On day 4, cells were washed 5 – 10 times with 37°C PBS and the DIA cocktail was added to degrade the POI. After 12 h of release, on day 5, the cells were treated with 25 μM of Apcin and 6 μM of ProTame for 2 h prior to fixation.

3.1.7 Live-cell imaging

All live-cell imaging (LCI) was conducted in temperature- and CO_2 -controlled chambers set to 37°C and 5% CO_2 except in the spinning disc microscope, which did not have a CO_2 control, where cells were kept in CO_2 independent media. Where incidated, SiR-DNA and SiR-Tubulin were incubated for a minimum of 2 h prior to LCI experiments. Cells were imaged either on a IX-71 microscope using fluorescence channels and/or differential inter-

ference contrast (DIC), or with the PerkinElmer Operetta microscope using fluorescence channels. Operetta LCI experiments were conducted using PerkinElmer 96-well Ultra plates, TC treated. Imaging conditions were set to below 10% for each laser.

3.1.8 Immunofluorescence

Cells were fixed using either 100% ice-cold or – 20°C methanol for 1 min or 4% Formaldehyde/PBS for 10 – 20 min at RT and washed with PBS. They were then permeabilised using PBS/NP-40 (0.1%) for 15 min and blocked with 3%BSA in PBS for 1 h. All primary antibodies were diluted in 3%BSA, at a dilution of 1 : 200, except γ-tubulin (1 : 1000) and incubated for 1.5 – 2 h at RT. Secondary antibodies were diluted in 3%BSA at 1 : 2000 and incuubated at RT for 1 h. Cells were then stained with DAPI for 10min and mounted using mounting media. If using 96-well plates for IF, the antibody dilutions were kept the same, but instead of mounting media, they were kept in PBS at 4°C and analysed within 7 days.

3.1.8.1 Immunofluorescence signal quantification

PerkinElmer Harmony 4.7 and 4.8 program was used for immunofluorescence analysis to measure phospho-antibody intensity. A single plane in the cell centre was selected for all instances and intensity measured manually for a minimum of 100 cells per condition.

3.1.9 Chromosome spreads

Cells were synchronised by a single thymidine block as described above, followed by a release and the addition of proTAME / Apcin. Cells were collected by shake-off and incubated in 37°C 75 mM KCl for 10 - 20 min at RT. Cells were then centrifuged and resuspended in methanol : acetic acid (3 : 1). Cells were incubated for 5 min and centrifuged. Cells were resuspended in 100 - 300 μl of the methanol : acetic acid solution and dropped on glass coverslips. The coverslips were allowed to dry prior to additional fixation in 4% Formaldehyde for 10 min at RT. Immunofluorescence analyses were then conducted as described above.

3.1.10 Immunoblotting

Cell pellets were washed 1 – 2 times with PBS and either frozen at -80°C or used for making the cell lysate. The pellets were resuspended in ice-cold lysis buffer, incubated on ice for 20 min and spun down at 4°C for 30 min. The suspensions were then sonicated at 39% amplitude for 10 s. Afterwards, sample buffer was added and cell lysates boiled at 95 for 3 – 5 min. This was then loaded into a SDS-PAGE gel. The SDS-PAGE gel was made according to the lab's protocol, ranging from 10% – 15%, depending on the POI size. The gels were run in a Bio-RAD chamber at 200 V for a minimum of 1 h, depending on the POI size.

Afterwards the proteins were transferred to a Amersham Hybond 0.45 μm PVDF transfer membrane (ThermoFisher) using a semi-dry transfer system. To assemble the tranfer sandwich, 3 sheets of 3MM paper (Whatman, UK) were soaked in Anode 1 bufer placed into a dry dish, followed by 2 sheets of 3MM paper soaked in Anode 2 buffer and placed on top. Then, a PVDF membrane was soaked in methanol and placed on top of the paper. The gel was disassembled and washed under tap water, and washed in MiliQ water prior to being placed on top of the PVDF membrane. Then, 5 sheets of 3MM paper soaked in Cathode buffer were placed on top. Bubbles were rolled out using a roller and the sandwich was placed into a transferring machine (Trans-Blot Turbo, Bio-RAD) where the proteins were transferred with a 20 mAmp current for 30 min. The membrane was then blocked in either 5% skimmed milk or 5% BSA (if probing for phosphorylated proteins) for 1 h; the solutions were made in PBS/NP-40 (0.1%). Primary antibody was incubated overnight (O/N) at 4°C, diluted in the same solution as was used for blocking at 1:1000 unless specified differently. The membranes were then washed three times with PBS/NP-40 (0.1%). Secondary antibody was also diluted in the blocking solution at 1:2000 and incubated for 1 h at RT. The membranes were again washed three times for a minimum of 5 min each time with PBS/NP-40 (0.1%). Membranes were then incubated for 1 min with either a luminol solution made according to the lab's protocol, or a Bio-RAD Luminol kit, and exposed to film prior to developing them using the developer facility in GDSC.

3.1.11 GFP-Trap Assay

Chromotek GFP-Trap beads were used to carry out immunoprecipitation following their protocol (GFP-Trap Magnetic Agarose, Chromotek, code: gtma-10). A fully confluent 10 cm plate or P75 flask of cells was collected using 200 μl IP buffer and diluted to 500 μl with Dilution buffer (prepared according to the provider's protocol). Afterwards, beads were incubated with the lysate overnight at 4°C. Following this, beads were washed three times with dilution buffer and resuspended in 100 μl 5X sample buffer. Afterwards they were boiled at 95°C for 10 min and analysed using western blotting.

3.1.12 EdU pulse-chase

Cells were seeded into a 6-well plate to reach confluency on day of EdU addition and collection. Cells were untreated or pre-treated for 4 h with the DIA cocktail. After this, 10 μM of EdU was added for 1 h. Following this, cells were washed with PBS 10 times and collected for the 0 h timepoint. After this, cells were collected every 3 h for the next 12 h to follow the cells through S phase to mitosis. They were collected and analysed according to the following protocol in Section 3.1.14.

3.1.13 FACS analysis

Cells were trypsinised and centrifuged to collect the pellet. The pellet was washed twice with PBS and resuspended in ¡ 100 μl of PBS. Then, the resuspended cells were mixed into 750 μl of 4°C 70% Ethanol (EtOH) and incubated O/N at 4°C. Cells were resuspended in 5 μg/ml of propidium iodide mixed with 12.5 μl/ml RNase A for a minimum of 2 h. Following this incubation, cells were filtered and analysed on a FACS Accuri machine. Data was analysed using BD CSampler software and Microsoft Excel.

3.1.14 EdU FACS analysis

Cells were prepared and fixed in EtOH O/N as above. Text morning, cells were washed twice with 3% BSA. Following this, cells were incubated for 30 min in dark in a Click-it cocktail, made according to the manufacturer's Click-it EdU protocol (ThermoFisher, cat. no. C10337). Afterwards cells were washed in 3% BSA and resuspended in 5 μg/ml of propidium iodide mixed with 12.5 μl/ml RNase A for a minimum of 2 h. Following

this incubation, cells were filtered and analysed on a FACS Accuri machine. Data was analysed using BD CSampler software and Microsoft Excel.

3.2 Poon lab materials and methods

3.2.1 List of solutions

Reagent	**Components**
BSA	2 mg/ml BSA
$CaCl_2$	2M $CaCl_2$
DMEM	1.35% w/v DMEM powder (Gibco, Carlsbad, CA, USA), 0.37% w/v $NaHCO_3$, pH 6.8
ECL	1.25 mM luminol, 2.25 mM 4-Iodophenylboronic acid, 100 mM Tris-HCl (pH 8.5)
H_2O_2	30% v/v H_2O_2
HBS (2X)	50 mM HEPES (pH 7.1), 280 mM NaCl, 1.5 mM, Na_2HPO_4
Hoechst 33258	Dilute 5 mg/ml stock 25000 fold in PBS
KCl	75 mM KCl
Lysis buffer	50 mM Tris-Cl pH 7.5, 2 mM EDTA, 1 mM DTT
PBS	0.8% w/v NaCl, 0.02% w/v KCl, 0.061% w/v Na_2HPO_4, 0.02% w/v KH_2PO_4, pH 7.2
PBS/T (For BrdU FACS)	PBS / Tween (0.1%) / BSA (3%)
Propidium iodide	4 mg/ml promidium iodide
Proteasome inhibitor cocktail	200 μg/ml Leupeptin, 400 μg/ml Aprotinin, 2 mg/ml Soybean trypsin inhibitor, 3 mg/ml Benzamidine, 2mg/ml Chymostatin, 2 mg/ml Pepstatin
RNase A	10 mg/ml RNase A
SDS-PAGE gel mix (15%)	15% w/v acrylamide, 0.086% w/v bisacrylamide, 375 mM Tris-Cl (pH 8.8)
SDS-PAGE running buffer	2.88% w/v glycine, 0.6% w/v Tris base, 0.1% w/v SDS
SDS-PAGE sample buffer	2% w/v SDS, 80 mM Tris-HCl (pH 6.8), 10% v/v glycerol, 0.02% w/v Bromophenol blue, 5% v/v β-mercaptoethanol

SDS-PAGE stacking gel mix	5% w/v acrylamide, 0.13% w/v bisacrylamide, 125 mM Tris-HCl (pH 6.8)
Transfer buffer A	300 mM Tris-Cl (pH 10.5), 20% v/v MeOH
Transfer buffer B	25 mM Tris-Cl (pH 9.4), 40 mM ϵ-amino-hexanoic acid, 20% v/v MeOH

Table 3.2.1: List of solutions used in the Poon lab. A table of solutions used in the Poon lab for work conducted in Chapter 4 (Section 4.4.1) and Chapter 7. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. The solvent was Mili-Q water (Millipore, Billerica, MA, USA), unless stated otherwise.

3.2.2 List of DNA plasmids

Name	**Vector**
CCNB1 CRISPR 5	pX330
CCNB1-mAID	pUHD SB (modified to work with the sleeping beauty transposase)
CCNB2 CRISPR 2	pX330
FLAG-3C-cyclin A2	pCDNA3.1
FLAG-cyclin B1-myc-H6	pEF6/myc-HisB
FLAG-cyclin B2	pUHD
H2B-GFP	pEF/Bsd
mRFP	pLNCX2
mRFP-cyclin A2	pLNCX2
myc-PLK1	pRcCMV
myc-PLK1 T210D	pRcCMV
Sleeping beauty transposase	pCMV (CAT) T7-SB100

Table 3.2.2: List of DNA plasmids used in the Poon lab. A table of DNA plasmids that were used in the Poon lab for work conducted in Chapter 7.

3.2.3 List of CRISPR-targeting oligonucleotides

Name	Oligo sequence	Description
CCNB1 CRISPR 5	CCTAATTGACTGGCTAGTAC	Used to knock-out CCNB1
CCNB2 CRISPR 2	caccGAGACTCTGTACATGTGCGT	Used to knock-out CCNB2

Table 3.2.3: List of CRISPR-targeting oligonucleotides used in the Poon lab. A table of CRISPR-targeting oligonucleotides that were used in the Poon lab for work conducted in Chapter 7.

3.2.4 List of antibodies

Name	Host	Code	Manufacturer
β-actin	Mu	A5316	Sigma-Aldrich
Alexa Fluor 488 Anti-Mu	Gt	A11001	Life Technologies
Alexa Fluor 594 Anti-Rb	Gt	A11012	Life Technologies
p-AURK(A,B,C)	Rb	D13A11	Cell Signaling Technology
BORA	Rb	12109S	Cell Signaling Technology
BUBR1	Mu	sc -47744	Santa Cruz
CDK1	Mu	A17	Gift from Hunt lab
CDK1	Rb	N/A	N/A
p-CDK1 (Y15)	Mu	612307	BD Transduction Laboratories
CDK2	Mu	D12	Santa Cruz
CDK2	Rb	N/A	N/A
p-CDK substrates [pTPXK]	Rb	14371S	Cell Signaling Technology
CDC27	Mu	610455	BD Technologies
Cyclin A2	Mu	AT10	Gift from Hunt lab
Cyclin B1	Mu	V152	Gift from Julian Giannon (CRUK)
Cyclin B1	Mu	GNS1	Santa Cruz (sc-245)
Cyclin B2	Mu	sc-28303	Santa Cruz
Cyclin B2	Rb	sc-22776	Santa Cruz

Cyclin E1	Mu	HE12	Santa Cruz
EMI1	Mu	37-6600	Zymed Laboratories
GFP	Mu	sc-9996	Santa Cruz
p-Histone H3	Rb	sc-8656R	Santa Cruz
HRP-Anti-Mu IgG	Rb	P161	DAKO
HRP-Anti-Rb IgG	Gt	P0448	DAKO
Lamin A / C	Mu	sc-7292	Santa Cruz
p-Lamin A / C (S22)	Rb	2026	Cell Signaling Technology
Myc	Mu	sc-40	Santa Cruz
Pericentrin	Mu	ab220784	Abcam
PLK 1	Mu	sc-17783	Santa Cruz
p-PLK1 (T210)	Rb	D5H7	Cell Signaling Technology
TCTP	Rb	D10F2	Cell Signaling Technology
p-TCTP (S46)	Rb	5251	Cell Signaling Technology

Table 3.2.4: List of antibodies used in the Poon lab. A table of antibodies that were used in the Poon lab for work conducted in Chapter 4 (Section 4.4.1) and Chapter 7. Manufacturer details are as follows: Abcam (Cambridge, UK), BD Transduction Laboratories (Franklin Lakes, New Jersey , USA), Cell Signaling Technology, (Danvers, MA, USA), DAKO (Agilent, Santa Clara, CA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma-Aldrich (St. Louis , MO, USA), Zymed Laboratories (San Francisco, CA, USA).

3.2.5 Cell culture and transfections

HeLa cells were grown in DMEM supplemented with 10% FBS and 1% Pen/Strep. All cells are grown in 37°C and 5% CO_2 unless stated otherwise.

HeLa cells were transfected using calcium phosphate. Cells were seeded into a 10 cm plate at 50 – 75% confluency a minimum of 6 h prior to transfection. Prior to transfection, 2X HBS and 2 M $CaCl_2$ were warmed up to RT or 37°C, and DNA was kept at RT. Distilled water, 10 – 20 μg of DNA, and 61 μl $CaCl_2$ were mixed to a total of 500 μl and incubated for 0 – 10 min. Next, 500 μl 2X HBS was added drop-wise while vortexing and incubated for 0 – 10 min prior to adding to cells by dropping the mixture onto the plate.

3.2.5.1 Drugs used in the Poon lab

Drugs used for this study and working concentrations were as noted here:

Abbreviation	**Drug name**	**Concentration**
IAA	Indole-3-acetic acid	100 μM
DOX	Doxycycline	2 μg/ml
thy	Thymidine	4 mM
NOCO	Nocodazole	0.1μg/ml
MK	MK1775	10 μM

Table 3.2.5: Drug names and concentrations. A table of drugs and their working concentrations as used in the Poon lab for work in Chapter 4 (Section 4.4.1) and Chapter 7.

3.2.5.2 Calcium phosphate transfection

HeLa cells were transfected using calcium phosphate. Cells were seeded into a 10 cm plate at 50 – 75% confluency a minimum of 6 h prior to transfection. Prior to transfection, 2X HBS and 2 M $CaCl_2$ were warmed up to RT or 37°C, and DNA was kept at RT. Distilled water, 10 – 20 μg of DNA, and 61 μl $CaCl_2$ were mixed to a total of 500 μl and incubated for 0 – 10 min. Next, 500 μl 2X HBS was added drop-wise while vortexing and incubated for 0 – 10 min prior to adding to cells by dropping the mixture onto the plate.

3.2.5.3 Retroviral transfection

Retroviruses were generated by Dr. Ken H. T. Ma by transfecting HEK-293 cells with CaP using the pLNCX viral transfection vector containing the gene of interest, previously cloned by Dr. Ken H. T. Ma. The media was collected and filtered after a minimum of 48 h transfection, and stored in 4°C for up to 4 weeks. To transfect HeLa cells, the cells were incubated with the filtered viral media by adding 1 ml of the media every 12 h, for 72 h in the presence of 5 μ/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Cells were then grown to a fully confluent 20 cm round dish, and sorted by FACS for their respective fluorescence signal, thereby generating a mix population which was used for future analyses.

3.2.5.4 Transfections with siRNA

Cells were grown in Ab-free media prior to trypsinisation for siRNA treatment. SiRNAs were incubated with Lipofectamine RNAiMAX reagent for 20 min at RT prior to incubation. After a minimum of 7 h incubation at 37°C and 5% CO_2, the media was replaced with an antibiotic-containing media. Cells were analysed after 24 – 48 h post-transfection.

3.2.6 Colony survival assay

Cells were counted and 150 cells were seeded into a 55 mm plate containing 5 ml media. Cells were grown for a minimum of 7 days and fixed with methanol : acetic acid (3 : 1) for 20 min at RT. Cells were then incubated with a crystal violet stain overnight (O/N) at RT. The next day, plates were washed with H_20 until no violet stain was being washed off, and dried.

3.2.7 Degron tag in combination with a TET-OFF promotor

The design of the degron tagging approach was not a topic of this thesis, but can be found in [387]. Briefly, HeLa cells that constitutively express tTA were used for the Poon lab mAID method. AID or mAID tags were fused to the N- or C-terminus of our POI that was under tTA TET-OFF controlled promotor. The POI was cloned to incorporate a silent mutation to make it resistant to CRISPR targeting. HeLa cells were transfected either with retroviruses to achieve a stable expression (AID-cyclin A2), or with several Sleeping Beauty plasmids delivered by a CaP method. The transfected plasmids were as follows: (i) tTA controlled cyclin B1-mAID, (ii) osTIR1, (iii) Cas9 with a gRNA, and (iv) H2B-GFP at the same time. The gRNA to target CCNB1 and CCNB2 were designed by Dr. Ken H. T. Ma. Then, 24 – 48 h post-transfection, cells were put into antibiotic selection for 7 days or more, depending on the efficiency of antibiotic selection. Afterwards, cells were seeded into 96-well plates and grown to a large population prior to checking by Western blotting and Sanger sequencing.

Adding DOX inhibits transcription by inactivating the tTA promotor, and IAA aids TIR1 with the recognition of mAID to target it for degradation (see Figure 3.2).

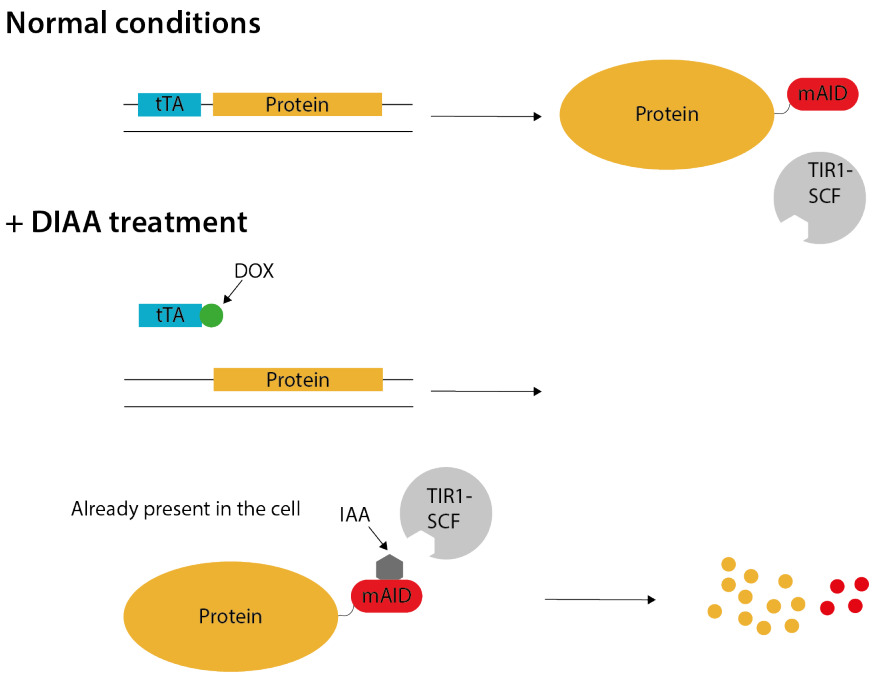

Figure 3.2: The degron tagging system used in HeLa cells. Cells express the tTA controlled protein tagged with an AID or a mAID and osTIR1. After DIAA (2 μg/ml doxycycline and 100 μM IAA) treatment, DOX binds with the tTA and inhibits gene transcription, and IAA promotes protein degradation by promoting osTIR1 recognition of the mAID or AID [387]. The lines on the top and middle left side represent the DNA that encodes for the tTA-controlled protein. Protein degradation is marked by small circles.

3.2.8 Synchronisation with double thymidine

HeLa cells were seeded at approximately 50% confluency into 10 cm or 20 cm plates and incubated with 2 mM of thymidine for a minimum of 14 h, followed by two washes with warm PBS and 8 – 10 h incubation with their normal growth media. Cells were then again incubated with 2 mM of thymidine for a minimum of 14 h and washed twice with warm PBS prior to incubation with their nornal media. For immunoblotting, cells were incubated with and without DIAA or re-seeded into 55 cm or 10 cm plates containing DIAA or not for harvesting at the indicated timepoints. For immunofluorescence analyses or live-cell imaging, cells were trypsinised and seeded at the second release from thymidine into DIAA containing or not 96-, 24-, 12-, or 6-well plates to approximately 50 – 75% confluency. The 96-well plates were TC-treated PerkinElmer CellCarrier plates used for live-cell imaging.

3.2.9 Live-cell imaging

Cells were seeded at a 50 – 75% confluency into either PerkinElmer CellCarrier 96-well plate or a 24-well TC treated polystyrene plates and imaged using CD7 Zeiss microscope at 37°C and 5% CO_2. Asynchronous cells were treated with DIAA for 6 h prior to imaging. Thymidine-synchronised cells were incubated with DIAA-containing media after the second release from thymidine and incubated for a minimum of 2 h prior to imaging. Cells were imaged either with Phase contrast, YFP, GFP or RFP settings, depending on the cell line. The laser intensities and exposure times were adjusted each time according to the cells, to a maximum of 35% laser power and 220 ms exposure time.

3.2.10 Immunofluorescence

Cells were either grown on coverslips in a 6-well plate, or in a 24-well plate. Cells were fixed with cold Methanol for 1min, washed with PBS, permeabilised for 10 – 20 min with PBS-Tween (0.1%), blocked with 3% BSA for 1 h and incubated with primary and secondary antibodies for 1.5 h and 1 h, respectively. Following staining, the cells were washed and incubated in 1 μg/ml Hoechst dye for 10 – 20 min prior to washing and mounting the coverslips using a mounting solution (), or left in PBS in 24-well plates. Cells were then analysed on CD7 Zeiss microscope within three days.

3.2.11 Chromosome spreads

Cells were synchronised by a double thymidine block as described above, followed by a release into normal or DIAA-containing media. Cells were collected by shake-off and incubated in 37°C 75 mM KCl for 10 - 20 min at RT. Cells were then centrifuged and resuspended in methanol : acetic acid (3 : 1). Cells were incubated for 5 min and centrifuged. Cells were resuspended in 100 - 300 μl of the methanol : acetic acid solution and dropped on glass coverslips. The coverslips were allowed to dry prior to incubation in 1 μg/ml Hoechst dye for 10 – 20 min. The coverslips were then mounted using a mounting solution as described above.

3.2.12 Immunoblotting

Cells were trypsinised and washed once with PBS and either frozen or immediately incubated with a lysis buffer for 30 min on ice. Afterwards, cells were centrifuged at 4°C and the supernatant concentration measured using the bicinchoninic acid (BCA) assay (Pierce, USA). Sample concentration was determined using BCA reagents (BioRAD) and compared against a BSA serial dilution standard by measuring the colour development at OD570 using a Opsys MR^{Tm} microplate reader (Dynex Technologies, Chantilly, VA, USA). Cell lysates were diluted to 2 μg/μl in 2X sample buffer. Unless indicated otherwise, a 15% SDS-PAGE resolving gel was prepared by adding 1% v/v APS, 1% v/v TEMED. After the gel was set, the stacking gel was prepared by adding 1% v/v APS, 1% v/v TEMED and poured on top of the resolving gel, including plastic combs to shape the wells for protein loading. Afterwards, 20 μg sample was loaded to each well. After running at 20 mAmp per gel for 1 h 12 min, the gels were sucbject to a semi-dry transfer.

A semi-dry transfer apparatus Semi-phor (Hoefer, Holliston, MA, USA) was used to transfer proteins from the polyacrylamide gel to Immobilon PVDF transfer membrane (Millipore, USA). A stack of 4 pieces of 3MM paper (Fisher Scientific, UK) was soaked in transfer buffer A and placed onto the bottom of the apparatus. A PVDF membrane (95 mm x 65 mm) was soaked in methanol and placed on top of the 3MM paper stack. The polyacrylamide gel was soaked in transfer buffer B and placed onto the PVDF membrane, covered by another stack of 3 pieces of 3 MM paper pre-soaked in transfer buffer B. A glass stick was used to roll the filter papers and membrane each step to eliminate air bubbles. The upper part of the apparatus was then assembled and a constant current of

55 mA was applied for 1.5 h.

Following this, membranes were blocked in either 5% milk (5% w/v skimmed milk powder diluted in TBS/T) or 5% BSA (5% w/v BSA powder diluted in TBS/T) if analysing phosphorylation status of proteins for 1 h. Membranes were then incubated in primary antibodies overnight at 4°C. Afterwards, membranes were incubated in a secondary antibody for 2 h at RT prior to ECL incubation and analyses using the Bio-RAD Chemi-doc system. Prior to ECL incubation, 0.01% v/v H_2O_2 was added. Images were analysed using the Image Lab software from Bio-RAD and arranged with Adobe Illustrator.

3.2.13 Immunoprecipitation

Cells were grown in 10 cm plates until fully confluent and collected. They were then washed and incubated with cell lysis buffer, as in Western blot analyses. Lysate concentration was then measured and 800 – 1000 μg of protein was incubated with 0.5-1 μl of primary antibody overnight at 4°C. Following this, beads were washed with a 1X beads buffer (Stock is 2X: 50 mM Tris-Cl pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 1 μg/ml Leupeptin, 2 μg/ml Aprotinin, 10 μg/ml Soybean trypsin inhibitor, 15 μg/ml Benzamidine), resuspended in 1X beads buffer and added to the supernatant for a min of 4 h at 4°C. The beads were then washed 3x with 1X beads buffer prior to incubation with 45 μl 2X SB and boiled at 95°C for 3 min. The samples were analysed using WB as described earlier.

3.2.14 BrdU pulse-chase

The cells were seeded into 55 mm plates to be fully confluent on the day of treatment. The cells were incubated with DIAA for 6 h prior to 1 h incubation with 10 mM BrdU. Following this, cells were washed three times with PBS and collected every 2 h according to the BrdU FACS analysis protocol. Cells were fixed in cold 80% EtOH added dropwise while vortexing and incubated overnight at 4°C.

3.2.15 BrdU FACS analysis

To stain for BrdU, cells were washed with PBS three times after overnight EtOH incubation. The pellets were then incubated with 2 M hydrochloric acid (HCl) for 20 min,

and sodium tetraborate for 5 min. Following this, the pellets were washed once with PBS and once with PBS/T. They were then incubated with mouse BrdU primary antibody in PBS/T for 1 – 2 h at RT. Afterwards, they were washed twice with PBS/T and incubated with a secondary Alexa Fluor 488 anti-mouse for 30 min at RT in the dark. Cells were then washed twice with PBS/T and incubated with propidium iodide as mentioned in Section 3.2.16.

3.2.16 FACS analysis

The cells were collected by trypsinisation and washed twice with PBS. The PBS was discarded and the pellets were resuspended in the residual supernatant. The pellets were then fixed using cold 80% EtOH added dropwise while vortexing and incubated overnight at 4°C. The pellets were then washed three times with PBS and incubated with 500 μl – 1 ml of mixed propidium iodide and RNAse in a tris-EDTA solution (TE), according to the size of the pellet, for 2 h at RT in the dark, or for 30 min at 37°C in the dark.

Following staining, the samples were analysed within one day using the BD FACS Diva machine running the BD FACS Diva software. The same software was used for any gating analyses.

Chapter 4

Roles of cyclin A2 in human cells

4.1 Summary

Using novel, previously established, degron tagging methods, striking differences in the necessity for cyclin A2 in two human cell lines were determined: cyclin A2 is essential for proliferation in a non-transformed epithelial cell line RPE-1, but not in a cervical cancer cell line HeLa. The importance of cyclin A2 was also demonstrated in two additional human cell lines using RNA interference.

In this Chapter, a distinct role for cyclin A2 in late S phase in RPE-1 was established. Cyclin A2 is essential for mitotic entry in RPE-1 cells as it promotes CDK1-cyclin B activation. Exogenous expression of nuclear, but not wild-type, cyclin B1 in cyclin A2 deficient RPE-1 cells promoted mitotic entry in half of the population, thus indicating that cyclin A2 has a dual role in mitotic entry – triggering nuclear events and activating CDK1 in the cytoplasm.

In contrast, this Chapter also shows that cyclin A2 is not essential for proliferation in HeLa cells. However, cyclin A2 is important for a faithful execution of the cell cycle in HeLa as its depletion induced delays in mitotic entry and progression. These data match previous observations where a non-essential role for cyclin A2 as been reported in the same cell line.

4.2 Introduction

Roles of cyclin A2 in the cell cycle Cyclin A2's functions, protein structure, regulation and implications in cancer have already been discussed in the Introduction part of this thesis (Chapter 2, Section 2.2.1). Notably, cyclin A2 is essential for mouse development [67], but not for the proliferation of mouse embryonic fibroblasts (MEFs) [91]. Previous work in HeLa cells has established that cyclin A2 contributes to mitotic entry, but its role may be redundant with cyclin B1 [12, 16]. Due to limitations of conventional protein depletion methods, and a high expression of cyclin A2 in each cell cycle, it has been difficult to achieve sufficient levels of depletion to elucidate and separate between its roles in different stages of the cell cycle.

Cyclin A2 has been indicated to promote mitotic entry, but the reports across different model organisms are not uniform. While some show that it is essential to establish mitosis [9, 56, 89, 90, 96], others disagree [12, 16] and further research is needed to define its functions. Cyclin A2's roles in mitosis are unclear, but it has been implicated to aid in cell reorganisation at the onset of mitosis by affecting RhoA and actin [123, 124], suppressing replication in mitosis [122] and contributing to APC/C inhibition to prevent premature degradation of cyclin B1 [97]. Moreover, following nuclear envelope breakdown (NEBD) in mitosis, specific roles for cyclin A2 have been shown [50, 51], but it is still not fully clear whether these are essential for the establishment of metaphase and a faithful mitotic division.

Role of cyclin A2 in DNA damage repair Gu et al. have shown cyclin A2's importance during Homologous Recombination (HR). Cyclin A2 promotes the expression of two key double strand break (DSB) recognition proteins, MRE11 and RAD50 [173]. MRE11 and RAD50 are part of the MRN complex important for recognising DSBs and recruiting HR machinery. Cells lacking cyclin A2 had a higher occurrence of DSBs due to defects in DSB recognition and HR [173]. Initial DSB recognition involves, apart from the MRN complex, ataxia-telangiectasia mutated protein (ATM), which promotes DSB signalling by phosphorylating a H2A histone variant, thus generating γH2A.X foci, which serve to amplify the DSB signal. A cascade of events then leads to p53-binding protein (53BP1) recruitment that further aids with the signalling pathway (reviewed in [388]).

Furthermore, 53BP1 is also involved in choosing the DNA repair pathway – cells can repair DSBs either by HR or by Non-Homologous End-Joining (NHEJ) – and 53BP1 presence at breaks favours NHEJ (reviewed in [389, 390]). More information on DSB recognition and repair is beyond the scope of this thesis, and there are a number of reviews available that discuss these complex mechanisms (reviewed in [388–390]). These events are described here to familiarise the reader with the indicated role of cyclin A2 in DSB repair.

Conventional depletion methods were previously used to study cyclins, but they take from one to two cell cycles to achieve sufficient levels of depletion. Since cyclin A2 is involved in more than half of the cell cycle, and has several roles, it is difficult to then separate and attribute the phenotypes observed to specific functions in a set phase. With the development of gene-targeting and protein degradation tags addressing the above questions has become more straightforward.

4.3 RPE-1

4.3.1 Questions to be addressed

As noted above, cyclin A2 has been implicated to play roles in different stages of mitosis. These will be investigated in this Chapter. The stages of mitosis where cyclin A2 has been implied to have a role are visualised in Figure 4.1. The model of mitotic progression separates the stages of mitosis by colour (prophase in red, prometaphase in purple, and metaphase in blue). Specifically, the contributions of CDK-cyclin A2 activity to the establishment of each of the marked stages are not entirely clear, which will be investigated in this thesis, and are emphasised by the question marks. The same model will be updated throughout this thesis, as the key requirements for the establishment of each mitotic stage in RPE-1 cells are defined. Notably, the model does not separate between CDK1-cyclin A2 and CDK2-cyclin A2 as both activities are present in cells and it is difficult to separate between their respective roles.

Prophase Cyclin A2's function in mitotic entry has been implied [9] but its exact contribution is unclear. I aim to determine whether it is essential for the establishment of prophase in RPE-1 and HeLa cells.

Prometaphase Cyclin A2's role in mitotic progression and entry into prometaphase is poorly defined, although it was shown to contribute to the proper timing of NEBD [16]. Characterised by NEBD, prometaphase is the stage where chromosomes are attached to microtubules, and cyclin A2 has also been implied to aid in these events, prior to its degradation during this stage [1, 50, 51].

Metaphase Even though cyclin A2 is degraded during prometaphase, it could aid in the proper timing of metaphase events by phosphorylating key substrates in the preceding stages, or by activating other mitotic kinases, such as PLK1 [51, 88]. I aim to analyse the effect of loss of cyclin A2 during this stage as well, although there is a possibility of defects from previous stages affecting mitotic progression.

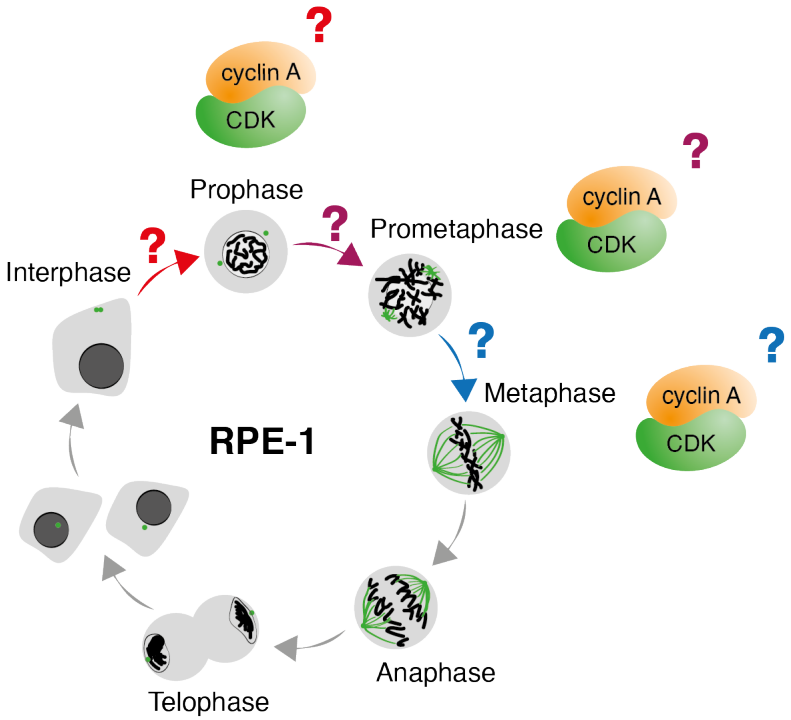

Figure 4.1: A schematic of possible roles for cyclin A2 in separate stages of mitosis in RPE-1 cells. The possible roles of cyclin A2 in prophase, prometaphase and metaphase are highlighted with question marks in red, purple, and blue, respectively. The possible functions have been implied by previous data, mentioned in this Section.

4.3.2 Cell line generation and characterisation

The reader is reminded that setting up the double degradation tag (double degron, ^{dd}) system was not a part of this study as it was conducted by Dr. Hegarat and Dr. Hochegger in the Hochegger lab [24, 386]. Thus, gene targeting and plasmid generation will not be described here, but for an easier understanding of the research shown in this Section, the method and some initial cell line characterisation experiments conducted by Dr. Hegarat will be discussed next.

Double degron tag method Endogenous cyclin A2 was tagged with two degron tags on its C-terminus by targeting the STOP codon of the *CCNA2* gene (gene targeting carried out by Dr. Hegarat [386]). Cyclin A2 was first tagged with a mini auxin-inducible-degradation tag (mAID), followed by a small molecule assisted shut-off tag (SMASh) downstream of the mAID (see Chapter 3 Materials and Methods, Section 3.1.4.4). The second tag, SMASh, is much larger than mAID, but it cleaves itself from the protein soon after translation in the absence of a specific small-molecule inhibitor asunaprevir (Asv), after which it is degraded by the proteasome [23]. The SMASh tag triggers protein degradation when it remains bound to its target, hence it is especially efficient for depletion of strongly expressed proteins.

Under normal conditions, the cyclin A2 protein is only tagged with the smaller mAID that is less likely to affect protein folding and mobility, as reported in a separate study with a different protein [391]. However, the same study showed that there is some basal depletion of mAID tagged proteins even in the absence of Auxin (IAA), which is required to direct the plant F-box protein TIR1 complexed with the SCF E3-ubiquitin complex to its target [391]. Mammalian cells do not express the plant-specific TIR1, thus an *Oryza sativa* TIR1 (osTIR1) was introduced into cells to allow mAID targeting. To bypass the basal degradation problem, osTIR1 was introduced into RPE-1 cells under a tetracycline inducible (TET-ON) promotor to express osTIR1 only when necessary to trigger mAID depletion in the cells. These cells, RPE-1 conditionally expressing osTIR1, were the parental cell line used to generate the degron tagged cells. A more detailed description of the generation of RPE-1^{dd} cell lines is also available in [386].

RPE-1 cyclin A2^{dd} depletion is complete within 4 h Cyclin A2's degradation is most efficient when both degron tags (mAID and SMASh) are targeted by the DIA cocktail (DOX, IAA, Asv, see Methods; this abbreviation will be used for both mAID and SMASh targeting throughout this thesis) (Figure 4.2 A, right panel). Protein depletion by targeting only a single tag, either mAID or SMASh, resulted in several cyclin A2 splice products remaining in cells for several hours (Figure 4.2 A, left and middle panels). It seems that while Asv prevented the SMASh tag from detaching from cyclin A2-mAID, the proteasome was ineffective in fully degrading these products, as noted by the appearance of bands of 100 and more kDa (Figure 4.2 A, left panel). The size of the SMASh tag is approximately 34 kDa [23], and the size of mAID ranges from 5 – 10 kDa, thus cyclin A2-mAID-SMASh protein amounts to approximately 85 – 90 kDa. One of the bands appears to be closer to 100 kDa and taking protein migration differences into account, it is likely that it represents the protein with both tags.

Notably, there is another band with a higher molecular weight, and this may be the protein fused with the antibiotic resistance that was introduced into the cells to allow the selection for cells that have incorporated the gene targeting cassette [386], although this was not confirmed. Regardless of persisting products when using Asv alone, activating the mAID alone or in combination with Asv showed a complete depletion of the larger protein products. Furthermore, incubating the cells with the DIA cocktail resulted in a complete depletion of the protein within 4 h (Figure 4.2 A).

The expression of osTIR1 is detectable by immunoblotting within two hours, with maximum expression after 6 h of DOX addition. Pre-treatment with DOX did not induce a faster mAID activation as even after 2 h of DOX, another 2 h of IAA and Asv incubation were necessary to achieve sufficient levels of depletion (data not shown). In this thesis, all double degron targeting experiments were conducted after 4 h incubation with the DIA cocktail.

Cyclin A2 is essential for RPE-1 cells Dr. Hegarat tested long-term cell proliferation in the absence of cyclin A2 and found that it is essential for survival in RPE-1 cells (Figure 4.2 B). I then utilised siRNA treatment in combination with DIA to assess the effects of cyclin A2 depletion in the parental RPE-1 osTIR1 without any degron tags and the daugther cell line with cyclin A2^{dd} (Figure 4.2 C, D). The cells were either untreated, treated with DIA or siCyclin A2, or with both (see Figure legend for more details). In-

terestingly, siCyclin A2 was more efficient in depleting cyclin A2^{dd}, than the untagged endogenous protein, as seen in Figure 4.2 C. This phenomenon was not addressed further due to a lack of time to pursue these investigations.

Next, siCyclin A2-transfected RPE-1 osTIR1 cells had residual levels of cyclin A2 remaining as made apparent by a higher exposure of the membrane. These cells accumulated in an 8N population following siCyclin A2 treatment, regardless of DIA addition. The accumulation of these cells in 8N is either due to residual amounts of cyclin A2 present during G_2 / M that promote a premature mitosis and the following cell cycle, or a consequence of reduplication that was previously shown to be triggered by a lack of cyclin A2 in *Arabidopsis* [181].

Depleting cyclin A2 by siRNA in RPE1 cyclin A2^{dd} cells resulted in a similar cell cycle profile when treated with siCyclin A2 alone or in combination with DIA (Figure 4.2 D, right). This result is likely due to a markedly stronger cyclin A2 depletion when treated with the siRNA, as compared with RPE-1 osTIR1. Nevertheless, it is clear that cells lacking cyclin A2 completely arrest in a 4N state, likely in G_2 or M.

Cyclin A2 has roles in several stages of the cell cycle, and to adhere to the sequence of events as they are carried out during the cell cycle, I will first discuss the roles of cyclin A2 in S phase. CDK2-cyclin A2 is an established driver of S phase [25], whereas the less abundant CDK1-cyclin A2 has specific roles in late origin firing [8, 44]. As mentioned previously, CDK1 can compensate for the loss of CDK2 [38], but the same has not yet been reported for CDK2 in RPE-1 cells. Notably, CDK2 also forms a complex with cyclin E in interphase, but it is unclear whether it is able to compensate for the specific CDK1-cyclin A2 roles in S phase.

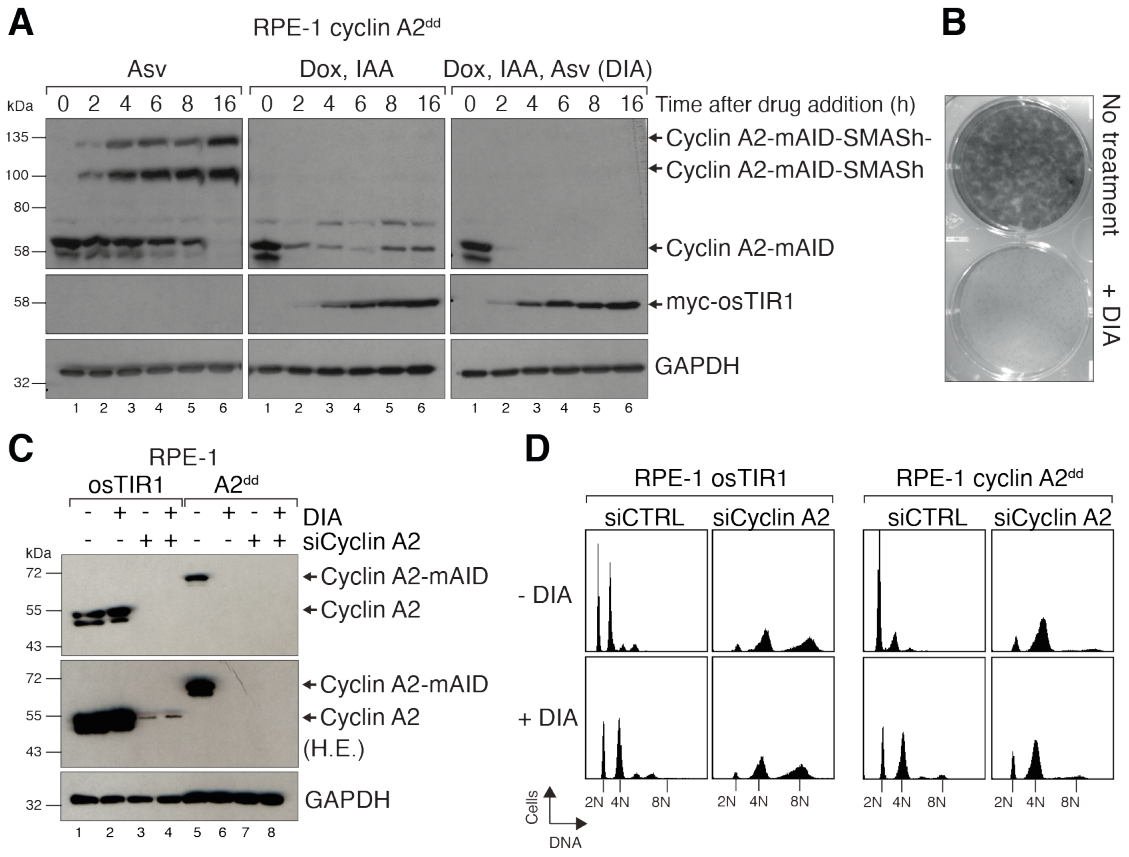

Figure 4.2: RPE-1 cyclin A2^{dd} cell line characterisation. A: Degron tag efficiency comparison. Asv is Asunaprevir used to trigger SMASh degradation, Dox is doxycycline that induces osTIR1 transcription and IAA is Auxin, which aids in the mAID-mediated degradation with osTIR1. The combined treatment of all three is termed DIA. Cyclin A2-mAID-SMASh- marks a protein that has a higher molecular weight, likely caused by the translation of an antibiotic resistance, although this as not been confirmed. The osTIR1 protein is myc tagged, here the anti-myc antibody was used. GAPDH is the loading control. B: Cell survival assay with untreated cells on top, and DIA-treated cells on the bottom well. C: Western blot of control or DIA treated and siCyclin A2 transfected or not RPE-1 osTIR1 and RPE1 cyclin A2^{dd} cells as indicated. Control cells were mock transfected with all reagents apart from siRNA. All cells were asynchronous and collected after 48 h of siRNA transfection. Cells also treated with DIA were pre-incubated with DIA for 24 h, and then transfected with siRNA for an additional 48 h in DIA-containing media. D: FACS analyses of cells in C. The DNA fluorescence was log-scaled. Figures A and B were generated by Dr. Hegarat [386].

4.3.3 Roles of cyclin A2 in S phase

EdU pulse-chase experiment outline To assess the mechanics of S phase progression in RPE-1 cells lacking cyclin A2, I used a thymine analogue, 5-ethynyl-2'-deoxyuridine (EdU), in a pulse-chase experiment (see Chapter 3 Materials and Methods, Section 3.1.12). Asynchronous cells were treated as indicated and collected for 12 h following the incubation with EdU (Figure 4.3 A). This experiment allowed a separation of populations according to the cellular DNA content, as detected by propidium iodide fluorescence, and replication status, as measured by EdU uptake.

Description of the cell cycle stages in the pulse-chase experiment FACS plots Figure 4.3 shows the FACS plots of cells subjected to EdU pulse-chase experiment in B, and analyses of these populations in C – E. The different populations of the asynchronous population will be described by explaining the representative gating shown in Figure 4.3 B (0 h time-point).

The cells that were in G_1 at the time of cyclin A2 depletion (blue, gate G_1) are characterised by a 2N DNA content and no replication, thus they are EdU-negative (on the bottom half of the y-axis). In contrast, the cells that have just entered into S phase are observed further up the y-axis (B), above the G_1 population as they have started to replicate their DNA, but have not yet increased their DNA content significantly (blue, gate G_1 / S1). Note that this gate will include the G_1 population after EdU-positive cells divide, which is why it is termed G_1 / S1.

Next, following this pattern, the cells that were already in S phase at the time of EdU addition had a higher amount of DNA, thus higher propidium iodide fluorescence, (further to the right on x-axis) and were also taking up EdU as they were replicating their DNA (top half of the y-axis). This stage is termed S2 (orange gate). The next population of cells is named S3 / G_2 (brown gate). While the initial time-points only include the late S phase population as they incorporate EdU during replication (S3), with time, these will have entered G_2 with high levels of EdU fluorescence.

On the other hand, cells that were in G_2 at the time of EdU addition did not incorporate any of the thymine analogue, and are thus EdU-negative, but have a 4N DNA content (brown gate, G_2). The only empty gate remaining is EdU-negative with an intermediate DNA content (orange, gate S). This population will represent cells that were

not replicating their DNA at the time of EdU pulse, thus it is empty to start with, as all the existing S phase cells are separated by the y-axis. With time, the G_1 cells will begin replication, thereby increasing their DNA content, but not incorporating EdU as it has been washed out after the 1 h pulse. This is apparent in later time-points (e.g. 6 h after EdU washout) as cells can be observed progressing from G_1 to G_2 on the x-axis.

RPE-1 cells lacking cyclin A2 are delayed in S phase progression Initially, five populations can be distinguished at the 0 h time-point of both – treated and untreated cells – G_1, early S (S1), mid S (S2), late S (S3) and G_2 phase (Figure 4.3 B). With time, these cells progress through the cell cycle, shown by the shift of signal on the FACS-plots (see explanation above).

Control cells (- DIA) that were in S phase during the EdU pulse (top half of the y-axis) progress through S phase as they continue to replicate their DNA, thereby increasing their DNA amount. These cells reach G_2 approximately 6 h after the EdU pulse washout and are able to successfully divide into two cells as they are next observed entering G_1 at the 9 h and 12 h time-points. On the other hand, the EdU-negative cells (bottom half of the y-axis) that were in G_1 at time of EdU addition begin entering into S phase 6 h after EdU washout and continue to progress through S in the next two time-points, some already reaching G_2 as seen at 12 h.

Cells lacking cyclin A2 (+ DIA) have a smaller population of EdU-positive cells in S3/G_2 phase at the 0 h time-point, as deduced from a falling curve in the S3 / G_2 population. This slower progression through late S phase of RPE-1 cells also apparent in time-points 6 and 9 h. These cells ultimately arrest in G_2 and none enter new G_1 stage, as observed by a lack of population in G_1 / S1 (top y-axis, 2N population on x-axis). This G_2 arrest phenotype will not be addressed in this Section (see Section 4.3.4).

Cells that were G_1 when cyclin A2 was depleted are able to normally enter and progress through early S phase without any apparent delays, as seen on the FACS plots of EdU-negative cells with increasing DNA content (bottom of the y-axis, progressing from 2N towards 4N on the x-axis). The latter was also analysed separately in Figure 4.3 E where I compared the amount of EdU-negative S phase populations at different times. I observed that the increase in replicating cells is similar regardless of cyclin A2's depletion, thus indicating that early S phase mechanics and S phase entry are not affected by the lack of

cyclin A2.

This experiment was repeated three times, and the analyses of the triplicate are shown in Figure 4.3 C, D, where I separated EdU-positive and EdU-negative cells, respectively. These graphs confirmed the above observations and showed that the cell cycle progression of cells lacking cyclin A2 is consistent. For instance, it is apparent from a comparison of G_2 phase populations between untreated and treated EdU-negative cells (Figure 4.3 D) that no cells lacking cyclin A2 exit G_2, as the population seems to remain unchanged for the duration of the experiment, whereas G_1 exit and S phase entry (reduction in the blue part of the barplot, increase in orange) are similar in both experiments. This is strikingly dissimilar to EdU-positive cells were the delay in S phase progression becomes more apparent by a large proportion of cells in S phase throughout the time-points (Figure 4.3 C). Here, the G_2 arrest is also very obvious, but this will be analysed after the next Section on other roles of cyclin A2 in interphase DNA damage signalling.

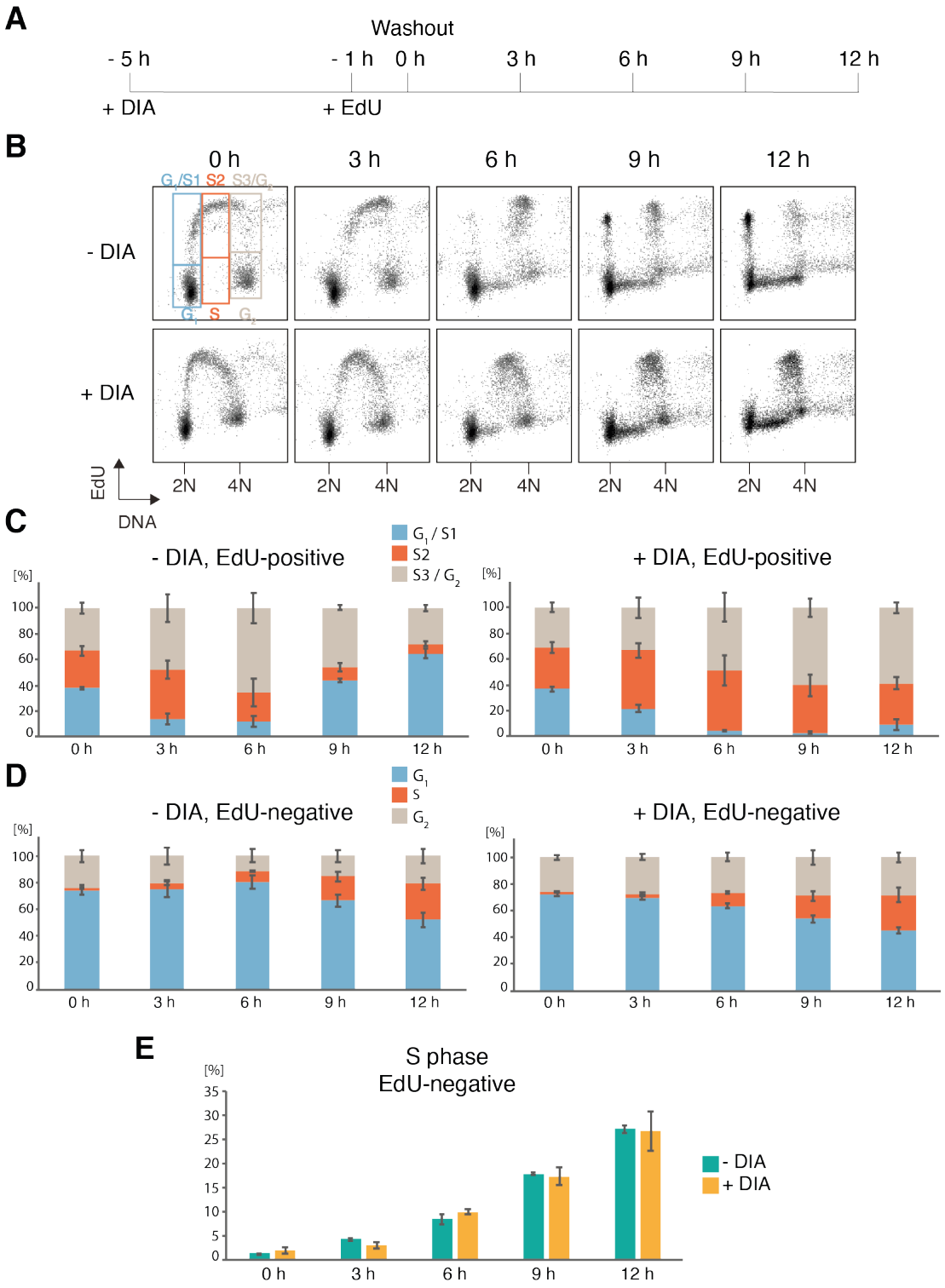

Figure 4.3: See caption on the following page.

Figure 4.3 *(preceding page)*: RPE-1 cyclin A2^{dd} EdU pulse-chase analyses. A: EdU pulse-chase experiment outline. Cells were treated with DIA for 4 h, followed by 1 h incubation with EdU (EdU pulse) with or without DIA and washed three times with warm PBS at 0 h. Cells were then re-incubated in normal or DIA-containing media, followed by collections for FACS analyses starting at EdU washout at 0 h, and then every three hours for 12 h after the EdU pulse. B: FACS plots of cells untreated or treated with DIA to deplete cyclin A2, representing one experiment. The gates in top left panel define the different populations, as described in Section 4.3.3. C: FACS analyses of the cell cycle of EdU-positive cells. Cells were gated as indicated in B, top left panel, and as described in Section 4.3.3. D: Same as C, but with EdU-negative cells. E: Analyses of S phase entry of EdU-negative cells that were in G_1 at time of cyclin A2 depletion (gating S in Figure B). FACS plots in B are representative of one experiment. Data from C to E were obtained from three independently repeated experiments and analysed using BD CSampler software. Data in C and D were normalised to total amount of EdU-positive or EdU-negative cells for each condition, respectively. All barplots indicate a mean of three experiments. Barplots in C and D show means of data that was normalised to the total amount of cells per each condition (EdU-positive or EdU-negative) and time-point. Error bars on all barplots throughout this Chapter indicate the standard deviation.

4.3.3.1 Role of cyclin A2 in DNA damage repair

Cells in Figure 4.4 were exposed to Ionising Radiation (IR) for either 0, 1 or 2 Grays and allowed to recover for 1 h, prior to fixation with ice-cold methanol and immunofluorescence staining (see Chapter 3 Materials and Methods). If cyclin A2 specifically contributed to the MRN complex expression in the instance of DNA damage, then lower amounts or fluorescence intensity of 53BP1 foci would be observed in the cells lacking cyclin A2.

According to preliminary data from Figure 4.4, it seems that there is no difference between treated and untreated cells in the abundance of a DSB signalling protein 53BP1 (analysis not shown). Comparing the treatments by Figures shows no apparent differences in these cells. This experiment was conducted twice, but similar observations were made both times.

Due to a lack of time I was not able to fully investigate the importance of cyclin A2 in DSB repair. Instead, this study focused on the mitotic roles of cyclin A2 and these will be discussed next.

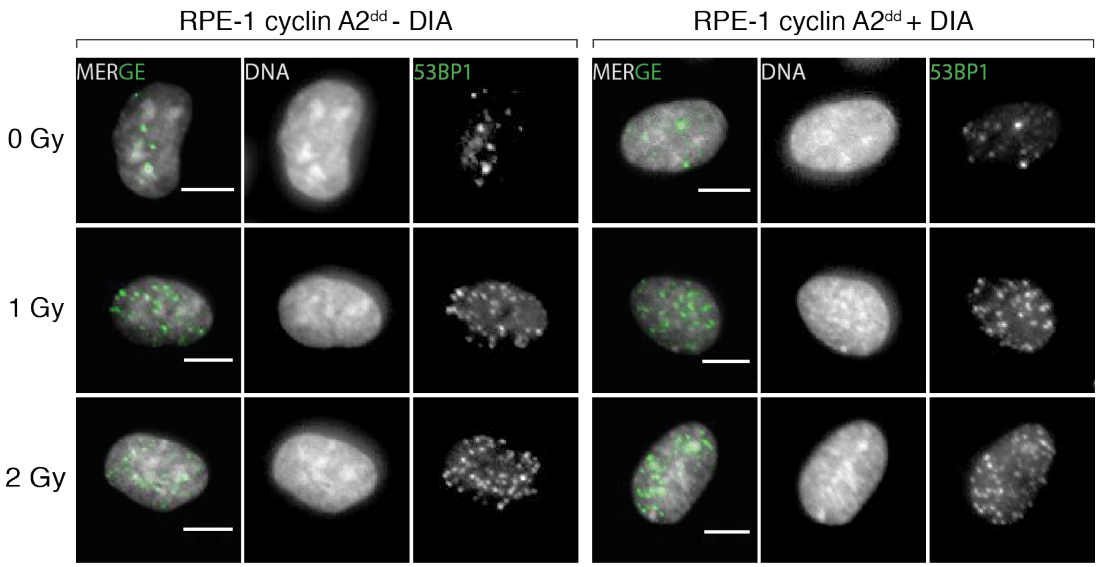

Figure 4.4: RPE-1 cells lacking cyclin A2 respond to DSBs normally. RPE-1 cyclin $A2^{dd}$ were untreated (left) or treated with DIA (right) and exposed to ionising radiation as indicated (Gy is gray). Cells were fixed and stained according to the immunofluorescence protocol (see Chapter 3 Materials and Methods, Section 3.1.8). Scale bars indicate 10 μm. DNA was stained with DAPI and is shown in white, 53BP1 in green.

4.3.4 Roles of cyclin A2 in mitosis

Figure 4.3 B, showing the EdU pulse-chase analyses, demonstrated that cyclin A2 deficient cells progress slower through late S phase, and eventually arrest in G_2. Furthermore, cells that were already in G_2 at time of cyclin A2 depletion (Figure 4.3 B, gate G_2) seem to stall in a 4N stage and do not enter the following G_1, as observed by a lack of an accumulation of a 2N population of EdU-negative cells (Figure 4.3 D, population G_1). It is possible that the cells arrest in G_2 / M due to errors accumulated from issues in earlier stages of the cell cycle as cyclin A2 is necessary for S phase progression. Thus, these cells were further analysed with live-cell imaging (LCI) analyses.

4.3.4.1 The G_2 arrest is not caused by defects from the preceding stages of the cell cycle

PCNA as a marker of replication in microscopy analyses Dr. Alexis Barr (ICR, London, UK) utilised the Hochegger lab RPE-1 cyclin $A2^{dd}$ cell line to establish cells stably expressing proliferating cell nuclear antigen fused with a red fluorescent protein (PCNA-mRuby). PCNA is a replication marker [85] that forms foci in S phase which disappear when cells enter G_2 [392] (Figure 4.5 A). This cell line enabled the distinction between S and G_2 phases of the cell cycle in LCI analyses.

Cyclin A2 is required in G_2 to trigger mitosis Dr. Maria F. S. P. Rodriguez, who was a PhD student in Hochegger lab at the time, analysed the cell cycle proliferation of cells lacking cyclin A2 using the cell line expressing PCNA-mRuby and found that cells that were in G_2 at the time of DIA treatment also arrested prior to M entry (Figure 4.5 B). These data showed that the arrest in 4N is not due to the accumulation of defects caused by cyclin A2's depletion during S phase, but uncovered cyclin A2 is (also) important specifically during G_2 to promote mitosis, agreeing with some previous observations [9, 12, 59, 60].

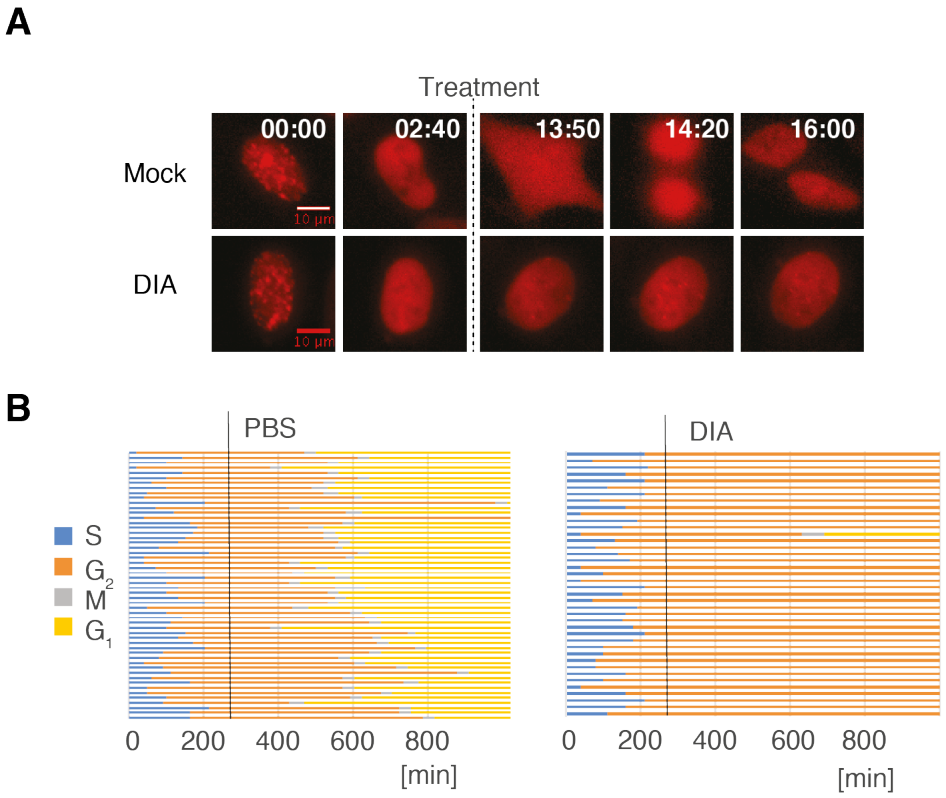

Figure 4.5: RPE-1 cells lacking cyclin A2 arrest in G_2. A: Snapshots of RPE-1 cyclin $A2^{dd}$ PCNA-mRuby, filmed with a widefield confocal microscope, only the mRFP is shown. Scale bar is 10 μm. The time-points are in hh:mm. B: Single-cell tracking analyses of cells in A, time of treatment with either PBS or DIA is indicated with the black line. Cell cycle stages were classified based on PCNA, and mitosis was judged by cell rounding and division, as observed by the mRFP. A and B were conducted and analysed by Dr. M. F. S. P. Rodriguez, cells were gifted by Dr. A. Barr.

4.3.4.2 Cyclin A2 triggers mitosis in RPE-1 by activating CDK1-cyclin B

Possible roles of cyclin A2 in mitotic entry As observed with LCI analyses using differential interference contrast (DIC) and a DNA-specific fluorescent dye (SiR-DNA), cells lacking cyclin A2 were alive and appeared to be in interphase for the duration of LCI analyses since they did not exhibit any obvious mitotic phenotypes like cell rounding, chromosome condensation, or cellular division (data not shown). These data implied that cyclin A2 is specifically required to trigger mitotic entry in RPE-1 cells.

Cyclin A2 could contribute to mitotic entry in three ways: (i) by promoting specific mitotic substrate phosphorylations, (ii) by contributing to the total amount of CDK activity required for mitotic entry, or (iii) by controlling CDK1-cyclin B's activity and thereby indirectly promoting the CDK substrate phosphorylations. The latter option was especially appealing as it is well-established that CDK1-cyclin B complex accumulates in G_2 in an inactive state, maintained by two inhibitory phosphorylations on T14 and Y15 [274, 312]. CDK-cyclin A2 could be required to trigger CDK1's activation by promoting the CDC25 family of phosphatases, and/or downregulating the WEE1 family inhibitors, as implicated previously [59, 309]. As it is more difficult to trigger the activation of CDC25 phosphatases, this was addressed by chemically inactivating the two WEE1 family kinases that inhibit CDK1. To this end, I used two small-molecule inhibitors that target either WEE1 or WEE1 / MYT1 kinases, MK-1775 (MK) and PD-166285 (PD), respectively (see Chapter 3 Materials and Methods, Section 3.1.4.1). MK is a highly specific WEE1 inhibitor [393] and PD is somewhat less specific, but potently inhibits receptor tyrosine kinases [394, 395] (the inhibitors will be described in more detail in the Discussion of this Chapter, Section 4.6).

Cyclin A2 triggers mitosis in RPE-1 by activating CDK1-cyclin B Dr. Hegarat previously demonstrated that cells do not enter mitosis when lacking cyclin A2, further supporting previous data shown in this Section (data not shown). Indeed, promoting CDK1 activation by inhibiting the WEE1 kinases resulted in a rescue of the G_2 arrest, and cells entered and progressed through mitosis while still lacking cyclin A2 (Figure 4.6 A). The drugs are very potent and fast-acting inhibitors and the rescue of mitotic entry was clearly observed within 4 hours of drug incubation. Notably, PD promoted mitotic entry stronger, likely due to its lower specificity.

Figure 4.6 C shows that cells treated with PD exited mitosis normally, and the amount of defective mitotic exit was negligible (data not shown). Interestingly, cells that entered mitosis without cyclin A2 spent significantly longer in metaphase, albeit no defects such as lagging chromosomes were observable by LCI (Figure 4.6 B, C). This is not the result of increased CDK1 activity as control cells completed mitosis normally or quicker.

Is mitotic progression in RPE-1 dependent on specific CDK activity, or its total amount? I previously mentioned three possible explanations for cyclin A2's role in mitotic entry. It is likely that either of the two, CDK specificity, or quantity, contribute to a timely metaphase progression. As there are two mitotic CDK-binding cyclin proteins present in mitosis, cyclins A2 and B, I set out to determine whether an increased quantity of mitotic cyclins promotes CDK activity by addressing if cyclin B is capable of compensating for the roles of cyclin A2.

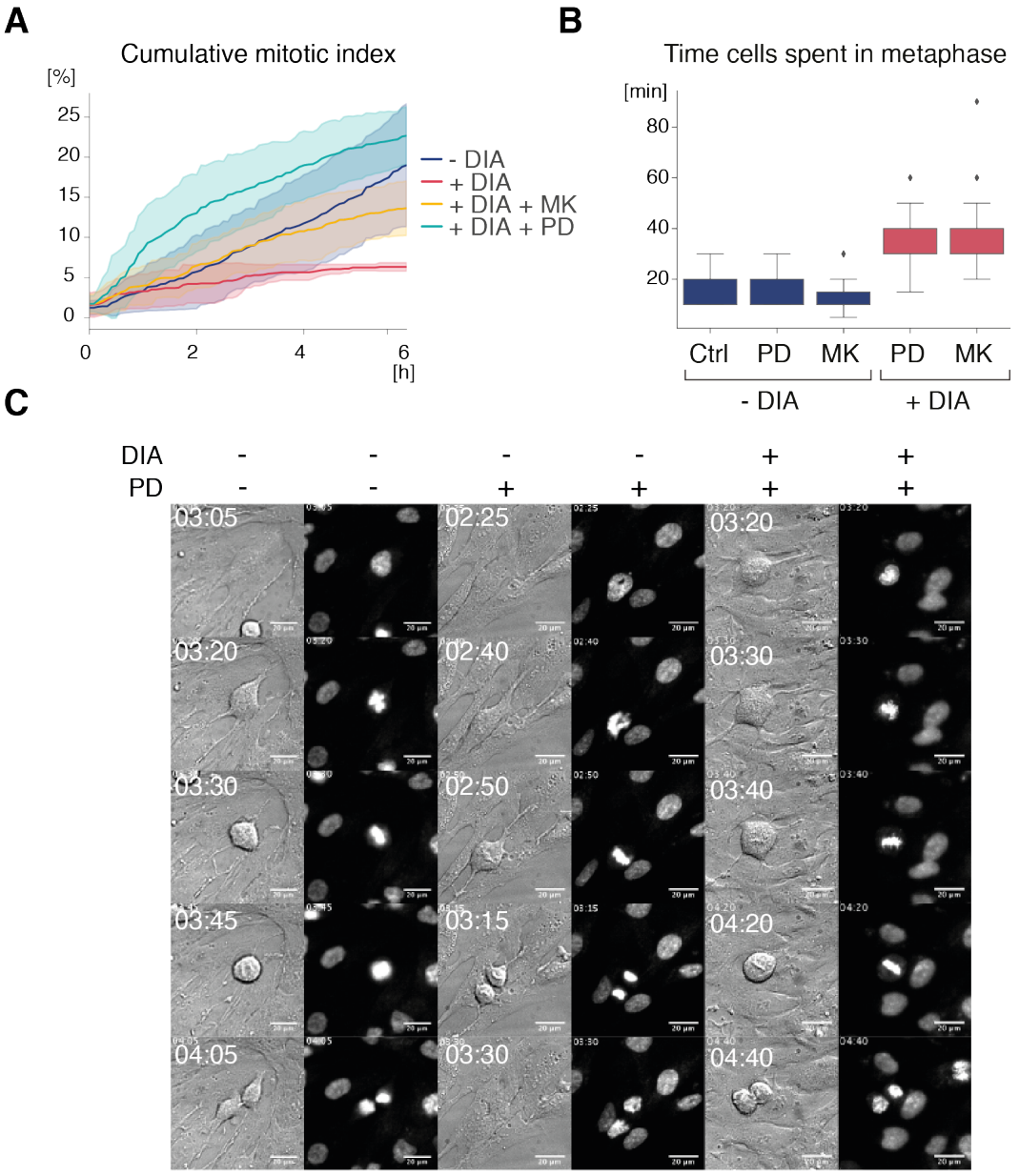

Figure 4.6: Cyclin A2 promotes CDK1 activation in RPE-1 cells. A: Cumulative mitotic index of RPE-1 cyclin $A2^{dd}$ treated as indicated. MK is MK-1775, WEE1 inhibitor. PD is PD-166285, WEE1 family inhibitor. Percentage total cells is shown on the y-axis. Time is on the x-axis, shown in hours. Data were obtained from three independently repeated experiments. The shaded area represents the standard deviation, and the line is representative of an average of three means in each time-point. B: Time cells in D spent in metaphase as observed by chromosome alignment in the equatorial plain. The boxplot indicates median, second and third quartile, and minimum / maximum values. The outliers are indicative of measurements not included in the boxplot. Time is shown in min. C: Snapshots of LCI analyses of cells in D and E with timestamps on the top left in hh:mm. Left channel is DIC, right channel is DNA, as visualised by SiR-DNA, shown in white. Scale-bars indicate 20 μm. Time-stamps are shown in hh:mm.

4.3.5 RPE-1 cells require a nuclear CDK activity to promote some mitotic events

Cyclin B1 preferentially binds with CDK1, but there have been reports of a CDK2-cyclin B1 complex in vitro [49]. It is likely that the majority of the endogenous cyclin B1 is bound by the inhibited CDK1 and it is thus unable to confer the necessary mitotic phosphorylations. Overexpressing cyclin B1 could result in an higher abundance of CDK2-cyclin B1 that is active before mitosis and not inhibited in late G_2 like CDK1, thus promoting key substrate phosphorylations.

Notably, cyclin B1 contains a cytoplasmic retention signal that was previously described (see Introduction). It is possible that mitotic events must be triggered inside the nucleus, as well as in the cytoplasm. To aid cyclin B1's localisation into the nucleus prior to prophase, I added a nuclear localisation signal (NLS) to the gene cassette that was generated for cyclin B1 gene incorporation (Figure 4.7 A).

Cell line generation To incorporate the gene cassette into RPE-1 cyclin A2^{dd} a novel transposase system termed Sleeping Beauty (SB) was used, that was shown to be highly efficient [385] (see Chapter 3 Materials and Methods, Section 3.1.4.2). Genes of interest were inserted into a vector containing a TET-ON promotor (pSB-tet-BP, obtained from Addgene [385]), which allowed the activation of gene transcription by DOX addition (along with triggering osTIR1 expression). This was of particular interest as a previous study found that cyclin B1 promotes replication when its levels were maintained in the nucleus during S phase [396], which could interfere with the normal replication mechanics in these cells. Furthermore, only promoting cyclin B1 expression when required also lowered the possibility of defects in mitosis as previous studies have shown that high levels of cyclin B1 after metaphase could induce mitotic catastrophe [258].

Cyclin B constructs to be overexpressed in cells Cyclin B1-YFP either fused with an NLS (BYN) or not (BY), as well as just YFP (Y) and YFP-NLS (YN) (Figure 4.7 A) were introduced into RPE-1 cyclin A2^{dd} cells by using the above mentioned transposase. I assessed the functionality and degradation mechanics of BY and BYN fusions separately using RPE-1 cyclin B2^{ko} / B1^{dd}, that will be described later in this thesis (see Chapter 5, Section 5.5.5).

Cell line characterisation Single clones of RPE-1 cyclin $A2^{dd}$ expressing the above proteins were then isolated and the level of BY and BYN expression following 4 h and 6 h of DIA incubation was assessed with western blotting (Figure 4.7 B, top). Out of the two clones tested for each construct, I chose BYN2 and BY5 for all future analyses, and they will be referred to as BYN and BY hereafter. Both achieve sufficient levels of cyclin A2 depletion and cyclin B1 overexpression within 4 h. Figure 4.7 B bottom shows that control plasmids YN and Y do not affect cyclin A2 depletion nor express exogenous cyclin B1-YFP. YFP expression and YN localisation was confirmed by western blotting and with fluorescence microscopy respectively (data not shown). Clone YN11 (hereafter YN) was used as the control for the following experiments with BYN and BY.

4.3.5.1 Overexpressing cyclin B1 in the nucleus promotes some mitotic events

LCI analyses demonstrated that a subpopulation of cells lacking cyclin A2 and overexpressing BYN is able to overcome the G_2 arrest, whereas cells expressing BY are less efficient (Figure 4.7 C). All cells were released from a single thymidine block and RPE-1 cells are less tolerant for this type of cell synchronisation, as also demonstrated by a significant population that does not re-enter the cell cycle within the duration of this experiment. Nevertheless, in comparison with the cells lacking cyclin A2, overexpressing BYN promotes mitotic entry in about half of the cycling population. On the other hand, BYN is approximately three-fold better in rescuing the G_2 block than the BY construct (Figure 4.7 C).

Cyclin A2 has specific roles in metaphase Cells entering mitosis thanks to cyclin B1's overexpression also spent much more time in metaphase (Figure 4.7 D) but still divided without obvious defects (data not shown). This was a further indication of cyclin A2's specific roles in mitosis, agreeing with other studies [8, 50], that can eventually be carried out by other mitotic players. However, cyclin B1 is apparently not able to promote the cyclin A2-specific events, as in this case the time spent metaphase would have reduced in conditions with double amounts of cyclin B1. Due to a lack of time I did not further investigate this phenotype.

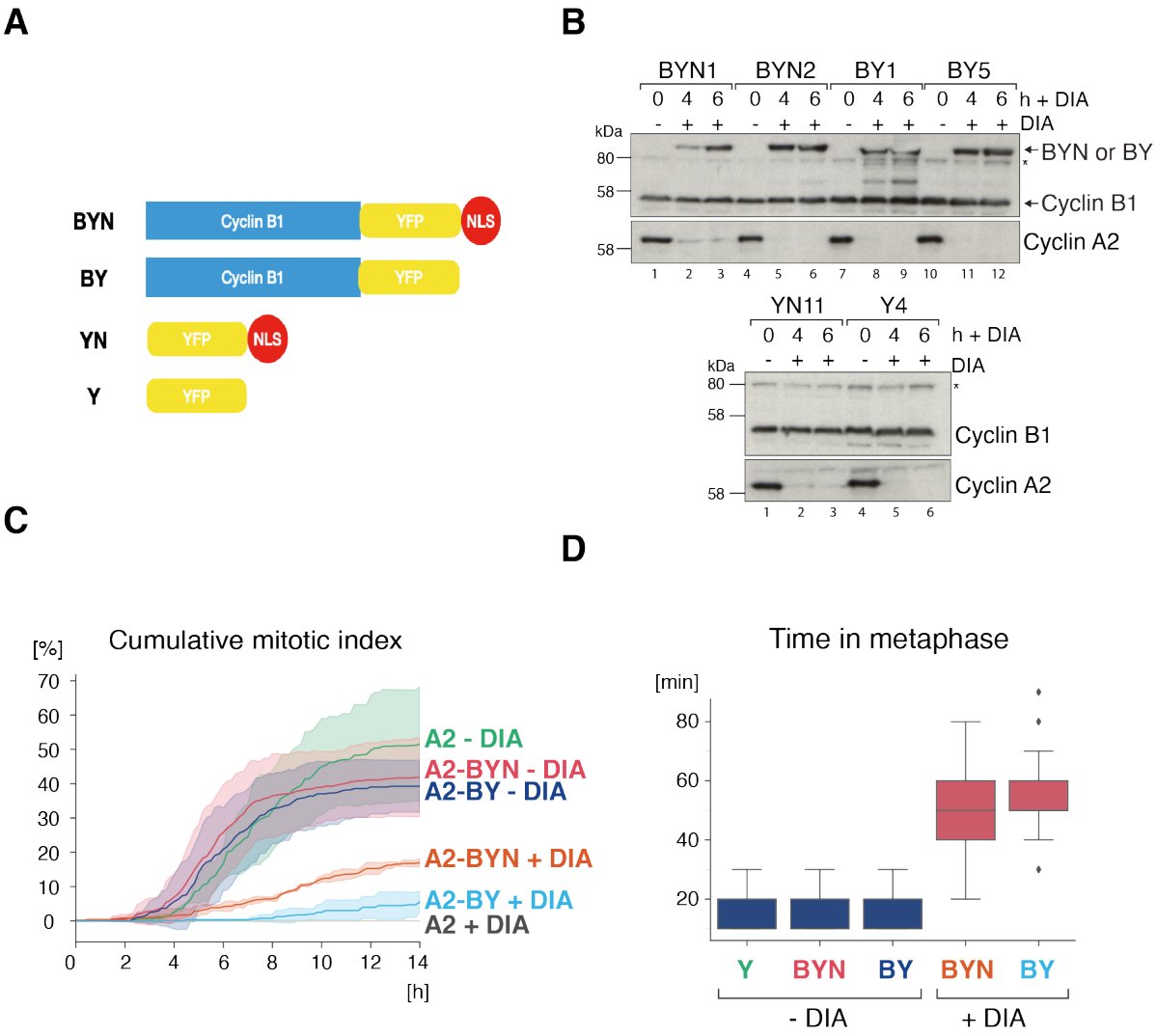

Figure 4.7: Generation of cyclin B1-overexpressing RPE-1 cyclin A2^{dd} cell lines. A: Schematic representation of gene cassettes used for protein expression and their names. B: Western blot of two clones of either BYN and BY, and a single clone of YN and Y. The endogenous cyclin B is the loading control, all cells are asynchronous. C: Cumulative mitotic index of all cells analysed, treated or not with DIA. Shaded area represents the standard deviation of three independently repeated experiments, and the line is the mean. D: Cells as in C, analyses of the time they spent in metaphase in minutes. Data were obtained from three independently repeated experiments, n > 50. The boxplot indicates the median, second and third quartile, and the minimum / maximum values.

4.3.5.2 Immunoprecipitation analyses show a minimal presence of CDK2-cyclin B1

To determine whether the overexpressed proteins are able to bind CDK1 with the same affinity as endogenous proteins and if they also bind with CDK2, I used a GFP-Trap assay that specifically immunoprecipitates GFP and YFP tags. Interestingly, GFP-Trap of BYN cells resulted in a significantly larger yield of cyclin B1, compared with BY (Figure 4.8). This phenomenon is surprising as the expression level of both proteins is very similar, as well as the pull-down of the GFP tag (Figure 4.8). Due to this, there was also more CDK1 and CDK2 observed in lanes 7 and 8, corresponding to the BYN pull-downs, compared to BY, lanes 9 and 10.

Nevertheless, these data clearly show that the introduced cyclin B1 fusion proteins are able to form a complex with CDK1, confirming that protein folding is not affected by the YFP tag on the C-terminus. Furthermore, it can be observed there are also CDK2-cyclin B1-YFP-NLS complexes present, as judging from a faint band in lanes 7 and 8 of Figure 4.8. Due to the lack of time, I was unable to optimise immunoprecipitation of endogenous cyclin B1 to assess the level of CDK2 that is normally bound with cyclin B1 in these cells.

The above data call for additional experiments assessing CDC25 and WEE1 / MYT1 activity in cells lacking cyclin A2 and in the above cells overexpressing cyclin B1 to further determine how CDK1 is activated in each case. However, this was not the focus of this thesis. Instead, I examined the cell cycle proliferation of the cells lacking cyclin A2 and overexpressing cyclin B1 that will be explained in the following paragraphs.

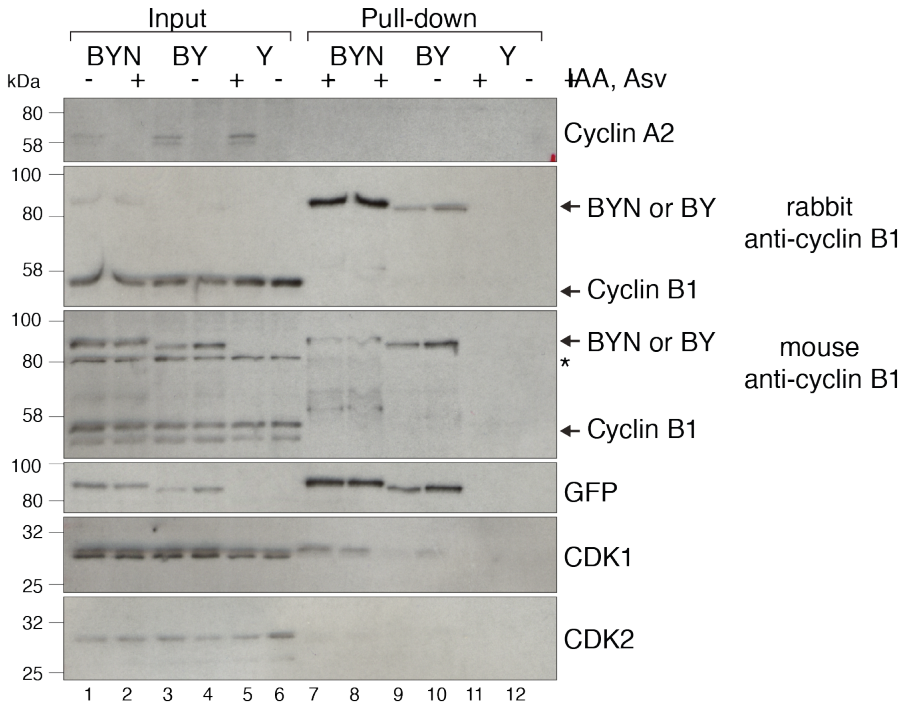

Figure 4.8: Immunoprecipitation analyses of the newly established cell lines. A Western blot showing indicated cells that were subjected to a GFP-Trap assay (see Chapter 3 Materials and Methods). Cyclin B1 was blotted with two different antibodies, a rabbit and a mouse one, as indicated. Blots also show cyclin A2, GFP, CDK1 and CDK2. * marks an unspecific band.

4.3.5.3 Analysing the cell cycle of cells lacking cyclin A2 and overexpressing cyclin B1

I utilised the EdU pulse-chase experiment, same as described previously in this Chapter, Section 4.3.3, to assess the cell cycle proliferation in RPE-1 cyclin A2^{dd} conditionally overexpressing BYN, BY, and YN. Figure 4.9 A shows an example of the FACS plots that were analysed, but only cells expressing BY and BYN, and lacking cyclin A2, are shown here for clarity. The gating is indicated in the same Figure and it was used for data analysis, like in the previously described EdU pulse-chase in Section 4.3.3. Analyses in Figure 4.9 B only show the DIA-treated BY and BYN conditions, as the untreated match the YN – DIA control.

A subset of cells expressing nuclear cyclin B1 successfully complete mitosis The cell cycle analyses further confirmed that a subset of BYN cells are able to complete mitosis and enter a new G_1 phase, but this is most apparent when following EdU-positive cells through time (Figure 4.9 A). Data analysis of EdU positive BY and BYN cells confirmed that these cells eventually cycle to the G_1 stage, but a larger amount of BYN cells enter G_1, as compared with BY (Figure 4.9 B, right panels, G_1 populations). While EdU-negative cells BYN cells lacking cyclin A2 also cycle, this is less obvious in a normalised population, albeit a decreased G_2 population in BYN that matches the untreated YN control supports that these cells are not arrested at this stage (Figure 4.9 B, left hand side, G_2 populations).

Cyclin B1 does not promote late S phase events Cyclin A2-deficient RPE-1 cells progress slower through late S phase, as noted in Section 4.3.3, thus I next aimed to determine whether cells overexpressing cyclin B1 could rescue this issue.

Regardless of cyclin B1's overexpression, cells are still slower in progressing through S phase (Figure 4.9 B, right panels, S2 population). The amount of cells in S phase gradually declines in untreated cells, whereas all instances of cyclin A2 depletion demonstrate a significantly larger population of S phase cells persisting throughout the course of this experiment. Furthermore, a quicker downward curve matching the cyclin A2 depleted cells in Section 4.3.3 was observed in all DIA-treated cells here as well (BY and BYN in Figure 4.9 A, YN not shown).

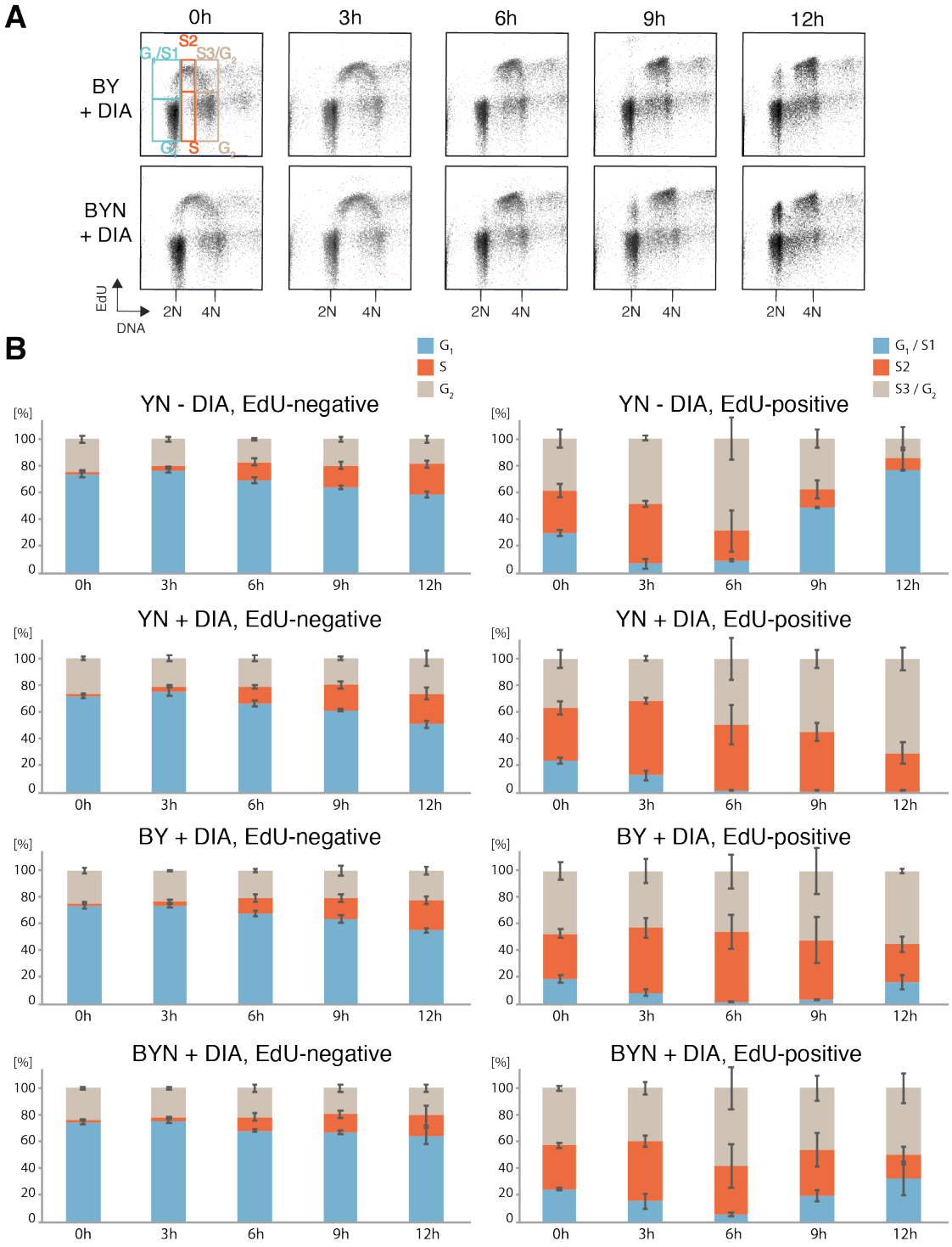

Figure 4.9: EdU pulse-chase analyses of RPE-1 cyclin A2^{dd} cells overexpressing YN, BY, or BYN protein fusions. A: FACS plots of BY or BYN cells treated with DIA to deplete cyclin A2. Top left plot indicates gating, as previously described (Figure 4.3) B: FACS analyses of the indicated populations, as in Figure 4.3. Data for FACS plots in A were from obtained from a single experiment. Data in B were obtained from three independently repeated experiments. Data in left or right column were normalised to either EdU-positive or EdU-negative of each condition, respectively. Barplots represent the mean of three experiments, error bars show standard deviation.

4.4 HeLa

4.4.1 Cyclin A2 is not essential in HeLa cells

After defining the roles of cyclin A2 in RPE-1 cells, I sought out to compare the effects of cyclin A2's depletion in a different cell line, using a similar degron tagging approach. This work was conducted in the Poon lab and the techniques used in this Section are noted in Chapter 3 Materials and Methods, Section 3.2.

HeLa cells constitutively expressing the tTA1 transcription factor (TF) that is required for TET-OFF controlled gene expression were used to establish the HeLa AID-cyclin A2 cell line by a PhD student in the Poon lab, Rita Ng (described in the following Section, published in [387]). I used these cells to compare the roles of cyclin A2 found previously in untransformed cells, to a cancer cell line. The cell line characterisation was carried out by Rita Ng, and I will summarise her findings below, followed by the cell cycle analyses of HeLa cells lacking cyclin A2.

4.4.1.1 HeLa AID-cyclin A2 cell line generation and characterisation

Poon lab degron tagging system HeLa cells are an intrinsically unstable cancer cell line that is notorious for its aneuploidy [397, 398], hence endogenous tagging of proteins is even more challenging. The method used in the Poon lab works by knocking out the endogenous gene of interest (GOI), and replacing it by incorporating an exogenous AID-tagged GOI. The GOI cassette that is introduced into the genome of these cells includes a TET-OFF promotor, that will stop gene transcription upon doxycycline addition (see Section 3.2.7, [387]). Here, osTIR1 is constitutively expressed as it can not be TET-ON controlled in the same setting where TET-OFF method is used, due to the use of a tTA1 TF.

The protein depletion is executed in two manners – halting the GOI's transcription, and inducing protein degradation by IAA addition. The treatment used in these cells consists of doxycycline and IAA, and will be termed as DIAA hereafter. Using this system, cyclin A2 was depleted within 6 – 7 hours (Figure 4.10). Comparing to the previously described RPE-1 system, this is almost double the time needed to reach cyclin A2 depletion, but still markedly less and more efficient as compared with the conventional RNAi depletion methods. Notably, there are several splice products of AID-cyclin A2

observed in these cells, but after DIAA treatment, these are all degraded (Figure 4.10). Genome sequencing done by Rita Ng confirmed the knock-out of endogenous protein (data not shown).

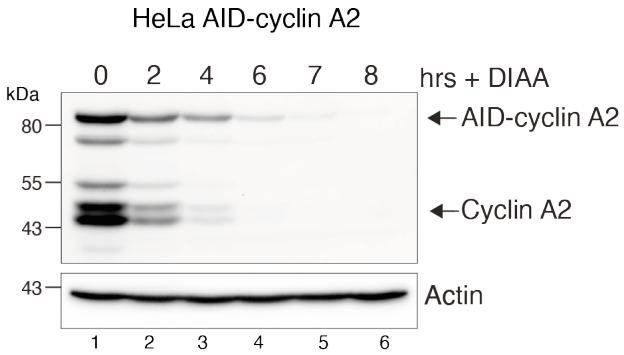

Figure 4.10: HeLa AID-cyclin A2 depletion efficiency. Western blot showing the depletion efficiency of AID-cyclin A2 after DIAA cocktail treatment for the indicated amounts of time. Actin is the loading control.

4.4.1.2 HeLa cells deficient in cyclin A2 are slightly delayed in their cell cycle progression

BrdU pulse-chase experiment outline To analyse the cell cycle progression of HeLa cells lacking cyclin A2, I decided to conduct a BrdU pulse-chase experiment. This experiment also complements the EdU pulse-chase data shown in RPE-1 cells (Section 4.3.3).

BrdU, instead of EdU, was used in these experiments as the reagents were readily available and previously optimised for use in the Poon lab. There are some key differences between the EdU and BrdU methods: (i) cells were collected every two hours, instead of three as in RPE-1 analyses, owing to a slightly shorter cell cycle of HeLa cells (according to LCI analyses, not shown here), (ii) the method of BrdU staining is different to the EdU protocol (see Chapter 3 Materials and Methods, Section 3.2.14) which may lead to some discrepancies, and (iii) the FACS machine and software were different as previously. Altogether, this resulted in some changes in the FACS plots, such as a higher background (Figure 4.11 B). This was obvious during triplicate analyses as seen by the larger standard deviations, indicated by error bars on the barplots (Figure 4.11 C – E).

Cyclin A2 controls the timing of mitotic events in HeLa Asynchronous HeLa AID-cyclin A2 cells were treated and collected as indicated in Figure 4.11 A. According to the FACS plots of a single experiment (Figure 4.11 B), cells that were in S phase at the time of BrdU pulse, lacking cyclin A2, progress slower through the cell cycle. This is most apparent in 6 and 8 h after the BrdU pulse, as the control cells (- DIAA) are seen re-entering G_1, but the treated cells (+ DIAA) are only starting to enter G_1 8 h post-BrdU pulse. This is also evident in the triplicate analyses (Figure 4.11 D, G_1 / S1 populations). The accumulation of G_1 cells is markedly slower, and most seem to reach late S / G_2 stage by the end of the experiment. This difference is less apparent in BrdU-negative cells (Figure 4.11 C), but the increase in standard deviation cells lacking cyclin A2 indicates more variability. Examining Figure 4.11 B and comparing all BrdU-negative cells, a slight difference in the time when the bulk of cells reach G_2 after 8 and 10 h of the pulse is observable, confirming previous observations that cells lacking cyclin A2 enter mitosis slower (according to Rita Ng, not shown).

Similar to RPE-1, S phase entry is not affected by the loss of cyclin A2 (Figure 4.11 E).

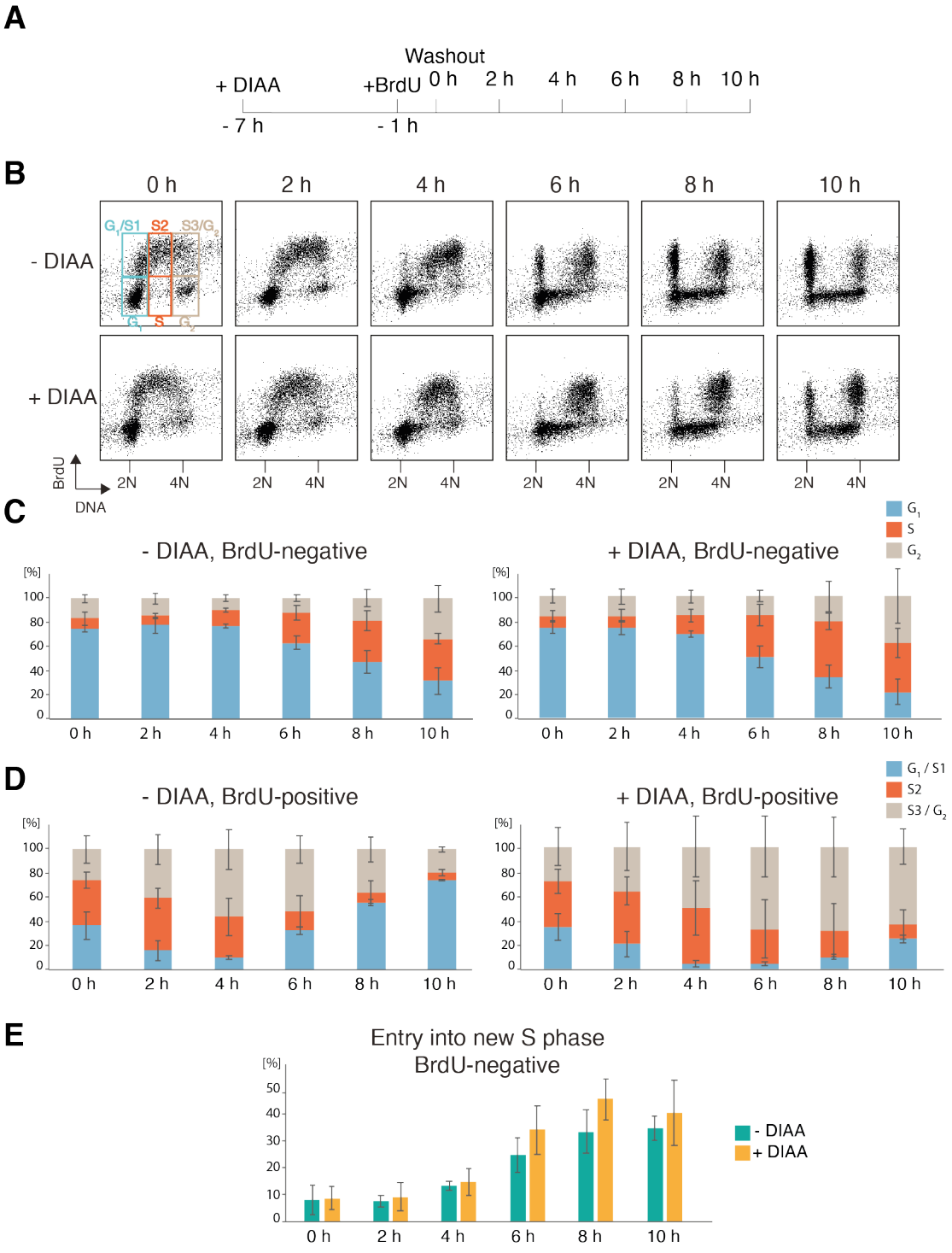

Figure 4.11: HeLa AID-cyclin A2 BrdU pulse-chase analysis. A: Outline of the BrdU experiment. Asynchronous cells were treated with DIAA for 6 h prior to BrdU addition, followed by three washes, and re-incubating in normal or DIAA-containing media. Cells were collected at washing, and every two hours afterwards. B: FACS plots of HeLa AID-cyclin A2. Gating as shown in untreated 0 h was used for analyses in C, D and E. C, D: Analyses of untreated or treated cells separated by their BrdU uptake. E: S phase entry of BrdU-negative treated and untreated cells side by side. Analyses in C – E obtained from three independently repeated experiments. Barplots are as in Figure 4.3.

4.4.1.3 Cyclin A2 is not required for mitosis in HeLa cells

The above information comes in agreement with Rita Ng's results, where she observed an approximately 2 hour delay in mitotic entry in cells lacking cyclin A2. To look further into this, I conducted live-cell imaging experiments with these cells.

Live-cell imaging analyses confirmed a delay in mitotic entry and progression Figure 4.12 A shows single-cell tracking graphs of an asynchronous population and it is evident that cells lacking cyclin A2 enter mitosis slightly slower. Furthermore, these cells spent more time in mitosis (Figure 4.12 B), thus indicating that cyclin A2 is important for mitotic events, although any defects from preceding stages of the cell cycle could also induce a slower mitotic progression. Apart from the delay, few other phenotypes were observed indicative of mitotic catastrophe, supporting data from Rita Ng (data not shown).

Further analyses in the sought out to determine whether other non-transformed and transformed cells show similar phenotypes, but due to the lack of time I could not set up the degron-tag system in these. Instead, I utilised conventional RNAi methods and analysed the roles of cyclin A2 in two additional human cell lines. These will be described in the next Section of this Chapter.

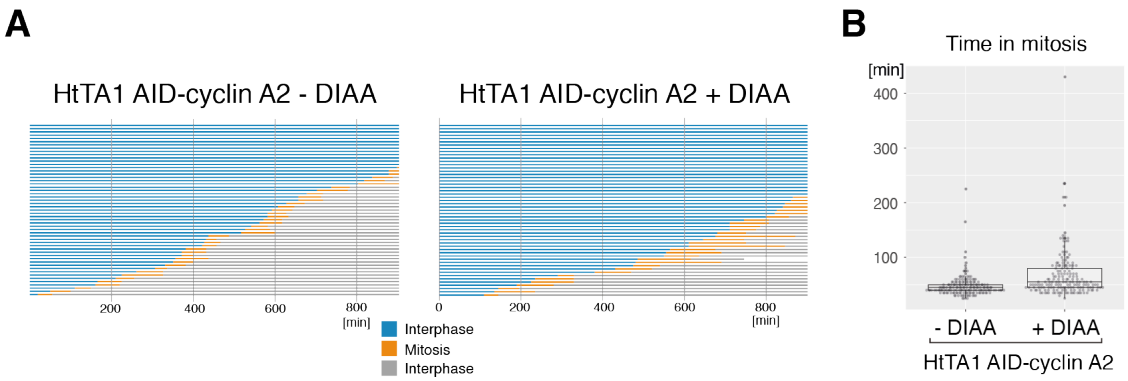

Figure 4.12: HeLa cells lacking cyclin A2 are slightly delayed in mitotic entry and progression. A: Single-cell tracking analyses of a single experiment, n = 52. B: Time cells spent in mitosis. Data in B obtained from three independently repeated experiments, n > 40. The boxplot indicates median, second and third quartile. Boxplot whiskers show the first and fourth quartile. Bee-swarm is overlayed, each dot represents an individual measurement.

4.5 Role of cyclin A2 in other human cell lines

Two additional human cell lines, a non-tumorigenic epithelial MCF10A and a breast cancer cell line MCF7 were used to analyse and compare the effects of cyclin A2's depletion. I utilised RNAi experiments for these analyses due to a lack of time to set up degron tagged cells.

4.5.1 Cyclin A2 depletion does not arrest cells in G_2

Figure 4.13 A shows the RNAi depletion efficiency after 48 h, and 72 h of treatment. These cells were analysed with FACS (Figure 4.13 B) where it is evident that lacking cyclin A2 leads to alterations in the cell cycle progression, as seen by the population surpassing the 4N DNA content. This is either due to previously described rereplication caused by a lack of cyclin A2 [181], or due to defects in mitotic exit that interfered with cellular division, but were sufficient to promote a new cell cycle entry. Importantly, the siRNA experiments highlighted the importance of cyclin A2 in two additional human cell lines.

Owing to a lack of time, I was unable to conduct further analyses with these cell lines. Future research is needed to confirm the findings of roles of cyclin A2, shown in RPE-1 and HeLa, in other human non-transformed and cancerous cell lines. Setting up degron tags in more human cell lines will allow for more detailed studies of this protein's functions.

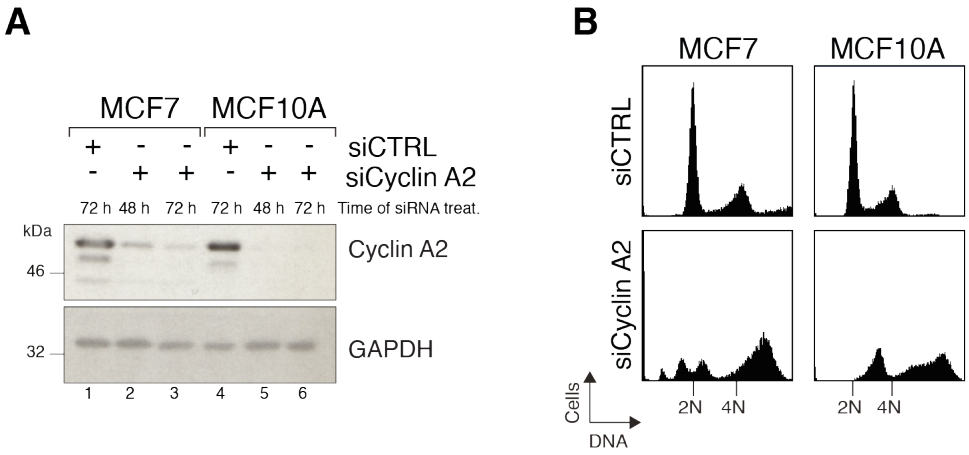

Figure 4.13: MCF7 and MCF10A cells exhibit cell cycle defects after cyclin A2 depletion, but do not arrest. A: Western blot showing cyclin A2 depletion after the indicated times of siRNA transfections. GAPDH is the loading control. B: FACS plots of cells in A treated with siRNA for 72 h. Cells were treated as described in Chapter 3 Materials and Methods, and analysed on a BD Accuri machine.

4.6 Discussion

This Chapter uncovered that there are remarkable differences in the roles of cyclin A2 in human cell lines by comparing two well-established human cell line systems, a retinal pigment epithelial cell line RPE-1 and transformed cancer cells, HeLa. The former are a hTERT immortalised cell line that is otherwise untransformed, whereas HeLa cells derive from a cervical tumour and are known for their genomic instability [397, 398]. Both cell lines were investigated with novel protein degradation techniques, the details of which have been mentioned in this Chapter, as well as in Chapter 3 Materials and Methods.

The functions of cyclin A2 that were defined here will be discussed separately for each cell line, starting with RPE-1 cells.

4.6.1 Roles of cyclin A2 in RPE-1 cells

Cyclin A2 is the key that unlocks mitotic entry in a human non-transformed retinal pigment epithelial cell line, RPE-1. Without it, cells are unable to promote any mitotic events as its main role is evidently to promote CDK1-cyclin B activation.

4.6.1.1 Cyclin A2 is required for the mitotic entry in RPE-1 cells, but not for progression through mitosis

This Chapter showed that cyclin A2 promotes mitotic entry by activating CDK1, thus defining its importance in the onset of prophase (Figure 4.14). According to the data shown in this study, CDK-cyclin A triggers mitosis by activating CDK1-cyclin B. It is possible that cyclin A2 also promotes phosphatase inactivation, but whether this must be achieved for prophase or prometaphase entry is not yet clear. Chapter 6 of this thesis will address this in more detail.

Furthermore, it is yet to be determined whether CDK1 activation is achieved by CDK-cyclin A2 inactivating WEE1 and MYT1, by promoting CDC25 phosphatases, or a combination of both. It is inevitable that one event will trigger the other, but knowing how this cascade of events is initiated is essential to pinpoint the roles of cyclin A2 in mitotic entry.

This Chapter also showed that activation of CDK1 promotes mitotic entry by treating the cells with two potent WEE1 family inhibitors, PD and MK. There were some

discrepancies in the rescue of mitotic entry, which will be addressed next.

Why do PD treated cells enter mitosis more drastically as compared with MK treatment? When comparing the mitotic entry of either MK or PD treated cells, I observed a larger amount of cells, even in comparison to the untreated cells, entering mitosis following PD treatment (Figure 4.6 A). This may be explained by the fact that MK is very specific for WEE1 inhibition [393], whereas PD was initially developed to study blood cell signalling, but its potent inhibition of receptor tyrosine kinases [394, 395] resulted in its use in cell cycle studies. Since PD also inhibits other receptor tyrosine kinases, and some have been implicated in a DNA-damage pathway that leads to CHK1 activation, it can promote mitotic entry more efficiently by bypassing the DNA damage checkpoint. Even though more mitotic catastrophe due to unrepaired DNA damage could be expected in this treatment, this was not observed in this setting. One explanation for this is that cells have already been arrested in G_2 due to the lack of cyclin A2 prior to small-molecule inhibitor treatment, and any errors that would have lead to mitotic defects in the case of checkpoint bypass have already been repaired in that time.

Cyclin A2 is specifically important for a faithful mitotic progression Cyclin A2's function during mitosis in RPE-1 is at least partially specific for CDK-cyclin A2. This was indicated by the persistent mitotic delay in cells that entered mitosis in the presence of a nuclear localised cyclin B1 (BYN). Notably, a subpopulation of cells with the overexpression promoted mitotic events, even though the cells all had the same level of BYN overexpression, implying that other factors likely affect the cells' decision to enter mitosis. Thus, cyclin A2 has specific roles that are more efficient in triggering mitotic entry. It is possible that CDK-cyclin A2 promotes mitotic events via PLK1, as implied previously [51], but it could also carry out some specific mitotic phosphorylations in the preceding stages that are essential for a faithful mitosis, albeit these can eventually be achieved by other mitotic kinases like CDK1-cyclin B, PLK1 or MASTL, hence the delay.

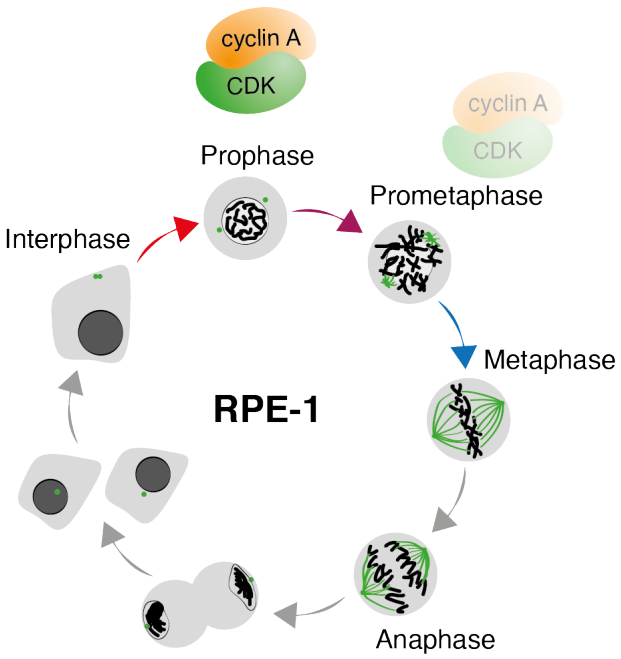

Figure 4.14: Cyclin A2 is essential for prophase and may aid in prometaphase. CDK-cyclin A2 (possibly CDK1 or CDK2) is essential for the establishment of prophase, but nuclear CDK-cyclin B can also promote these events (not shown). Cyclin A2 has additional roles in prometaphase, but it is not required for metaphase. The inactivation of phosphatase could also contribute to these events, but this is not yet clear.

4.6.1.2 Cyclin A2's CDK partner in mitosis is undetermined

It is not certain whether cyclin A2 activates CDK1-cyclin B in complex with CDK1 or CDK2. While the functions of the CDK2-cyclin A2 complex are well established and its activity is known to be controlled differently to CDK1 [25], there is much less known about CDK1-cyclin A2. This complex is already active in late S phase [8] and CDK1 activity at that level does not trigger mitotic events [44], thus indicating that CDK1-cyclin A2 is regulated differently, and has a separate function, to CDK1-cyclin B. Defining the roles of CDK1-cyclin A2 and CDK2-cyclin A2 is essential especially with the development of novel CDK2-cyclin inhibitors. Regardless of CDK2-cyclin A2 / E inhibition, cyclin A2 could possibly carry out its function by binding with CDK1, as it is established that CDK1 can take over for other interphase CDK proteins [38].

4.6.1.3 Nuclear localisation of cyclin B can trigger mitosis

Overexpression of cyclin B1 promotes mitotic events in approximately half of the proliferating population, however it must be fused with an additional NLS signal to trigger mitosis. Interestingly, a small amount of wild-type cyclin B1-YFP overexpression also rescued the G_2 arrest. Cyclin B1 shuttles between the nucleus and the cytoplasm prior to mitotic entry and it is actively exported from the nucleus by a cytoplasmic retention signal (CRS) [203]. Therefore, overexpressing cyclin B1-YFP increases the amount that is being shuttled and some cells may lack the capacity to export this additional quantity of cyclin B1. I reasoned that there could be enough cyclin B1 present in the nucleus at some point to trigger mitotic events in some cells overexpressing BY. BYN rescues mitotic entry more efficiently, implying that mitotic events must be triggered from inside the nucleus. Cyclin A2 is normally present in both the nucleus and the cytoplasm [88], allowing it to promote mitotic events in the nucleus while triggering activation of CDK1 in the cytoplasm. Not much is known about which CDK is activated by cyclin A2, and further research is needed to separate between functions of CDK2-cyclin A2 and CDK1-cyclin A2 in mitosis.

4.6.2 Roles of cyclin A2 in HeLa cells

In contrast, human cervical cancer cells HeLa have re-defined their cell cycle by abolishing the necessity for cyclin A2, albeit the cellular proliferation is slowed without it. Lacking cyclin A2 delays their mitotic entry and progression, but does not arrest them, nor induces mitotic catastrophe. Furthermore, HeLa cells do not require cyclin A2 to aid in late S phase, unlike RPE-1 cells. This information raised questions on whether these cells have adjusted from a qualitative to a quantitative CDK-cyclin activity requirement – are specific cyclins necessary, or are they fully redundant if others can achieve sufficient levels of required CDK activity? This question will be further addressed in Chapter 7.

4.6.3 Comparison of cyclin A2's roles in RPE-1 and HeLa

Interestingly, both HeLa and RPE-1 cells lacking cyclin A2 spent a significantly longer amount of time progressing through mitosis, but this has not yet been investigated further. Previous studies have exposed the roles of cyclin A2 in kinetochore-microtubule attachments [50, 51] and linked cyclin A2 with PLK1's activity [51]. Concomitant with this, cyclin A2's effect on PLK1 in mitotic entry has been reported recently [88], thus showing that this interaction is possible, and may extend into mitosis. Further analyses with cyclin A2 degron cells could help to cement the roles of cyclin A2 in mitosis.

This Chapter contributed to the current knowledge of cyclin A2 in the human cell cycle and demonstrated how it triggers mitotic entry. To date, this was unclear due to analyses with incomplete depletion of cyclin A2, but owing to a higher level of protein degradation, achieved by two different degron tagging systems, the role of cyclin A2 was further elucidated.

In the next Chapter, the roles of cyclin B in RPE-1 cells will be assessed, using the same system as for RPE-1 cyclin $A2^{dd}$

Chapter 5

Roles of cyclin B in RPE-1 cells

5.1 Summary

Cyclin B is crucial for mitosis in RPE-1 cells, but not essential for mitotic entry or nuclear envelope breakdown. Cyclin B contributes to some specific substrate phosphorylations that were identified with phospho-proteomic analyses, however these are apparently crucial for metaphase events as no chromosome alignment to the equatorial plane was observed in cells lacking cyclin B. These phosphorylations are largely dependent on cyclin B as maintaining the endogenous levels of cyclin A2 in metaphase did not promote a chromosome plane alignment. RPE-1 cells rely on other activities, such as cyclin A2 and MASTL, to promote CDK substrate phosphorylations in early stages of mitosis in the absence of cyclin B. Inhibition of CDK1 triggered mitotic exit, implying that CDK1-cyclin A2 activity is sufficient to maintain the mitotic state in cyclin B deficient cells.

5.2 Introduction

Cyclins B1, B2, and B3 belong to the cyclin B family and their similarities along with some key differences were previously outlined in Chapter 2 of this thesis, Section 2.2.2, and will thus not be reviewed here. The reader is reminded that cyclin B3 is not somatic and since this thesis focuses on the roles of cyclins in human somatic cells cyclin B3 will not be mentioned hereafter. One study compared the effects of depletion of either cyclin B1, cyclin B2, or both, in RPE-1 and HeLa cells and demonstrated their redundancy; this study also showed that either somatic B-type cyclin is required for a faithful mitotic entry and progression in human cells [11]. Regardless of the difference in their localisation, after a short delay in mitotic progression in cells lacking cyclin B1, cyclin B2 is sufficient to promote a successful mitosis [11, 194]. On the contrary, during mouse development only cyclin B1 can compensate for the loss of cyclin B2 [193], further implying that cyclin B1 is crucial for a faithful execution of the cell cycle. Given their compensatory roles, further mentions of cyclin B in this thesis comprise both cyclins B1 and B2, unless specifically noted.

Cyclin B has several roles in mitosis Cyclins confer specificity of CDKs to promote and direct their substrate phosphorylations [39, 40, 42, 194]. To date, specific roles of CDK1-cyclin B and CDK1/2-cyclin A2 complexes in human cells are not yet clear due to the difficulty of separating between the precise roles of these two essential cyclins, and cyclin A2's ability to bind both, the mitotic CDK1 [8] and the interphase CDK2. Furthermore, cyclin B can also activate CDK2 in vitro [49] and while this has not yet been shown in vivo, it indicated that an additional CDK-cyclin complex could also contribute to the proper execution of the cell cycle.

Mitotic spindle establishment Cyclin B1 is localised to kinetochores [19, 223–225] and the mitotic spindle after nuclear envelope breakdown (NEBD) [17, 227]. As noted previously, CDK1-cyclin B1 has roles in centrosome separation and spindle assembly by promoting the kinesin family EG5 motor protein [20, 228, 231, 232]. EG5 directly drives centrosome separation in early mitosis [399] and is regulated by a microtubule nucleation factor, TPX2 [400], that is also activated by CDK1-cyclin B [21, 401]. TPX2 also controls KIF15, likely via AURKA [402], that also promotes the spindle assembly independently

of EG5 [233].

The above paragraph is a mere indication of the complex pathways involved in the spindle formation, and CDK1-cyclin B may be crucial to promote these events.

Interplay between cyclins A and B in mitosis Data in Chapter 4 established the role of cyclin A2 in RPE-1 cells. While it is essential for initiating mitotic events in RPE-1 cells, I successfully bypassed this requirement by activating CDK1, implying that a CDK-cyclin B activity is sufficient to carry out the progression through mitosis, albeit there may be a specific requirement for cyclin A2 to coordinate the timing of metaphase. Cyclin A2 likely controls mitotic events either by activating downstream kinases, or by contributing to mitotic phosphorylations. It appears that the latter option is less probable, as overexpressing cyclin B1 partially rescued mitotic entry but not the time those cells spent in mitosis. Thus, it appears that the majority of mitotic events are carried out by CDK-cyclin B, though the level of compensation between the two cyclins is yet to be determined.

5.3 Questions to be addressed

A model of mitotic entry and progression in RPE-1 cells was introduced in the previous Chapter. The focus of this Chapter is on the roles of cyclin B in mitotic progression. For clarity, the proposed model of mitotic progression in Figure 5.1 only shows the roles that I have established for CDK-cyclin A2 previously in this thesis, and the implied roles of CDK-cyclin B. The shaded CDK-cyclin A2 in prometaphase indicates that it is not essential, but may contribute to these events.

Prophase entry While cells are unable to trigger mitosis without cyclin A2, as shown in Chapter 4, promoting CDK1's activation resulted in a successful progression through mitosis, thus implying that cyclin B can promote prophase events. It is unclear whether this was achieved by CDK-cyclin B directly, or by other mitotic activities such as phosphatase inactivation or other mitotic kinase activation. This Chapter will elucidate the requirement of cyclin B in prophase.

Prometaphase progression Following prophase exit, marked by NEBD, cells enter prometaphase. Prometaphase is a highly dynamic stage of mitosis during which cells organise chromosomes to the equatorial plain, eventually establishing metaphase. The roles of CDK1-cyclin B in the start of prometaphase have been indicated when the translocation of CDK1-cyclin B into the prophase nucleus was observed [13]. Whether it really contributes to NEBD is not yet clear and I aim to address the requirements for cyclin B in NEBD in this Chapter.

Metaphase establishment Next, the roles of CDK1-cyclin B are implied by its localisation to the mitotic spindle [17], but the importance of this complex to establish metaphase is unknown. Current data suggests that CDK1 activity is crucial for mitosis and since cyclin A2 is degraded prior to cyclin B, this implies that it has key roles during this stage. But what these may be has not yet been clarified and is also going to be addressed below.

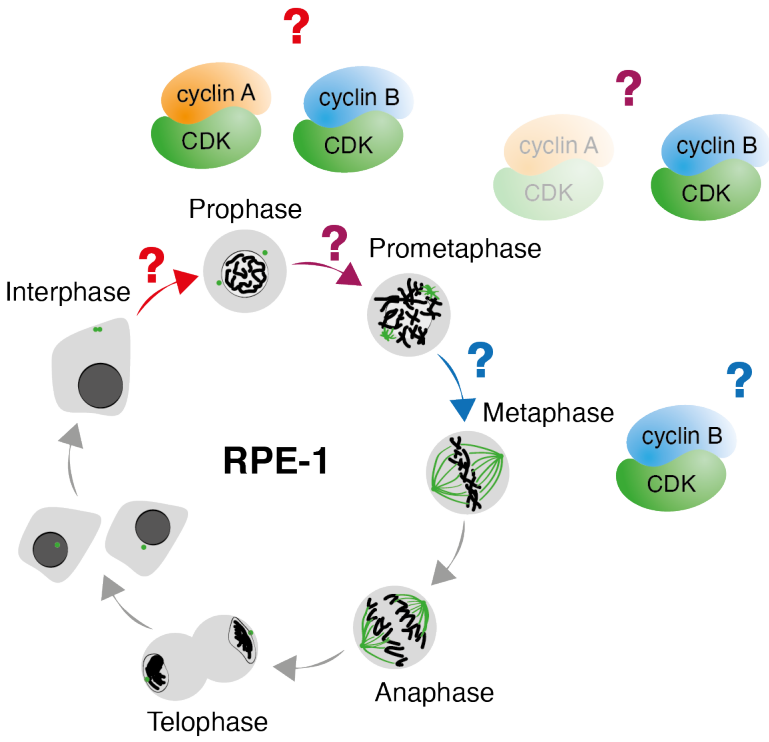

Figure 5.1: A proposed model addressing the requirements of cyclin B during separate stages of mitosis in RPE-1 cells. CDK-cyclin A2 (possibly CDK1 or CDK2) is essential for the establishment of prophase, but the role of CDK-cyclin B is not yet clear. This Chapter will address the roles of cyclin B in prophase, prometaphase and metaphase.

5.4 Cell line generation and characterisation

Similarly to the previously described RPE-1 cyclin A2^{dd}, the cyclin B1^{dd} was also established by Dr. Nadia Hegarat and Dr. Helfrid Hochegger in the Hochegger lab [386]. Owing to this, several parts of the analyses were conducted by Dr. Hegarat. Key findings will be described here to provide the basis for further work understanding cyclin B's key functions in RPE-1 cells. Experiments that were conducted by Dr. Hegarat are noted in the main text and in Figure legends.

The reader is also reminded that while cyclins B1 and B2 can compensate for each other, cyclin B2 is not essential. For clarity, cyclin B2 was knocked out (confirmed by Sanger sequencing and western blotting, data not shown), and the cyclin B1^{dd} was established in cells lacking cyclin B2 (RPE-1 B2^{ko}, B1^{dd}), and in the parental RPE-1 osTIR1 cell line which still contain the wild-type cyclin B2 (RPE-1 B1^{dd}). The double degron system is as described in Chapter 3 Materials and Methods, Section 3.1.4.4, and in Chapter 4 Section 4.3.2. Any references to cyclin B hereafter include both cyclins B1 and B2, unless stated differently.

5.4.1 Endogenous Cyclin B1 is degraded within 4 h

The degradation of cyclin B1^{dd} is similar to the previously described cyclin A2^{dd} (Chapter 4 Section 4.3.2). Activating only SMASh-mediated degradation by Asunaprevir (Asv) results in two extra bands migrating at 80 or higher kDa that are likely the full-length cyclin B1-mAID-SMASh protein and alternative splice products including the antibiotic resistance cassette (Figure 5.2 A, left panel). Inducing protein depletion by targeting only mAID with doxycycline and IAA resulted in residual levels of cyclin B1 remaining until the end of this experiment, up to 16 h after treatment (Figure 5.2 A, middle panel). Finally, degrading the protein by activating both degron tags with the DIA cocktail led to a highly efficient depletion of cyclin B1, where protein levels were undetectable by western blotting 4 h after DIA treatment (Figure 5.2 A, right panel).

Depleting only cyclin B1 already induced some defects in long-term cell proliferation (Figure 5.2 B, left side), supporting previous observations noting the importance of cyclin B1 over B2 [193], but cells were unable to survive without both B-type cyclins.

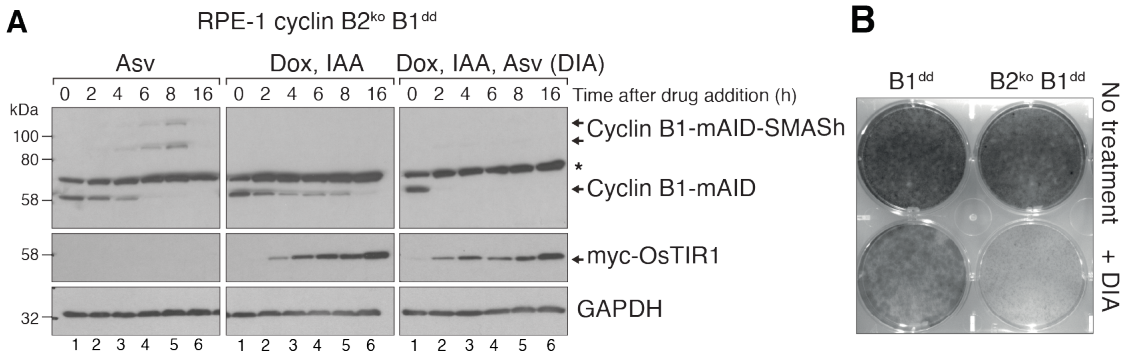

Figure 5.2: RPE-1 cyclin B1^{dd} is efficiently depleted within 4 h. A: Western blot showing the effect of different degron tag drug treatments on asynchronous cells, Asv targets the SMASh tag, and Dox, IAA activate mAID degradation. The DIA treatment is a combination of all three, Dox, IAA, and Asv, same as previously. B: Survival assay of RPE-1 cells with cyclin B1^{dd} in a cyclin B2 wild-type (B1^{dd}) or knock-out (ko) background (B2^{ko} B1^{dd}). Both A and B were done by Dr. Hegarat [386]. * marks an unspecific band.

5.4.2 Cyclin B is essential for a faithful mitosis

According to the survival assay (Figure 5.2 B), the presence of one type of cyclin B is crucial for proliferation in RPE-1 cells, confirming previous observations from RNAi experiments [11]. To determine cyclin B's function in these cells, the cells were further analysed with live-cell imaging (LCI) analyses.

Cells lacking cyclin B enter mitosis normally Mitotic entry of asynchronous cells lacking cyclin B was unhindered, according to the cumulative mitotic index of RPE-1 cyclin $B2^{ko}$ $B1^{dd}$ treated or untreated with DIA (Figure 5.3 A). The depletion interfered with later stages of mitosis, as noted by mitotic exit analyses in Figure 5.3 B. These cells were filmed with differential interference contrast (DIC), and SiR-DNA fluorescent dye was added to visualise the DNA with fluorescence.

Cyclin B is essential for a successful mitotic exit The majority of cells lacking both types of somatic cyclin B exited mitosis after nuclear envelope breakdown (NEBD), but did not segregate their DNA contents into two cells. A significant proportion of cells exited mitosis with defects during cytokinesis, marked by cytokinesis failure. These cells eventually segregated their DNA, separating them from the 'no segregation' phenotype. A subpopulation of cells exited mitosis from prophase, prior to NEBD, as defined by cell rounding and chromosome condensation, followed by decondensation of the DNA and cells observably re-adhering to the surface. These cells did not break down their nuclear envelope, as observed by the shape of the nucleus.

Cyclin B2 is sufficient for the first mitosis after cyclin B1 depletion To assess the defect in proliferation caused by cyclin B1 depletion alone (Figure 5.2 B), the ability to complete the first mitosis following DIA treatment was compared in the following cell types: (i) parental cells without an mAID or SMASh tag (RPE-1 osTIR1), (ii) cyclin $B1^{dd}$ cells, and (iii) cyclin $B2^{ko}$ $B1^{dd}$ cells. Cyclin B2 completely compensated for the roles of cyclin B1 and only cells missing both B-type cyclins were unable to complete cell division (Figure 5.3 B).

According to these data, RPE-1 cells can proliferate normally with just one type of somatic cyclin B, however even when missing both cyclins B1 and B2, more than 60 % of the mitotic population progress past NEBD before exiting from mitosis with defects.

These data contrast previous observations noting the importance of cyclin B in mitotic entry of mouse embryos [10], but support other studies demonstrating the redundancy of cyclin B in M entry [11, 12, 19].

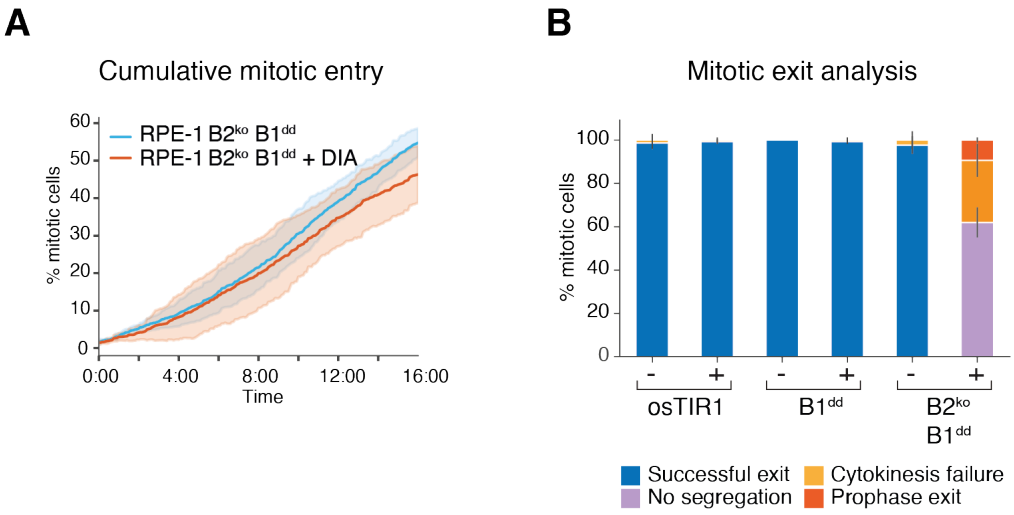

Figure 5.3: Cells lacking cyclin B enter mitosis normally, but cannot exit without defects. A: Asynchronous cells were pre-treated or not with DIA for 4 h prior to analysis with live-cell imaging. Cells were classified as mitotic based on their rounding as seen with DIC. Data were obtained from three repeats (n > 500), standard deviation indicated by shaded area. B: Asynchronous cells were imaged with DIC and SiR-DNA and only mitotic cells were analysed. The mitotic exit phenotypes were defined as successful, prophase exit where no NEBD was observable according to the DNA fluorescence, no segregation where cells progressed past NEBD but did not separate their DNA contents, and cytokinesis failure where cells progressed past NEBD and separated their DNA contents into two or more cells with observable defects as noted by DIC and SiR-DNA fluorescence. Data were obtained from three repeats (n > 50), the barplots signify the mean of three experiments. Error bars in all barplots indicate standard deviation. Data in A were obtained by Dr. Hegarat [386].

5.5 Roles of cyclin B in mitosis

5.5.1 Cyclin B is necessary for the establishment of metaphase

Experimental outline Time-lapse microscopy analyses in the previous Section showed that cells lacking cyclin B progress into prometaphase undisturbed, but could not establish the metaphase plate alignment. To investigate this further it was crucial to synchronise the cells in order to obtain a larger mitotic population for future analyses. To this end, Dr. Nadia Hegarat and I tested various synchronisation protocols. In the end, I synchronised the cells with a single thymidine release followed by an arrest in mitosis. Proteasome inhibition or microtubule poisons could trigger a mitotic arrest; however a proteasome inhibitor could not be used for this purpose due to the activity required to degrade the mAID-SMASh tagged cyclin B1. Additionally, microtubule poisons require the maintenance of a spindle assembly checkpoint (SAC) which has previously been shown to require cyclin B [11, 12]. Instead, the anaphase promoting complex / cyclosome (APC/C), essential to trigger mitotic exit (as reviewed in Chapter 2 Section 2.2.1.2), was inhibited by Apcin and proTAME. The two inhibitors were previously found to be efficient in blocking the cells in metaphase [403] and since the cells are unable to establish the proper metaphase plate alignment, the inhibitors could be used to examine the specific defects that occur prior to metaphase. Moreover, the APC/C inhibitors could further aid with the maintenance of a mitotic arrest by stabilising cyclin A2, as cells lacking cyclin B have been shown to exit mitosis sooner when stalled in mitosis [11, 12]. In the presence of Apcin and proTAME, the APC/C targets will not be degraded, including cyclin A2 and securin, which is necessary for the maintenance of sister-chromatid cohesion. Indeed, cyclin A2 is stable for the duration of the experiments utilised in these analyses (data are shown in Chapter 6 Section 6.3.1.1).

Cyclin B is necessary for a metaphase alignment of chromosomes Immunofluorescence analyses confirmed that while the APC/C inhibitor treatment was effective in arresting cells still expressing cyclin B1 in metaphase (Figure 5.4 A, - DIA), no metaphase plate alignment was observed in the population lacking cyclin B (+ DIA). Mitotic phenotype analyses showed that the majority of cells without cyclin B were either still in prophase, or progressed past NEBD but did not establish metaphase (Figure 5.4 B).

These cells were classified as prometaphase, kinked or monopolar as indicated in the Figure legend.

Figure 5.4 A shows one example of the peculiar kinked phenotype in the middle panel, and one of a cell classified as prometaphase on the right. Cells that were not distinctly kinked or monopolar were counted as prometaphase. They are characterised by a formed mitotic spindle with separated centrosomes.

Cyclin B is involved in mitotic spindle organisation Several cells with disoriented astral microtubules were observed, as can also be seen in Figure 5.4 A, right panel. These were analysed separately by Dr. Hegarat and will not be shown here. Briefly, the defect in regulation of astral microtubules in prometaphase was confirmed by a cold treatment analysis where elongated microtubules facing the opposite direction of chromosomes were detected in cells lacking cyclin B [386].

The next Section discusses how depleting cyclin B resulted in these drastic phenotypes on a molecular level.

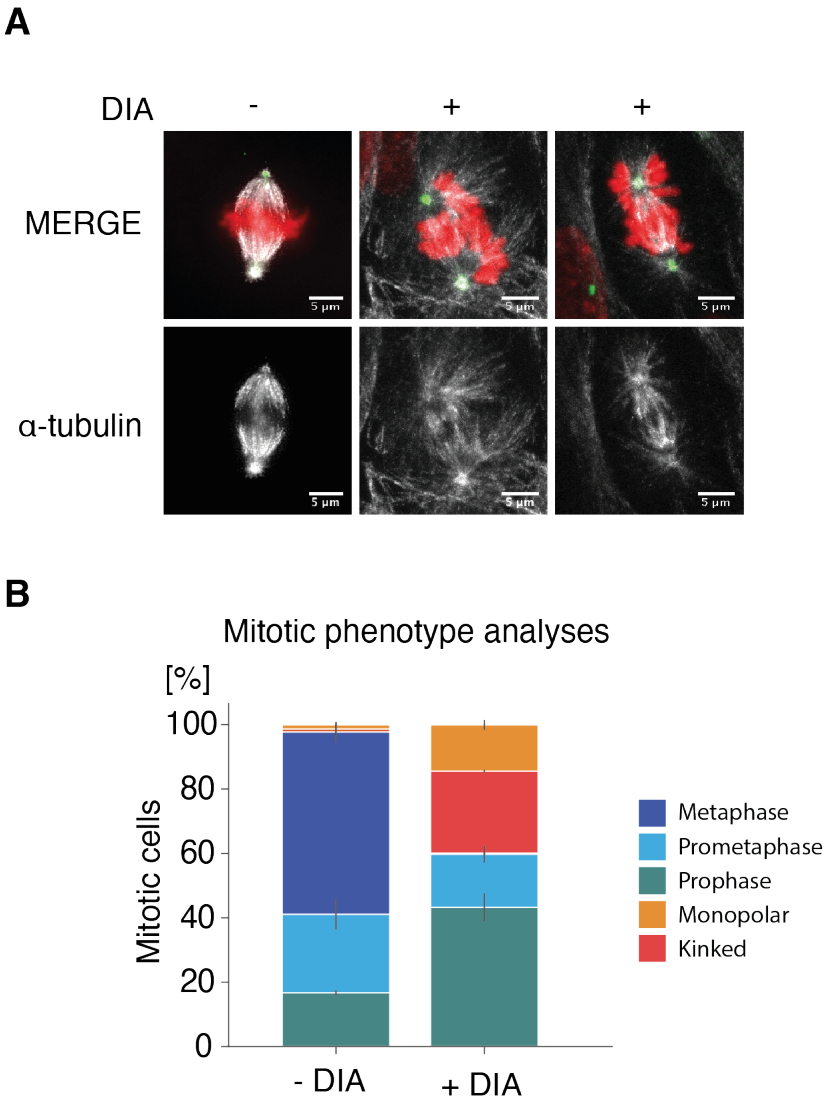

Figure 5.4: RPE-1 cells lacking cyclin B fail to establish metaphase. A: Cells were synchronised with a single thymidine block, untreated or treated with DIA, and arrested in mitosis with Apcin and proTAME treatment (see Methods). Afterwards, they were fixed and analysed with immunofluorescence staining for α-tubulin, pericentrin, and DAPI (MERGE, DAPI in red, pericentrin in green, α-tubulin in white). B: Quantification of mitotic phenotypes of cells in A ($n > 50$). Cells were divided into five categories, according to DAPI, tubulin, and pericentrin staining. Metaphase: Chromosomes condensed, aligned in the equatorial plain, centrosomes separated, mitotic spindle established (see A, far left). Prometaphase: As metaphase, but chromosomes not aligned into a plain (see A, far right panel). Prophase: chromosomes are condensed but confined into a circular shape by the nuclear envelope. Spindle is not yet established and centrosomes are starting to be separated. Monopolar: NEBD has occured but centrosomes are not separated, characterised by microtubules emanating from a single point. Kinked: NEBD has occured, chromosomes are condensed, centrosomes separated, but the spindle is not in alignment with centrosomes and DNA is mostly found on one side of the cell (see A, middle panel). Data were obtained from three independently repeated experiments, barplots show the mean, and error bars indicate standard deviation.

5.5.2 Cyclin B is only responsible for a subset of mitotic phosphorylations

The phospho-proteome of treated and untreated mitotic RPE-1 $B2^{ko}$ $B1^{dd}$ cells was analysed with the help of Hochegger lab collaborators in Angus Lammond's group at the University of Dundee (see Hegarat et al. for more details and methodology [386]). Mass spectrometry analyses were conducted separate to this thesis and only some key findings are included here. Briefly, phospho-proteome analyses demonstrated that cyclin B is not specifically required for over 90% of mitotic phosphorylations in RPE-1 cells, and only the remaining 10% were significantly changed after cyclin B depletion (Figure 5.5 A). Sites that were significantly affected by the loss of cyclin B are shown in orange whereas the rest are pictured in green (Figure 5.5 A). The Hochegger lab looked into several substrates that were identified using this approach [386], but as this was not the focus of this thesis, only one will be described below to provide an example for the reader.

KI67's localisation is controlled by cyclin B KI67, a chromatin remodeller that has roles in interphase and is found to coat the chromosomes during mitosis [359], was detected as a possible target with a phosphorylation status significantly dependent on cyclin B (Figure 5.5 B shows the KI67 peptides that were identified in blue). To determine how CDK1-cyclin B controls this protein, cells were analysed by mitotic spread analyses, followed by immunofluorescence (see Chapter 3 Materials and Methods).

Even though the mitotic spread technique is known to be a rigid method that normally depletes proteins, since KI67 coats the chromosomes, it is likely to remain tethered to the DNA even after this treatment. Indeed, after preparation of mitotic spreads, KI67 was found on the surface of chromosomes (Figure 5.5 C, left panel). This was confirmed by quantifying the fluorescence intensity in a line over one chromosome arm and comparing the amount of DAPI and KI67 signals (Figure 5.5 D). Cells lacking cyclin B do not have KI67 localised to the chromosomes, as seen in Figure 5.5 C, right panels, and quantified in D where it is readily observable that KI67 has lower fluorescence intensity and its pattern does not coincide with previously observed KI67 peaks in untreated cells.

5.5.2.1 Cyclin B regulates specific mitotic substrates

Apart from KI67, other proteins affected by the loss of cyclin B were identified. Dr. Hegarat and Dr. Hochegger further confirmed a selection of substrates, including Repo-Man (recruits PP1 to the chromatin in mitosis) [358, 404], TOP2B (decatenating enzyme) [405], AURKB (chromosome passenger complex component) [111], and TPX2 (involved in spindle assembly and function) [400]. All of the above were affected by the loss of cyclin B [386], but they will not be shown here as this work was beyond the scope of this thesis.

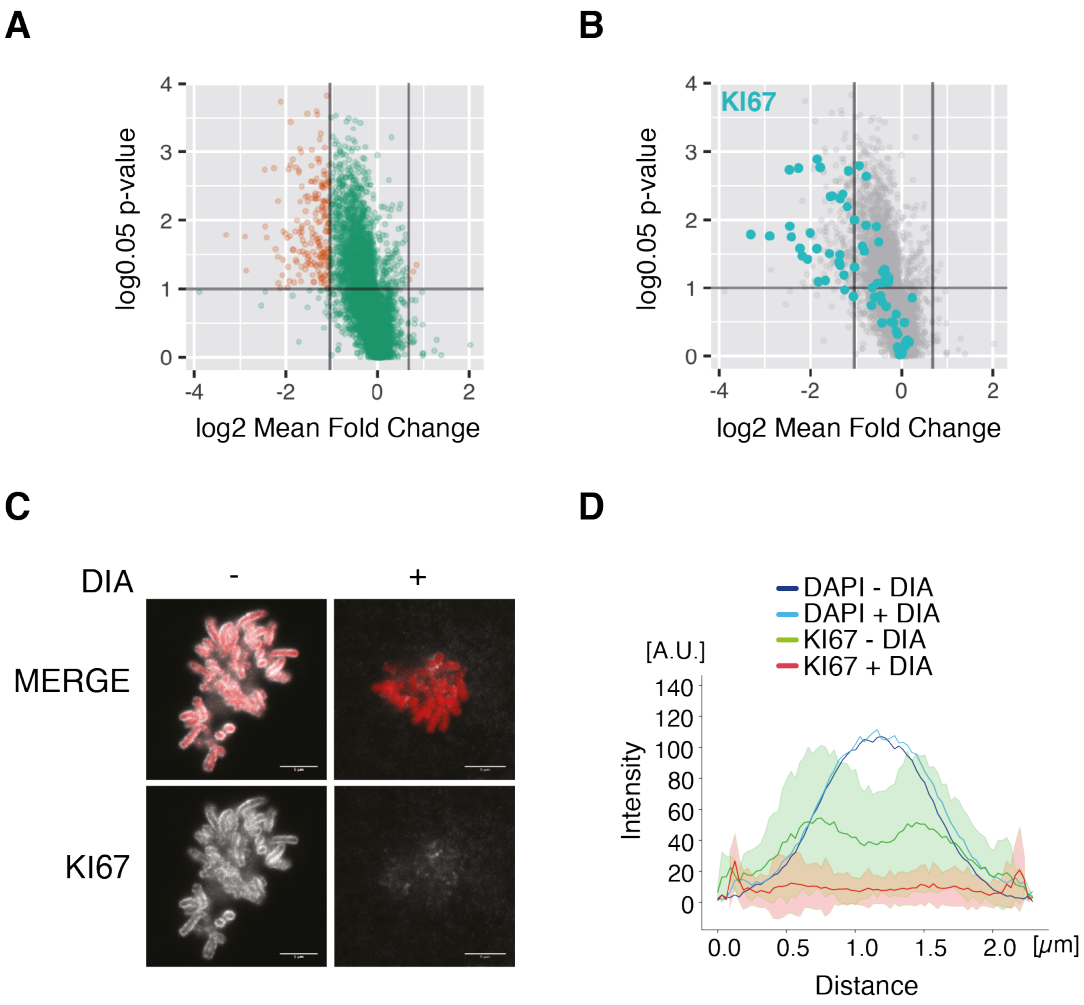

Figure 5.5: Cyclin B controls only a subset of mitotic CDK substrate phosphorylations. A: Phospho-proteomic analyses of specific mitotic substrate phosphorylation changes conducted with the Lammond lab. Sites significantly affected by the loss of cyclin B are shown in orange (p = 0.05). B: A schematic view of phosphorylation sites on KI67 (blue) that were identified in mass spectrometry analyses. C: Cells were treated as in A, and fixed according to the chromosome spread protocol (see Chapter 3 Materials and Methods) followed by immunofluorescence analyses to detect KI67 and DNA. D: Quantification of fluorescence intensity of DAPI or KI67 in a line over one chromosome using FIJI. DAPI is shown in blue, KI67 in green and orange, DIAA-untreated or treated, respectively. Standard deviation, represented by a shaded area, is shown only for KI67 measurements. Data were obtained from three independently repeated experiments (n > 50).

5.5.3 Anti-CDK1 phosphatase activity is affected by the loss of cyclin B

The mitotic phosphorylation activity is constantly counteracted by phosphatases. Two key mitotic kinases PP1 and PP2A are inactivated during mitosis to ensure that mitotic CDK substrates are not prematurely dephosphorylated. The phosphatase activity in cells lacking cyclin B will be addressed in this Section.

The below experiments were conducted together with Dr. Helfrid Hochegger. Cells were trapped in mitosis and forced to exit by the addition of a potent CDK1 inhibitor, flavopiridol [406]. Immunoblotting analyses show that cells lacking cyclin B dephosphorylate their mitotic substrates faster as compared with - DIA cells (Figure 5.6 A, compare lanes 3, 4 to 7, 8). This could be due to a slightly lower total amount of CDK1 phosphorylations, or hyperactive phosphatases. Comparing the relative change of the signal intensity confirmed that the dephosphorylation mechanics are not significantly changed in cells lacking cyclin B (Figure 5.6 B), thus the difference between dephosphorylation mechanics was attributed to a slightly lower starting amount of CDK1 phosphorylations.

Specific changes in substrate phosphorylations were undetectable by immunoblotting analyses for total phosphorylated CDK1 substrates ((K/H)p-SP motif).

Cells without cyclin B inhibit PP2A Next, the activity of two key mitotic phosphatases, PP1 and PP2A, was analysed. Interestingly, cells lacking cyclin B altered the control of PP2A regulators - MASTL and consequently of ENSA / ARPP19, two inhibitors of PP2A (Figure 5.6 C). Notably, ENSA and ARPP19 are two strongly similar proteins and the antibodies typically recognise both proteins (as observed previously in the Hochegger lab). It appears that MASTL is dephosphorylated slower, while ENSA's activity increases in cells lacking cyclin B, thus indicating that PP2A inhibition is maintained in these cells, and PP1 may be the main phosphatase triggering mitotic substrate dephosphorylation.

During mitotic entry PP1 is inhibited by a phosphorylation on T320 by CDK1 and this phosphorylation is removed upon mitotic exit [407]. According to the western blot in Figure 5.6 C, PP1 is inhibited during mitotic entry but after flavopiridol treatment the phosphorylation is removed, although the activation of PP1 is apparently at a slightly

lower level in cells lacking cyclin B. Nevertheless, cyclin B depletion does not affect the phosphatase inhibition during mitotic entry, indicating that there is an additional CDK1 activity that controls PP1's inhibition during mitosis. The persistent ENSA phosphorylation that suppresses PP2A may thus be compensated for by PP1 activation in cells without cyclin B. This comes in agreement with previous observations noting the redundancy of PP2A in mitotic exit in *Xenopus* [362], and a recent study on PP1's function during mitotic exit [408].

APC/C is hyperactive in cells lacking cyclin B Cells activate APC/C gradually during mitosis in different forms, as described in the Introduction part of this thesis (see Chapter 2, Section 2.2.1.2). Two APC/C components, CDC27 and APC1, were found to be active in cells lacking cyclin B, according to their phosphorylation status, thus showing that the E3 ubiquitin ligase complex is active and would normally trigger the depletion of its substrates (e.g. cyclin A2) and likely promote mitotic exit. These data further highlight the importance of APC/C inhibitors in this study, which enabled a mitotic synchronisation of these cells, regardless of the ubiquitin ligase's activity.

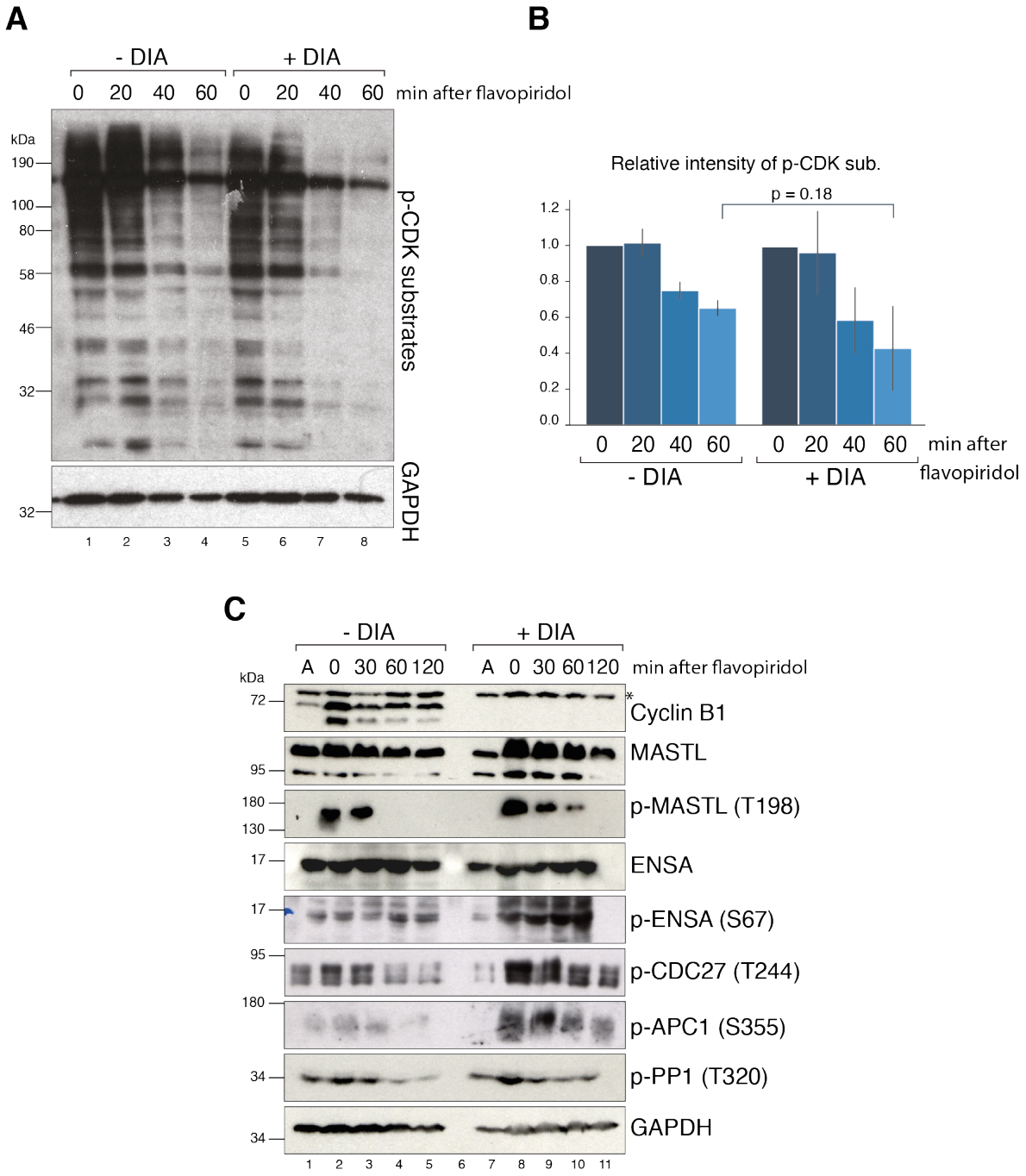

Figure 5.6: Few mitotic phosphorylations are dependent on cyclin B. A, B: cells were treated with a single thymidine block and arrested in mitosis with Apcin and pro-TAME (see Chapter 3 Materials and Methods), followed by 1 μM flavopiridol treatment for the indicated time before harvesting and analysing with immunoblotting. Cells were treated and harvested by Dr. Hochegger. Relative intensities of the western blot in A were analysed from three repeats and normalised to the corresponding GAPDH loading control. Bars indicate means, error bars indicate standard deviation. C: Immunoblotting analyses of cells treated as in A, probed with the indicated antibodies. Additionally, p-CDK substrates detects the (K/H)p-SP motif, GAPDH is the loading control and * marks an unspecific band.

5.5.4 Stabilising cyclin A2 does not rescue defects in metaphase alignment

Cyclin A2 likely promotes the majority of CDK phosphorylations when cyclin B is absent. I have already shown that cyclin A2 has an essential role in mitotic entry in the previous Chapter. It is not entirely clear why cyclin A2 is unable to establish metaphase in these cells, but this could be explained by the cyclin's specificity for earlier mitotic substrates (prophase and prometaphase) and / or owing to its degradation pattern that precedes cyclin B. To address this, I decided to further stabilise cyclin A2 in these cells.

An essential APC/C subunit was depleted using siRNA against CDC27, as an additional inhibition of APC/C activity (Figure 5.7 B shows siRNA efficiency after 72 h). Then, cells were synchronised with a single thymidine block and released into normal or DIA-containing media. Afterwards, the cells were incubated with Apcin and proTAME for an additional 2 h. Here, I quantified all kinked or monopolar phenotypes as prometaphase cells since siCDC27 treatment could additionally extend the time in mitosis, due to to defects from the preceding cell cycles and possible premature mitotic entry. This and the previously described treatments are thus not entirely comparable when analysing the specific prometaphase phenotypes of cells lacking cyclin B. However, I theorised that I could still detect any discrepancies between cells containing or lacking CDC27 by simply comparing the amounts of prometaphase and metaphase cells. Immunofluorescence analyses showed no cells in metaphase when cyclin B was depleted in siCTRL or siCDC27 cells (Figure 5.7 B).

I next checked whether proTAME treatment alone stabilises cyclin A2 by treating the cells with two metaphase-arresting compounds, a microtuble depolymerisation agent nocodazole [409] or an EG5 inhibitor STLC [410]. Notably, Gavet et al. have previously shown that cyclin A2 becomes depleted during a nocodazole arrest [220].

Cells were incubated with the arresting agents for 12 h, followed by 6 hours of treatment with DIA and ProTame. An increase in the amount of cyclin A2 was observed (Figure 5.7 C), showing that the degradation is halted, but the phenotype caused by cyclin B depletion remained unchanged (data not shown).

The above findings demonstrated that cells require cyclin B to promote specific mitotic events, regardless of cyclin A2's presence. To ensure that these cells were not additionally

modified after CRISPR targeting, and that cyclin B is indeed the cause of these phenotypes, I decided to supplement them with an exogenous version of cyclin B and assess their proliferation.

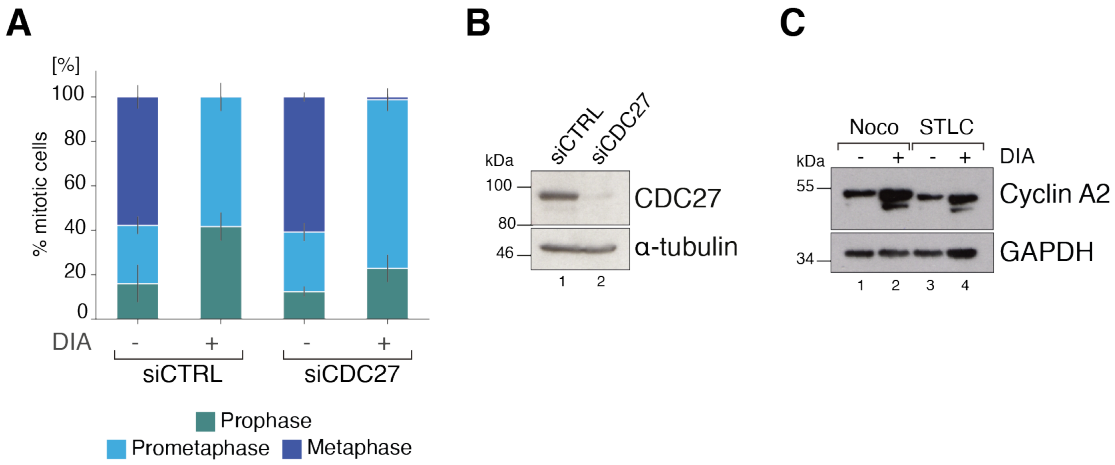

Figure 5.7: Additional inhibition of APC/C did not promote a metaphase establishment. A: Cells were transfected with siRNA for 40 h, followed by a single thymidine block for 24 h, and a release into media containing DIA or not for 13 h. Afterwards, Apcin and proTAME were added for an additional 2 h prior to fixation and immunofluorescence analyses. Cells were quantified as prophase, prometaphase, or metaphase (see main text for explanation). Data were obtained from three independently repeated experiments. Error bars indicate standard deviation. B: Western blot of asynchronous siRNA transfected cells for 72 h. C: Western blot of cells treated or not with 18 h of Nocodazole (Noco) or STLC (EG5 inhibitor). After 12 h, DIA (where indicated) and proTAME were added for the last 6 h of treatment. GAPDH is the loading control.

5.5.5 Overexpressing exogenous cyclin B rescues only short-term defects

5.5.5.1 Cell line establishment and characterisation

To confirm that the phenotypes observed above are due to the loss of cyclin B, I established new cell lines with conditional expression of cyclin B1 constructs - cyclin B1-YFP-NLS (BYN), cyclin B1-YFP (BY), YFP-NLS (YN) , and YFP (Y) (Figure 5.8 A) – previously described in Chapter 4, Section 4.3.5. I utilised these constructs here to also ensure that they are fully functional.

Confirmation of cyclin B1 overexpression after DIA treatment Two clones of each overexpression, BYN and BY, were tested with western blotting analyses following 8 h of DIA treatment in an asynchronous population (Figure 5.8 B). In untreated cells, these constructs are undetectable as they are TET-ON controlled, but there is a dramatic increase after 8 h of DIA treatment in all tested cell lines. While I tried to find clones with similar levels of overexpression as the endogenous mAID-tagged protein, I was unable to isolate clones with a lower level of expression (data not shown).

Clones expressing BY have such high levels of protein overexpression that the lab-made luminol solution depleted too quickly even with short times of membrane exposure, characterised by an empty space surrounded with a dark circle where luminol is still being processed (lanes 9 and 12, 80 kDa bands). Nevertheless, the western blot still shows that the cyclin B protein is being expressed, and a difference in size between BYN and BY is readily observable by a shift in the band. Blotting for GFP signal confirmed the existence of the YFP tag.

In parallel, two control constructs lacking cyclin B were also tested, YN1 and Y1 (Figure 5.8 C). The western blot analysis showed that cyclin B1-mAID is still depleted as normal, and the constructs only express either YN or Y, as seen by the GFP signal. The clone expressing YFP is apparently promoting YFP even in the absence of doxycycline, however this did not appear to affect cell growth (data not shown).

5.5.5.2 Exogenous cyclin B overexpression cannot rescue long-term proliferation

Cells are unable to survive in a survival assay lacking endogenous cyclin B, regardless of overexpressed versions of cyclin B1 (Figure 5.8 D). I hypothesised that it is possible that high levels of cyclin B interfere with cellular proliferation, so I checked their growth in the presence of both, endogenous and the newly introduced version of cyclin B1. Figure 5.8 E shows that cells where only doxycycline was added exhibited proliferation defects, even when just YFP was overexpressed (clone Y). Doxycycline promotes osTIR1 expression, which has been linked with basal depletion of mAID-tagged protein in the absence of IAA [391]. Partial depletion of endogenous cyclin B1 could have lead to the decrease in the survival rate of these cells, indicating that the overexpression did likely not cause cellular proliferation defects. These data implied that it is likely that the above cells are unable to survive in a long-term proliferation assay because they have lost the control over this cyclin B1's expression levels.

For future analyses, clones BYN1 and BY3 were used and will be referred to as BYN and BY, respectively, unless stated otherwise. Out of the two control constructs lacking cyclin B tested clone Y1 was used for future analyses, termed Y.

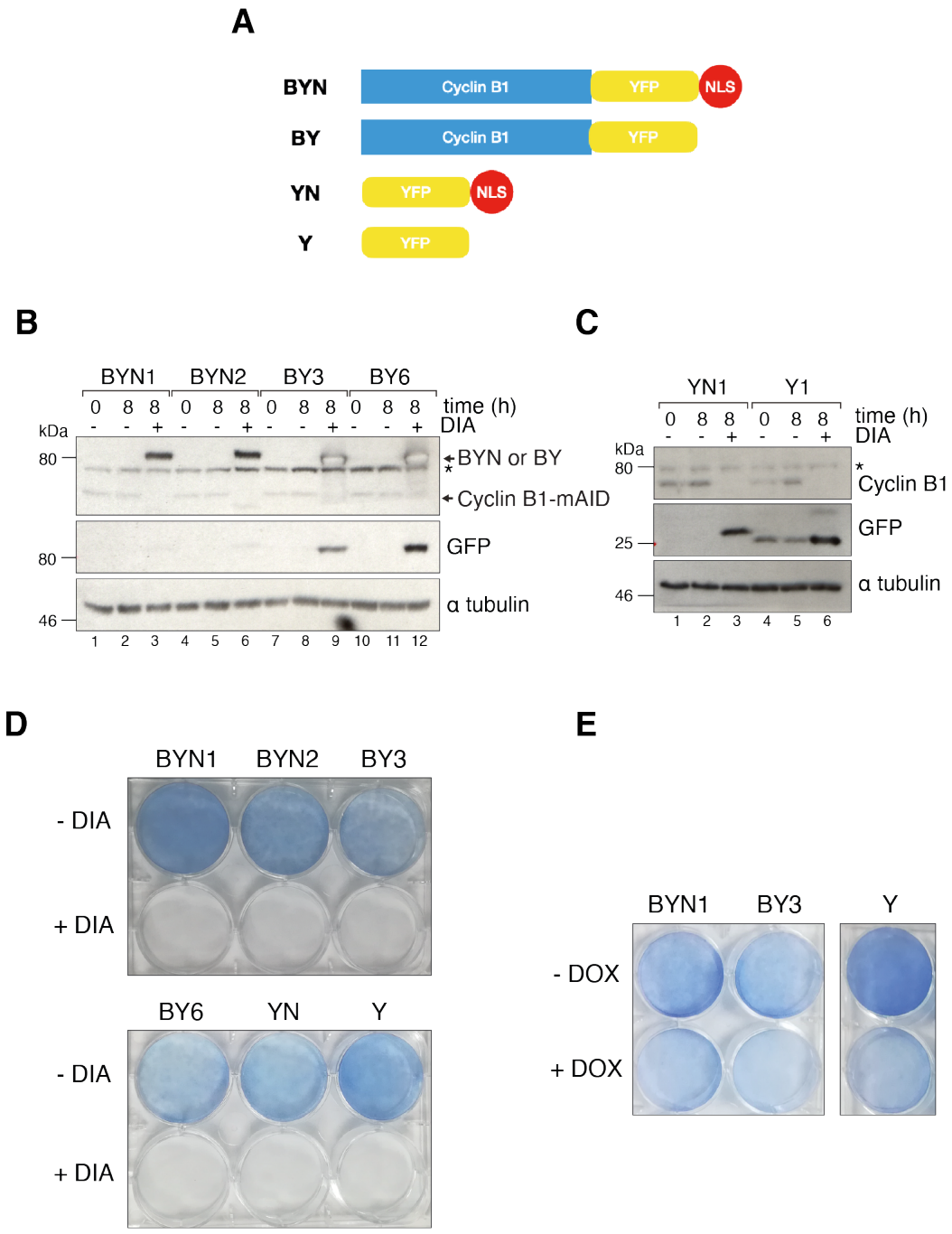

Figure 5.8: Cyclin B1 overexpression does not promote long-term survival. A: A schematic view of constructs that were introduced into these cells. B, C: Western blots of the newly established cell lines treated or not with DIA for the indicated amounts of time. D: Survival assays of clones in A and B. Cells were seeded at $1x10^4$ and incubated with or without DIA for 5 days. E: Survival assay of indicated cells, treated or not with doxycycline. Cells were seeded as in D.

5.5.5.3 Overexpression of cyclin B rescues short-term mitotic defects

Even though cells overexpressing cyclin B1-YFP constructs lacking the endogenous protein were unable to survive in a long-term assay, I speculated that this could be due to a lack of transcriptional control, or insufficient degradation of the overexpressed proteins during mitosis.

As noted previously, the presence of cyclin B1 after metaphase can induce mitotic catastrophe in anaphase [3, 260]. The degradation mechanics of cyclin B1-YFP were addressed using live-cell imaging experiments, which allowed for further insights into mitotic entry and exit following cyclin B1's depletion. Furthermore, these analyses also enabled the visualisation of the localisation of cyclin B1-YFP and will show whether the NLS signal in BYN is sufficient to trigger a nuclear localisation even in the presence of an active cytoplasmic retention signal (CRS).

BYN is nuclear, and BY is cytoplasmic Cells lacking the endogenous cyclin B and overexpressing the BYN or BY are both able to promote mitotic exit, regardless of their subcellular localisation (Figure 5.9 A). The efficiency of the NLS tag to induce a nuclear localisation is demonstrated in Figure 5.9 B as the YFP signal of the BYN clone is readily detectable in the nucleus, whereas the BY protein is constrained to the cytoplasm, as judged by the lack of YFP signal in the nuclear area, made apparent by the DNA signal.

BYN and BY localise to the spindle during mitosis Regardless of the interphase localisation of BYN and BY, the YFP signal was be detectable on the spindle and centrosomes of both BYN and BY clones, and also uniformly distributed in the cytoplasm (Figure 5.9 B). These analyses further confirmed the unhindered function of BY and BYN constructs, matching previously established information on cyclin B1's localisation [15]. This pattern of YFP signal distribution was not observed in control YFP or YFP-NLS expressing cell lines (data not shown).

Overexpressed cyclin B1 becomes degraded at anaphase onset Next, I aimed to determine the degradation mechanics of BYN and BY constructs. The overexpressed cyclin B1 proteins are degraded at the same time as the endogenous cyclin B1 is normally destroyed (Figure 5.9 B, time-points 00:20 onwards) [144, 244].

The depletion of YFP signal in BYN and BY cells confirmed that persisting levels of cyclin B1 do not interfere with mitotic exit in these cells. The disappearance of YFP is not due to an inherent instability of the YFP tag as the signal was unchanged in cells expressing YN or Y regardless of cell cycle stage (data not shown).

The above data indicate that transcriptional control of cyclin B1 may play a more important role in cell cycle progression, as cells apparently degrade the exogenous cyclin B1-mAID but are still unable to survive for prolonged periods..

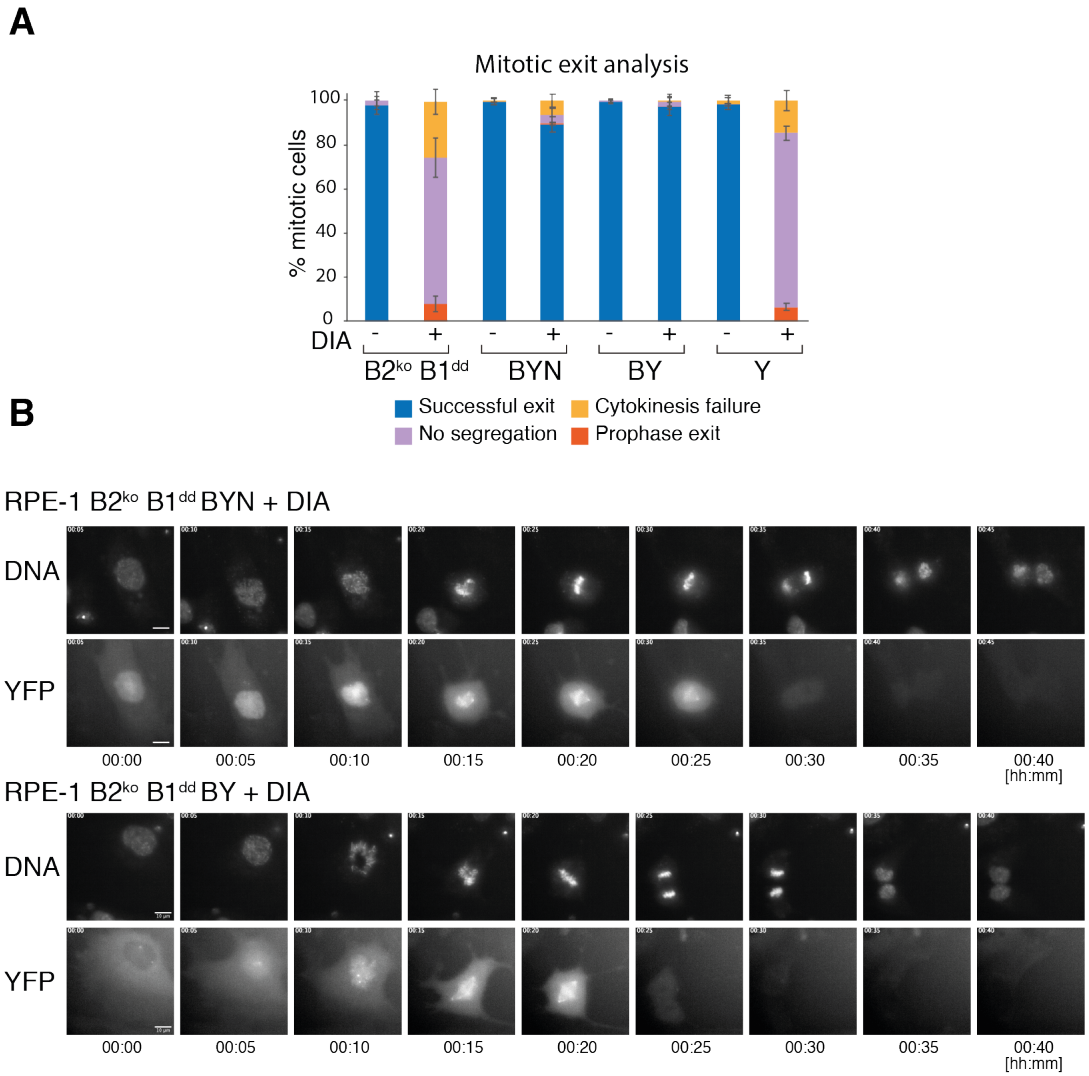

Figure 5.9: Overexpressing cyclin B rescues mitotic phenotypes regardless of its localisation. A: Mitotic exit analyses of three independently repeated live-cell imaging experiments (n > 50). Mitotic phenotype classification is as noted previously. B: Time-lapse imaging of the indicated cells. Asynchronous cells were pre-treated with SiR-DNA for a minimum of 4 h, and DIA for 4 h, before the start of live-cell imaging. Cells in A were imaged with an IX-71 wide-field microscope. Cells in B were filmed with the PerkinElmer Operetta system. Scale bar indicates 10 μm. Time-stamps below the snapshots are shown in hh:mm. Barplots show means of three experiments. Error bars show standard deviation.

5.6 Discussion

5.6.1 Roles of cyclin B in RPE-1 cells

This Chapter showed that cyclin B is crucial for the establishment of a metaphase state in human RPE-1 cells. Cyclin B directs CDK1 to specific substrates responsible for organisation of the mitotic spindle and consequently chromosomes. Additionally, several substrates that do not have known roles in the mitotic spindle assembly were also identified using phospho-proteomic analyses. Of those, KI67, Repo-Man, TPX2 and AURKB were confirmed as valid targets for CDK1-cyclin B1 phosphorylations, but this Chapter desribed the validation of one target, KI67, as others were conducted by a postdoc in the Hochegger lab, Dr. Nadia Hegarat [386].

5.6.1.1 How RPE-1 lacking cyclin B maintain mitotic substrate phosphorylations

Cells can inactivate PP2A independently of cyclin B Immunoblotting analyses showed that CDK1 substrate phosphorylations are not strongly affected by the loss of cyclin B, and phospho-proteomic analyses further confirmed that only a subset of substrates are reliant on cyclin B's presence. Interestingly, even without CDK1-cyclin B's activity, these cells activate ARPP19 and ENSA, two PP2A inhibitors, which likely contribute to the establishment and maintenance of CDK1 phosphorylations. Moreover, the phosphatase inhibition in the absence of cyclin B implies that other mitotic players promote its inhibition, likely the CDK1 trigger, CDK-cyclin A2.

In addition, the existing CDK1 substrate phosphorylations could be dependent on cyclin A2, given that this is the only other CDK-binding mitotic cyclin.

Do RPE-1 cells rely on specific, or total amounts of any cyclins to drive mitosis? Either due to the cyclin A2's specificity or owing to its prometaphase degradation, it is unable to promote metaphase establishment. The latter seems unlikely as the use of APC/C inhibitors, Apcin and proTAME, which blocked the degradation of cyclin A2 and stabilised the protein, did not revert the metaphase defects. However, the quantity of cyclin A2 versus cyclin B is unknown, and it is possible that the endogenous levels are insufficient to promote the same amount of CDK1 phosphorylations in mitosis. Indeed,

preliminary data from the Hochegger lab suggested that overexpressing cyclin A2 did promote some mitotic phenotypes, including a metaphase alignment, but this was not investigated further. These data calls for more research to determine the requirement for cyclin specificity versus quantity for mitotic progression in RPE-1 cells.

5.6.1.2 A proposed model for mitotic progression in RPE-1 cells

Below is an updated version of the mitotic progression model that has been shown previously in this thesis.

Mitotic entry Cells lacking cyclin B enter into prophase and progress past NEBD unhindered, thus showing that cyclin B is not essential for mitotic entry in RPE-1 cells. While the importance of CDK-cyclin A2 for mitotic entry has already been shown, the status of PP2A activity has not yet been addressed – perhaps CDK-cyclin A2 promotes mitosis by activating CDK1 and also inactivating PP2A. Previous data showing that a CDK1 complex activates MASTL [100], and that PP2A was inhibited in cells lacking cyclin B1, support these claims. Further work is required to confirm this hypothesis.

Prophase to prometaphase progression It is unclear which activity promotes NEBD to drive the cells into prometaphase, but either CDK-cyclin A, CDK1-cyclin B, or PP2A inhibition are likely candidates.

This thesis has shown that cells lacking either cyclin A2 or cyclin B successfully break down the nuclear envelope, but the status of PP2A has not yet been addressed in these. It is possible that either cyclin activity or PP2A inactivation is required for this step, or an interplay between both CDK activity and PP2A inactivation. This is the second question that remains to be addressed.

Prometaphase to metaphase progression CDK1-cyclin B activity is essential to allow the cells to enter metaphase and organise their chromosomes and the mitotic spindle. This seems dependent on a subset of substrates specific for cyclin B-mediated CDK1 activity. Using phospho-proteomic analyses, several specific targets were identified and validated. PP2A is inhibited at this stage, but it is not yet clear whether that is an essential requirement for a metaphase alignment, so it is not shown on this model yet.

Mitotic exit In the end, to allow mitotic exit, phosphatases must be re-activated and CDK1 activity depleted. Interestingly, cells lacking cyclin B are unable to activate PP2A, and PP1 is apparently sufficient to dephosphorylate mitotic substrates with high efficiency, so I conclude that either PP1 or PP2A are sufficient for mitotic exit.

What is the role of PP2A in metaphase? Cells are apparently entirely dependent on cyclin B for a faithful chromosome alignment and spindle assembly. Phosphatases are thought to contribute significantly to mitotic events (reviewed in [235]), but even PP2A inhibition, in combination with stabilised CDK1-cyclin A2, were insufficient to establish metaphase in cells lacking cyclin B.

However, these data do not imply that the PP2A inhibition is not required for metaphase as this has not yet been investigated. It is possible that a highly active PP2A could counter the effects of cyclin B. Thus, the importance of PP2A inhibition for entry into metaphase remains to be shown. Furthermore, as outlined previously in this thesis, the timing of PP2A inhibition is yet to be established – is the inactivation of PP2A required for prophase or prometaphase events?

The next Chapter will address the importance and timing of phosphatase (in)activity and the interplay between key mitotic players in RPE-1 cells.

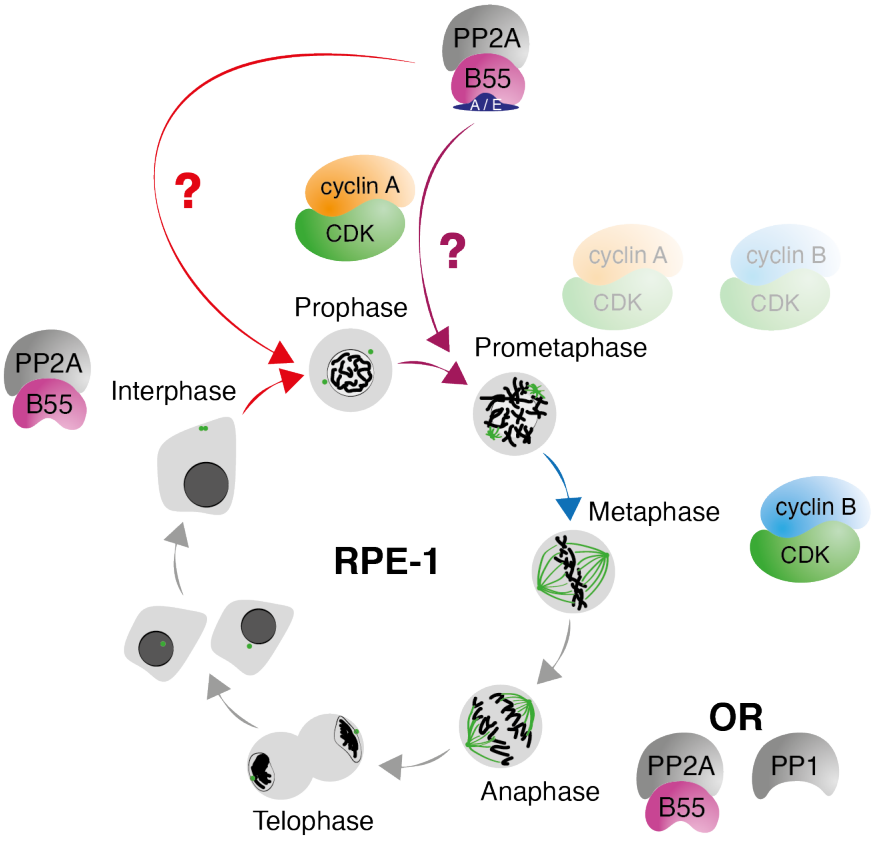

Figure 5.10: Updating the mitotic progression model: cyclin B is essential for metaphase establishment. Cyclin B's roles in prometaphase are not entirely clear, but it is crucial for the establishment of metaphase, regardless of phosphatase activity. The PP2A phosphatase must be inactivated, although it is unclear whether this is required for prophase or prometaphase entry. Either PP1 or PP2A must be active to promote mitotic exit and chromosome segregation in anaphase.

Chapter 6

A novel prophase-steady state defined in RPE-1 cells

6.1 Summary

RPE-1 cells require only cyclin A2-mediated CDK activity to enter prophase. Blocking the mitotic phosphatase inactivation and removing cyclin B resulted in a novel prophase-steady state with intermediate CDK substrate phosphorylations, resembling normal prophase cells. Cells can arrest at this stage for long periods of time, which is uncharacteristic for mitosis, especially in RPE-1 cells. This is the first description of a prophase-steady state in RPE-1 cells and this work further underlines the crucial roles of cyclin A in early mitosis, but also demonstrates that there is an additional requirement that needs to be fulfilled for nuclear envelope breakdown. In addition to maintaining CDK activity, cells must also inactivate the mitotic PP2A phosphatase to enter prometaphase in the absence of cyclin B.

6.2 Introduction

Mitotic entry and progression are highly regulated to prevent errors in cell division and subsequent genome instability that could promote cell death. Mitosis is a particularly stressful stage that requires a lot of energy and the checkpoints such as the spindle assembly checkpoint (SAC) serve to arrest the cells in case of errors during the spindle assembly. Researchers have developed drug treatments that can arrest the cells in mitosis for several hours - such as Nocodazole, a microtubule depolymerasing agent [409], and MG-132, a potent proteasome inhibitor [411] - but these conditions do not occur in a normal cell cycle, and are eventually extremely damaging.

A point of no return in mitosis? Dose-dependent inhibition of CDK1 in HeLa cells resulted in a steady prophase arrest, as observed previously in the Hochegger lab [190], and cells were shown to base the decision on continuation of mitosis on the level of CDK1 activity. The prophase-steady state was defined by a translocation of CDK1-cyclin B into the nucleus, an event previously implicated to mark a point of no return in mitosis [59]. In contrast, others have suggested anaphase as the marker for irreversibility of mitosis [412], but one study demonstrated a possible reversal of anaphase / telophase back into metaphase [413], thus questioning the significance of 'no return'. Reports may vary based on the definition of 'no return', as some studies may classify this as a point that marks mitotic events, while others may specifically define it as a point after which cells are unable to return back to a G_2-like stage.

Thus, there are actually two points of no return in mitosis, depending on the chosen explanation: one that marks a mitotic stage, but cells could still exit 'back' into G_2, and a second point that signifies the end of the cell cycle as cells are unable to revert back into G_2 once they have passed this point. Even by this definition, different groups may still disagree in the events that mark each point.

In this thesis, mitosis is defined as prophase entry characterised by chromosome condensation and CDK1 activation, followed by CDK1-cyclin B translocation. The second point that marks the end of the cell cycle is nuclear envelope breakdown (NEBD) as cells can no longer return back to G_2.

Studies have so far been unable to separate the requirements for the two events in

RPE-1 cells.

6.2.1 Questions to be addressed

The topic of this Chapter was already implied in the Discussion of Chapter 5, Section 5.6.1.2. The interplay of key mitotic players that have been previously introduced will be addressed here. Again, these are highlighted in a proposed model of mitotic progression in Figure 6.1.

Mitotic entry Firstly, highlighted with red, the critical requirements for mitotic entry of RPE-1 cells will be investigated. Cyclin A2 is necessary for mitotic entry and previous Chapter shows that PP2A becomes inactivated at some stage of mitosis regardless of cyclin B's presence. However, it is unclear whether this is required for the entry into prophase or prometaphase, hence there are two suggested times when PP2A is inhibited in the mitotic progression model.

It is possible that cyclin A2 triggers mitotic entry in two ways - by activating CDK1-cyclin B, and by triggering phosphatase inactivation (indicated in the model as ARPP19 / ENSA binding to B55. A / E in dark blue). Other studies have demonstrated that MASTL, which leads to PP2A inactivation, can be targeted by CDK1 in vitro [189, 375], thus it is a valid hypothesis that cyclin A could directly activate MASTL to achieve a quick and efficient PP2A inactivation. Furthermore, a recent study uncovered that CDK1-cyclin B directly phosphorylates PP2A to additionally regulate mitotic entry and exit [414].

Prophase to prometaphase transition Next, the importance of mitotic players that could trigger nuclear envelope breakdown in RPE-1 cells, and entry into prometaphase, is unknown. In the model, this is highlighted by purple question marks. Cells lacking cyclins B or A2 both enter prometaphase, but it is not yet clear whether PP2A (in)activity plays a role in this event. It is tempting to imagine that there may be an interplay between a CDK activity and phosphatase inactivation. CDK activity is shaded at this Section because its importance is not yet determined.

Metaphase establishment Cyclin B is crucial for metaphase events, but PP2A was already inactive at that point. It is likely that there is an interplay between the two mitotic

players as premature substrate dephosphorylations could lead to an untimely mitotic exit and increase the chances for mitotic catastrophe. Indeed, other studies have attributed important roles for PP2A in mitotic exit [361, 363], but one report showed that PP2A's role in cytokinesis may be redundant in *Xenopus* [362] as it could be compensated by other phosphatases. Data in the previous Chapter of this thesis show that the activation of PP1 is sufficient to promote mitotic exit even if PP2A is inactive, but the necessity PP2A inhibition prior to anaphase has not yet been investigated.

Studies that depleted MASTL and thus promoted PP2A activity showed an increase in mitotic catastrophe [371, 373], but it is necessary to keep in mind that PP2A has crucial roles controlling the timing of mitotic events already during mitotic entry [235].

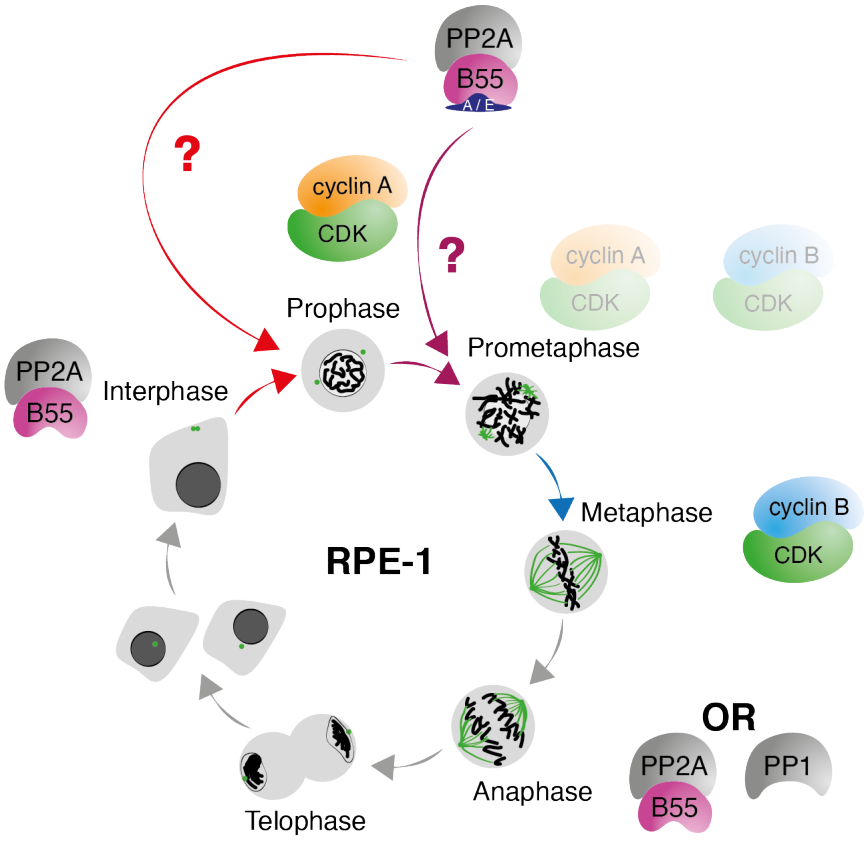

Figure 6.1: A schematic view ot the possible interplay of mitotic players that likely drive mitotic progression. CDK-cyclin A2 triggers mitotic entry and ensure the progression through early and mid mitosis by activating CDK1-cyclin B and MASTL, which activates the two small proteins ARPP19 and ENSA (A / E) that directly inhibit the PP2A phosphatase. The timing of PP2A activation and roles of cyclin B in prometaphase are not yet clear.

6.3 RPE-1 cells arrest in a novel prophase-steady state

6.3.1 Analysing the interplay between phosphatases and kinases in mitotic entry and progression

6.3.1.1 Cells enter and progress into prophase with only CDK-cyclin A2

Experimental design To assess the roles of cyclin B and phosphatase inactivation in RPE-1 cells, I further characterised the RPE1 B2^{ko} B1^{dd} cell line. While cyclin A2's function in mitotic entry is clear, the importance and timing of PP2A's activation that could be promoted by the CDK-cyclin A2 - MASTL pathway is not yet known. To address this question, I incorporated siRNA treatment with the previously described single thymidine block (Figure 6.2 A). The efficiency of siRNAs that were used in this Chapter was tested separately, shown using western blotting in Figure 6.2 B – D. Cyclin B depletion in combination with siMASTL and APC/C inhibitors (Apcin and ProTAME) that were used for mitotic arrest in the previous Chapter is also shown in Figure 6.2 E. Here, it is readily observable that cyclin A2 is stable in cells arrested with Apcin and ProTAME.

Maintaining high PP2A activity does not affect mitotic entry in RPE-1 cells Small interfering RNA (siRNA), siMASTL or depletion of its downstream regulators siARPP19 and siENSA (siA / E), efficiently deplete the targeted proteins (Figure 6.2 B, C). The siRNA transfections were combined with a single thymidine release (Figure 6.2 A) to keep the cells from completing mitosis in the preceding cell cycles with low levels of MASTL that have previously been shown to induce mitotic catastrophe [361, 363, 415] (reviewed in [235]). Similar phenotypes were observed in this study when asynchronous cells were treated with siRNA for 48 h (data not shown).

Cells were treated as indicated in Figure 6.2 A, fixed with ice-cold methanol, and subject to immunofluorescence analyses (see Chapter 3 Materials and Methods). While depleting cyclin B alone (+ DIA) slightly reduces the amount of mitotic cells, siMASTL or siA / E did not result in a lower mitotic index (Figure 6.2 F), concomitant with previous observations [415], but contrasting initial siMASTL studies where cells ceased to enter

mitosis after its depletion [335]. In the latter study, it is possible that cells stopped entering mitosis due to the accumulation of defects from the preceding cell cycles.

Cyclin B and phosphatase inactivation are not required for mitotic entry I hypothesised that an active PP2A, combined with the slightly reduced CDK1 activity caused by the loss of cyclin B, will result in a G_2 arrest. However, surprisingly, cells still entered mitosis, albeit this was slightly hindered when cyclin B was depleted (Figure 6.2 F), consistent with siCTRL data.

Depleting the regulatory subunit PP2A:B55 alone resulted in more cells entering mitosis (Figure 6.2 D), regardless of an additional MASTL depletion (Figure 6.2 F), confirming that MASTL activity is upstream of B55. This result was not unexpected as cells are known to readily enter mitosis if lacking phosphatase activity (reviewed in [236]). Combining the siB55 treatment with cyclin B degradation did not affect mitotic entry (Figure 6.2 F), further highlighting the redundancy of cyclin B for the establishment of mitosis.

Cyclin A2 alone is sufficient to establish prophase, but not prometaphase As noted above, cells lacking cyclin B with a highly active phosphatase, thus only having cyclin A2-related activity, still enter mitosis (Figure 6.2 F). These cells appear to establish prophase, as initially characterised by chromosome condensation, centrosome separation, and the presence of a nuclear envelope as observed by lamin A / C staining (Figure 6.2 H). Quantification analyses revealed that approximately 50% of cells lacking either cyclin B1 or actors that inhibit the PP2A, progress past NEBD into prometaphase (Figure 6.2 G), however this dropped to 10% or lower when the treatments were combined. Depleting the B55 subunit in cells lacking MASTL and cyclin B then partially reverted this phenotype with about 30% cells entering prometaphase, as characterised by NEBD. These data showed that, when cells are lacking cyclin B1, PP2A inhibition is necessary for prometaphase entry.

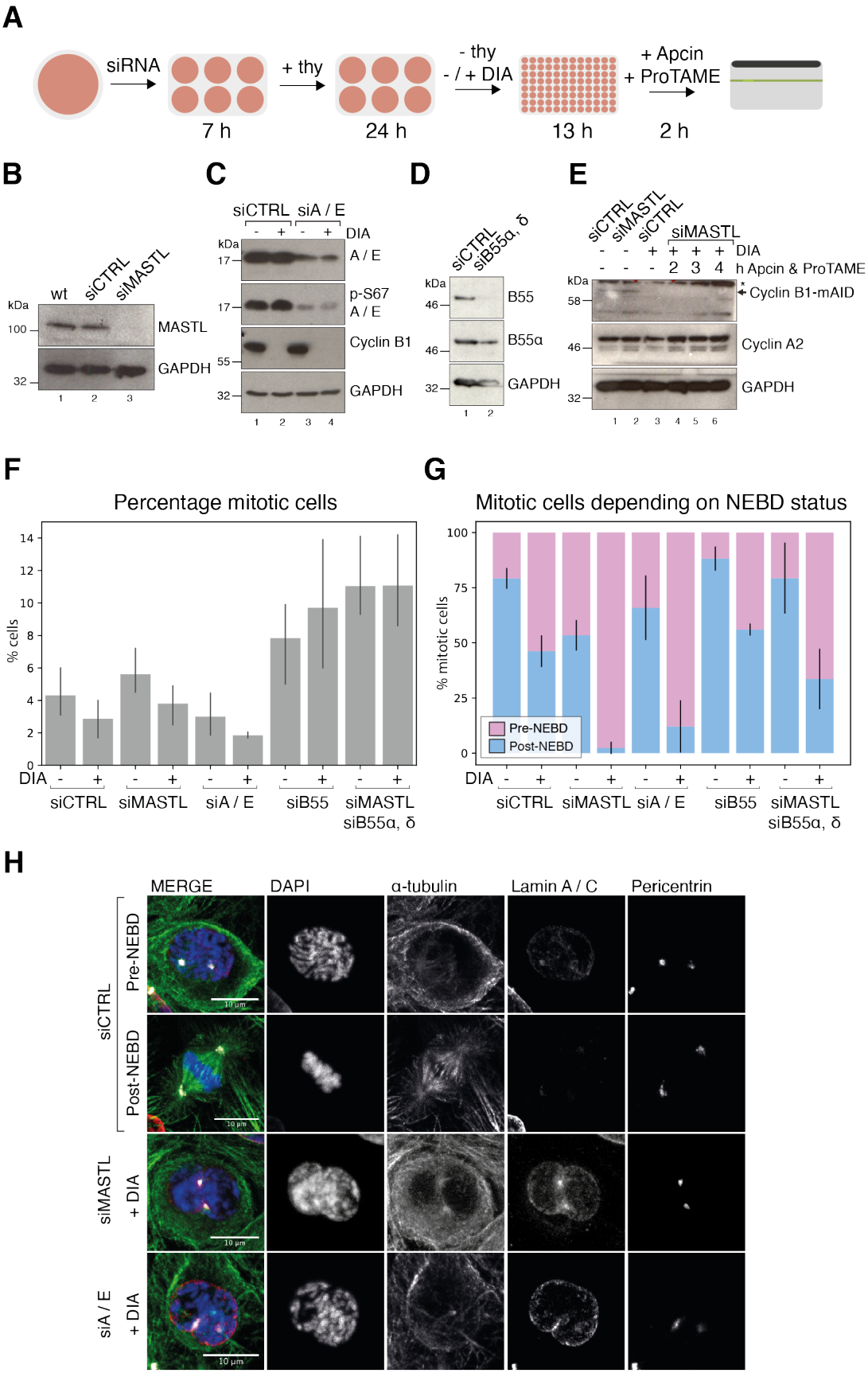

Figure 6.2: See caption on the following page.

Figure 6.2 *(preceding page)*: RPE-1 cyclin B2^{ko} B1^{dd} must inactivate the phosphatase to promote NEBD. A: A schematic representation of the experimental outline used for immunofluorescence analyses in this Chapter. B – D: Western blot analyses of the indicated siRNA transfections after 48 h. In C, si A/ E stands for a combination of siARPP19 and siENSA treatment. In D, a mixture of siB55 subunit α and siB55 subunit δ was used. E: Western blot analyses of cells treated as indicated in A, with siMASTL or siCTRL. Different times of Apcin and ProTAME treatment were applied as noted. F: Percentage mitotic cells out of total treated as in A were quantified. G: Mitotic cells treated as in A were classified depending on their nuclear envelope status. Cells with nuclear envelope still present as judged from Lamin A / C staining (see H) were counted as pre-NEBD, and cells lacking the nuclear envelope were judged as post-NEBD. H: Immunofluorescence figures of cells treated as in A treated with the indicated primary antibodies. On the MERGE figure DAPI is in blue, α-Tubulin in green, Lamin A / C in red, and Pericentrin in white. Scale bar indicates 10 μm. Data for analysis in F and G were obtained from three independent repeats, n $>$ 100. PerkinElmer Operetta system was used for imaging, cells were classified and counted manually. Barplots here and throughout this Chapter show the mean of three experiments, error bars indicate standard deviation.

6.3.1.2 Live-cell imaging analyses of the prophase-steady state

The prophase-steady state phenotype is not a side effect caused by temporal constraints The information shown in the previous Section was obtained with immunofluorescence analyses and it is possible that more cells were found in prophase simply due to them requiring more time to establish the necessary CDK1 substrate phosphorylations that exceeds the 2 h APC/C inhibitor treatment used in this setting. To determine whether this is true, I utilised live-cell imaging analyses and found that cells were indeed unable to promote NEBD when lacking both cyclin B and MASTL (data not shown), or cyclin B and ARPP19 / ENSA (Figure 6.3 A). Control cells, as well as cells lacking either of the mitotic players entered mitosis and progressed past NEBD, as judged by the DNA and tubulin reorganisation (Figure 6.3 A).

Cells lacking cyclin B with active PP2A apparently entered into a prophase state but did not continue past NEBD. Cells normally maintained this state for a minimum of one hour, as characterised by cell rounding, tubulin reorganisation and chromosome condensation (Figure 6.3 B). In those, a pressure imposed on the nucleus was observed, according to the microtubules contracting around the centre of the nucleus, and this may be the source of the pinched nucleus shape that was commonly observed during immunofluorescence analyses (Figure 6.2 H). Moreover, the live-cell imaging revealed that some cells may attempt to separate their DNA prior to NEBD (Figure 6.3 A). Other live-cell imaging analyses further confirmed that microtubules were pressuring and contracting the nucleus, in some even centrosome movement around the nucleus was apparent (data not shown).

Cells spent a substantial amount of time in prophase The above data demonstrate that RPE-1 cells can establish a prophase-like state with just cyclin A2 and a highly active PP2A phosphatase, as judged by chromosome condensation, centrosome separation, and attempted mitotic spindle organisation.

Prophase or antephase? Previous observations have defined a stage between G_2 and M called antephase that allows cells to revert back to G_2 in the presence of specific mitotic stress, but not DNA damage [416–418] (reviewed in [419]). Activation of p38 during this stage leads to a halt in mitotic events by downregulating CDC25B [418]. Much work has

been conducted to better define this stage, however, to date it was unknown that cells may enter a mitotic-like stage simply by lacking specific mitotic players.

To shed more light on the newly observed stage, I decided to further characterise these cells, but it was termed prophase-steady state, as the definition for antephase includes an existence of mitotic stressors, which were not used in these studies.

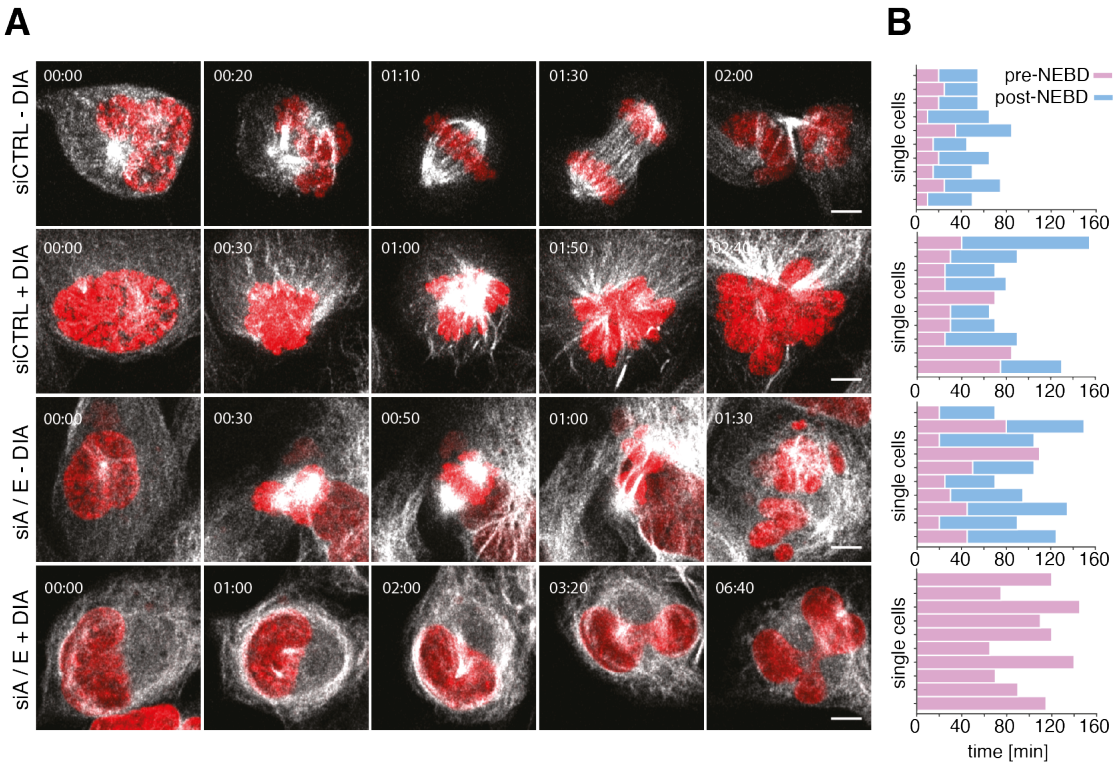

Figure 6.3: Cells lacking cyclin B and ARPP19 / ENSA spend substantial amount of time in prophase. A: Snapshots of cells transfected with siCTRL or siARPP19 / ENSA (siA / E) for 48 h and treated or not with DIA for 4 h prior to imaging as indicated. RPE-1 $B2^{ko}$ $B1^{dd}$ stably transfected with H2B-mRFP were treated with SiR-tubulin for a min of 18 h prior to imaging. Times of each frame are indicated. Scale bar is 10 μm. LCI acquired with an Airy scan confocal microscope. Time-stamps on top left are shown in hh:mm.

6.4 Characterisation of the prophase-steady state

Experimental outline Immunofluorescence analyses were utilised to determine the level of CDK1 mitotic substrate phosphorylations. The experimental outline is as described earlier (Figure 6.2 A), but instead of pericentrin, here I used phospho-antibody staining.

As noted previously, RPE-1 cells are difficult to synchronise, implying that immunoblotting analyses could not offer the same level of accuracy as immunofluorescence analyses, especially in control cells where a synchronisation of cells in prophase is extremely challenging. Microscopy analyses enabled a higher degree of separation by cell cycle stages based on chromosome condensation and the separation into pre- or post-NEBD depending on nuclear envelope existence as observed by lamin A / C staining (Figure 6.4 A).

The PerkinElmer Operetta system runs on a Harmony software, which can measure fluorescence intensity of a selected area. The DNA was selected as the area where the average fluorescence intensity was measured. The difference in signal may be more obvious by measuring the entire cell, but owing to a lack of channels to include a cytoplasmic marker, the DNA was the obvious choice. Some discrepancies are observable because of this, especially clear in post-NEBD cells, where p-CDK substrate fluorescence is observable on the cellular periphery and on the microtubules (Figure 6.4 A). However, the DNA-measuring method was still showing a significant increase in the fluorescence signal (Figure 6.4 B). Therefore, this approach was determined as appropriate to measure and compare the intensity of phospho- antibodies between different stages of mitosis.

6.4.1 Levels of phosphorylated CDK1 substrates correspond to a prophase stage

CDK substrate phosphorylations increase during mitosis Quantification analyses of cells stained with a p-CDK substrate antibody (6.4 A) that recognises the phosphorylated TPXK motif show a clear separation between interphase, pre-, and post-NEBD cells based on the intensity of antibody fluorescence (Figure 6.4 B). All conditions, regardless of their treatment, had similar levels of interphase fluorescence intensity, which served as an internal control for immunofluorescence staining.

A strong shift in fluorescence signal intensity was observed when comparing interphase and pre-NEBD cells, but this was still distinctly less than the post-NEBD signal. Notably, only prometaphase and metaphase cells were measured as post-NEBD cells, as the phosphorylations begin to dissipate during anaphase (data not shown).

Depletion of cyclin B or PP2A inhibitor pathway reduces the total amount of phosphorylated CDK substates A very minor decrease of fluorescence intensity was observed in cells depleted of cyclin B (Figure 6.4 B), concomitant with the findings in the previous Chapter. Next, transfecting the cells with either siMASTL or siA / E further reduced the amount of fluorescence intensity in pre- and post-NEBD cells (Figure 6.4 B, compare all - DIA conditions), as expected due to the highly active PP2A phosphatase.

Prophase-steady state cells have prophase levels of CDK substrate phosphorylations Interestingly, combining the cyclin B depletion with either siMASTL or siA / E did not result in a marked decrease of p-CDK substrate fluorescence intensity during prophase, as observable when comparing all the pre-NEBD cells under different conditions (Figure 6.4 B). While the phosphorylation intensities of cells lacking cyclin B and MASTL or ARPP19 / ENSA are notably lower than the control untreated cells, they are maintained at a similar level as compared with either + DIA or siMASTL and siA / E cells.

This comparison revealed that the observed novel stage is indeed a non-interphase stage, as judged by this condition. I next aimed to look at another phospho-antibody to further characterise the prophase-steady state.

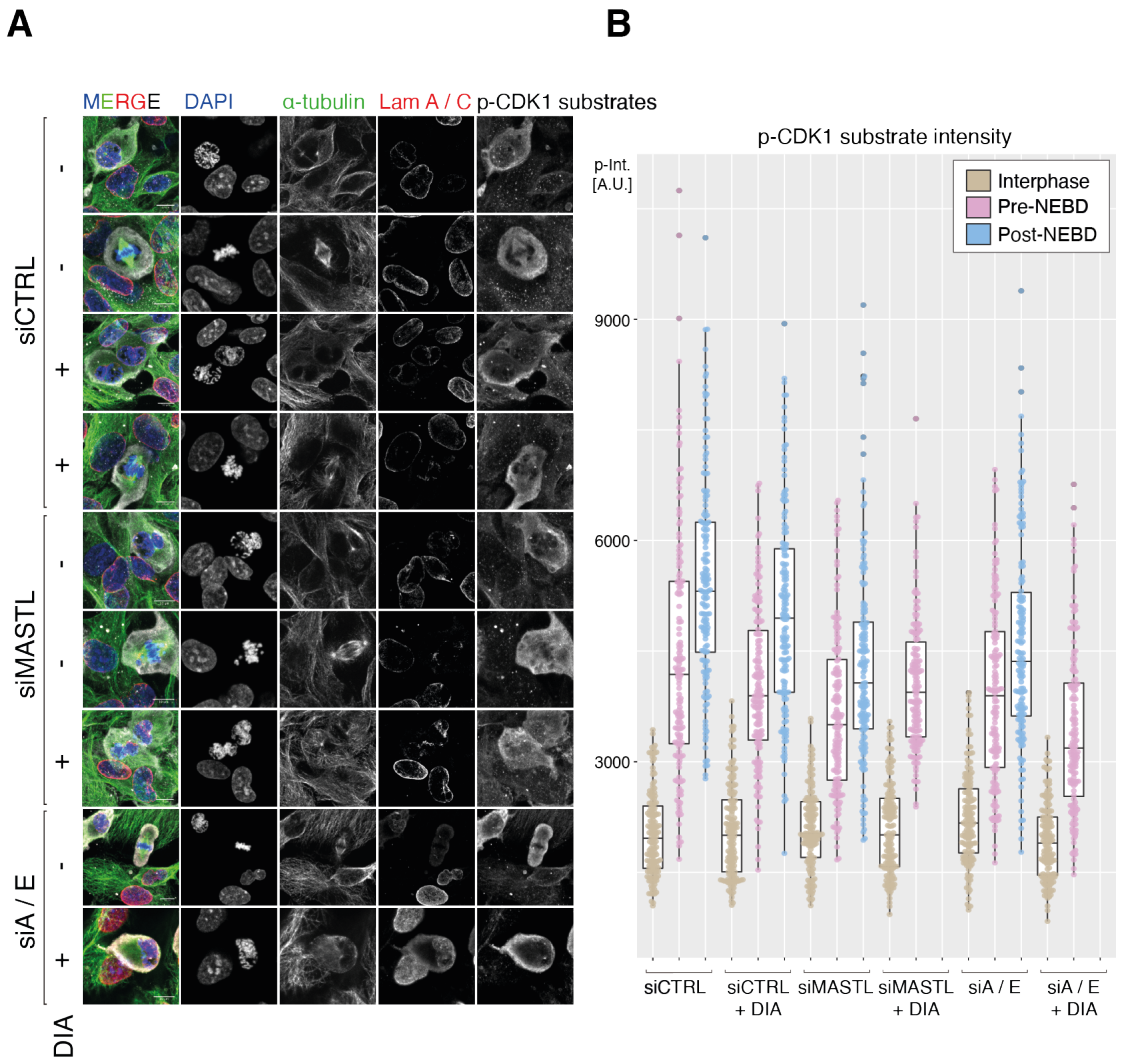

Figure 6.4: A comparison of fluorescence intensity of phosphorylated CDK substrates. A: Immunofluorescence images obtained with the LSM 880 Airy Scan microscope. Scale bars indicate 10 μm. Colours in MERGE correspond to the channel colours as indicated by the name at the top, DAPI in blue, α-tubulin in green, lamin A / C in red, and p-CDK substrates in white. B: Analysis of the fluorescence signal of p-CDK substrate in indicated cells. Interphase and mitotic cells were separated by chromosome condensation and tubulin staining, while pre-NEBD and post-NEBD cells were further separated by the existence of a nuclear envelope as observable by lamin A / C staining. Only cells that were in prometaphase or metaphase were counted as post-NEBD, and not anaphase or later, as the phosphatases become re-activated at that stage. Cells were treated as in Figure 6.2 A, and analysed with PerkinElmer Operetta microscope and the accompanying software Harmony 4.6. Signal intensity was measured as average signal in the DNA area corresponding to the DAPI staining. Data were obtained from three independently repeated experiments, n > 50 per condition. Graph in B was generated with R, boxplots indicate the median, and the second and third quartile. Boxplot whiskers indicate the first and fourth quartile. Outliers mark data that was not included in the boxplot. Bee-swarm shows each individual measurement from all three experiments.

6.4.2 Lamin phosphorylation is similar in all mitotic cells

Lamins build the nuclear envelope To continue the prophase-steady state analyses I addressed the properties of the nuclear envelope. There are three subtypes of lamin, A, B, and C and they polymerase to build the nuclear envelope. Lamins A and C are phosphorylated by CDK1 at several sites, but one of those, S22, is known as the mitotic site that regulates its degradation (reviewed in [420]). This site was not identified as a substrate specific for cyclin B-mediated CDK1 phosphorylations, according to the phospho-proteomic analyses mentioned in Chapter 5. Nevertheless, I aimed to see whether the prophase-steady state cells are lacking this phosphorylation, which could block their nuclear envelope degradation. The cells were treated as in the previous Section, using a phospho-S22 lamin A / C antibody.

Lamin is similarly phosphorylated in all mitotic cells Regardless of NEBD, all cells maintain the lamin S22 phosphorylation during mitosis. Interestingly, while control untreated cells appear to lose significant amounts of p-S22 lamin signal after NEBD, only slight shifts of fluorescence intensity were observed in all other pre- and post-NEBD comparisons (Figure 6.5 B). This was attributed to the temporal differences in mitotic events. Control cells do not lack any key mitotic activity, progressing through prometaphase and metaphase unhindered, including a quick and efficient nuclear envelope disassembly. Treated cells experience difficulties in prometaphase and metaphase which also likely interfere with the timing of nuclear envelope degradation, likely resulting in a prolonged p-lamin presence in the cells and emitting a higher fluorescence signal.

Pre-NEBD levels of p-S22 lamin are uniform across all conditions, regardless of cyclin B's presence or PP2A activity. This further indicated an existence of a true prophase state and implied that this substrate that is highly specific for CDK1-cyclin A2 phosphorylation, given that other CDK activity is not present in this condition.

As noted previously, peculiar spindle movements were observed with live-cell imaging analyses. As an additional control of the stage in mitosis these cells are in, I next decided to analyse centrosome separation mechanics.

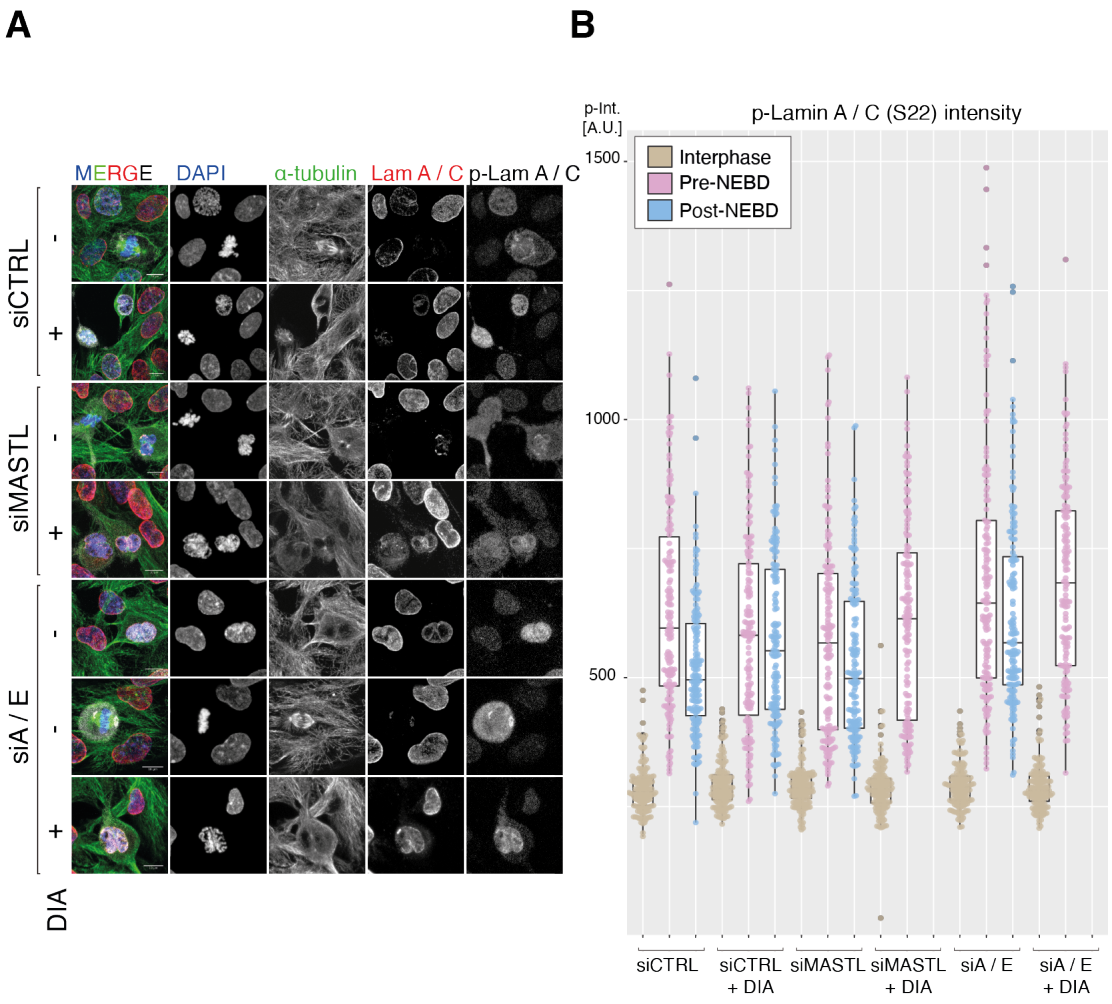

Figure 6.5: A comparison of fluorescence intensity of phosphorylated S22 of lamin A / C. A: IF images obtained with the LSM 880 Airy Scan microscope. Scale bars indicate 10 μm. Colours in MERGE correspond to the channel colours as indicated by the name at the top, DAPI in blue, α-tubulin in green, lamin A / C in red, and p-lamin S22 in white. B: Analysis of the fluorescence signal of p-lamin S22 in indicated cells. Cells were treated as in Figure 6.2 A, and analysed with PerkinElmer Operetta microscope and the accompanying software Harmony 4.6. Signal intensity was measured as average signal in the DNA area corresponding to the DAPI staining. Data were obtained from three independently repeated experiments, n $>$ 50 per condition. Boxplot as in Figure 6.4.

6.4.3 Centrosome separation mechanics are not significantly affected

Centrosomes separate during early stages of mitosis when the mitotic spindle starts being organised. CDK1 has been shown to control EG5 activity and its localisation by phosphorylation [20, 421, 422] and PP2A to counteract it during mitotic exit [423]. If CDK1-cyclin B is necessary for this activity, then the centrosome separation dynamics will be impaired in all conditions where cells lack cyclin B. Similarly, if PP2A directly dephosphorylates the residues important for this activity during mitotic entry, then the siMASTL or siA / E should counteract the separation as well.

Similar to the above experiments, cells were assessed by immunofluorescence analyses, with the exception of using pericentrin staining, which is a centrosome scaffold [424], to visualise centrosomes.

Early centrosome separation is mainly controlled by CDK1-cyclin A Centrosome distance was measured in pre- and post-NEBD cells (Figure 6.6 A). Interphase measurements are not included as they either had a singular centrosome or unseparated centrosomes. According to the quantification in Figure 6.6 B, mitotic cells start the centrosome separation in prophase, and further continue this after NEBD as the difference in pre- and post-NEBD cells is apparent in all conditions observed. The lack of cyclin B, but not the increase in PP2A activity, interferes with centrosome separation dynamics in post-NEBD cells. However, comparing all pre-NEBD cells, it is clear that the centrosome separation dynamics are similar in all observed conditions, thus indicating that these early events are not specifically controlled by CDK1-cyclin B or PP2A (in)activity. This comes in agreement with [423], where the authors show that these proteins do not interact in early stages of mitosis.

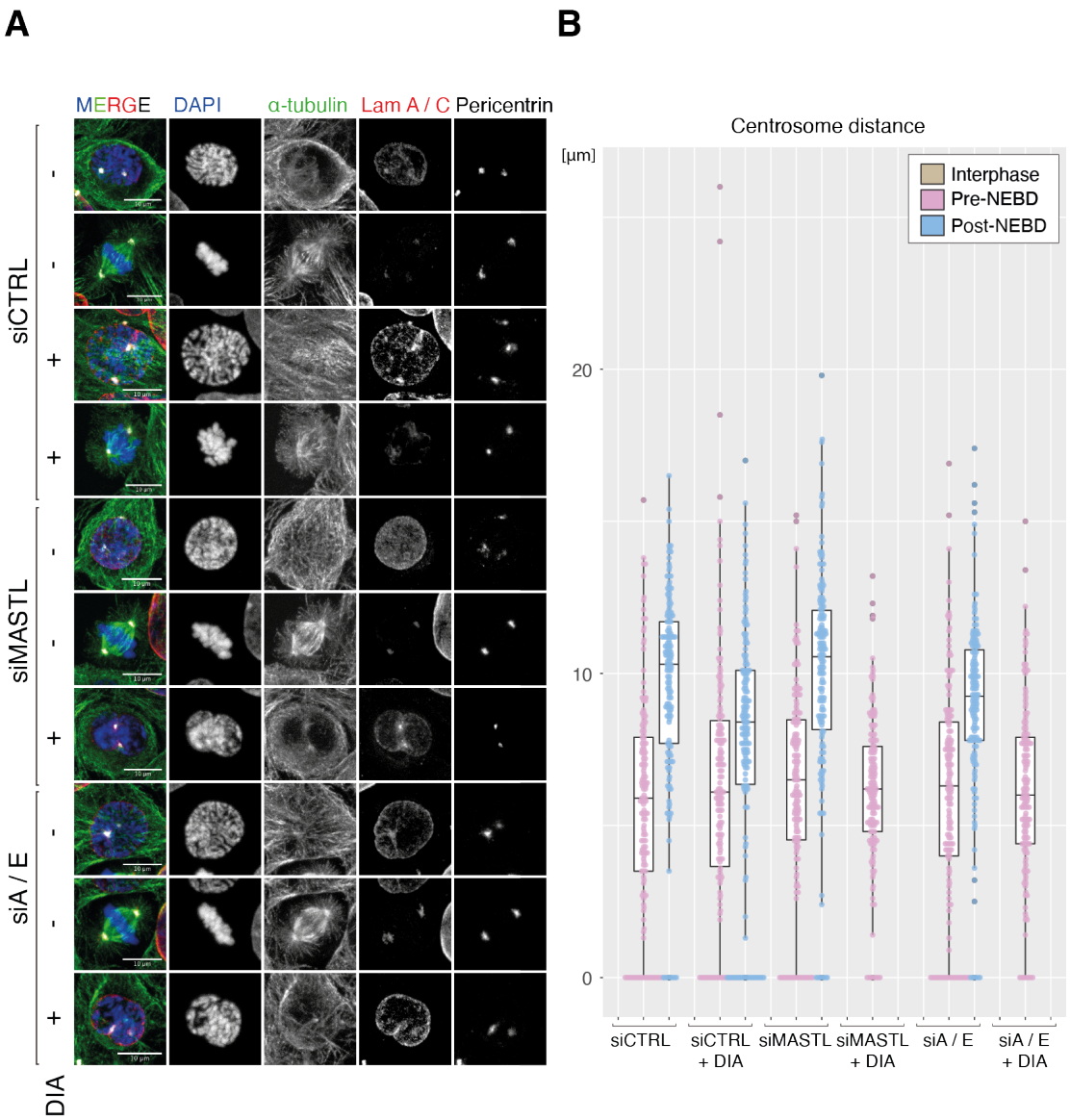

Figure 6.6: A comparison of centrosome distances in mitotic cells pre or post-NEBD. A: Immunofluorescence images obtained with the LSM 880 Airy Scan microscope. Scale bars indicate 10 μm. Colours in MERGE correspond to the channel colours as indicated by the name at the top, DAPI in blue, α-tubulin in green, lamin A / C in red, and pericentrin in white. B: Analysis of centrosome distances in μm of indicated cells. Cells were treated as in Figure 6.2 A, imaged with PerkinElmer Operetta microscope and analysed with ImageJ software. Centrosome distances were measured manually. Data were obtained from three independently repeated experiments, n $>$ 50. Boxplot as in Figure 6.4.

6.5 Discussion

Prophase-steady state is similar to antephase arrest The interplay between cyclins A2, B and PP2A was analysed in this Chapter using degron tagging and RNA interference methods. A novel prophase-steady state has been characterised in RPE-1 cells that is unlike a previously described antephase.

Antephase was previously defined as an intermediate stage between prophase and G_2, during which the centrosomes separate and APC/C^{CDH1} is activated in response to DNA damage with subsequent degradation of D-box containing proteins, including cyclins [425]. Mitotic stressors were found to be the key to antephase onset [418, 419, 425] and Feringa et al. show that cells in antephase eventually attempt cytokinesis [425], however, the prophase-steady state cells did not typically segregate their DNA contents into two cells, but some have succeeded in splitting the nucleus in two parts. Further characterisation is required to better understand the prophase-steady state, and its importance in the cell cycle.

6.5.1 A prophase-steady state exists in RPE-1 cells

In this Chapter, the prophase-steady state was defined by analysing the level of mitotic substrate phosphorylations, lamin A / C phosphorylation status, and centrosome separation dynamics. These data show that RPE-1 cells only require CDK-cyclin A2 activity to enter mitosis. Notably, PLK1 is a key downstream mitotic player that has been linked with cyclin A2 activity. Due to the lack of time, the contribution of PLK1 to the establishment of prophase was not assessed here, but it is probable that it also plays a role. It is apparent that mitotic progression of RPE1 cells is dependent on the interplay and specificity of mitotic kinases and phosphatases.

6.5.2 There are three types of mitotic substrates

With the help of Hochegger lab collaborators in Bela Novak's lab at the University of Oxford, we were able to design computer models describing the key activities affected by the conditions applied in this Chapter (Figure 6.7). Here, we separated mitotic substrates into three subtypes, each responsible for a specific stage of mitosis. Figure 6.7 A shows how concentrations of different types of phosphorylated substrates vary depending on the

condition marked on the left.

Early mitotic substrates The data in this Chapter show that there are some mitotic substrates that are efficiently phosphorylated by CDK-cyclin A2, and these are sufficient to trigger mitotic entry and the establishment of prophase. These early substrates (pS_{early}) are distinct from others by their capability to be activated by CDK-cyclin A, but are less susceptible to PP2A dephosphorylation activity. While the cells exit this prophase-steady state eventually, they enter and establish a mitotic stage for a substantial amount of time (Figure 6.3 B).

Intermediate mitotic substrates Nuclear envelope breakdown (NEBD) marks the entry into prometaphase. This is controlled by a second, intermediate, subset of mitotic substrates (pS_{inter}). Assuming that CDK-cyclin A2 is present to allow prophase entry, either PP2A inhibition, or CDK1-cyclin B activity are required for the progression into prometaphase.

Late mitotic substrates The establishment of metaphase is apparently reliant on a third subset of mitotic substrates (pS_{late}) that are much more specific for CDK1-cyclin B mediated phosphorylations, regardless of PP2A activity. This is clear as only cells without cyclin B cannot organise their mitotic spindle or chromosomes. Stabilised cyclin A2 did not contribute to this event, as shown in Chapter 5, however it is possible that its levels were insufficient to promote the phosphorylations.

Activity of key mitotic players Figure 6.7 B shows how the activities of some key mitotic players respond to the conditions used in this Chapter. Lacking either pro-mitotic activity results in no change in pS_{early}, but triggers a delay in pS_{inter} and leads to a lack of pS_{late}. Furthermore, the lack of CDK1-cyclin B activity, in combination with an active PP2A, promotes an entry into a prophase-steady state where only cyclin A2 activity rises, and thus promotes pS_{early}, but other CDK substrate phosphorylations are kept at a low level.

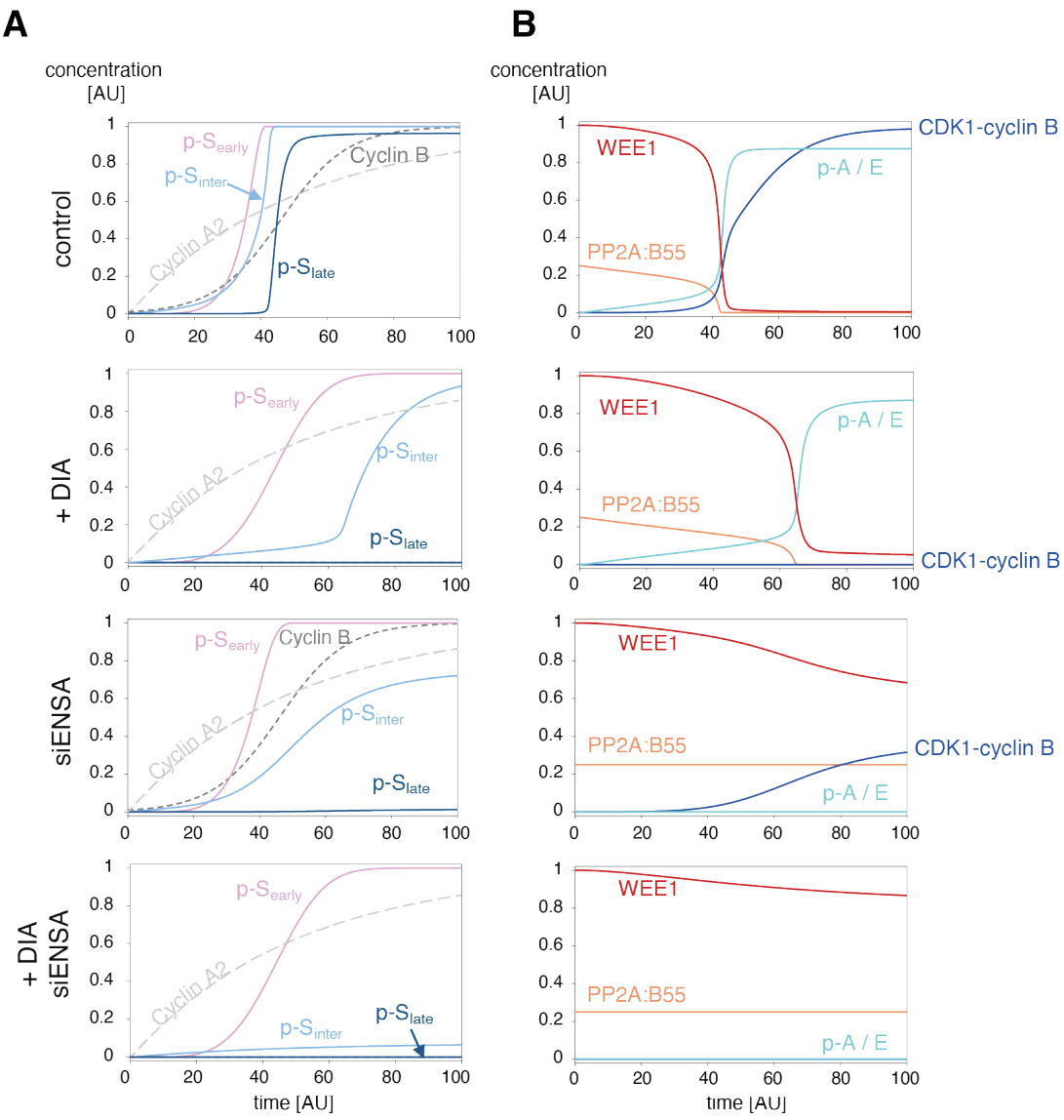

Figure 6.7: A numerical model of the three types of mitotic substrates based on the conditions applied in this Chapter. A: Cyclin activity and the levels of mitotic substrate phosphorylations divided into early (pS$_{early}$), intermediate (pS$_{inter}$), and late (pS$_{late}$). The condition is noted on the left side and also matches the corresponding graphs in B. B: Activity of some key mitotic players in mitosis, depending on the condition marked in A. Note: MASTL is not included, but it acts through ARPP19 / ENSA (A / E). Models were generated with the help of Hochegger lab collaborators in Bela Novak's lab.

6.5.3 Updating the model of the requirements for mitotic entry in RPE-1 cells

The model defining the roles for key players in mitosis in RPE-1 cells has been updated according to the information shown in this Chapter (Figure 6.8). To avoid repetition, the model will be described here only briefly.

The data shown in this thesis confirmed that CDK-cyclin A2 is sufficient to trigger mitotic entry, regardless of PP2A's inhibition. To progress into prometaphase, RPE-1 cells require either CDK1-cyclin B activity or PP2A inactivation. As indicated in Chapter 5, cyclin B is crucial for a metaphase alignment of chromosomes, and PP1 or PP2A must be re-activated for mitotic exit. This was implemented into the model for mitotic progression (Figure 6.8).

This Chapter concludes the work done in RPE-1 cells. The importance of cyclin B was also investigated in HeLa cells, which is the topic of the following Chapter.

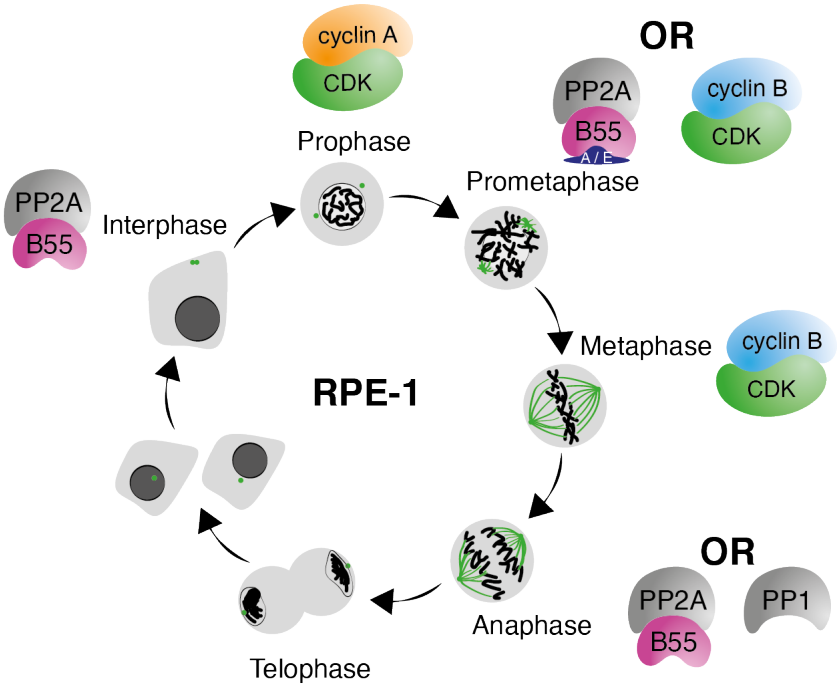

Figure 6.8: Updated model: Two main pathways promote mitotic progression in RPE-1 cells. CDK1-cyclin A2 or CDK2-cyclin A2 trigger mitotic entry and ensure the progression through early and mid mitosis by activating CDK1-cyclin B and MASTL, which activates two small molecules that, in turn, inhibit the PP2A phosphatase (as depicted by the blue protein, marked A/E, that inhibits the B55 substrate-binding pocket). Either PP2A inhibition, or CDK-cyclin B are sufficient to trigger NEBD and prometaphase onset, but cyclin B is crucial for the establishment of metaphase. Either PP1 or PP2A activity promotes mitotic exit (shown in Chapter 5).

Chapter 7

Roles of cyclin B in HeLa cells

7.1 Summary

The work in this Chapter determined that cyclin B is essential for mitotic progression in HeLa cells in earlier stages of mitosis than in RPE-1 cells. A novel intermediate stage between G_2 and M that is distinct from a previously described antephase has been uncovered and characterised.

Further work revealed that cyclin A2 is sufficient to establish most of CDK substrate phosphorylations that result in a prophase-like arrest. Overexpressing cyclin A2 dose-dependently promotes separate stages of mitosis. This implied that HeLa cells rely on the total amount of CDK activity to progress through mitosis, thereby establishing a quantitative model of mitotic progression in this cell line.

7.2 Introduction

The redundancy between mitotic CDK-binding cyclins has been addressed throughout this thesis. Cyclin A2 is not relevant for mitosis or cell cycle progression in HeLa cells. These data led to questions regarding the roles of cyclins in HeLa cells. I hypothesised that the cells could rely heavily on the other mitotic CDK-binding cyclin, cyclin B, or the two cyclins may compensate for each other to promote mitotic events.

Previous research implied that cyclin B is only required for late mitotic events, including chromosome alignment and segregation [12, 187], but other studies have also found important roles for cyclin B in mitotic entry [220, 426]. The redundancy between the two mitotic CDK1-binding cyclins has been a long-standing question in the field. I have shown previously that cyclin A2 is not required in HeLa cells (Chapter 4, Section 4.4.1). It has been implicated that cyclin B plays important roles in mitosis, but the possibility of the interplay between the two CDK-binding mitotic cyclins has also been noted [12, 16]. Similar to RPE-1, the specific roles or redundancy between cyclins A and B in HeLa cells have not yet been elucidated.

Cyclin B has already been described in detail in the Introduction part of this thesis (Chapter 2), and in Chapter 5, thus further reviews of this protein will not be provided here. This thesis has also shown that RPE-1 cells control mitotic entry and progression with specific mitotic substrate phosphorylations. Cyclin B is largely redundant until later stages of mitosis in RPE-1 cells, whereas cyclin A2 is necessary for early events. While these are both human cell lines, HeLa cells are a highly transformed cancerous cell line and it is possible that they have adapted to utilise cyclins in a different manner to ensure their growth and proliferation, similar to some other cancerous tumours described previously [261–265, 269]. This is also supported by the information presented in Chapter 4, showing that cyclin A2 is not important for mitosis or cell cycle proliferation in HeLa cells.

The reader is reminded that the work in this Section was conducted in the Poon lab at the HKUST, so all methods used are described in Chapter 3 Materials and Methods, Section 3.2.

7.3 Cell line generation and characterisation

7.3.1 Cell line establishment

Cyclins B1 and B2 are highly redundant [11, 192]; their compensation has been demonstrated in RPE-1 cells, albeit cyclin B1 was more efficient at promoting long-term cellular proliferation than cyclin B2, agreeing with a previous study [193]. These findings were described in Chapter 5 of this thesis. This Chapter investigates whether the observations from RPE-1 cells are consistent in HeLa cells.

Previous reports in HeLa cells have already indicated that there may be a similar compensation between cyclins B1 and B2 [11, 187]. Cell lines to study the roles of cyclin B in HeLa were established in a similar manner as with RPE-1 cyclin B2^{ko} B1^{dd}, described in Chapter 5. Due to the complexity of endogenous degron-tagging in this highly transformed cell line, I utilised the Poon lab degron method to knock-out the endogenous gene(s) and replace them with a TET-OFF controlled version (see Chapter 3 Materials and Methods, Section 3.2.7). This method was also described previously in Chapter 4, Section 4.4.1.1.

Initial immunoblotting analyses of selected clones The sleeping beauty transposase system, that has been mentioned previously in this thesis (Chapter 4 Section 4.3.5), was utilised for gene of interest (GOI) incorporation in parallel with CRISPR-targeting (see also see Chapter 3 Materials and Methods). HeLa cells with a constitutive tTA1 expression (HeLa, wt) were utilised to generate the following cell lines: cyclin B2 knock-out (cyclin B2^{ko}), cyclin B1 knock-out supplemented with cyclin B1-mAID (B4, B5), or cells knocked out for both cyclins B1 and B2, and overexpressing cyclin B1-mAID (BB1, BB3). An osTIR1-myc gene was also introduced into cells that include the cyclin B1-mAID in parallel with the above transfections. The western blot presented in Figure 7.1 is representative of several isolated clones.

Interestingly, the inhibitory p-Y15 phosphorylation on CDK1 is closely linked with the presence and amount of cyclin B1. Furthermore, the phosphorylation is not observable in cells lacking both types of cyclin B.

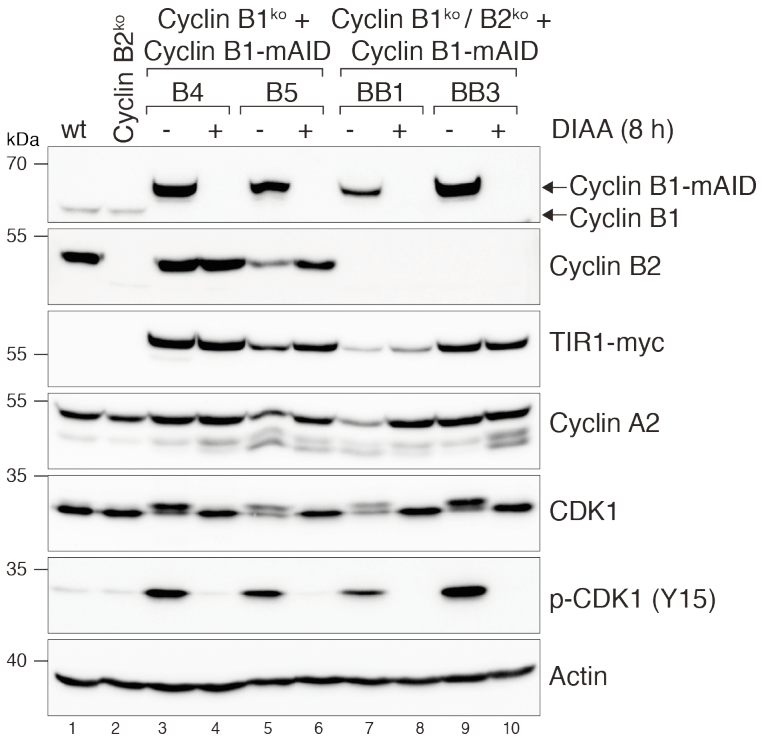

Figure 7.1: Western blot showing selected cell lines generated to study the roles of cyclin B in HeLa. Cells were treated or not with DIAA for 8 h and subjected them to immunoblotting analyses. HeLa parental cells are marked as wt and used as a control. The membrane was probed for for cyclin B1, cyclin B2 and myc to detect the osTIR1-myc expression. Cyclin A2, CDK1 and p-CDK1 (Y15) are included for a more detailed analyses. Actin is the loading control.

7.3.2 Initial characterisation of the newly established clones

The above cells were subject to initial proliferation analyses. I first tested the cyclin B1-mAID depletion efficiency (Figure 7.2 A). All of the analysed clones were found to deplete cyclin B1-mAID to levels below western blotting detection in less than 6 h. There are some variations between them, but their proliferation was assessed prior to selecting one of each clone for further research.

7.3.2.1 Cyclin B is essential for cell proliferation

A survival assay showed that all clones overexpressing cyclin B1-mAID are slightly hindered in growth, as judged by the colony size of untreated cells (Figure 7.2 B). Interestingly, similar to RPE-1, cells lacking cyclin B1 are poorer in their long-term proliferation even when cyclin B2 is still present (see clones B4 and B5, + DIAA). On top of this, clone B5 is additionally slower in its growth as observed by the number of cells and cell death when culturing the cells. The survival assay of the B5 clone also showed that the cells form much smaller colonies, as observed upon a closer investigation of the plate, but the starting number of these cells was also likely lower due to the amount of death of this clone; the underlying cause for the increased apoptosis is unclear, but it could be due to off-target CRISPR effects. Next, cells lacking both types of cyclin B (BB1 and BB3 + DIAA) are unable to proliferate, as observable by the lack of colonies in the colony survival assay.

Cells lacking cyclin B arrest in G_2 Short-term proliferation of the B4 clone is not affected by the loss of cyclin B1, as judged by their cell cycle profile (Figure 7.2 C). Interestingly short-term proliferation defects are more readily observable in clone B5, as compared with B4.

Next, both BB1 and BB3 accumulate as a 4N population within 24 h of DIAA treatment and remain arrested until eventual cell death that is most apparent after 72 h of incubating with the DIAA cocktail. FACS analyses of the forward and side scatter indicated a change in cellular morphology and implied that cells continue growing after arresting. The morphological characteristics of BB1 cells were addressed separately in Section 7.5.1.1.

Selection of clones for further analyses Based on the above information, clones B4 and BB1 were selected for further analyses. B4 was chosen as the cyclin B2 expression is unaffected, unlike in clone B5, and the cyclin B1 depletion is equally efficient in both. There are apparent differences in their growth and short-term defects, hence this was an additional reason to select B4. Next, clone BB1 was selected as it has a lower level of cyclin B1-mAID expression, which is closer to the protein level in parental HeLa cells. The naming B4 and BB1 will be maintained throughout this Chapter. All analyses in this Chapter were conducted after a minimum of 6 h of DIAA treatment.

Confirmation of CRISPR targeting The CRISPR efficiency of cyclin B1 and B2 knock-out efficiency were assessed in the BB1 clone with Sanger sequencing (data not shown) and by immunoblotting analyses of cyclins B1 and B2 with two different antibodies (data not shown).

Live-cell imaging (LCI) analyses were utilised for further investigation of the arrest of BB1 cells.

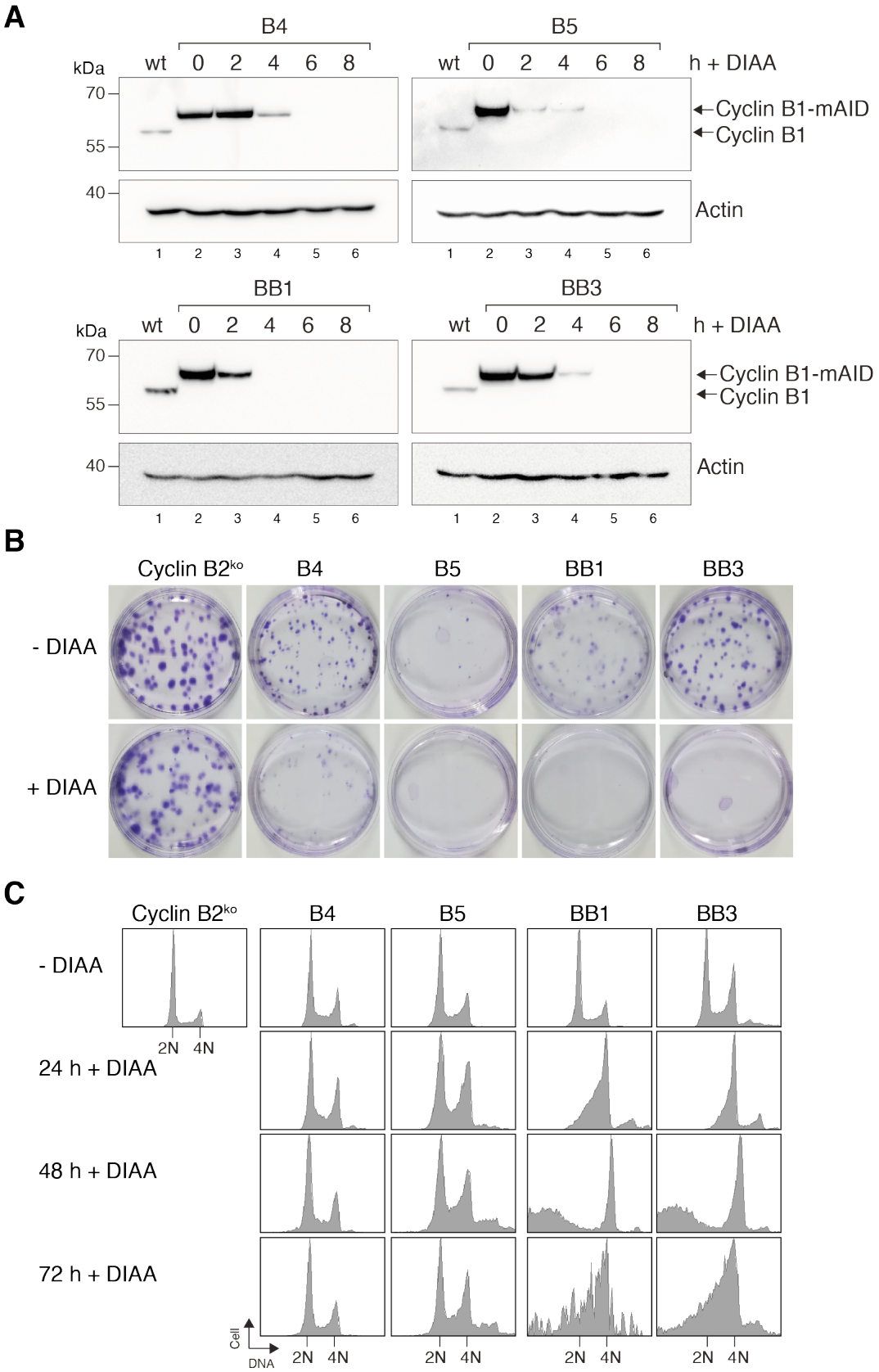

Figure 7.2: See caption on the following page.

Figure 7.2 *(preceding page)*: Cyclin B is essential for cellular proliferation. A: Western blots of the HeLa clones treated with DIAA for the indicated amount of time to assess the eficiency of cyclin B1-mAID depletion. HeLa wt is included for reference. B: Colony survival assays showing in the presence or absence of DIAA. For each condition 150 cells were seeded and subject to fixing and crystal violet staining after 11 days (according to the confluency of untreated cells). C: FACS analyses of cells stained with propidium iodide (see Chapter 3 Materials and Methods), treated or not with DIAA for the indicated amounts of time, gated for the living population as in untreated cells. The y-axis corresponds to the maximum number of cells in each plot (not shown).

7.4 Cells lacking cyclin B enter a prophase-like stage

7.4.1 Live-cell imaging analyses revealed a novel phenotype of cells lacking both types of cyclin B

The cell cycle progression of asynchronous cells deficient in cyclin B1 was assessed in this Section. Single-cell tracking analyses of one experiment are shown as an example (Figure 7.3 A). The cyclin B2^{ko} clone was included as a control. Cells lacking cyclin B2 (B2^{ko}) progress through mitosis normally (HeLa wild-type data not shown), concomitant with previous observations.

The loss of cyclin B1 does not interfere with cellular division of B4 cells, according to single-cell tracking analyses (Figure 7.3 A), supported by mitotic entry and exit phenotype analyses (Figure 7.3 B, C). Cells lacking both types of cyclin B (BB1) exhibited a much more drastic phenotype where no cells completed cellular division even though a mitotic entry was observable as judged by the rounding of cells, as typical for mitosis (see Section 7.5.1.1). Furthermore, cells that apparently exited mitosis were morphologically distinct as compared with interphase cells (prior to their entry into mitosis), which will also be discussed in more detail in Section 7.5.1.1. All instances of mitotic exit where cells remained undivided were classified as mitotic slippage (M slippage), although further investigation with additional mitotic markers is needed to better determine this phenotype.

Cells lacking both types of cyclin B exhibit mitotic defects According to Figure 7.3 B, the majority of analysed cells entered mitosis, apart from cells lacking both types of cyclin B. There is also a slight decrease in the total mitotic entry of B4 cells lacking cyclin B1, which was investigated further in the following Section. As evident in Figure 7.3 C, cells lacking either cyclin B1 or B2 mostly exited mitosis normally, whereas all cells lacking both types of cyclin B endured mitotic slippage, apart from a subpopulation that have not exited mitosis within the LCI analyses.

Cells lacking both types of cyclin B spend more time in mitosis Compared with cyclin B2^{ko}, B4 cells treated or not with DIAA spent a similar amount of time in mitosis, as did untreated BB1 cells (Figure 7.3 D). However, BB1 cells treated with the DIAA cocktail spent distinctly more time in mitosis, and the times vary significantly,

especially compared with other conditions.

Further analyses with the B4 and BB1 clones are necessary to characterise the mitotic exit phenotype. In subsequent experiments, the cyclin B2^{ko} clone was no longer analysed, with the untreated B4 and BB1 cells utilised as controls instead.

To achieve a greater level of detail, H2B-GFP was stably expressed in B4 and BB1 clones to provide an additional layer of detail during live-cell imaging analyses.

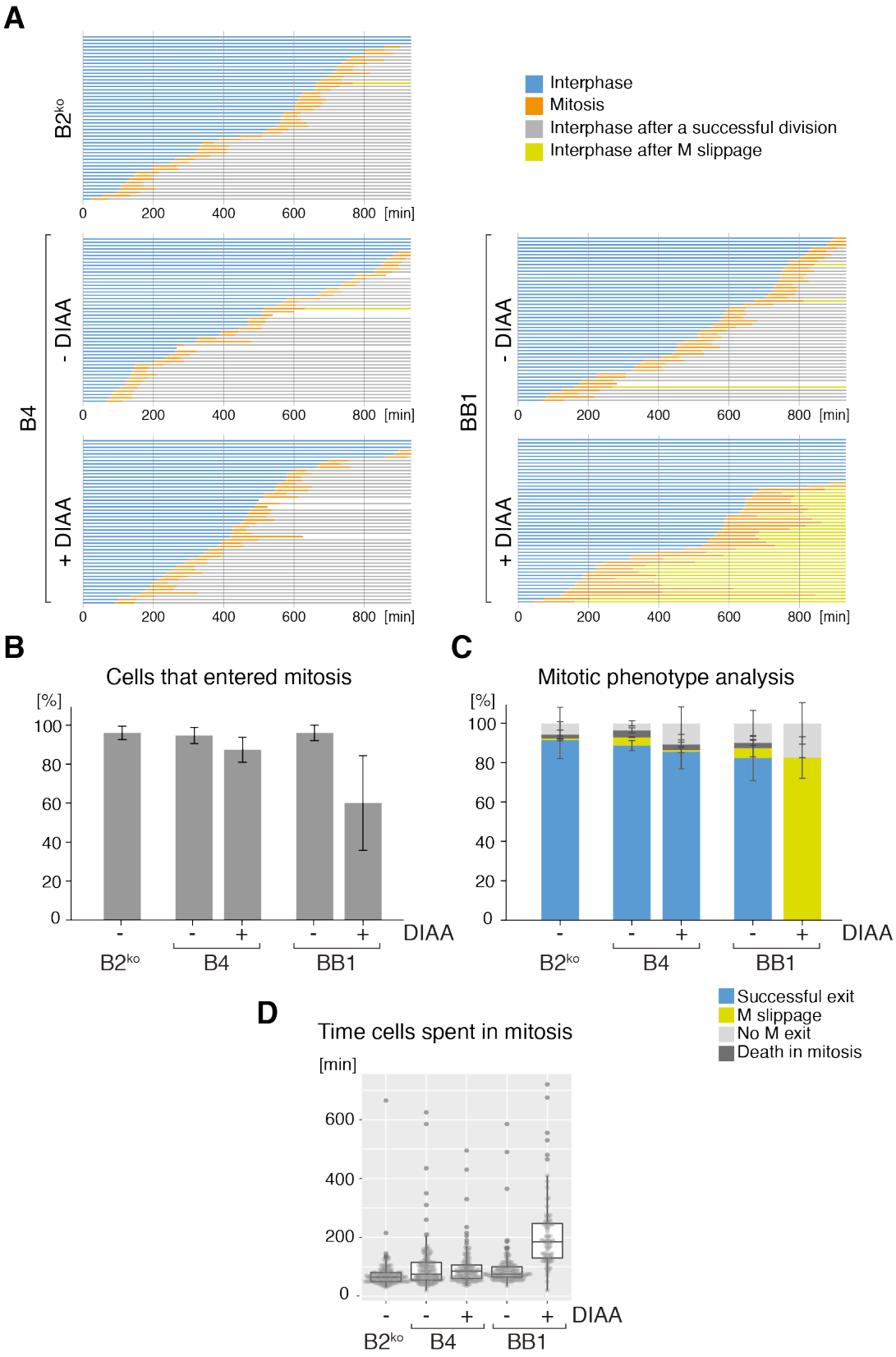

Figure 7.3: See caption on the following page.

Figure 7.3 *(preceding page)*: Depletion of both types of cyclin B affects mitotic entry and progression. A: Single-cell tracking graphs of live-cell imaging analyses of indicated cell lines (n = 50). Asynchronous cells were seeded into a 24-well plate and DIAA was added a minimum of 6 h prior to live-cell imaging analyses. Microscopy was conducted with a Zeiss Cell-Discoverer 7 microscope throughout this Chapter, using Phase-contrast, and / or fluorescence channels where indicated. Cells were filmed for 16 h, time is noted in min on the x-axis. Mitotic cells were classified either into a successful division, or M slippage if they exited mitosis with any defects. B: Analysis of mitotic entry of indicated cells as judged by cell rounding. Data were obtained from three independent repeats (n = 50). C: Analysis of cells in B, classified as in A. Data were normalised to total mitotic cells in each condition. D: Analysis of time cells in B and C spent in mitosis as judged by cell rounding to cell re-adhering to the surface (total n > 80). Data in B, C and D were obtained from three independent repeats, and only cells that exited mitosis within the analysed time-frame were assessed in D. Cells that entered mitosis but did not exit within the analysed times are marked as No M exit in C. All barplots here and in the following Figures of this Chapter indicate the mean measurement of three independently repeated experiments, unless stated otherwise. The error bars signify standard deviation of three experiments. In D, and in the rest of the Figures in this Chapter that include a boxplot, the boxplot whiskers mark the first and fourth quartile, and the box marks second and third quartile. The horizontal line in the boxplot is a mean measurement of all data points. Bee-swarm indicates each individual data point.

7.4.2 Mitotic entry mechanics are similar in all B4 and BB1 cells

Mitotic entry is slightly delayed in the absence of cyclin B1 To determine the mechanics of mitotic entry, cells were synchronised with a double thymidine block (see Chapter 3 Materials and Methods) and released the cells into normal or DIAA-containing media. Cell tracking analyses revealed that cells lacking only cyclin B1 (B4 + DIAA) are slightly slower in mitotic entry (Figure 7.4 A), concomitant with previous observations. Interestingly, the mitotic entry of BB1 cells resembles the entry mechanics of B4 cells, including the slight delay of both DIAA-treated populations. Furthermore, nearly all cells entered mitosis following the thymidine release, regardless of cyclin B's depletion, thus showing that cyclin B1 may contribute to the proper timing of mitotic entry, but it is not essential for the appearance of some mitotic markers.

Cyclin B deficient HeLa cells apparently exit mitosis prior to NEBD The phenotypes of mitotic exit were analysed next. The cells were now classified into four categories, depending on the nuclear envelope breakdown (NEBD) and cellular division: (i) successful exit, (ii) pre-NEBD exit, (iii) post-NEBD exit, and (iv) failed division. These are distinct by the following characteristics: pre-NEBD exit includes cells that exit mitosis without NEBD as observed by the shape of their nuclei and chromosomes. Cells that exit mitosis after NEBD but are unsuccessful in division and re-adhere to the surface as a singular cell are classified as post-NEBD exit. Cells that progressed past NEBD and divided into two cells with readily observable defects such as lagging chromosomes, or any divisions into three cells, were classified as failed division. This categorisation will be used for the rest of the Chapter.

Approximately 80% of B4 cells divide successfully irrespective of cyclin B1's presence, although some failure in division was observed (Figure 7.4 B). This is an intrinsic property of HeLa cells and their high level of genome instability is well documented [397, 398] but some increase in defective mitotic exit could also be due to the overexpression of cyclin B1-mAID, or due to the knock-out of the endogenous gene. It is clear that BB1 + DIAA cells enter into a mitosis-like stage that resembles prophase, but do not fully condense their DNA into chromosomes, nor progress past NEBD. These cells apparently exit mitosis as observed by their partial re-adhering to the surface, however they are unlike interphase

cells, judging by their appearance, which will be discussed in the following Section.

Cells lacking cyclin B consistently spend more time in mitosis Concomitant with the information above, BB1 + DIAA cells spent a much longer amount of time in mitosis, and are distinctly less uniform in this property (Figure 7.4 C), as already noted previously.

So far I have distinguished between a mitotic and a non-mitotic stage in BB1 cells, but recent observations demonstrating the lack of chromosome condensation in these cells have posed questions on whether these cells truly enter a mitotic stage, as the appearance of chromosomes is one of the key markers of this stage.

First, an analysis of the morphological properties of BB1 cells will be discussed, followed by microscopy and immunoblotting analyses of the arrest observed in the BB1 cells. Given that the peculiar phenotype is characteristic for BB1 cells only, further analyses will be conducted with this cell line, and B4 cells will only be included where the additional control was deemed necessary.

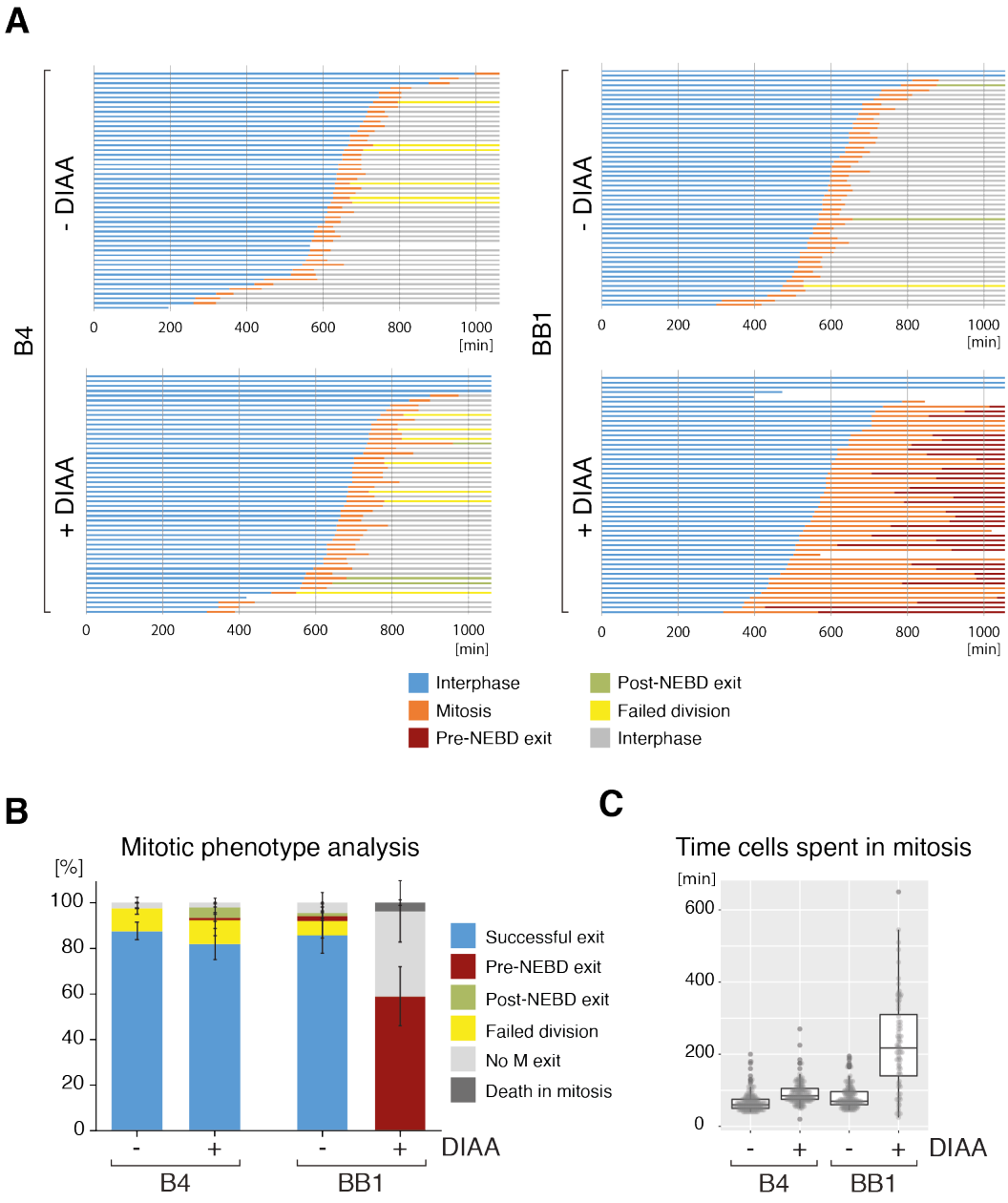

Figure 7.4: Cells lacking both types of cyclin B exit mitosis prior to NEBD. A: Single-cell tracking graphs of live-cell imaging of indicated cell lines stably expressing H2B-GFP (n = 50). Cells were released from a double thymidine block, followed by seeding into a 24-well plate containing DIAA or not for live-cell imaging. B: Analyses of mitotic exit phenotypes as indicated in Section 7.4.2. Data were obtained from three independent repeats, (n > 42). Cells that entered mitosis but did not exit within the analysed times are marked as No M exit. C: Analyses of time cells spent in mitosis as judged by cell rounding and chromosome condensation to cell re-adhering to the surface and chromosome decondensation (total n > 72). Data obtained from three independent repeats, and only cells that exited mitosis within the analysed time-frame were assessed. Barplots and boxplots are as in Figure 7.3.

7.5 Characterisation of a novel prophase-like stage

7.5.1 Immunofluorescence and live-cell imaging analyses of BB1 cells

7.5.1.1 Cells lacking cyclin B are morphologically distinct and have phosphorylated lamins A and C, but cannot trigger NEBD

Lamins A and C are phosphorylated on their mitotic site There are three subtypes of the lamin family, A, B, and C and they polymerise to build the nuclear lamina (described previously in Chapter 6, Section 6.4.2). Lamins A and C are phosphorylated by CDK1 at several sites, but one of those, S22, is known as the mitotic site that contributes to the regulation of their degradation during NEBD (reviewed in [420]). I aimed to determine whether the nuclear envelope is indeed intact or if initial steps towards its degradation have been taken. Previous information obtained in this thesis showed that lamins A and C are phosphorylated in RPE-1 cells in prophase, regardless of cyclin B's absence (see Chapter 6, Section 6.4.2), and the same antibody was utilised here for more detailed analyses. Immunoblotting analyses showed that the mitotic site on lamin A / C (S22) is indeed phosphorylated throughout the entire time that these cells were followed after the thymidine release (Figure 7.5 A). Notably, a faint top band can be detected in BB1 – DIAA cells (lanes 3 and 4) but not in DIAA-treated cells.

Total and phosphorylated lamin A / C levels decrease after NEBD in untreated cells but this is not observable in DIAA-treated cells. The maintenance of lamin A / C S22 phosphorylation in BB1 + DIAA cells supports an existence of a separate mitotic state in these cells.

Prophase-like cells are morphologically distinct when compared with interphase and mitotic cells During live-cell imaging analyses, a peculiar change in the morphology of BB1 cells treated with DIAA was observable. The cells appeared more rounded and 'mitotic' approximately 8 h after the thymidine release (Figure 7.5 B, 8 h), eventually followed by cells apparently adhering back to the surface, but not to the level of interphase cells (Figure 7.5 B, 16 h). Untreated cells are in comparison distinctly less uniform and more crowded due to cells dividing by 16 h of imaging. Towards the end of LCI analyses, cells untreated with DIAA are over-populated with some floating,

interphase and mitotic cells. However, cells treated with DIAA have remained in the previously described shape and visibly increased their size as compared with the earlier time-frames (Figure 7.5 B, 24 h).

The difference in their morphology was observable each time the cells were incubated with the DIAA cocktail for a sufficient amount of time, and it also served as a marker of the drug treatment efficiency throughout these analyses. According to the H2B-GFP fluorescence that is stably expressed in these cells, I speculated that the nuclear envelope does not dissipate in these cells since the distinct prometaphase chromosomes were not observed. Further details of the cells lacking both types of cyclin B will be provided next.

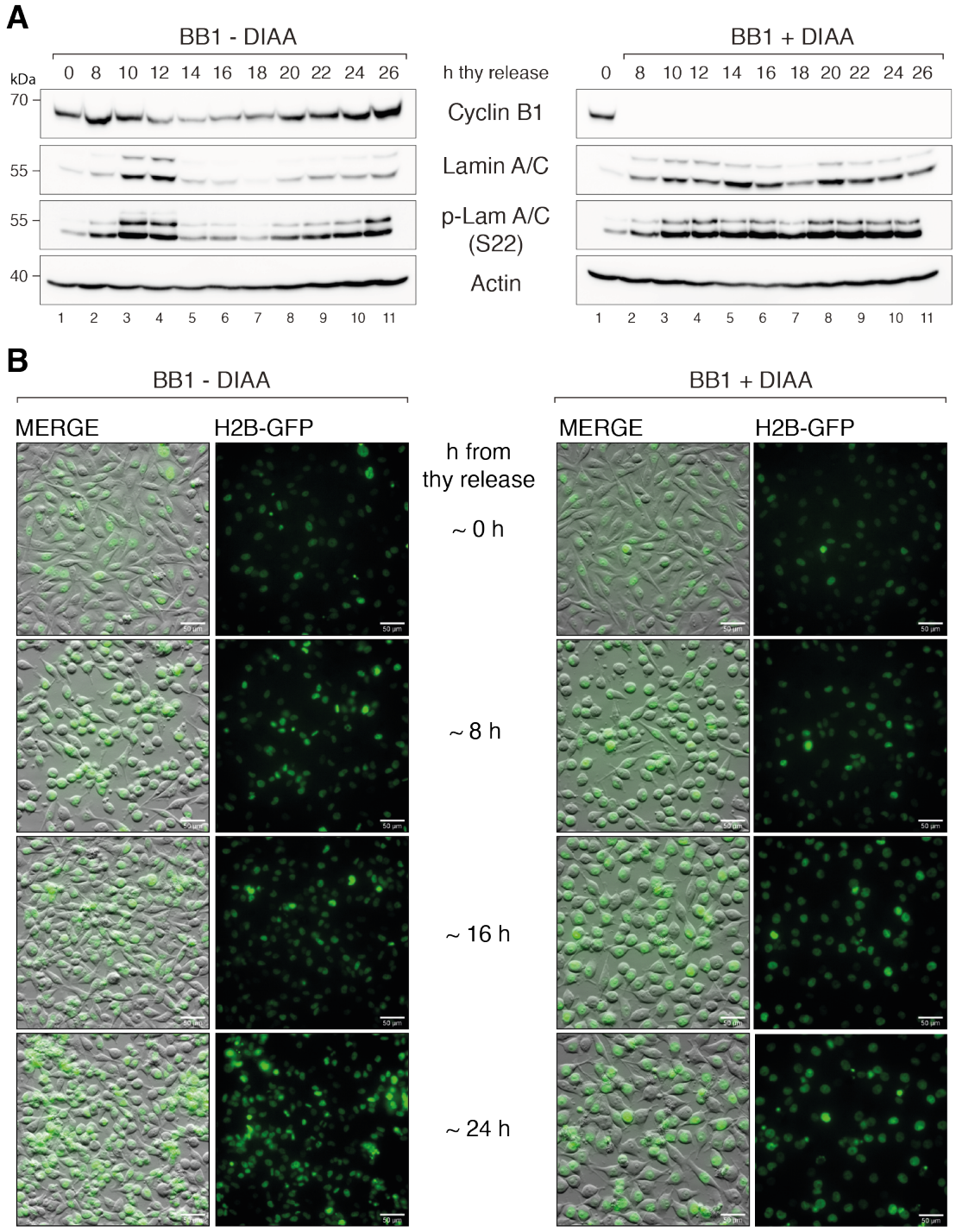

Figure 7.5: BB1 cells maintain phosphorylated lamins A / C and change their morphology when lacking cyclin B. A: Western blots of the indicated cells. The membranes were probed with the indicated antibodies. Actin is the loading control. B: Snapshots of BB1 cells in the approximate times of thymidine release. Cells stably express H2B-GFP. Scale bar is 50 μm.

7.5.1.2 The majority of prophase-like cells have two centrosomes

The centrosome cycle is regulated in a separate manner to the cell cycle. CDK-cyclin A has been implicated in the control of centrosome duplication [84] and immunoblotting analyses show that the protein is stabilised in cells lacking cyclin B (see Section 7.5.2.2). I aimed to determine whether cells lacking cyclin B also halt their centrosome duplication cycle.

Figure 7.6 A shows the images of asynchronous BB1 cells treated with DIAA for 24 h, prior to fixation and immunofluorescence analyses. Quantification of centrosome numbers in cells showed that the majority of BB1 cells lacking cyclin B have two centrosomes, unlike others where a singular centrosome is prevalent, but this was expected as the cells arrest in a G_2-like stage. Interestingly, a small proportion of arrested cells had four, typically unseparated, centrosomes. Centrosome numbers were not observed at later time-points due to a lack of time. Instead, the morphology of these cells was analysed further, this time with immunofluorescence analyses, following a thymidine release to detect any distinct properties of arrested cells.

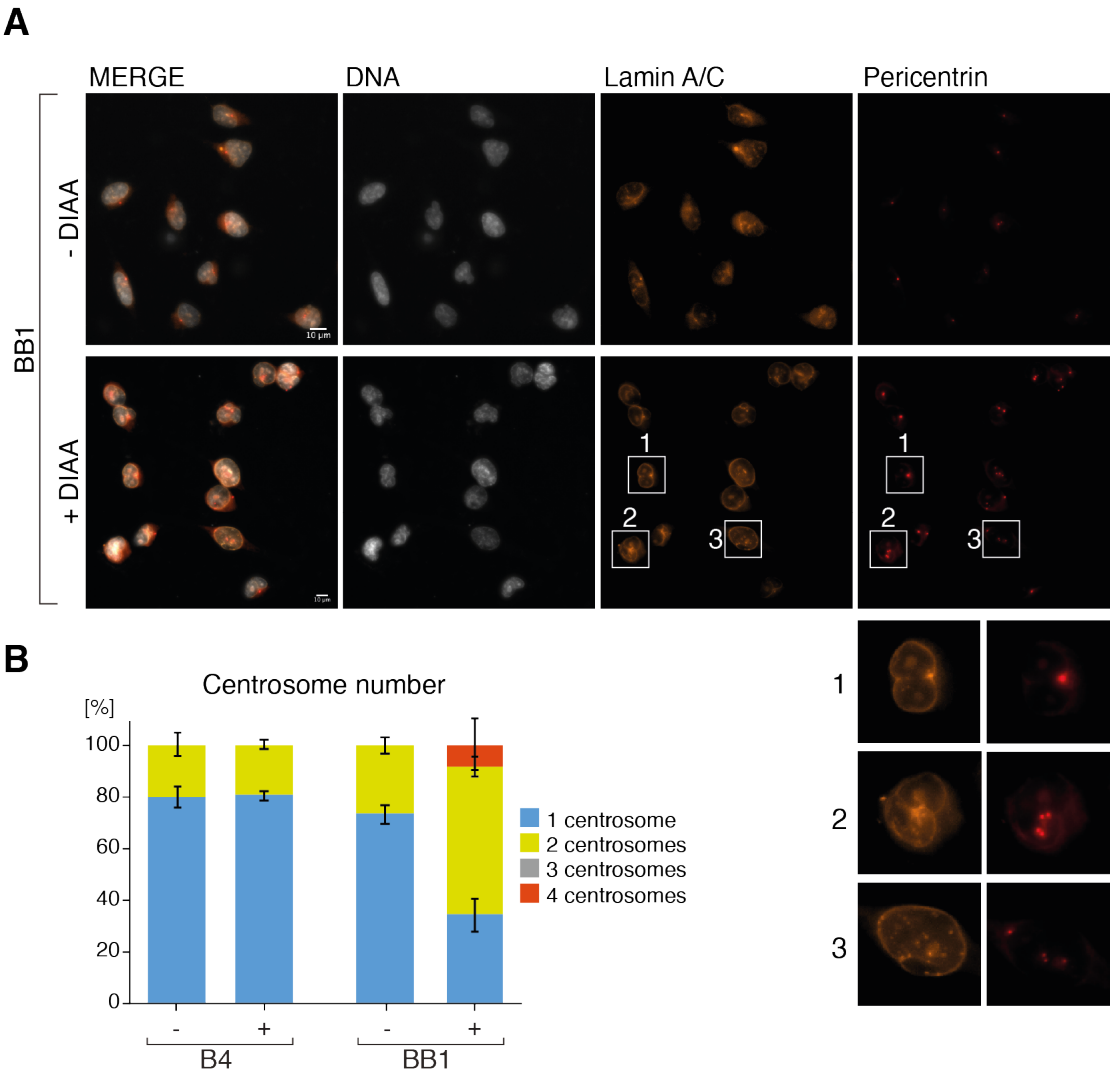

Figure 7.6: Immunofluorescence analyses of centrosome numbers in BB1 cells. A: Asynchronous BB1 cells were treated with DIAA for 24 h prior to immunofluorescence analyses. Scale bar indicates 10 μm. B: Analysis of chromosome numbers in the indicated cells. Data obtained from three repeats (n > 100). Barplots are as in Figure 7.3.

7.5.1.3 BB1 cells treated with DIAA do not break down the nuclear envelope, but some attempt to separate their nuclei

A small subpopulation of cells where the nucleus is apparently being pulled into two parts was observed in immunofluorescence analyses following a 22-hour thymidine release in the presence of DIAA treatment (Figure 7.7 A). These cells still appear to have a nuclear envelope present, despite the near-complete separation of their DNA into two nuclei. The images are only shown as a representation of the phenomenon observed, as the majority of cells did not display this phenotype. The level of centrosome separation in these varies strongly, as can be observed by the pericentrin staining in this Figure, thus the distance between centrosomes was not analysed. The state of DNA condensation of thymidine-released cells was assessed next.

I hypothesised that some details of DNA condensation could have been missed with basic immunofluorescence analyses, so I utilised a mitotic spread protocol (see Chapter 3 Materials and Methods). Cells were released from a double thymidine block for different amounts of time to assess their phenotype in different stages of the observed arrest (Figure 7.7 B is representative of two time-points, 16 h and 24 h). Signs of chromosome condensation were clear in a few cells, whereas the majority appeared to have an interphase-like nucleus (as observed during immunofluorescence analyses). According to the above, immunofluorescence analyses were insufficient to determine the nuclear properties of these cells. They were instead subjected to further live-cell imaging analyses, using newly established cell lines.

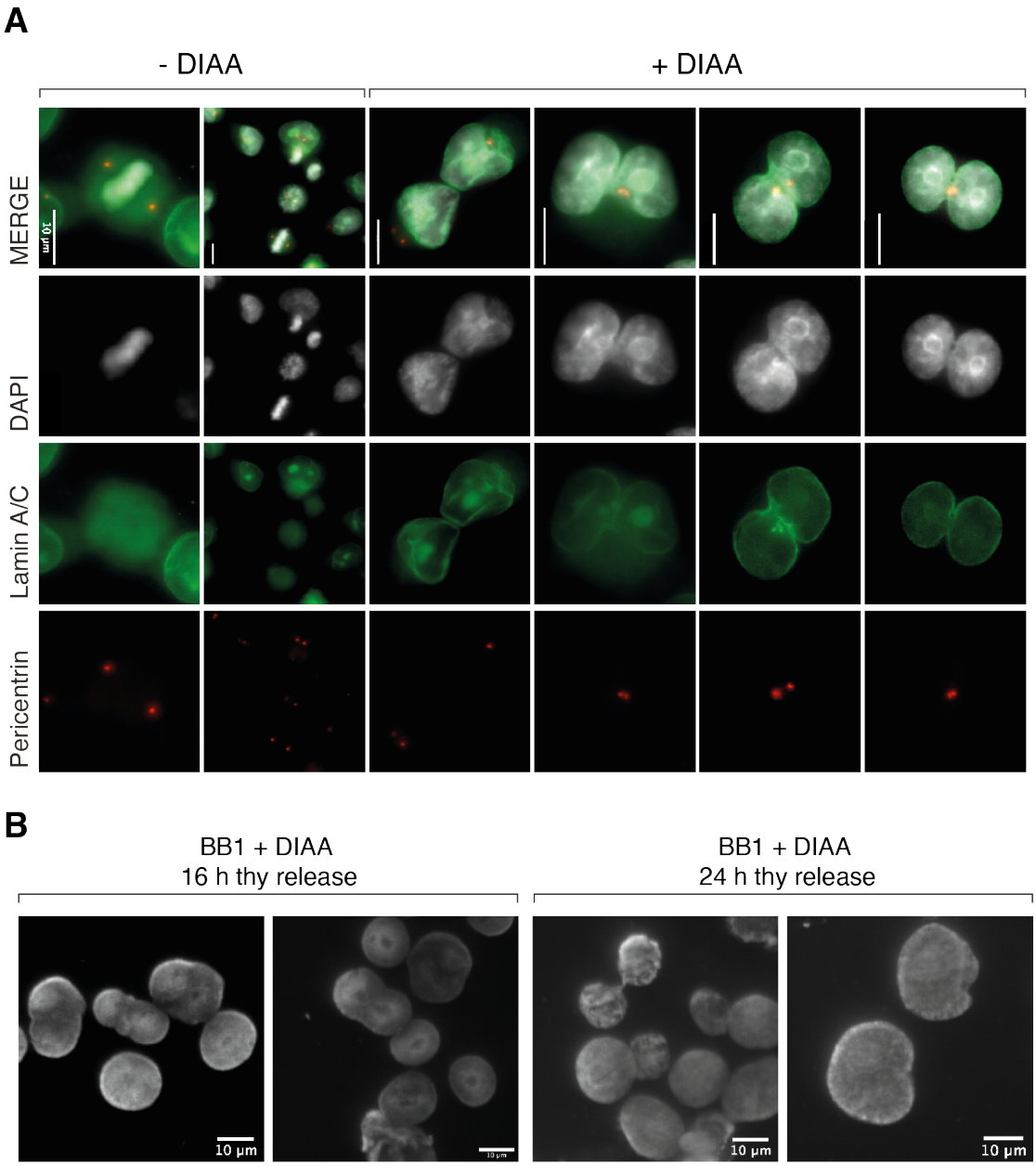

Figure 7.7: Immunofluorescence analyses of synchronised cells reveal peculiar cellular phenotypes. A: Cells were released from thymidine for 22 h prior to fixation and immunofluorescence analyses. B: Cells were released from thymidine for the indicated amount of time and subject to chromosome spread protocol. Scale bar indicates 10 μm.

7.5.1.4 The nuclear disassembly is not initiated without cyclin B regardless of phosphatase activity

One of the first events during nuclear envelope disassembly is the widening of specific nuclear pores that allow the nuclear and cytoplasmic contents to mix (reviewed in [427]). To assess whether the arrested cells promote nuclear envelope disassembly, a new cell line stably expressing H2B-GFP and mRFP-NLS was established. This approach allowed the visualisation of the mRFP signal flooding the entire cell at the onset of prometaphase in control cells (Figure 7.8 C, top panels). This was not detectable in cells lacking cyclin B as the signal remained strictly nuclear.

Furthermore, it is possible that the anti-mitotic phosphatases are highly active in HeLa cells, thus stalling the cells due to a constant removal of mitotic phosphorylations. Concomitant with this, high activity of PP2A triggered a prophase arrest in RPE-1 cells lacking cyclin B, as discussed in the previous Chapter of this thesis.

Phosphatases do not play major roles in mitotic progression of prophase-like cells Assuming the phosphatases are highly active, two well-established phosphatase inhibitors were utilised to suppress their activity. Figures 7.8 A and B show cells that progressed past NEBD, according to the mRFP-NLS signal. Cells in A were treated with Okadaic acid, a potent PP1 and PP2A inhibitor [428, 429]. While the phosphatase inhibition slightly accelerated the onset of NEBD in untreated cells, all cells treated with DIAA were unable to progress into prometaphase. In Figure B, PP2A was specifically inhibited with LB-100, a potent phosphatase inhibitor used in clinical trials for cancer treatments [430], but again no NEBD was detectable in cells lacking cyclin B (Figure 7.8 B, C). These data indicated that, in HeLa, phosphatases work closely with CDK1-cyclin B, so after the loss of this activity, phosphatase inhibition seems to be less relevant for early mitotic progression, unlike the data shown previously in RPE-1 cells (see Chapter 6).

Next, the mitotic markers and levels of phosphorylated CDK substrates were assessed using immunoblotting analyses.

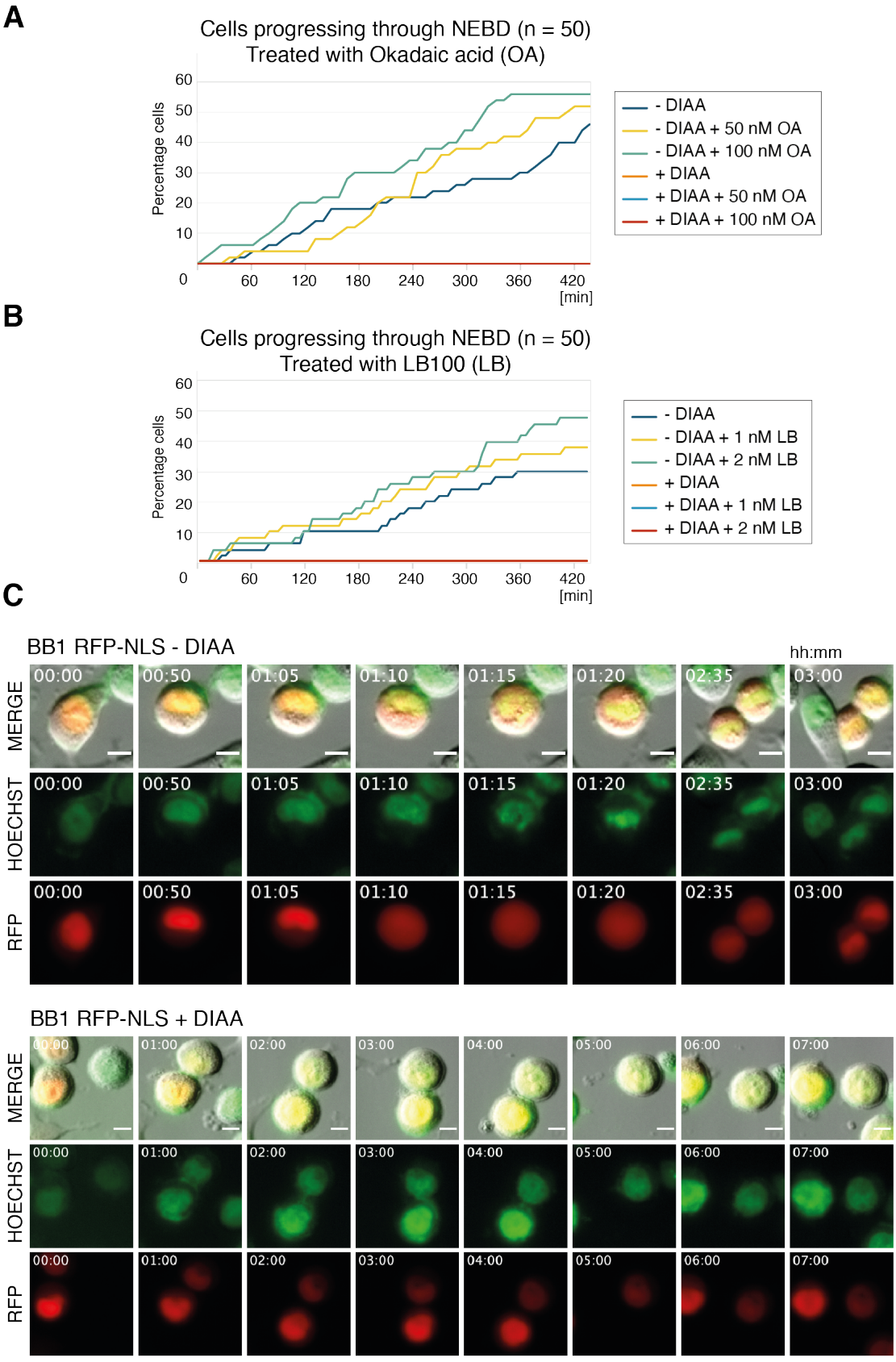

Figure 7.8: See caption on the following page.

Figure 7.8 *(preceding page)*: HeLa cells lacking cyclin B do not progress past NEBD, regardless of phosphatase inhibition. A and B: Graphs representing cumulative percentage of cells that have progressed past NEBD, as judged by the flooding of RFP-NLS signal. Data obtained from a single experiment (n=50) for each condition. C: Snapshots of cells in B, both treated with 2 μM LB-100, and treated or not with DIAA as indicated. Scale bars indicate 10 μm. DNA was visualised with Hoechst DNA dye that was added 1 h prior to the start of imaging, which caused some background signal towards the end of the film.

7.5.2 Analyses of the prophase-like stage by immunoblotting for key cell cycle markers

7.5.2.1 Experimental outline

Given the differences in cellular morphology observed between mitotic initiation and the novel arrested stage, I theorised that the cells may showcase different expression of cell cycle markers. B4 and BB1 cells were synchronised with a double thymidine block, released from the second block for 8 h, and collected at 2-hour intervals for an additional 18 h in order to capture an entire cell cycle. At 8 h post-thymidine release, most cells are in late G_2 as mitotic entry is detectable at 10 h post-thy release (according to LCI analyses). This experimental outline enabled the detection of key changes during late G_2, mitotic entry, and the subsequent cell cycle or prophase-like arrest of cells lacking cyclin B. Cells were then subjected to immunoblotting analyses.

Figure 7.9 A (left panel), shows western blots of B4 cells untreated with DIAA which serve as a control. Cyclins B1, B2 and A2 are destroyed by the 12-hour time-point, indicating that the majority of cells have completed mitosis and are progressing into the next G_1 stage. Cyclins slowly accumulate again, and at the last time-point (26 h), cyclin levels are increasing again marking the second G_2 phase, thus confirming that one full cell cycle has been captured with these analyses. Other mitotic markers like the loss of p-Y15 on CDK1 matches the timing noted above, as well as the increase in phosphorylated histone H3 and Aurora A, B, and C during mitosis.

7.5.2.2 Cells lacking cyclin B1 that still have cyclin B2 progress slower through the cell cycle

Analyses of clone B4 treated with DIAA, which still expresses the endogenous cyclin B2, demonstrated that cells exit mitosis at a similar time-point as control untreated cells, based on the timing of cyclin depletion and other mitotic markers. However, the subsequent re-accumulation of cyclins B2 and A2 occurs slower in cells also lacking cyclin B1 (Figure 7.9 A, right panel). The slight delay in the first mitotic entry following cyclin B1-mAID depletion that was observed with live-cell imaging is not apparent here, likely due to the lack of detail caused by 2-hour time-points. Nevertheless, it is apparent that the subsequent cell cycle occurs later in these cells, which may contribute to the poorer

long-term survival of these cells.

Interestingly, the p-Y15 CDK1 is undetectable in these cells even though cyclin B2 is still present. It is unclear how the mitotic entry timing is regulated in these, but the loss of this phosphorylation may additionally contribute to poor survival as it could lead to untimely mitotic entry.

Figure 7.9 B, left panels, shows untreated BB1 cells progressing through the cell cycle. The depletion of cyclins B1 and A2 corresponds to mitotic exit as above, although these cells do not deplete cyclin B1 as efficiently as B4 cells. Apart from this, other mitotic markers match the timings as observed in control untreated clone B4.

Cells lacking both types of cyclin B maintain high levels of cyclin A2 but lack some mitotic markers BB1 cells treated with the DIAA cocktail (Figure 7.9 B, right panels) maintain high levels of cyclin A2 for the duration of this experiment. No increase in phosphorylated histone H3 are apparent, which is a marker for mitotic chromosome condensation [431]. This was not surprising, as the absence of chromosome-condensation was previously observed with live-cell analyses and immunofluorescence imaging.

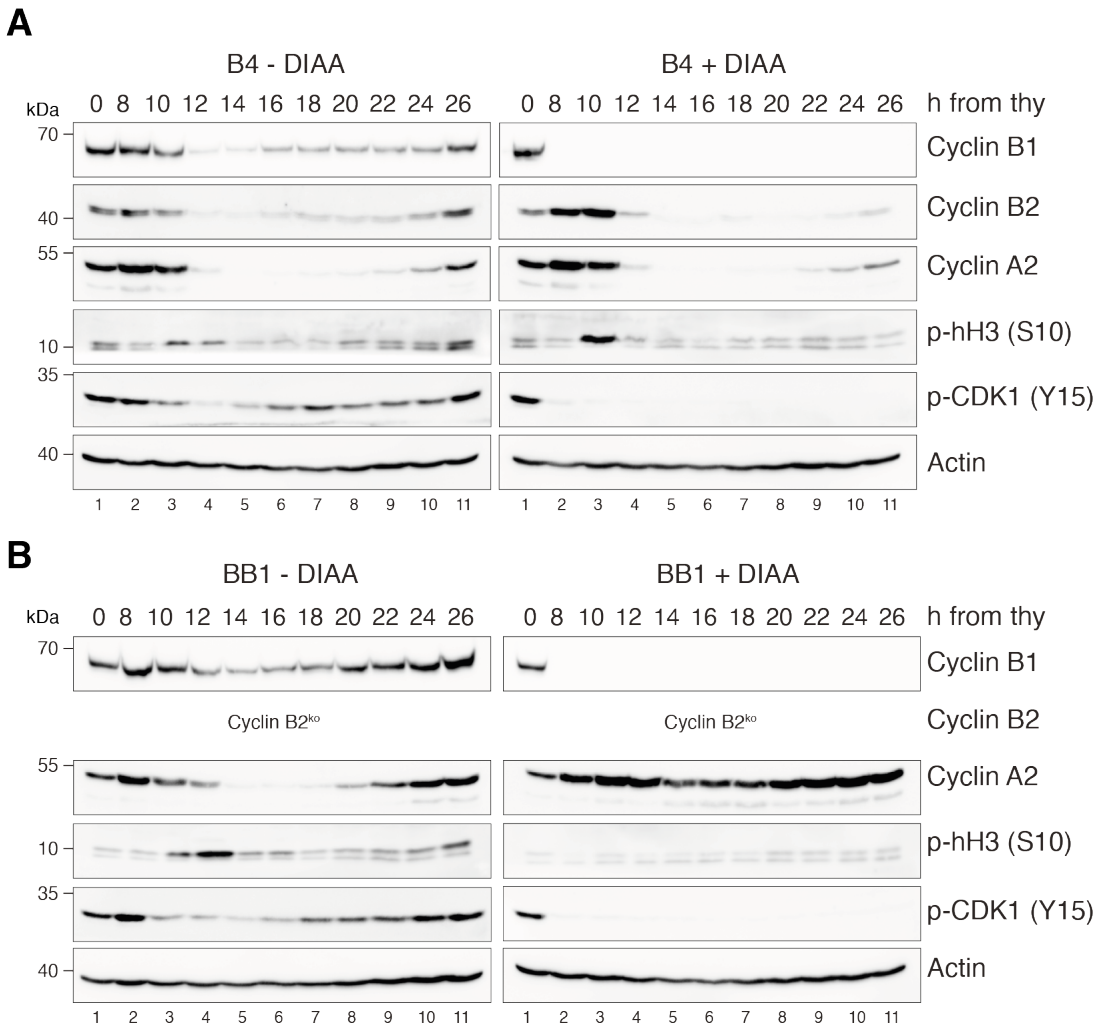

Figure 7.9: Immunoblotting analyses of thymidine-released cells. A: Western blots of thymidine released B4 cells treated or not with DIAA. The blot exposure was adjusted according to the 0 h time-point as it is the same in both conditions. B: Western blots as in A of BB1 cells. The membranes were probed with the indicated antibodies. Actin is the loading control, already shown in a previous experiment with the same samples (Figure 7.5).

7.5.2.3 Analyses of phosphorylated CDK1 substrates

The same lysates were next analysed as above with a phospho-antibody detecting the phosphorylated TPXK motif. These residues are typically phosphorylated by several CDKs [432, 433], but the phosphorylations are found to be highest during mitosis, as shown previously in this thesis (see Chapter 5, Section 5.5.3).

Figure 7.10 A shows that B4 cells, irrespective of DIAA treatment, accumulate and deplete these phosphorylations at timings that correspond to mitosis, and mitotic exit respectively, as observed in Figure 7.9 A. Similarly, BB1 cells untreated with the DIAA cocktail also exhibit a similar substrate phosphorylation signature on the immunoblot (Figure 7.10 left panel).

Cells lacking cyclin B are missing some key mitotic substrate phosphorylations Analyses of DIAA-treated BB1 cells showed that these cells accumulate and maintain the majority mitotic substrate phosphorylations for the duration of this experiment, but are missing some specific sites (Figure 7.10 B, right panel).

It would seem that these cells maintain a significant amount of mitotic CDK substrate phosphorylations, which is distinctly higher than what is typical for a G_2 stage (8 h post-thymidine release, analyses not shown). However, specific bands appear to be missing at approximately 34 kDa and 50 kDa. This stage is termed here as a prophase-like stage, owing to some key similarities that will be shown in this Chapter. Immunoblotting analyses of RPE-1 cells lacking cyclin B did not detect specific changes of mitotic CDK substrate phosphorylations (see Chapter 5, Section 5.5.3), although these were implied with phospho-proteomic analyses. The above data agree with the previous data suggesting cyclin B may control a specific subset of mitotic substrates in human cell lines, thereby promoting separate mitotic events.

Next, the activity of other mitotic players will be assessed with similar immunoblotting analyses.

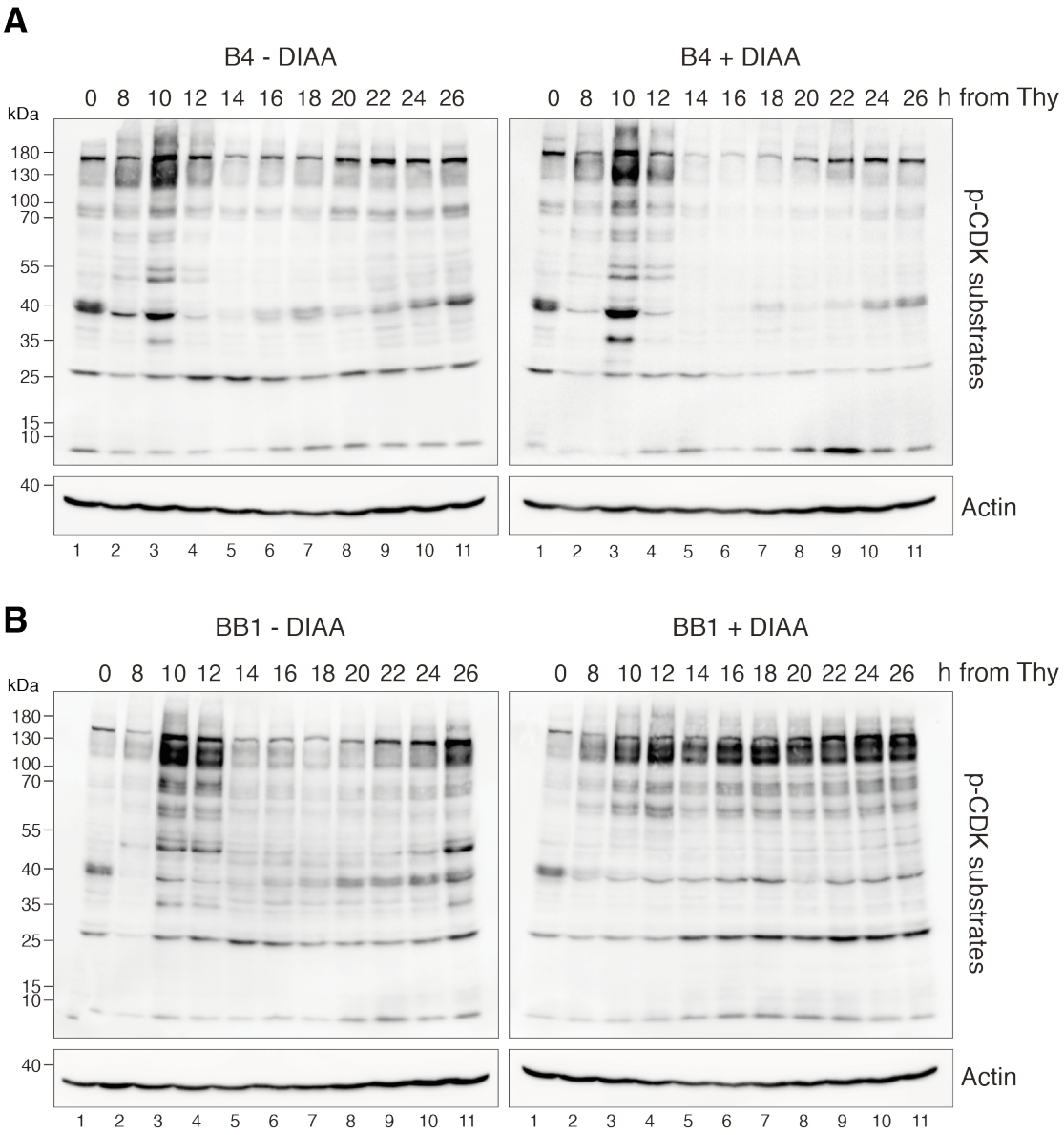

Figure 7.10: Immunoblotting analyses of phosphorylated CDK substrates of thymidine-released cells. A: Western blots of thymidine released B4 cells treated or not with DIAA. The blot exposure was adjusted according to the 0 h time-point as it is the same in both conditions. B: Western blots as in A of BB1 cells. All westerns shown here were probed with a p-TPXK phospho-antibody. Actin is the loading control, same as previously in Figure 7.5 as they were the same lysates.

7.5.2.4 Western blot analyses of key players in mitotic entry

One of the defining characteristics of this novel prophase-like stage is the significant duration of a mitotic-like stalling. To assess the mitotic players throughout this arrest, immunoblotting analyses were again utilised to analyse cells released from thymidine for 8 – 26 h, as previously described. Here, I focused only on the BB1 clone, as the BB1 – DIAA is a sufficient control for cell cycle progression, according to previous analyses (Figure 7.10 B).

Aurora kinases are not strongly phosphorylated when cells lack cyclin B (Figure 7.11). However, the immunoblots that will be shown in later parts of this Chapter consistently show the existence of p-AURKA in BB1 cells treated with DIAA (Figure 7.18, Figure 7.22 B, Figure 7.27 A), presumably due to a high amount of background, also conferred by the instability of this specific antibody, this was likely undetectable in this blot.

Another kinase thought to contribute to mitotic entry and progression, the polo-like kinase 1 (PLK1) is active in prophase-like arrested cells, as judged by its activatory phosphorylation on T210 [347], as well as the phosphorylation status of PLK1's direct substrate, TCTP, on S46 [434, 435].

The above data support that some mitotic events are triggered in the prophase-like BB1 cells. Previous descriptions of antephase, a stage between late G_2 and early M, identified two markers by which this phase could be distinguished: centrosome separation and APC/C^{CDH1} activation with subsequent degradation of D-box containing proteins, including cyclins [425]. Centrosome separation was addressed separately, but did not provide any conclusive information (see Section 7.5.1.2), so I next looked into the activity of APC/C.

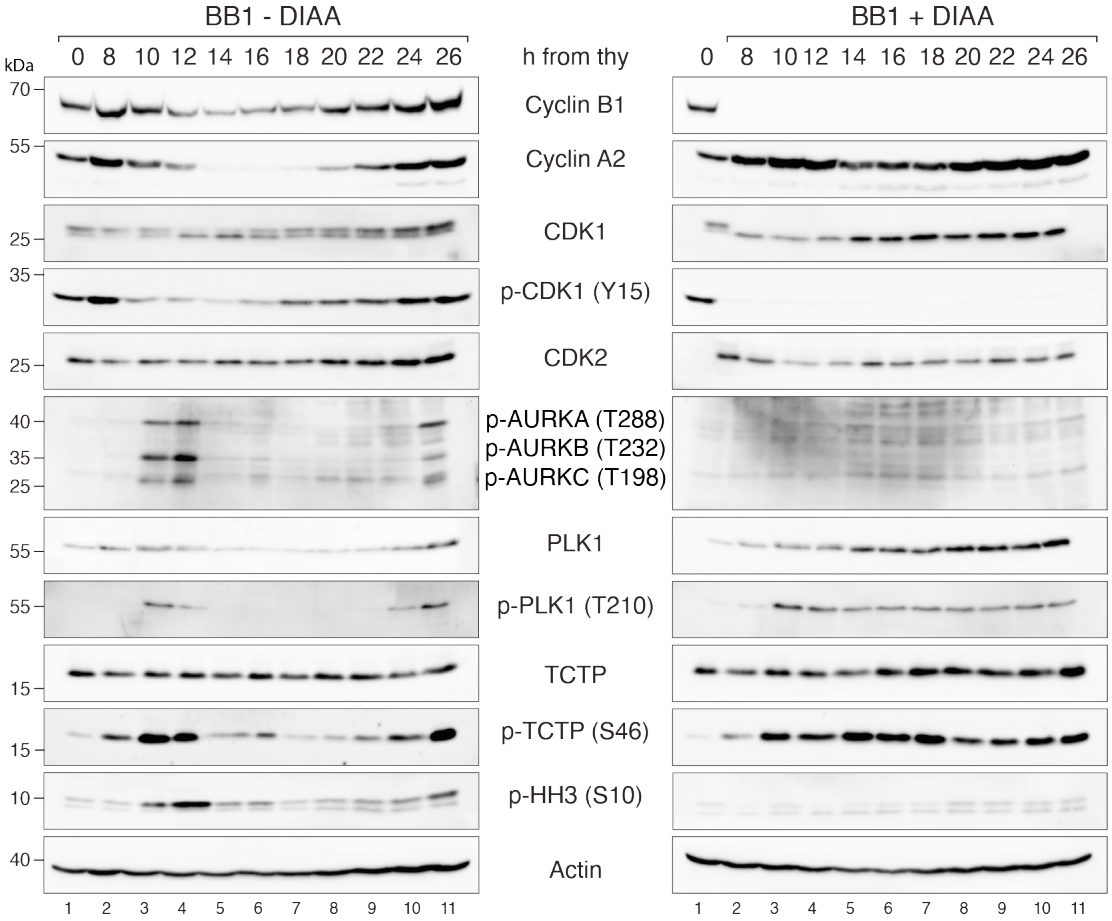

Figure 7.11: Immunoblotting analyses of key mitotic players in thymidine-released BB1 cells. Cells were synchronised with a double thymidine block and collected as previously described. The membranes were probed with the indicated antibodies. Actin is the loading control same as previously in Figure 7.5 as they were the same lysates. Cyclin B1 and cyclin A2 are also same as previously, Figure 7.9, shown here for clarity.

7.5.3 The activity of APC/C in prophase-like cells

7.5.3.1 Background information

The anaphase-promoting complex / cyclosome (APC/C) ubiquitin ligase is inactivated prior to S phase and re-activated during prometaphase and metaphase as reviewed in the Introduction part of this thesis (Chapter 2, Section 2.2.1.2). EMI1 is an APC/C inhibitor that becomes degraded in early mitosis, thereby allowing APC/C's activation [160] and Feringa et al. have demonstrated that EMI1 is degraded during antephase and could serve as a marker of this stage [425].

Immunoblotting analyses have consistently shown that cyclin A2 does not become degraded in cells lacking cyclin B, so EMI1's levels were assessed next. The western blot analyses showed that EMI1 is indeed degraded (Figure 7.12), questioning why cyclin A2 could not be targeted for degradation. There are two possible explanations for this: (i) APC/C is inactive regardless of EMI1 degradation, or (ii) a physical barrier between the two – the nuclear envelope – could interfere with APC/C's access to cyclin A2. It is unclear whether APC/C contains a signal controlling its localisation, but as the complex is only active after NEBD, it seems likely that a nuclear localisation signal is redundant. Besides, the nuclear envelope becomes increasingly porous, thereby allowing the proteins to pass freely before the actual NEBD (reviewed in [427]). Previous analyses using an mRFP-NLS indicated that the nuclear envelope is not increasingly permeable in arrested cells (see Section 7.5.1.4). It is unclear whether the nuclear envelope could indeed interfere with APC/C's activity.

7.5.3.2 Assessing APC/C's activity with immunoblotting

EMI1 degradation and CDC27 phosphorylation status point to an active APC/C The degradation of EMI1 is the first step in APC/C's activation and it does not confirm that APC/C is active. APC/C has to bind with its coactivators, either CDC20 or CDH1, that recognise D-box motifs (reviewed in [117] and in Section 2.2.1.2). APC/C's activity is also accompanied by its core subunit phosphorylation, CDC27, which is achieved by CDK1 [436]. Western blot analyses of CDC27 show a shift of the top band, which indicates the increase of p-CDC27, as seen in BB1 – DIAA, lanes 5, 6, and 7. The times of high phosphorylation coincide with the degradation of cyclins A2 and B1, further

confirming it as a potent marker for APC/C's activity. Interestingly, BB1 + DIAA cells also have an observable shift of this signal, which would imply that APC/C is active (lanes 5, 6, and 7), at least by this measurement.

MCC components imply an inactive APC/C Next. the presence of BUBR1, a component of the mitotic checkpoint complex (MCC), the effector of the spindle assembly checkpoint (SAC) (reviewed in [118]), was analysed. BUBR1 binds to CDC20's degron-binding sites, thus interfering with its substrate recognition [117]. BUBR1 is in turn degraded by the APC/C^{CDC20} once the SAC has been satisfied [437]. While BUBR1's levels are slightly decreased in BB1 – DIAA cells, as observed by the change in signal in 10 and 12 h post-thymidine release, this does not occur in BB1 + DIAA treated cells. The presence of BUBR1 indicates that APC/C could not be active as CDC20 substrate recognition is blocked.

Given that APC/C's activity was implied by two markers, further work sought out to investigate whether the nuclear envelope is a physical barrier that is protecting the predominantly nuclear cyclin A2 from degradation.

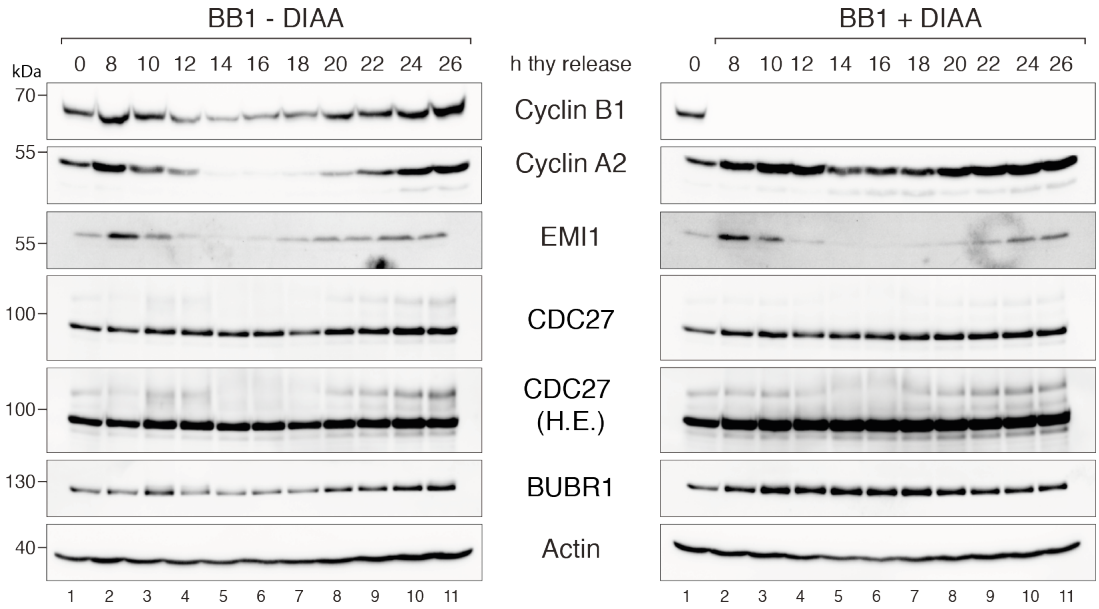

Figure 7.12: Western blot analyses of some APC/C and MCC components. Cells were released from a double thymidine block and collected at the indicated time-points. The membranes were probed with the indicated antibodies. Actin is the loading control, same as previously in Figure 7.5 as they were the same lysates. Cyclin B1 and cyclin A2 are same as in Figure 7.9, shown here for clarity.

7.5.3.3 Cyclin A2 is present in both the nucleus and the cytoplasm, and not degraded

Recent reports have shown that cyclin A2 has separate functions in the cytoplasm at the onset of mitosis [88], so I theorised that if APC/C is active only in the cytoplasm in these cells, only nuclear localisation of cyclin A2 could be observed.

Cyclin A2 is not degraded anywhere in the cell Figure 7.13 shows that the anti cyclin A2 antibody is specific to this protein as judged by the lack of signal in the cyclin A2 channel in cells that have recently divided in B4 + DIAA panels, as observable by the α-tubulin's localisation to the cellular midbody.

Cyclin A2 is present in the nucleus and cytoplasm of BB1 + DIAA treated cells, as compared with the overlay where the green signal clearly extends past DAPI fluorescence. This implied that APC/C is not active, despite EMI1's degradation and p-CDC27 status.

To ensure that APC/C is inactive, these cells were further analysed with live-cell imaging analyses of a newly established APC/C reporter cell line.

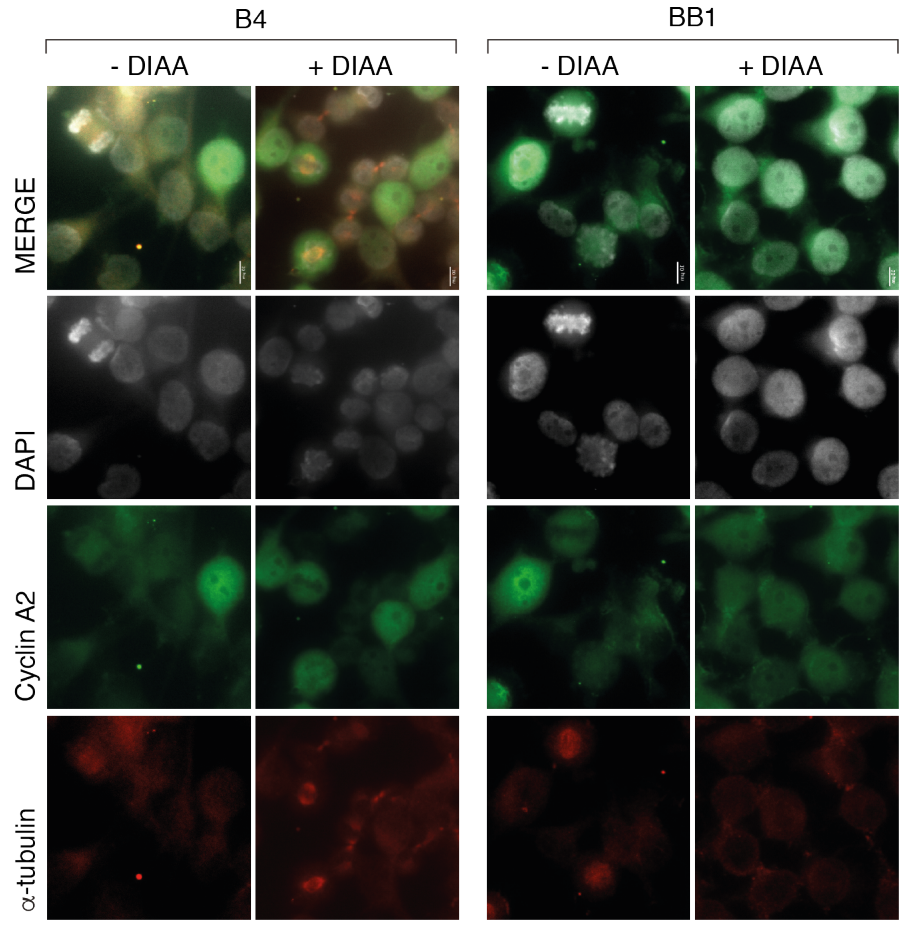

Figure 7.13: Cyclin A2 is localised to the nucleus of arrested cells. Cells were released from thymidine for 11 h and subject to immunofluorescence analyses. The immunofluorescence images show α-tubulin in red, cyclin A2 in green, and DAPI in gray. Scale bar indicates 10 μm.

7.5.3.4 APC/C reporter

Cyclin B1's D-box motif was isolated and fused with an mRFP signal (provided by Dr. Ken H. T. Ma), thereby generating an APC/C reporter by detecting the mRFP fluorescence in cells (hereafter referred to as APC/C reporter-mRFP). The mix population cell line was established by Dr. Ken H. T. Ma using retroviral transfection (see Chapter 3 Materials and Methods).

The mRFP signal disappears when APC/C becomes activated as shown in asynchronous cells stably expressing H2B-GFP and the APC/C reporter by live-cell imaging analyses in Figure 7.14, top panels. Simultaneously, cells were pre-treated with DIAA and imaged, with the treated cells shown on the bottom panels. Cells without cyclin B eventually accumulate in the prophase-like stage, as observed by cell rounding in the later time-points. Cells were imaged for up to 17 h but APC/C reporter-mRFP signal degradation was not observable, which further confirmed that the ubiquitin ligase is inactive.

Cells do not have uniform levels of the GFP and mRFP fluorescence as they were established as a mix population and not isolated as single clones (see Chapter 3 Materials and Methods). Regardless of their level of mRFP, towards the end of imaging, an accumulation of mRFP signal is observed in all cells expressing mRFP.

The activity that promotes and maintains the CDK substrate phosphorylations in prophase-like cells was analysed in the following Section.

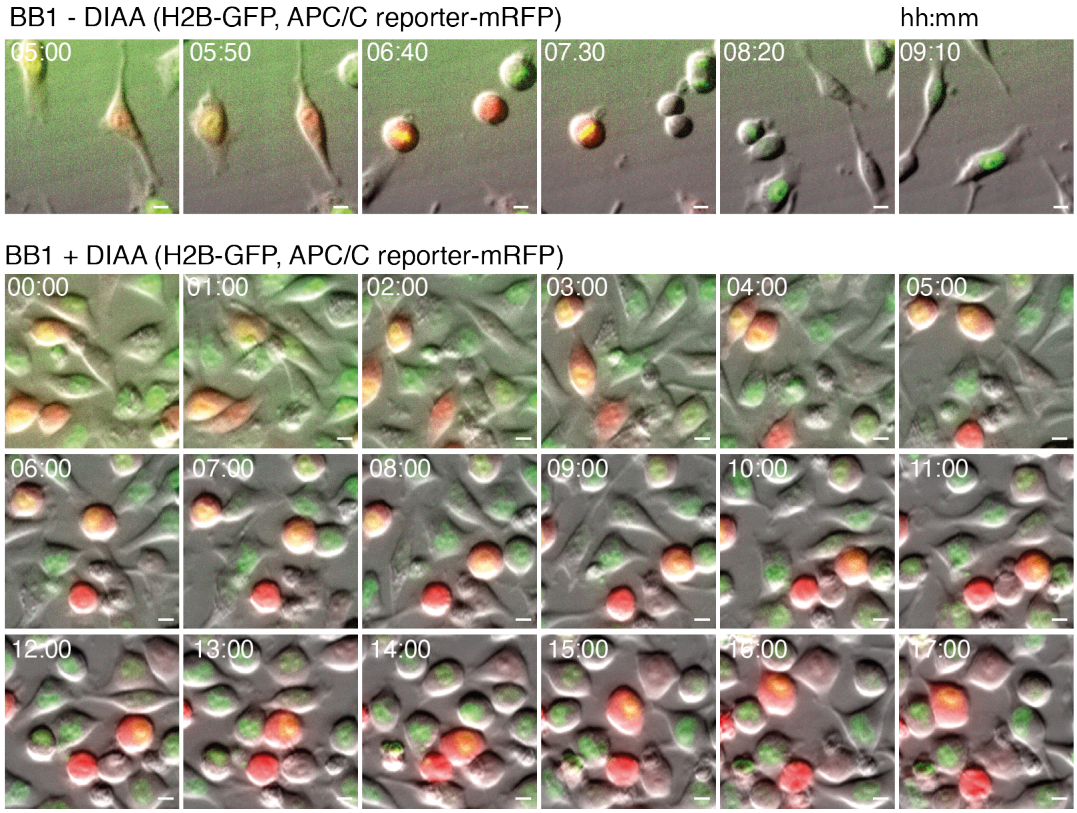

Figure 7.14: Live-cell imaging analyses demonstrating the potency of the APC/C reporter-mRFP to detect APC/C's activity. Asynchronous BB1 cells expressing H2B-GFP and the APC/C reporter-mRFP (cells were established as a mix population – different levels of both H2B-GFP and APC/C reporter-mRFP expression) were pre-treated with DIAA and subject to live-cell imaging. Scale bar indicates 10 μm. Snapshots show merged phase contrast, H2B-GFP in green, and APC/C reporter-mRFP in red. Time-points are as indicated in hh:mm.

7.6 Cyclin A2 is responsible for the establishment of the prophase-like stage

7.6.1 Analyses of CDK1 complexes in cells lacking either cyclin B2 and / or cyclin B1

The next goal was to determine the source of the majority of CDK substrate phosphorylations. Firstly, I analysed the role of CDK1 in these cells and found that it is essential for the maintenance of the prophase-like stage. Secondly, the CDK binding partner required for the establishment of observed mitotic substrate phosphorylations (Figure 7.10) was also undetermined and cyclin A2 seemed like the obvious candidate for this role owing to it's previously established roles in mitotic entry of RPE-1 cells (see Chapters 4 and 6).

7.6.1.1 CDK1 is essential to maintain CDK substrate phosphorylations during the prophase-like stage

Cells were treated or not with DIAA for 24 h, followed by treatment with a specific CDK inhibitor, RO3306. RO3306 targets CDK1 specifically at low dosage, but off-target effects to other CDKs have been reported, such as CDK2 and CDK4/6, leaving the possibility of inhibition of untargeted CDK proteins [438].

Using a low dose of 10 μM, which has been routinely used in the Poon lab and is thought to mostly target CDK1, I observed that BB1 cells, treated or not with DIAA, lose all of their p-TPXK motif CDK substrate phosphorylations (Figure 7.15). Interestingly, cells treated with DIAA depleted the levels of cyclin A2 throughout the time of RO3306 treatment, implying that CDK1 (and/or CDK2) are necessary to stop the activation of APC/C in cells arrested in the prophase-like stage. Future analyses call for additional investigation of APC/C targets in this condition to determine whether these cells exited the cell cycle into the following G0 or backwards into G2. The cells untreated with DIAA appear to arrest in G2, as is common for RO3306 treatment.

Additional proteins that were found to be maintained during the prophase-like stage were also dephosphorylated, including PLK1 and lamin A/C.

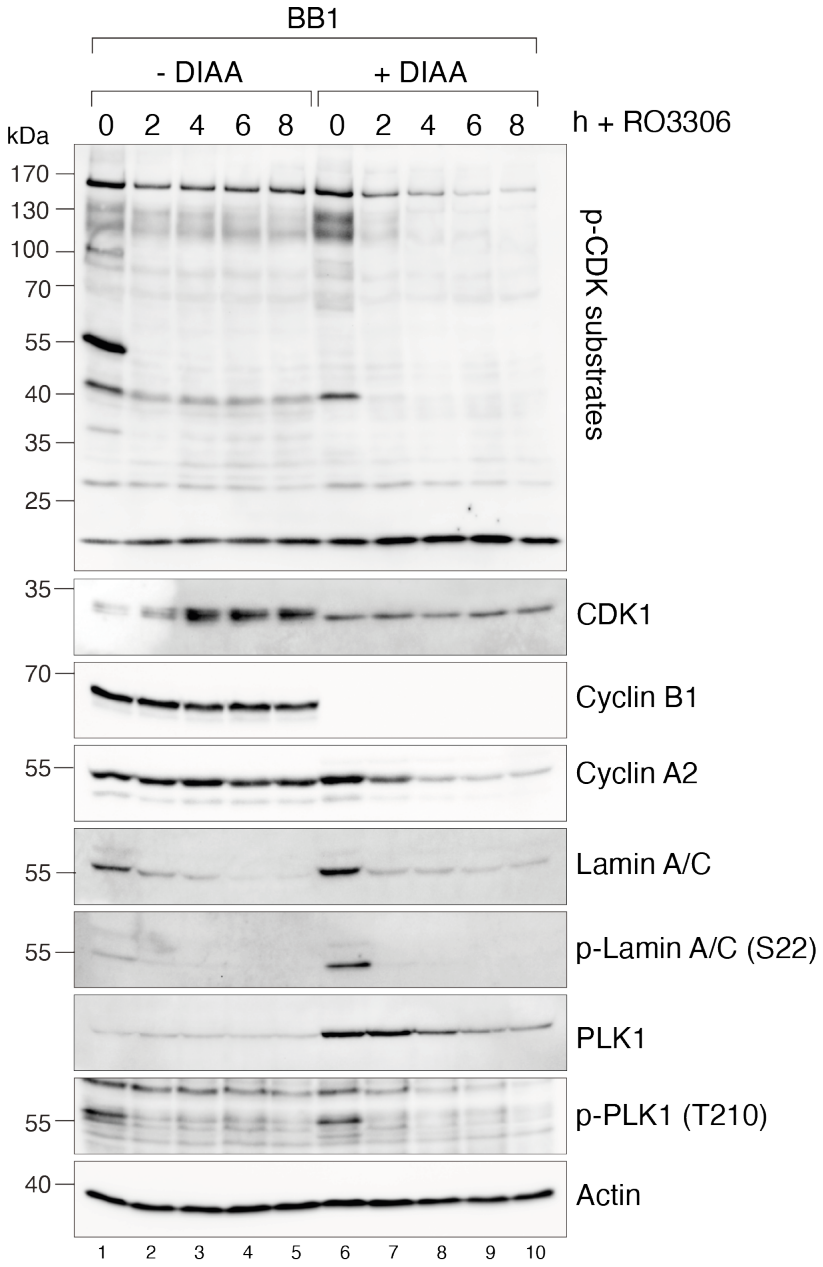

Figure 7.15: Immunoblotting analyses of CDK1 inhibition in BB1 cells. Western blot analyses of cells pre-treated with DIAA or not for 24 h, followed by addition of RO3306 for 2 – 8 h. Immunoblots show p-CDK substrate phosphorylations as detected with the p-TPXK CDK substrate motif antibody that was used previously in Figure 7.10. Other targets are as indicated next to the immunoblots. Actin is the loading control.

7.6.1.2 Cyclin A2, but not cyclin E1, binds with CDK1

Amounts of CDK1-cyclin complexes were analysed in the three cell lines that were established at the start of this Chapter. Figure 7.16 A shows immunoprecipitation analyses of $B2^{ko}$, B4 and BB1 cells. All cells were released from a double thymidine block for 8 h and thus analysed in their last common point, G_2, as the cell cycle progression to that point is similar, as judged by the immunoblotting analyses of the p-CDK substrates (Figure 7.10). Cyclin E is undetectable in the pull-down of CDK1, whereas cyclin A2 apparently increases to almost double only in B4 cells treated with DIAA. A similar observation was made in Figure 7.16 B, where more time-points following a thymidine release of B4 and BB1 were analysed. This property of B4 cells will be described in more detail below.

BB1 cells do not have a marked increase of CDK1-cyclin A2 in G_2, 8 h post-thy release (Figure 7.16 A and B), however analyses of later time-points show an accumulation of this complex. These data confirmed the presence of CDK1-cyclin A2 in both B4 and BB1 cells, so the importance of cyclin A2 in these cells was investigated next.

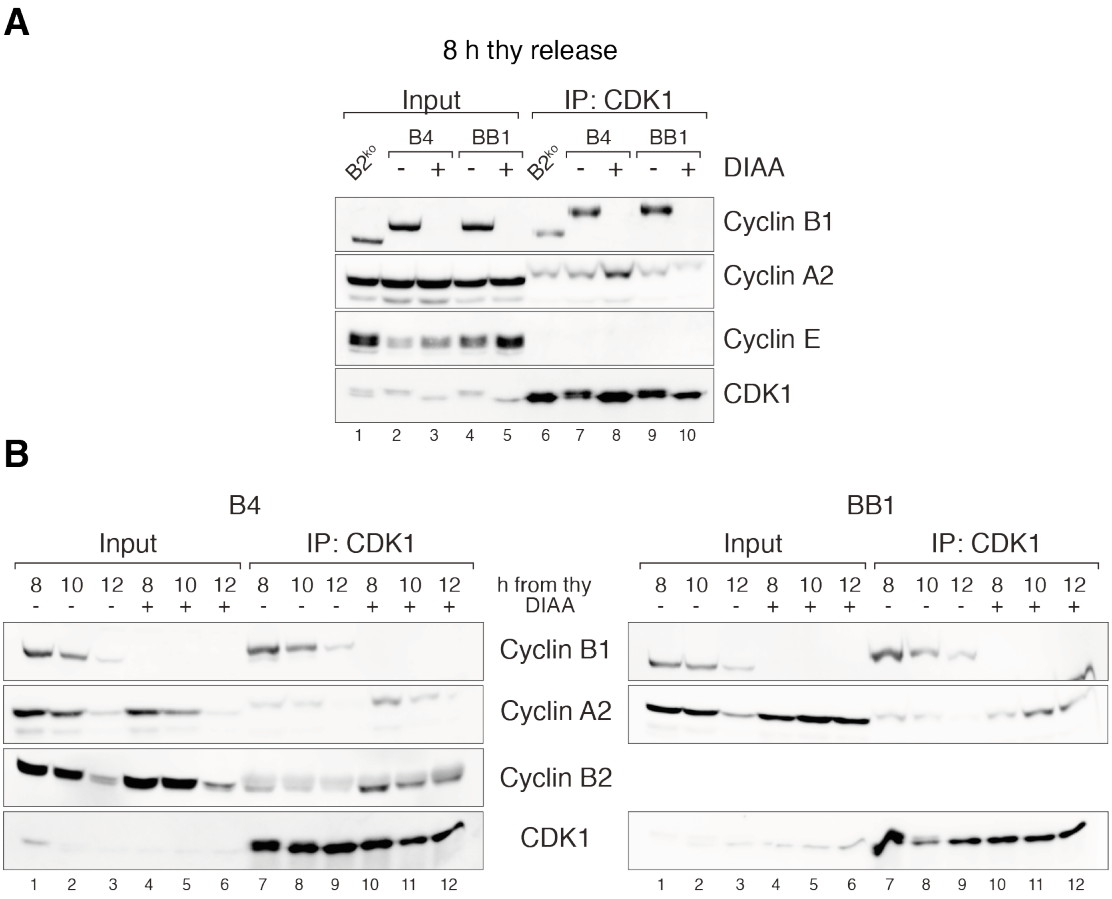

Figure 7.16: Immunoblotting analyses of thymidine released cells. A: Western blots of immunoprecipitates. Cells were released from a double thymidine block for 8 h, treated or not with DIAA. B: Same as A, but more time-points included. The membranes were probed with the indicated antibodies. CDK1 or CDK2 are controls for the pull-down efficiency. Cyclin B2 is knocked out in BB1 cells, thus no blot is shown here.

7.6.2 Cyclin A2 promotes the establishment of a prophase-like stage in HeLa cells

Experimental outline Here, siRNA analyses were used to determine whether cyclin A2 contributed to the existing CDK1 substrate phosphorylations. Cells were transfected with an siRNA against cyclin A2 (hereafter siCCNA2, in the interest of space in Figures), followed by a double thymidine synchronisation. They were then subjected to immunoblotting analyses, similar as above. Only the first mitosis after the thymidine release is investigated here, so cells were harvested at 8, 10, and 12 h post-thymidine release. Western blot analyses showed that siCCNA2 is efficient in cyclin A2 protein depletion (Figure 7.17 A, panels of blotting for cyclin A2).

7.6.2.1 Cyclin A2 is responsible for the majority of CDK substrate phosphorylations in the absence of cyclin B1

B4 cells untreated with DIAA have a normal p-CDK1 substrate signature, regardless of cyclin A2's depletion. Interestingly, depleting cyclin A2 leads to them accumulating these phosphorylations slightly earlier than expected, but the exit is still comparable. It is not clear why they could trigger these phosphorylations earlier as the loss of cyclin A2 consistently delayed mitotic entry according to Chapter 4 of this thesis. One possible explanation is that these cells have higher levels of cyclin B1 (due to the cyclin B1-mAID overexpression) as compared with wild-type HeLa cells, which could contribute to the rescue of cyclin A2's depletion phenotype.

Depleting both cyclins A2 and B1 resulted in a loss of the majority of CDK1 substrate phosphorylations, implying that cyclins A2 and B2 normally act together to ensure mitotic entry and progression in the absence of cyclin B1. It has not been determined whether mitotic timing is affected in these, and it is possible that they eventually still enter mitosis, beyond the time analysed in this experiment.

Cells without cyclin B and depleted of cyclin A2 cannot accumulate any mitotic substrate phosphorylations BB1 cells were analysed next, and data show that cyclin A2 depletion only affects cells in the absence of cyclin B. BB1 cells treated with DIAA consistently lose the specific bands that were mentioned earlier, at 34 kDa and 50 kDa, but additional depletion of cyclin A2 resulted in a loss of the majority of other mi-

totic substrate phosphorylations as well, at least to levels undetectable by western blotting (Figure 7.17 A).

Cell cycle progression is not affected by cyclin A2's depletion alone

B4 cells Figure 7.17 B shows cell cycle profiles of asynchronous cells transfected with siCCNA2 for 48 h, and treated or not with DIAA for the last 24 h. Cell cycle progression does not seem affected by the loss of cyclin A2, unless combined with the DIAA treatment. Surprisingly, B4 cells transfected with siCCNA2 and treated with DIAA have a somewhat normal cell cycle profile, albeit an increase in a G_2 population is observable. This implied that the cells are delayed in their cell cycle progression or arresting in G_2. These analyses explain the lack of CDK1 substrate phosphorylations observed above. Further analyses of these cells side by side on a western blot show that the signal is again significantly depleted (Figure 7.17 C).

BB1 cells Similar as previously, BB1 cells were subject to FACS and western blotting analyses (Figure 7.17 B, C). The cells consistently arrest in G_2 every time DIAA is added, but siCCNA2 alone or in combination with DIAA does not seem to induce any additional defects. Immunoblotting analyses of asynchronous cells treated with DIAA for 24 h further confirmed the decrease of the majority of CDK substrate phosphorylations detectable with the p-TPXK antibody.

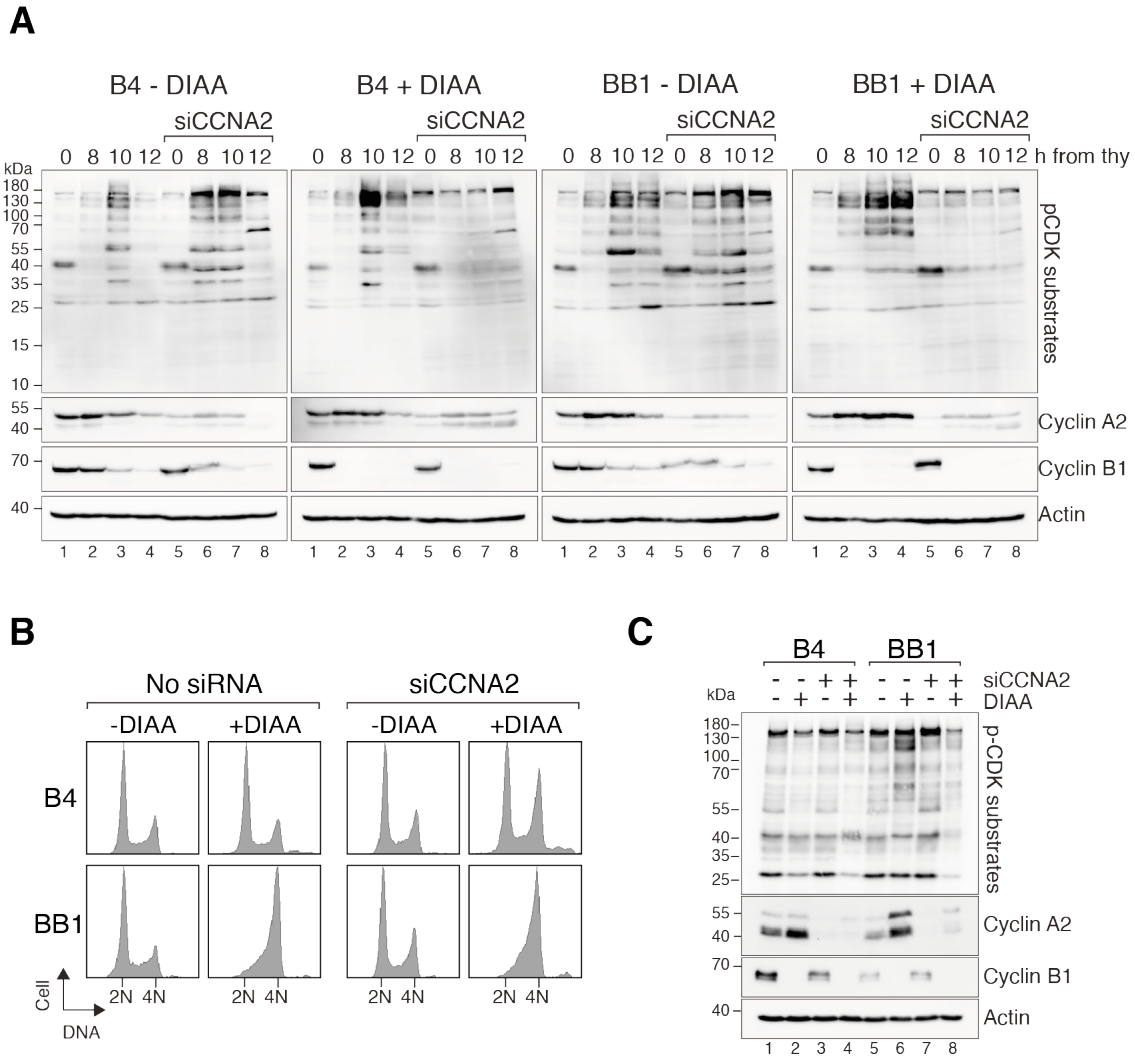

Figure 7.17: Cyclin A2 is responsible for the majority of CDK1 substrate phosphorylations in cells lacking cyclin B1. A: Western blots showing cells untreated with siRNA, or treated with siCCNA2 released from thymidine. Cells were incubated with siCCNA2 for a minimum of 7 h prior to the first thymidine block, and synchronised as previously. B: FACS plots of asynchronous cells treated or not with siCCNA2 for 48 h, and DIAA where indicated for the last 24 h. A minimum of 10,000 cells were analysed for each condition. C: Western blot of cells in B. The membranes were probed with the indicated antibodies, p-CDK substrates is the previously described antibody that detects the phosphorylated TPXK motif. Actin is the loading control.

7.6.3 Additional characterisation of BB1 cells lacking cyclin A2

The effect of cyclin A2's depletion in BB1 cells was investigated further in this Section, to determine its role in cells deficient in cyclin B.

Immunoblotting for key mitotic players thought to be linked with cyclin A2
Western blot analyses showed that PLK1 is present, but inactive in cells lacking cyclins B and depleted of cyclin A2, according to the lack of a key activatory phosphorylation at T210. In agreement with the above, PLK1's substrate TCTP is also unphosphorylated in these. The above data show that cyclin A2 is important for PLK1's activation in HeLa cells as it is clearly present in all other conditions. The pathway that leads to PLK1's activation was assessed next, to determine which steps become interrupted in these cells.

It is currently believed that a CDK1-cyclin complex activates BORA, which then promotes AURKA activity, and that results in PLK1's activation [352, 439]. BORA is apparently phosphorylated, as noted by the shift of the band in all mitotic cells (antibody specificity confirmed in Poon lab, data not shown). As soon as BORA activity is detectable, phosphorylation of aurora kinases is also observable by western blotting. BORA activation apparently occurs even in the absence of cyclin B and when cells are depleted of cyclin A2. As shown previously, cells lacking cyclin B only have AURKA active, but this phosphorylation is undetectable by immunoblotting when cells also lack cyclin A2, indicating that the PLK1-activatory pathway becomes interrupted at this stage.

The observable levels of BORA activation were somewhat surprising, but it is possible that BORA could be a very specific substrate of cyclin A2 and the siRNA mediated depletion may be insufficient to abolish this phosphorylation.

Next, phosphorylation of S10 on histone H3, a marker of chromosome condensation and a substrate of AURKB [431], is not detectable in any cells lacking cyclin B. Cyclin A depletion alone already decreased and delayed this phosphorylation.

The combination of siCCNA2 transfection and DIAA incubation further decreased the levels of phosphorylation for another mitotic marker, lamin A / C, demonstrating that these cells become arrested at an earlier stage that has even less mitotic markers than the prophase-like stage.

Live-cell imaging confirmed a previously observed phenotype of cyclin A2's depletion in HeLa cells Cells were assessed using live-cell imaging analyses, which showed that cells lacking cyclin B do not enter mitosis when also transfected with siCCNA2, as judged by cell rounding in phase contrast (Figure 7.17 B). The frame showing no siRNA + DIAA cells shows the change of cellular morphology of BB1 cells after they have exited from mitosis, but remain in a prophase-like stage.

Figure 7.18 C shows the cumulative mitotic entry of cells shown in panel B. A slight delay in mitotic entry of cells transfected with siCCNA2 was observed, which was initially missed in the immunoblotting analyses, likely due to the lack of detail when analysing cells at 2-hour time-points. Data showing a delay in mitotic entry when cells lack cyclin A2 are concomitant with previous information shown in Chapter 4, Section 4.4.1. The lack of mitotic entry in siCCNA2-transfected BB1 cells additionally confirmed that the CDK substrate phosphorylations, conferred by cyclin A2 in the absence of cyclin B, contribute to the establishment of an intermediate state between G_2 and M, here termed the prophase-like stage.

To determine potent candidates for mitotic progression in the absence of cyclin B, I designed transient transfection experiments that will be the focus of the following Section.

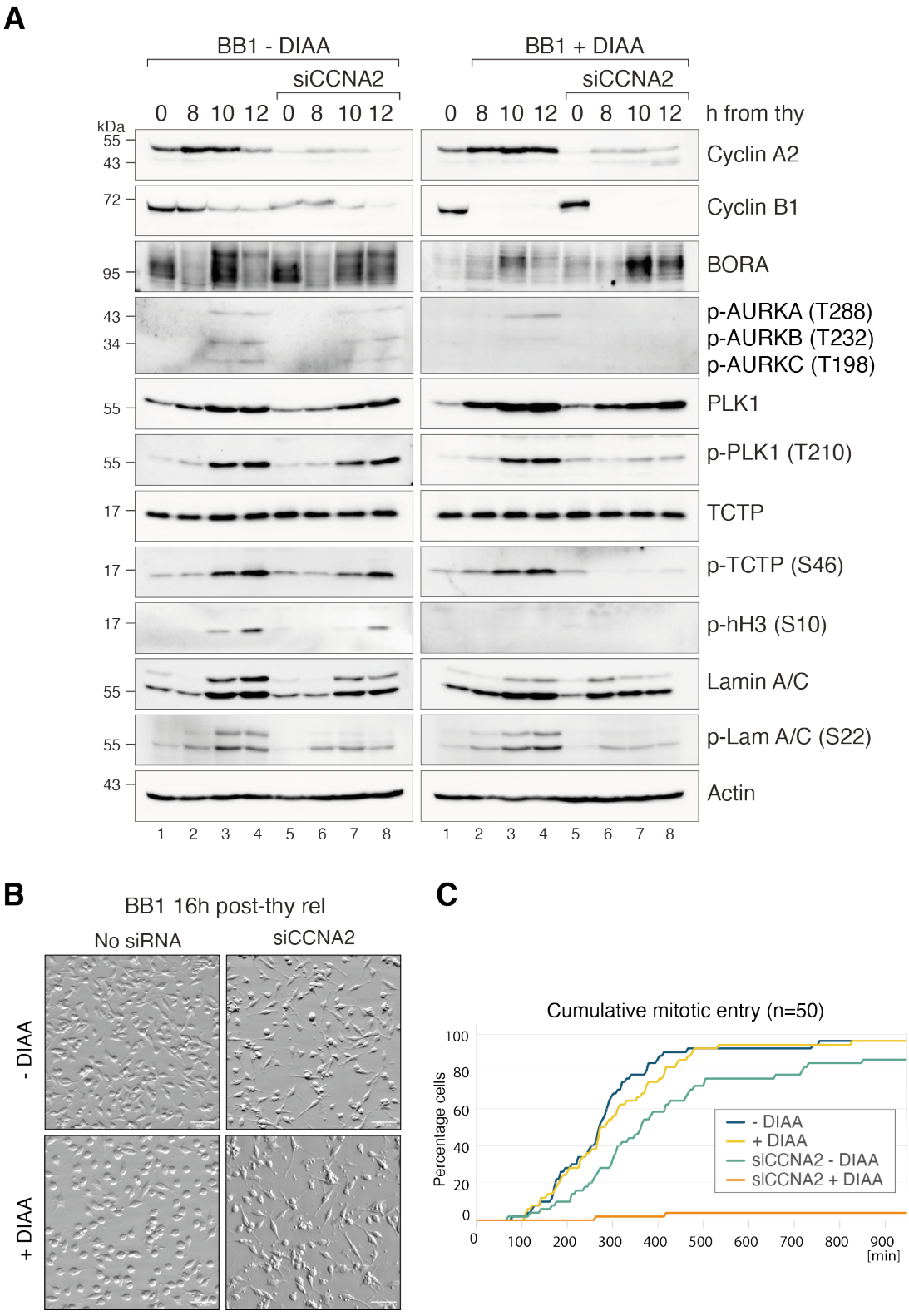

Figure 7.18: Cyclin A2 promotes CDK substrate phosphorylations in the absence of cyclin B1. A: Western blots of the indicated conditions with antibodies as marked on the right. Actin is the loading control, same as in Figure 7.17. B: Snapshots of cells treated as in Figure 7.17 A, subjected to live-cell imaging, 16 h post-thymidine release. Scale bar indicates 25 μm. C: Cumulative mitotic entry of cells treated as in B as judged by cell rounding (n = 50).

7.7 Assessing the roles of other key mitotic players in the presence or absence of cyclin B

7.7.1 Establishing a system for a straightforward analysis of potent candidates that could supplement cyclin B's role in mitotic entry

Background information and experimental design Knock-out studies typically confirm the phenotypes observed after eliminating a protein of interest by supplementing the cells with an exogenous version. In this work, this was done by supplementing the BB1 cells with cyclin B1-mAID which successfully replaced the endogenous version of cyclin B. Next, I aimed to analyse whether other mitotic players such as cyclin A2 and PLK1 could compensate for any roles of cyclin B in mitosis. To quickly determine which candidates are promising for further studies, transient transfections, followed by FACS analyses, were conducted. As a verification of the system's efficacy, plasmids encoding cyclin B1 and cyclin B2 were tested first.

7.7.1.1 Overexpression of cyclin B1 successfully rescues the G_2 arrest and verifies the usability of the transient transfection system for future analyses

Experimental outline Cells were analysed using a transient transfection calcium phosphate method to co-transfect a gene of interest with H2B-GFP (see Chapter 3 Materials and Methods). The transfection reagents were washed off after 16 h and cells were treated with the DIAA cocktail for 24 h (Figure 7.19 A). This amount of time was previously shown to induce a complete G_2 arrest, and the overexpression of a selected protein are also the most efficient in 24 – 48 h post-transfection. The level of overexpression was assessed by western blotting, and the cell cycle profile was analysed with FACS analyses, by separating the GFP-positive and GFP-negative populations (Figure 7.19 B), according to an untransfected control (data not shown).

Testing the transient expression system with cyclin B1's overexpression While the cells expressing only H2B-GFP arrest in G_2, regardless of GFP expression,

cells where cyclin B1 was co-transfected rescue the G_2-arrest phenotype (Figure 7.19 C). What is more, separating GFP-positive and GFP-negative cells allowed the visualisation of the difference between transfected and untransfected populations, confirmed by the rescue observable only in the GFP-positive population.

Transfection efficiency was confirmed by western blotting (Figure 7.19 D). In this particular case, the overexpression of cyclin B1 appears to be barely detectable, but this was attributed to the normal cyclin degradation mechanics in cells, especially as most cells are in G_1, when cyclin B1 is consistently degraded.

On this western blot, a band corresponding to cyclin B1-mAID is seen in + DIAA treated cells. The cause of an impaired cyclin B1-mAID depletion is unclear, especially as the band is not observed in control cells transfected with H2B-GFP. Nevertheless, the following Section showed that residual levels of cyclin B1-mAID still induced the 4N-arrest phenotype (Figure 7.20). Moreover, the FACS plot of GFP-negative cells also serves as an internal control, confirming the efficiency of the DIAA treatment.

Cyclin B1 rescue was additionally confirmed by generating a stable cell line overexpressing cyclin B1-YFP (established by Dr. Ken H. T. Ma) which will be shown after this Section (see Figure 7.24).

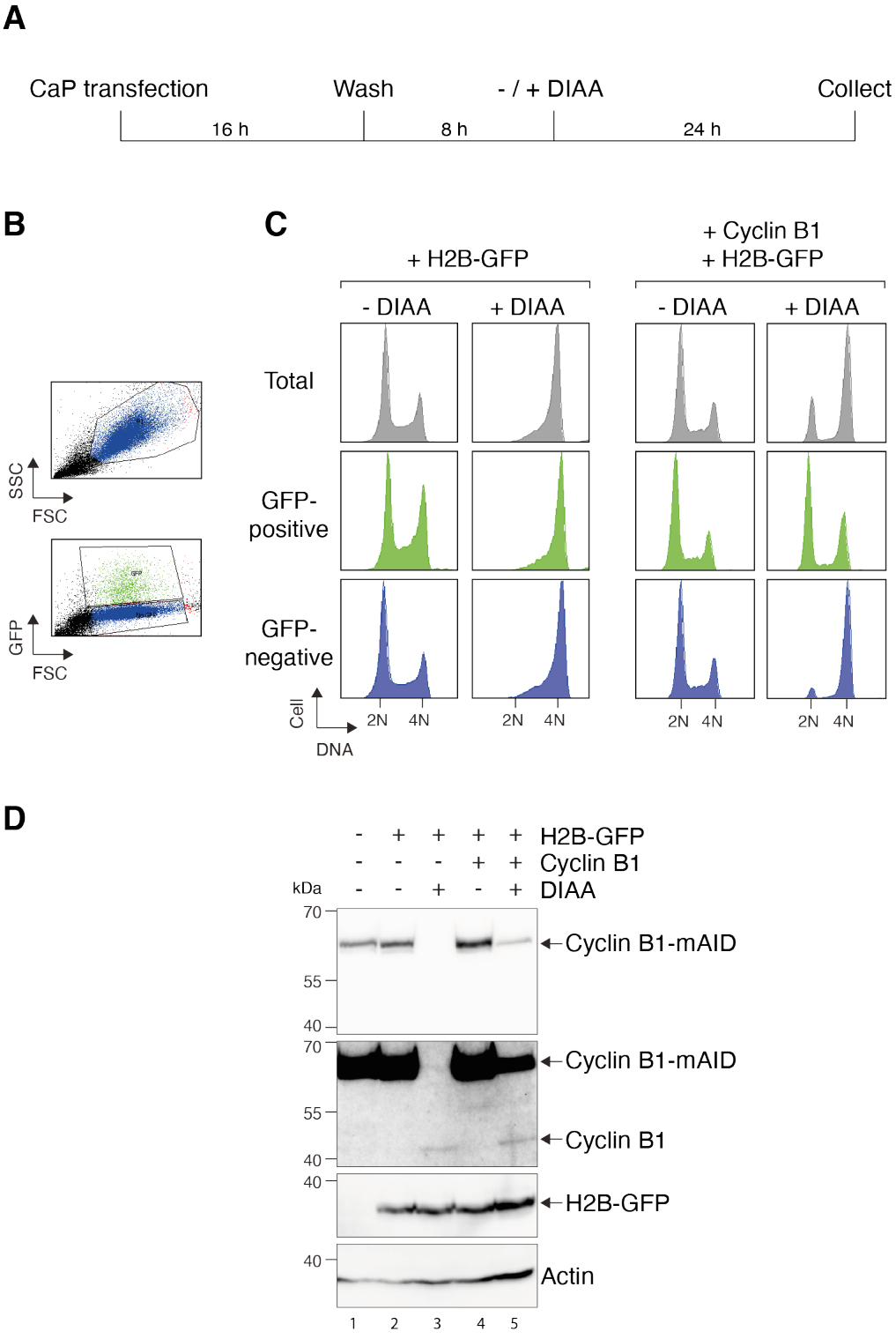

Figure 7.19: See caption on the following page.

Figure 7.19 *(preceding page)*: Verification of a transient transfection system to test the efficiency of mitotic players in rescuing the G_2 arrest. A: An outline of the experimental treatment. B: A FACS plot of GFP on the y-axis and FSC on the x-axis. Cells were first gated for live cells at the time of fixation based on their forward and side scatter. Then, as seen on the bottom plot, GFP-positive cells were gated, shown in green, and GFP-negative in blue. C: FACS-plots of cells treated as in A, analysed as in B. Total amount is the population selected in B, top plot. DIAA-treated cells were gated for their live population, adjusted as mentioned earlier in Section 7.5.1.1. The FACS plots are log-scaled. A minimum of 10,000 cells were analysed for each condition. D: Western blots of cells in C, blots show the protein of interest, GFP and actin. The transfected plasmids are indicated on the top, as well as DIAA treatment. FIX shifted 2N measurement.

Cyclin B2 overexpression promotes cell cycle progression in BB1 cells Cells expressing just cyclin B2 can proliferate somewhat normally during the first few cell cycles. In theory, cyclin B2 overexpression should also lead to the rescue of the initial 4N arrest in BB1 cells.

Notably, cyclin B2 was under a TET-OFF controlled promotor, thus only IAA and not doxycycline could be used instead of the traditional DIAA treatment. Control cells that were transfected with H2B-GFP and treated with IAA alone indeed arrested in G_2, regardless of persisting amounts of cyclin B1-mAID, further supporting the efficacy of this treatment for these types of studies. Cyclin B2's overexpression rescued the G_2-arrest phenotype of the GFP-positive population, but not of the GFP-negative. This assay again outlined the redundancy of cyclins B1 and B2 in short-term analyses.

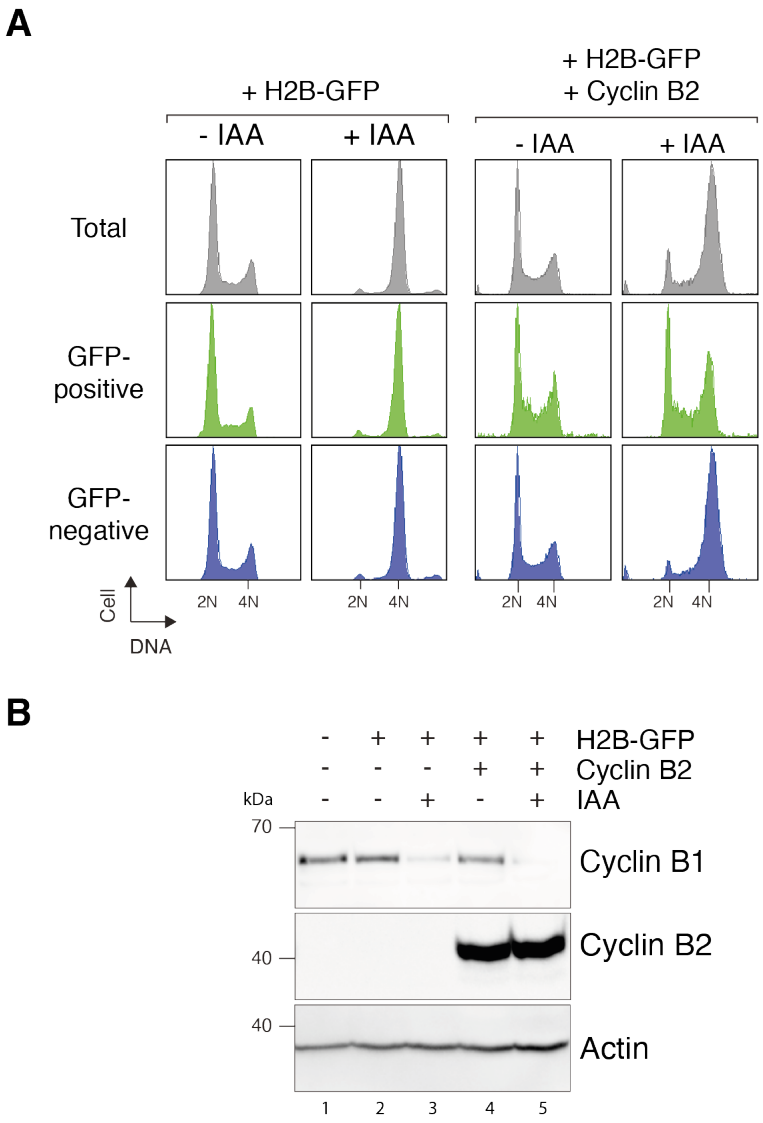

Figure 7.20: Overexpression of cyclin B2 also promotes the rescue of mitotic defects in BB1 cells. A: FACS-plots of cells treated as previously. Total amount is the population that was live at fixation. IAA-treated cells were gated for their live population, adjusted as mentioned earlier in Section 7.5.1.1. FACS-plots of H2B-GFP cells are log-scaled, but the cyclin B2-overexpressing plots are not. A minimum of 10,000 cells were analysed for each condition. B: Western blots of cells in A, showing cyclin B2, cyclin B1, and actin. Plasmids that were transfected, and IAA addition, is shown on top of the western blot.

7.7.1.2 Cyclin A2's overexpression promotes some mitotic events

Cyclin A2 overexpression results in an 8N population Next, the overexpression of cyclin A2 in BB1 cells was assessed. B4 cells are included here as a control since cyclin A2's overexpression could interfere with the normal cell cycle's progression.

Comparing all GFP-positive plots of B4 cells, it is clear that overexpressing cyclin A2 does induce some defects as noted by the population of cells passing the 4N mark (Figure 7.21 A). This could be due to defects in mitotic exit and cytokinesis, or re-replication issues.

However, BB1 cells lacking cyclin B accumulate in an 8N population drastically and this does not compare with the phenotype that the overexpression promoted in B4 cells. The western blotting analyses confirmed the efficiency of cyclin A2's overexpression (Figure 7.21 B). The exogenous cyclin A2 is FLAG-tagged, and is therefore observable slightly higher than the endogenous protein.

These FACS-plots are log-scaled to better visualise the accumulation of cells in 8N. Non-log scaled graphs were also checked, and a persisting amount of cells were present after the 4N population, up to 8N (data not shown).

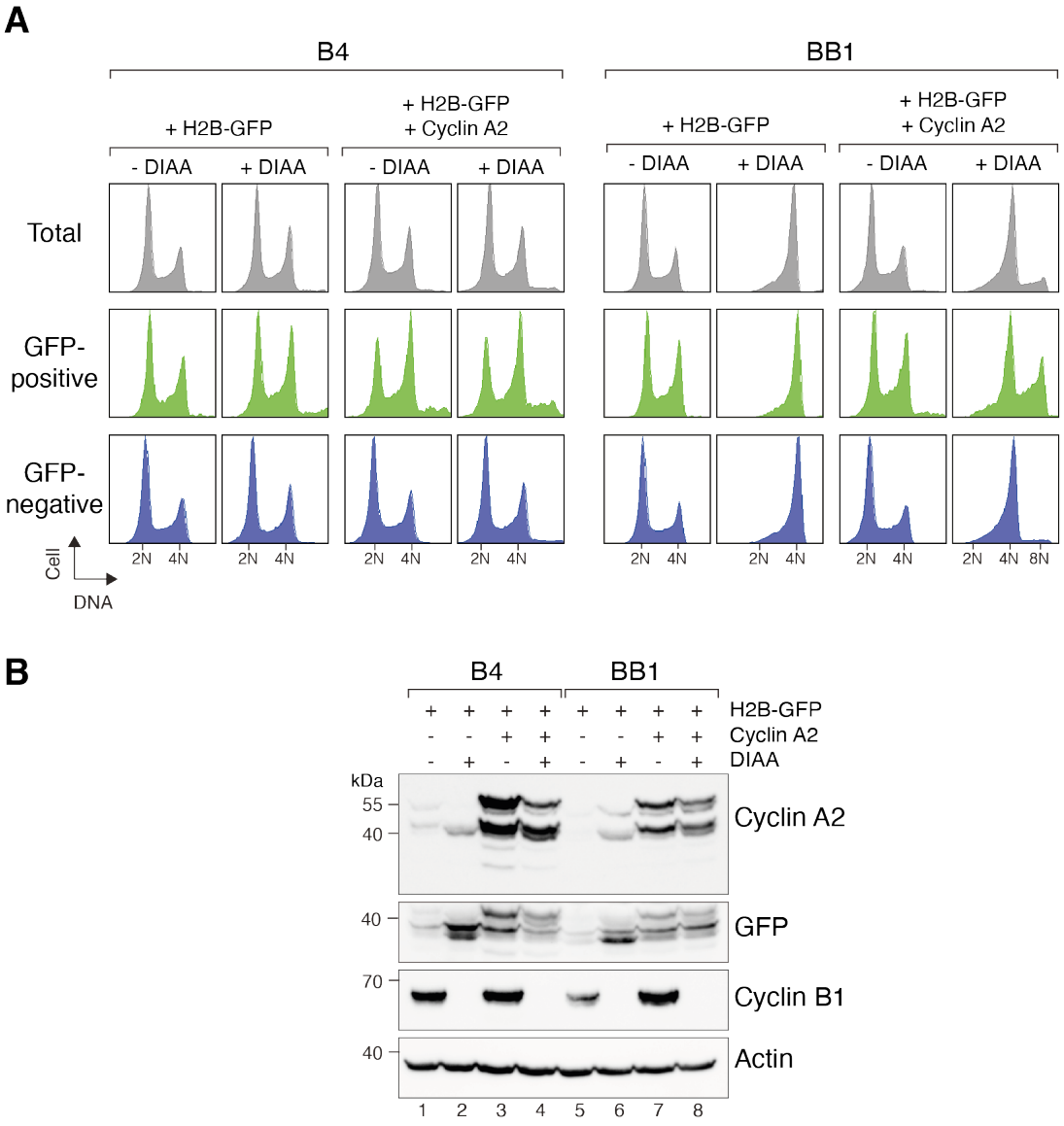

Figure 7.21: Overexpression of cyclin A2 leads to an 8N accumulation in cells lacking both types of cyclin B. A: FACS-plots of cells treated as previously. Total amount is the population that was live at fixation. FACS-plots are log-scaled. A minimum of 10,000 cells were analysed for each condition. B: Western blots of cells in A, showing cyclin A2, GFP, cyclin B1, and actin. Plasmids that were transfected, and DIAA addition, is shown on top of the western blot.

Cyclin A2 overexpression promotes some mitotic CDK1 substrate phosphorylations and activation of other key mitotic markers The characteristics of the 8N population observed in cells lacking cyclin B but overexpressing cyclin A2 were further looked into in this part. Live-cell imaging analyses were conducted, but the transfection stress, in combination with fluorescent microscopy resulted in a difficulty to analyse sufficient amounts of cells to draw valid conclusions. Instead, immunoblotting analyses were conducted with B4 and BB1 cells, but only BB1 is shown below for simplicity.

BB1 cells were synchronised by a double thymidine block, and transiently transfected during the first thymidine release, to maximise the transfection efficiency (Figure 7.22 A). The level of cyclin A2 was also checked to confirm the transfection efficiency of this system (Figure 7.22 B).

Western blots of p-CDK1 substrate antibody demonstrated that the bands at 34 and 50 kDa that are normally completely missing in BB1 + DIAA cells are now observable when cyclin A2 is overexpressed, however they are less prominent, compared with cells that do not overexpress cyclin A2. Moreover, phosphorylated AURKB and a faint band indicating p-AURKC have also appeared in BB1 cells with higher levels of cyclin A2. Concomitant with the rise in p-AURKB, levels of phosphorylated histone H3 are readily observable in these cells.

Together, these data imply that HeLa cells promote more mitotic events with higher CDK activity, regardless of which specific mitotic CDK-binding cyclin is present. Further investigations are required to determine the role of cyclin A2 levels in these cells. Thus, one potent candidate for the rescue of mitotic progression in BB1 cells has been found, and it will be investigated separately after this Section. First, PLK1 was overexpressed to assess its efficacy in promoting mitotic events with this system.

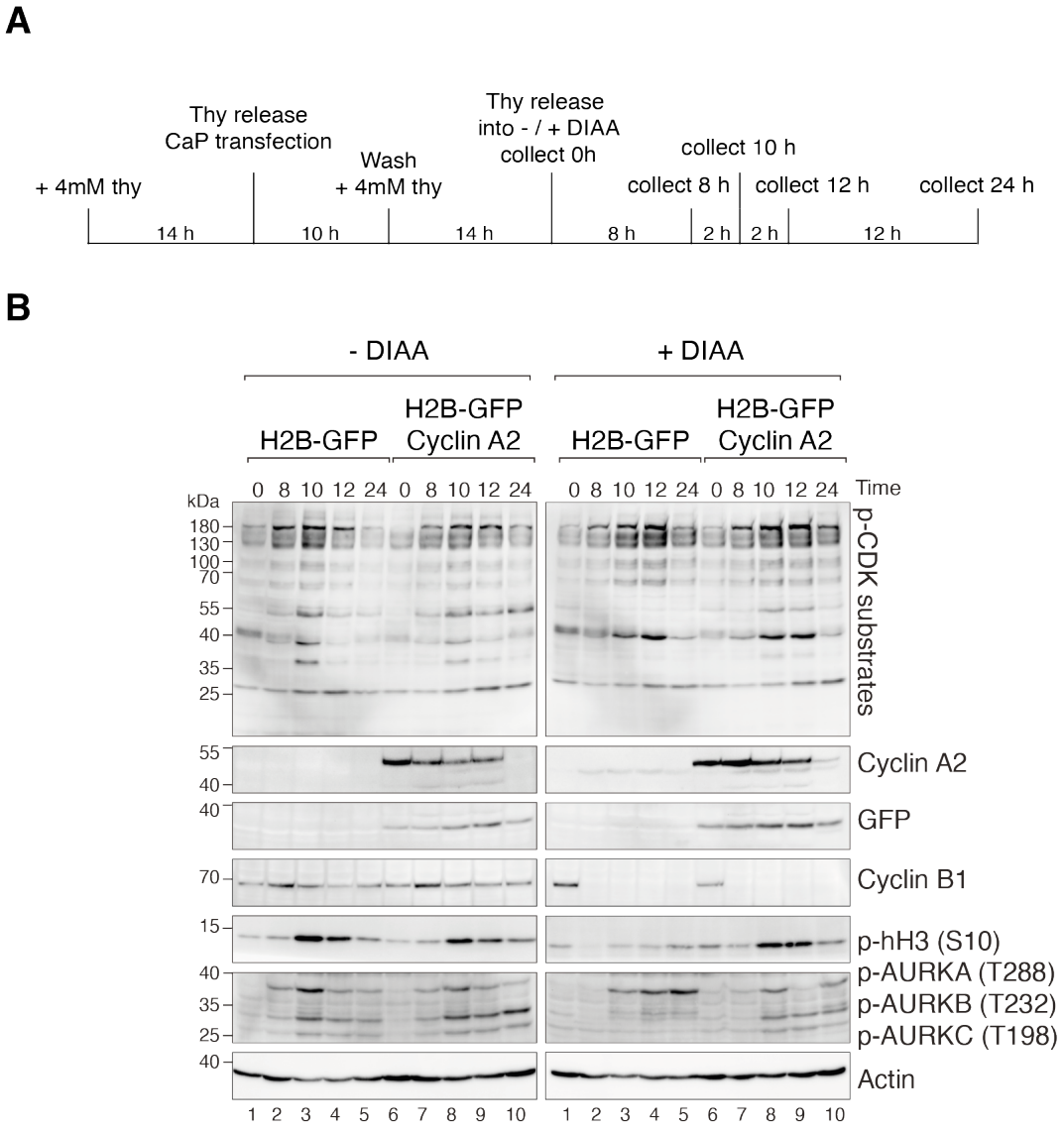

Figure 7.22: Immunoblotting analyses of cells transiently overexpressing cyclin A2. A:Experimental outline used to analyse transiently transfected cells with a thymidine block. Cells were transfected with CaP after the first release from thymidine. The transfection reagents were washed off after 10 h, followed by re-incubation with thymidine. After the release from the second thymidine block, cells were collected at the indicated time-points. B: Immunoblotting analyses of cells treated as in A. Western blots show p-CDK1 substrates (p-TPXK), cyclin A2, cyclin B1, and mitotic markers p-AURK A, B and C, as well as p-histone H3. Actin is the loading control.

7.7.1.3 PLK1 cannot promote mitotic events, regardless of its activity

Even though previous data showed that PLK1 is active regardless of cyclin B's presence, as judged by its phosphorylation on T210 (Figure 7.11), I theorised that a higher level could promote more mitotic events, similar to cyclin A2.

This was not the case as observed by FACS analyses (Figure 7.23 A), even though PLK1 overexpression was successfully achieved, as seen by western blotting. The exogenous protein is myc-tagged, making it clearly distinct from the endogenous PLK1 by immunoblotting analyses (Figure 7.23).

This showed that PLK1 is not a candidate for further investigation.

Future analyses Cyclin A2 was the only candidate identified that promotes mitotic events in cyclin B's absence, so this was investigated further. There is a possibility of other mitotic players promoting these events, however I lacked the time to investigate other potential pathways further. For instance, MASTL overexpression could also promote these events, although PP2A inhibitor analyses implied that the phosphatase activity does not play a significant role in the 4N arrest of BB1 cells.

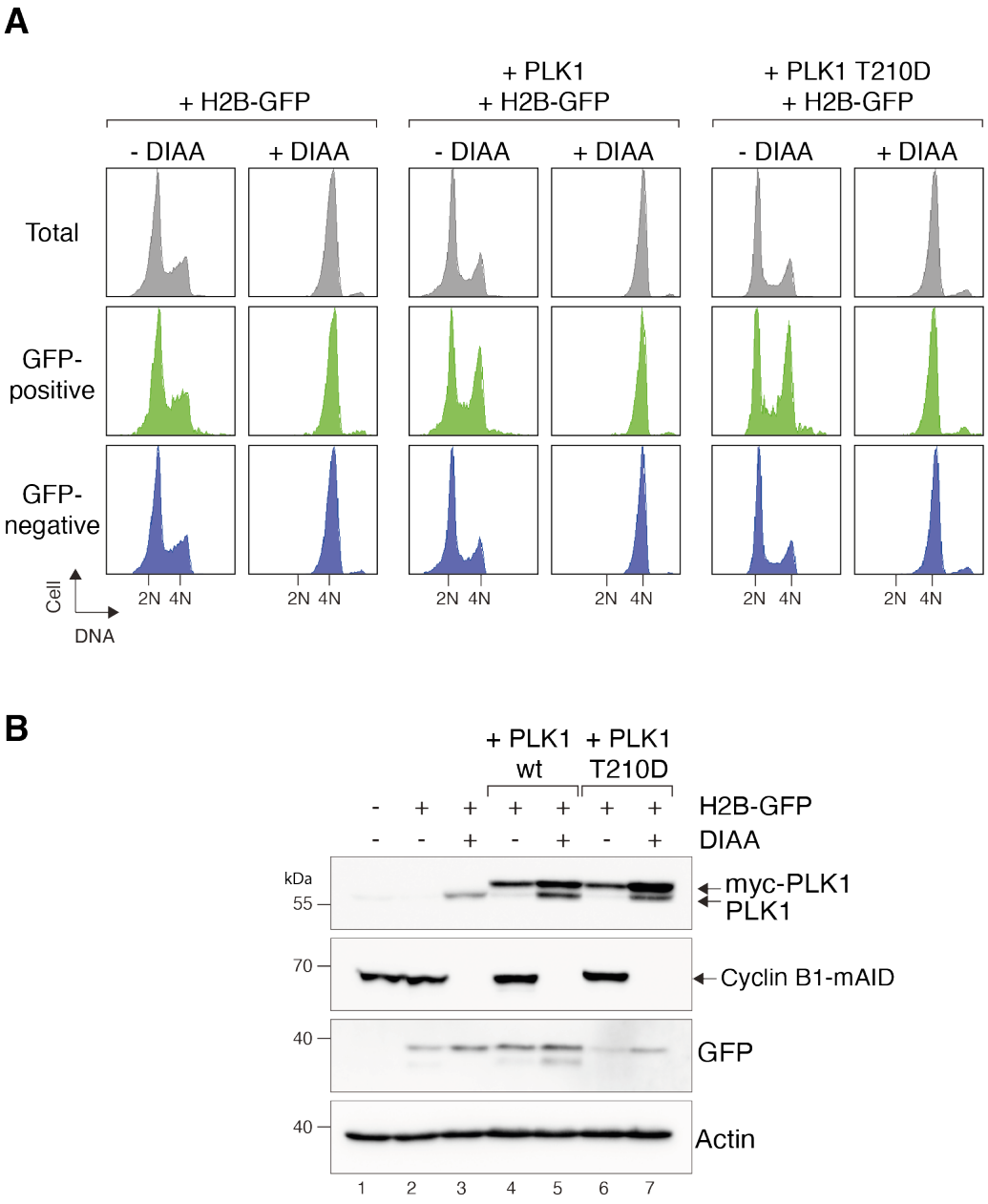

Figure 7.23: Overexpression of PLK1 does not promote mitotic events in BB1 cells. A: FACS-plots of cells treated as previously. Total amount is the population that was live at fixation. FACS-plots are log-scaled. A minimum of 10,000 cells were analysed for each condition. B: Western blots of cells in A, showing PLK1, cyclin B1, and actin as the loading control.

7.8 HeLa cells depend on the increase of CDK activity, but not CDK levels, to progress through separate stages of mitosis

Given the information that cyclin A2's overexpression leads to an accumulation of cells in an 8N population, I theorised that the protein can rescue some, but not all mitotic events. Two things must be considered next: (i) cyclin A2 preferentially binds with CDK2, and the CDK1-cyclin A2 complex accumulates in cells slowly, so it is possible that the overexpressed version lead to more CDK1-cyclin A2 complexes in these cells, and (ii) transient transfections cause additional stress to cells so it is possible this phenotype was also promoted by uncontrollable events.

The first option was addressed by stably overexpressing either CDK1 or CDK2 and assessing cellular proliferation. I hypothesised that even though CDK1 is present in these cells, increased levels could lead to more CDK1 activity, as it could increase the binding to other cyclins present in the cells. In parallel with this, the possibility of CDK2 rescuing the prophase-like arrest was also investigated.

First, similar as in transient transfections, the efficiency of using mix-populations of stable overexpression was tested by establishing mix populations of cells expressing cyclin B1-YFP, and a YFP alone as a control. These cell lines were established using retroviral transfections (see Chapter 3 Materials and Methods) by Dr. Ken H. T. Ma and the depletion efficiency of cyclin B1-mAID is shown in Figure 7.24 A. Live-cell imaging analyses of asynchronous cells confirmed that cyclin B1-YFP overexpression rescues the mitotic arrest as well as the time that these cells spend in mitosis (Figure 7.24 B, C). These findings confirmed the efficacy of the stable overexpression system and also showed that the phenotypes observable in these cells are indeed due to the lack of cyclin B.

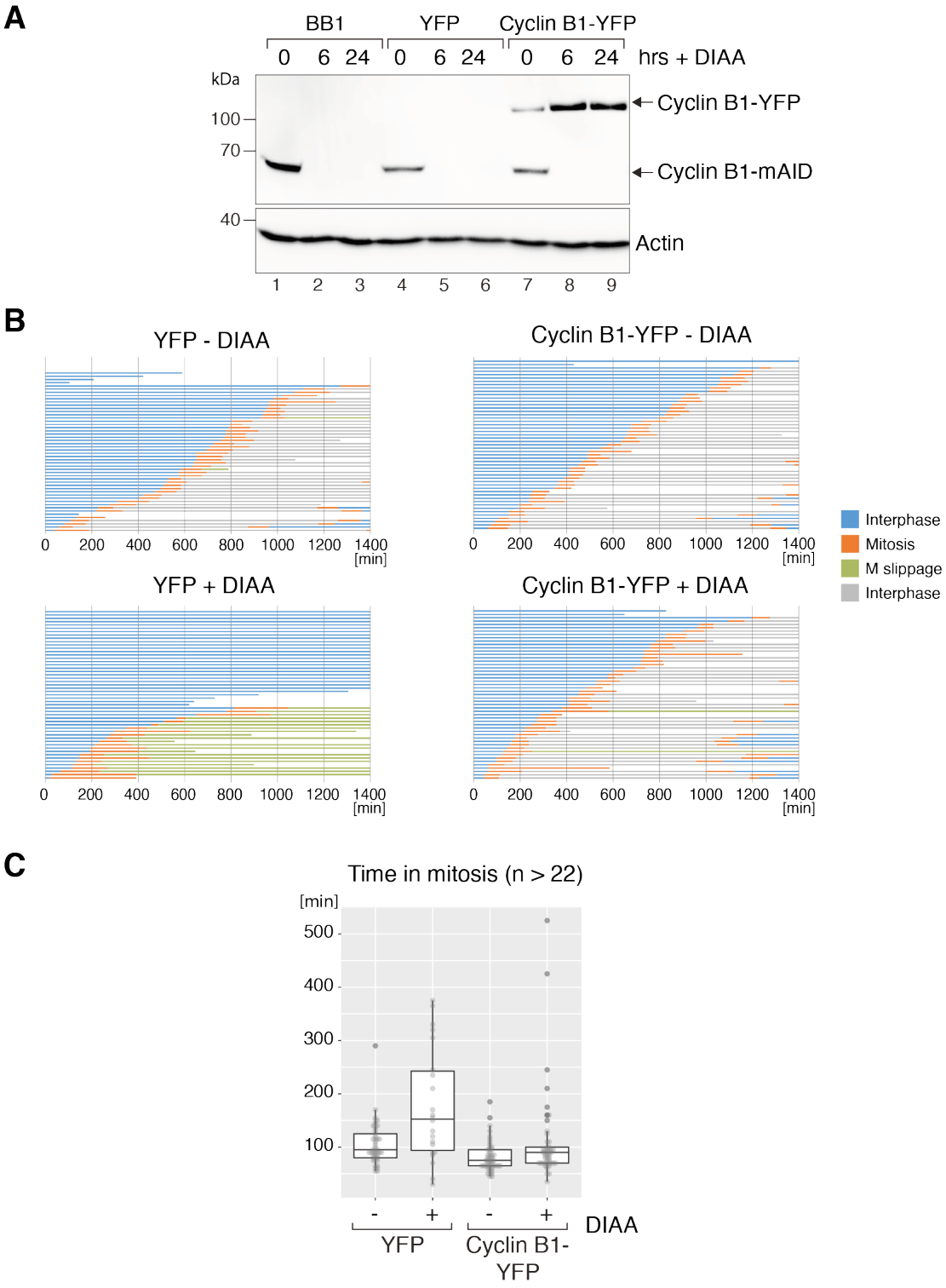

Figure 7.24: Stable overexpression of cyclin B1 rescues the mitotic arrest phenotype. A: Immunoblotting analyses of the asynchronous mix populations of BB1 cells expressing either YFP or cyclin B1-YFP either untreated or treated with DIAA for 6 or 24 h, as indicated. B: Single-cell tracking graphs of a live-cell imaging analysis. Cells were classified either as mitotic or interphase, or interphase following M slippage (marked as M slippage) due to the lack of H2B-GFP to assess NEBD. C: Time cells in B spent in mitosis in min, boxplots as in Figure 7.3. Data were obtained from a single experiment, n > 22.

7.8.1 CDK1 and CDK2 overexpression does not promote mitotic events

Dr. Ken H. T. Ma generated additional mix-populations of CDK2-mRFP and CDK1-mRFP BB1 cells in BB1 cells stably expressing H2B-GFP by FACS-sorting cells positive for mRFP. Figure 7.25 A shows immunoprecipitation analyses of either CDK2 in CDK2-mRFP (Figure 7.25 A) or CDK1 in CDK1-mRFP (Figure 7.25 B). According to this western blot, overexpressing CDKs does not significantly increase the amount of CDK-cyclin complexes. FACS analyses also demonstrated that these cell lines are unable to promote cell cycle progression (Figure 7.25 C). These cells were also assessed by live-cell imaging analyses where it was found that their phenotypes are not different to the parental BB1 H2B-GFP cells, as judged by cellular morphology and the absence of chromosome condensation following cyclin B's depletion.

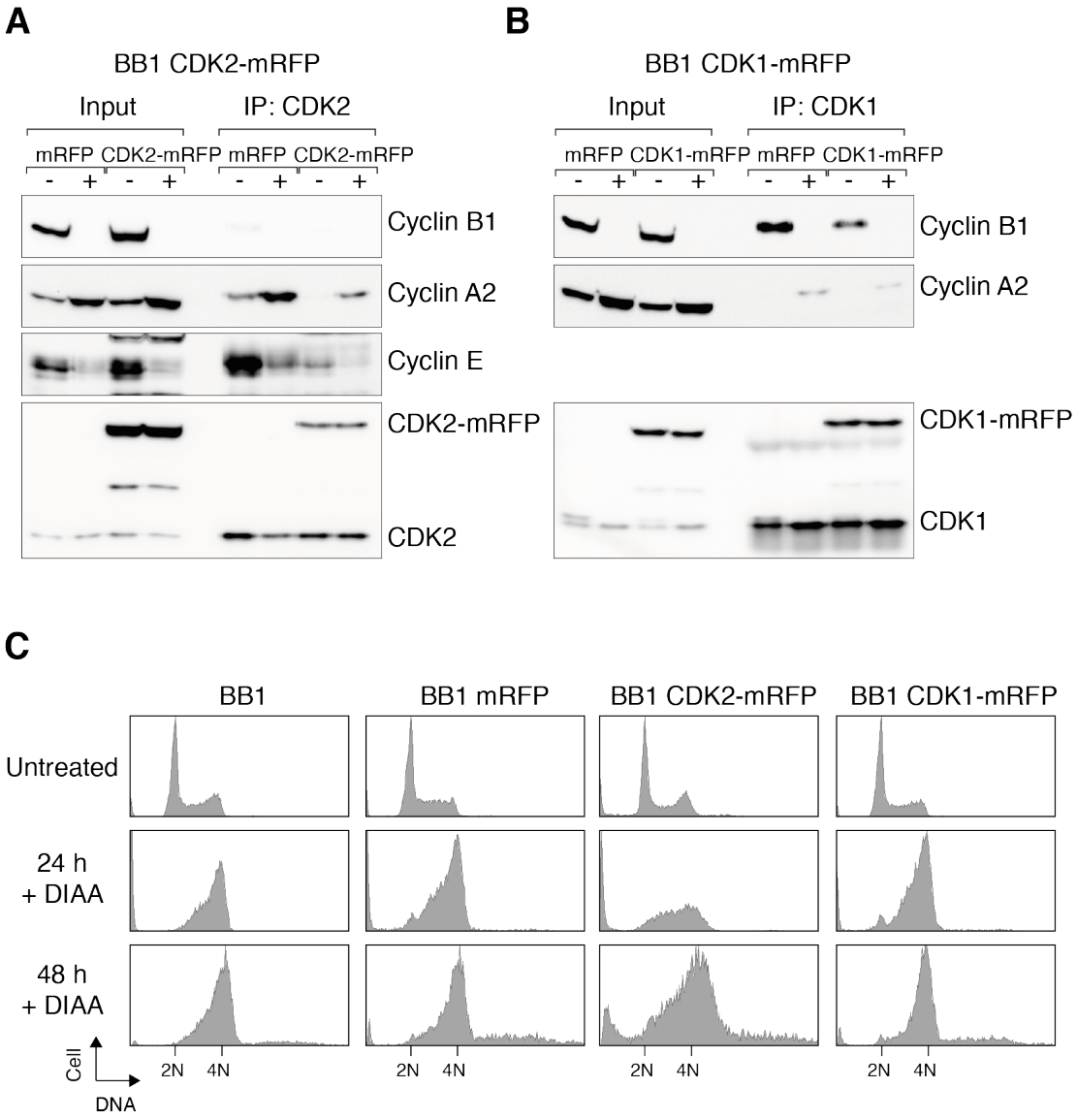

Figure 7.25: Stable overexpression of CDK1 or CDK2 does not promote mitotic events in BB1 cells. A: Immunoprecipitation analyses of CDK2-mRFP cells, treated or not with DIAA for 24 h, using an anti-CDK2 antibody to control for pull-down efficiency. B: Same as A, but overexpressing CDK1-mRFP and using an anti-CDK1 antibody. C: FACS-plots of cells treated with DIAA for 24 or 48 h. A minimum of 10,000 cells were analysed for each condition. Cells were gated for the living population as defined by untreated cells. The gating was maintained throughout this experiment.

7.8.2 Overexpression of cyclin A2 promotes mitotic events

7.8.2.1 Stable overexpression of cyclin A2 results in a higher amount of CDK2-cyclin A2 complexes

Immunoblotting analyses confirm an overexpression of mRFP-cyclin A2 Cells stably overexpressing H2B-GFP and mRFP-cyclin A2 were generated by Dr. Ken H. T. Ma for further analyses of cyclin A2's role in the absence of cyclin B. Immunoblotting analyses confirmed mRFP-cyclin A2's expression (Figure 7.26 A). This is a mix-population generated by FACS-sorting, so all cells do not have the same amount of cyclin A2 overexpression, but only the total amount across the entire population is measured here. Levels of cyclin A2 were also checked after 6 and 24 h of DIAA treatment, which demonstrated a slight accumulation of the endogenous cyclin A2 (as shown previously in this thesis), and of the overexpressed protein, as judged by the increase of signal on the western blot.

FACS analyses confirm previous findings from transient overexpressions The cell cycle profile of these cells was assessed after 24 and 48 h of DIAA treatment (Figure 7.26 B). Cells overexpressing cyclin A2 appear to steadily increase in their DNA fluorescence until they reach an 8N amount, thereby confirming previous findings. Notably, a significant amount of cells apparently remain in a 4N state and a small subpopulation is still observable as a G_1 peak. More research is needed to determine the reason for this peculiar phenotype. The FACS-plots here are slightly different to the previously shown analyses as they are not log-scaled, which showed that the shift from 4N towards an 8N population is steady, corresponding to an asynchronous cycling population.

Cyclin A2-mRFP preferentially binds with CDK2 rather than CDK1 Immunoprecipitation analyses showed that the majority of mRFP-cyclin A2 is bound with CDK2 (Figure 7.26 C), and not CDK1 (Figure 7.26 D), although a very faint band corresponding to mRFP-cyclin A2 can be seen in CDK1 pull-down analyses.

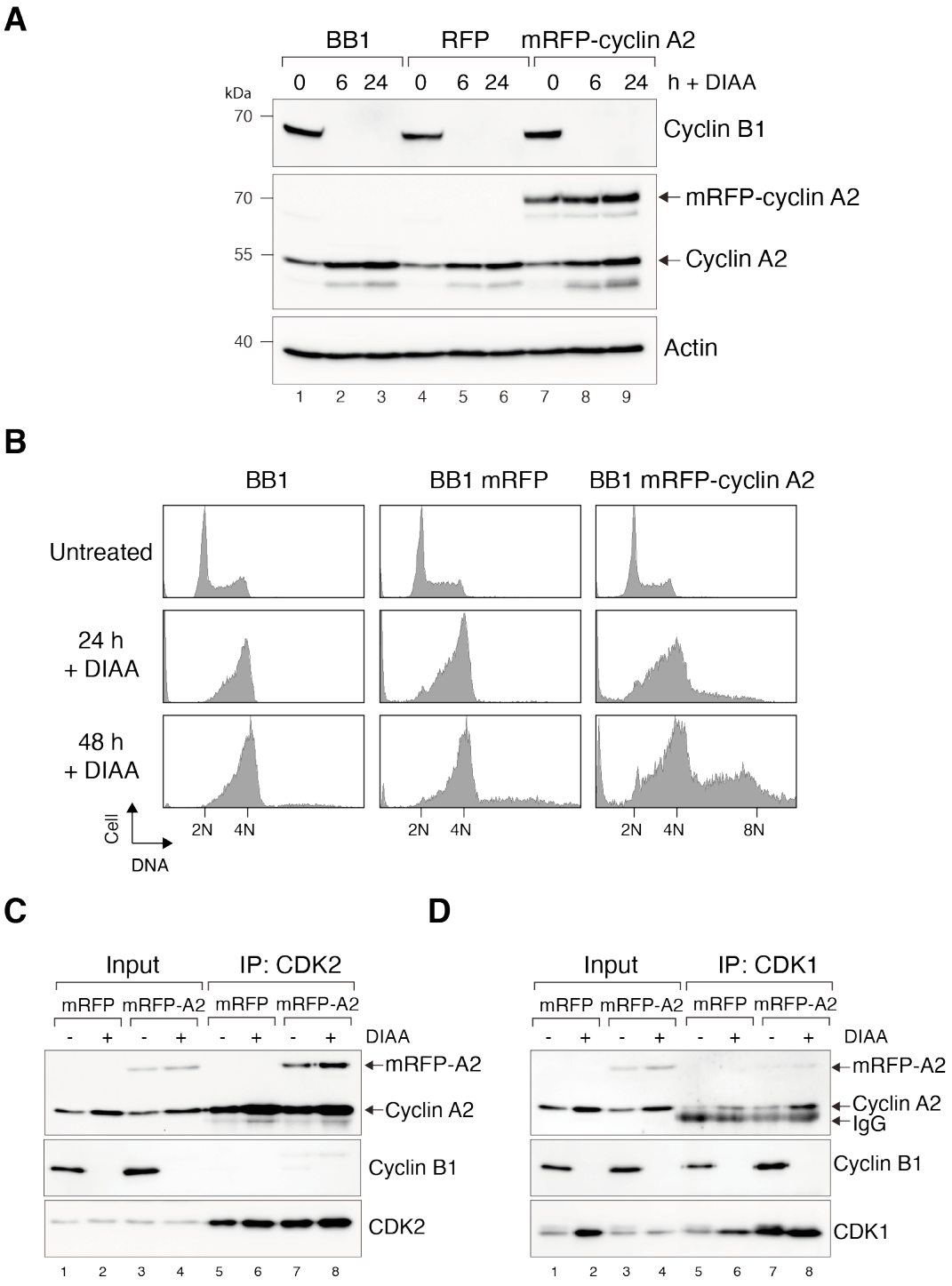

Figure 7.26: Stable overexpression of cyclin A2 promotes some cell cycle progression in BB1 cells. A: Western blot comparing the overexpression of cyclin A2 in either untransfected, mRFP, or mRFP-cyclin A2 cells. B: FACS-plots of cells treated with DIAA for 24 or 48 h. A minimum of 10,000 cells were analysed for each condition. Cells were gated for the live population at fixation in untreated cells. C, D: Immunoprecipitation analyses of mRFP-cyclin A2 cells, treated or not with DIAA for 24 h, using either an anti-CDK2 or anti-CDK1 antibody for pull-downs.

Cyclin A2's degradation mechanics are apparently hindered in cells overexpressing mRFP-cyclin A2 The above cells were further characterised by immunoblotting analyses of cells that were subject to a double thymidine synchronisation (Figure 7.27). The main focus lies on the first mitosis to see why some progress further and resume DNA replication, so time-points from 8 to 20 h post-thy release were harvested as indicated. The mRFP-expressing cell line was included as the control (Figure 7.27 A), but these are essentially the same as previously described BB1 cells and will not be explained separately here. Notably, the BB1 mRFP cells have a slightly higher amount of cell death as observed when culturing these cells. This could be either due to the mRFP signal causing additional stress to the cells, but the same was not observed with other cells expressing mRFP-tagged constructs. However, the level of protein overexpression in those was lower, according to observations made during microscopy analyses (data not shown). Regardless of this observation, these cells, as well as their parental BB1 cells stably expressing only H2B-GFP were routinely used as a negative control in the remaining experiments.

Cyclin A2 is normally degraded in prometaphase [50, 154, 163] (Figure 7.27 A), and its high levels are observable throughout the prophase-like arrest in BB1 cells treated with DIAA. Interestingly, overexpressing mRFP-cyclin A2 seems to interfere with the degradation mechanics of cyclin A2 and mRFP-cyclin A2 after mitosis and during the subsequent G_1 phase in untreated cells, as judged by immunoblotting analyses (Figure 7.27 B, – DIAA panels). The hindered degradation of this protein could promote cell cycle defects and genome instability [1, 4], but this was not obvious when observing the cell cycle profile in the untreated mix population (Figure 7.26 B). The incomplete degradation mechanics will be further elaborated in later parts of this Chapter.

Inhibitory CDK1 phosphorylation becomes more detectable The inhibitory phosphorylation of CDK1, p-Y15, was also more easily detectable in cells with higher levels of cyclin A2, implying that a larger proportion of the mitotic kinase is inactive, unlike in prior analyses where the phosphorylation could not be observed in cells lacking cyclin B. This was somewhat surprising, as previous data showed that there is an increase of CDK1-cyclin A2 in BB1 cells regardless of cyclin A2's overexpression, but no observable p-Y15 on CDK1 was detected. It is possible that the levels of the complex were too low for the CDK1 p-Y15 antibody to detect the signal.

Mitotic markers indicate clear mitotic entry and exit Phosphorylations of some key mitotic players, including aurora kinases and histone H3, were partially rescued in cells overexpressing cyclin A2. The phosphorylations more closely resemble the mitotic entry and exit timings of control cells, as judged by the oscillations in their phosphorylation signal. It appears that the levels of phosphorylated AURKB and AURKC are lower as in control cells, but still distinctly higher as compared with mRFP + DIAA cells.

The level of phosphorylated mitotic CDK substrates was also observed using the antibody recognising the p-TPXK motif that was used for previous analyses (Figure 7.10). However, there were no distinct differences observed in cells overexpressing cyclin A2. Instead, live-cell imaging analyses were utilised to investigate these cells further.

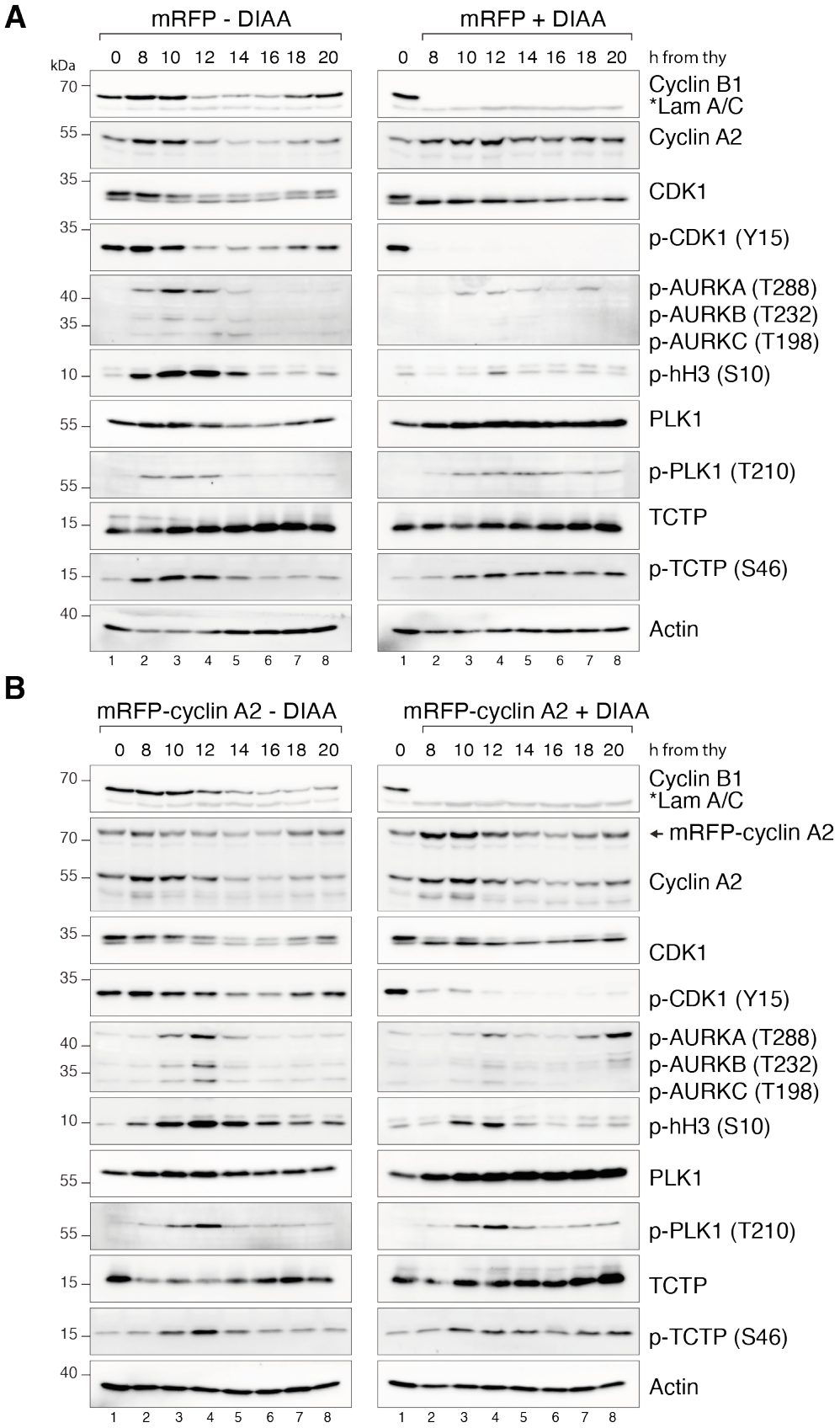

Figure 7.27: See caption on the following page.

Figure 7.27 *(preceding page)*: Immunoblotting analyses of synchronised BB1 cells expressing either mRFP or mRFP-cyclin A2. A, B: Western blots of BB1 cells expressing mRFP or mRFP-cyclin A2, respectively. Cells were synchronised with a double thymidine block and collected at the indicated times, starting at thymidine release at 0 h. DIAA was added at thymidine release, thus the 0 h time-point is the same in - and + DIAA treated populations. Western blots show cyclin B1, cyclin A2, CDK1, p-CDK1 (Y15), phosphorylated aurora kinases, histone H3, PLK1 and p-PLK1 as well as its substrate, TCTP and p-TCTP. Actin is the loading control.

7.8.2.2 Cells overexpressing cyclin A2 either exit mitosis in prophase or prometaphase

Mitotic entry of cells overexpressing mRFP-cyclin A2 is more similar to control cells Figure 7.28 A shows single-cell tracking analyses of BB1 cells overexpressing mRFP-cyclin A2, representative of one experiment. To compare the mitotic entry mechanics, I analysed the cumulative mitotic index of mRFP and mRFP-cyclin A2 cells treated or not with DIAA. The comparison showed that the timing of mitotic entry in cells overexpressing mRFP-cyclin A2 and lacking cyclin B, is more similar to the control cells that still contain cyclin B, than the control mRFP-expressing cells lacking cyclin B (Figure 7.28 B). Furthermore, cells expressing mRFP-cyclin A2 also spent less time in mitosis, regardless of the outcome (Figure 7.28 C). Notably, chromosome condensation was more readily detectable in these cells by microscopy analyses, regardless of cyclin B's depletion, correspodning to the above observable higher levels of phosphorylated S10 on histone H3 (Figure 7.27 B).

Overexpression of mRFP-cyclin A2 gives rise to two common phenotypes Further LCI analyses showed that approximately 40% of mitotic cells exit prior to NEBD, and a similar population exit mitosis after NEBD by either de-condensing their unseparated chromosomes, or are unable to divide into two cells (Figure 7.28 D). Two additional small populations of cells were either successfully exiting mitosis, or divided with observable defects, including lagging chromosomes or division into three cells.

Overexpressed mRFP-cyclin A2 does not degrade in prometaphase According to the mRFP signal in live-cell imaging analyses, a significant depletion of signal during or after prometaphase was not observable, further indicating that cyclin A2's degradation mechanics are impaired and should be taken into consideration when drawing conclusions on the role of this protein in mitosis.

The fate of these populations could be pre-determined by the different amounts of mRFP-cyclin A2 that is overexpressed in these cells, but this was not clear by microscopy analyses (not shown). To investigate this further, single clones with different levels of mRFP-cyclin A2 expression were isolated for further analyses.

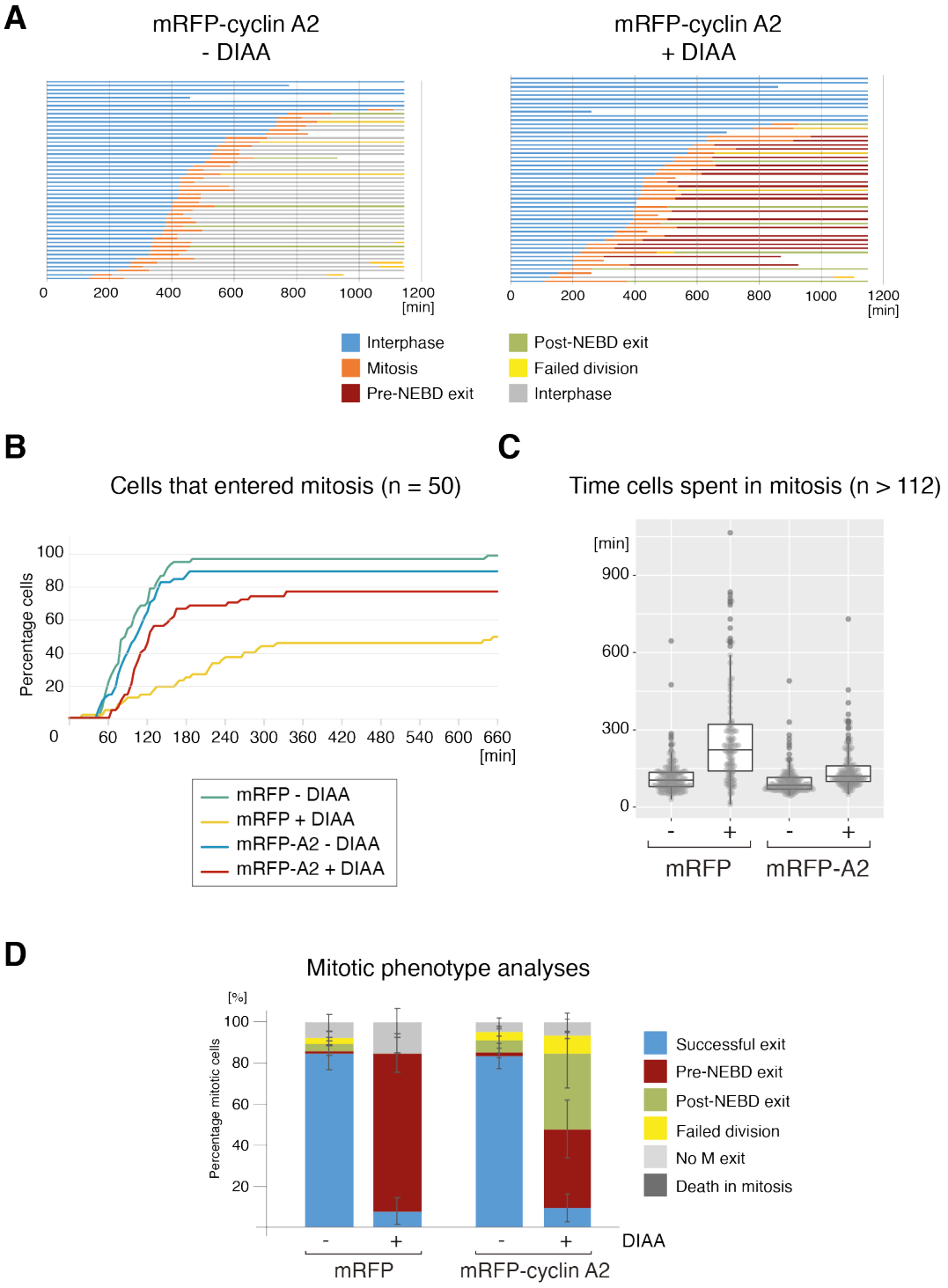

Figure 7.28: There are two possible outcomes of mitosis in cells lacking cyclin B with high levels of cyclin A2. A: Single-cell tracking analyses of mRFP-cyclin A2 cells either untreated or treated with DIAA. B: A comparison of mitotic entry, as judged by cell rounding, in cells expressing either mRFP or mRFP-cyclin A2, the latter shown in A. C: Analysis of time cells in B spent in mitosis. D: Analyses of mitotic phenotypes of BB1 cells expressing either mRFP or mRFP-cyclin A2 (Classification as in Section 7.4.2). Data were obtained from three repeats (n > 50). Barplots and boxplots are as in Figure 7.3.

7.8.3 Mitotic progression of HeLa cells is dependent on the total amount of CDK activity

7.8.3.1 Cell line generation and initial analyses

Establishment of clones overexpressing different amounts of mRFP-cyclin A2 Three clones with different levels of mRFP-cyclin A2 overexpression were isolated. Figure 7.29 A shows the western blots following 0 or 6 h of DIAA treatment. The intensity of mRFP-cyclin A2 compared with the endogenous cyclin A2 in mRFP-expressing cells was quantified. Clones that were isolated have either low (approximately 140% total amount), medium (approximately 380% total amount) and high (approximately 810% total amount) amounts of cyclin A2 (Figure 7.29 B). Interestingly, several bands below the size of endogenous cyclin A2 were detected with the cyclin A2-specific antibody. These bands normally increase after DIAA treatment, but it is not entirely clear what these are. One option is that these are degradation products of mRFP-cyclin A2, but this was not investigated further.

It is noteworthy that the cells overexpressing just mRFP, and low or medium amounts of mRFP-cyclin A2 experience more deficiencies in proliferation, compared to the high-expressing clone, as judged by a higher amount of cell death observed when culturing these cells (not shown), and during LCI analyses (data not shown). It is possible that the gene incorporation in cells expressing low or medium amounts of mRFP cyclin A2 interfered with their survival, as the cell line was generated by retroviral transfection, and not by targeted overexpression or the use of sleeping beauty transposase, or the gene expression products conferred some additional instability in these. Given the seemingly normal cell cycle profile, as observed with FACS analyses (Figure 7.29 C), these cells were utilised for immunoblotting and immunofluorescence analyses, mainly to control for the effects of the clone expressing high levels of mRFP-cyclin A2.

Cell proliferation is dependent on the amount of mRFP-cyclin A2 overexpression FACS analyses have shown that the different levels of mRFP-cyclin A2 overexpression have strikingly dissimilar phenotypes when lacking cyclin B. It seems that cells with low levels of mRFP-cyclin A2 arrest in 4N, cells with medium levels of cyclin A2 eventually accumulate as an 8N population, and cells with high levels resume proliferation according to a normal cell cycle profile (Figure 7.29 C). The latter phenotype was

not readily observable in previous analyses of the mRFP-cyclin A2 mix population. The rescue of cyclin B's depletion by mRFP-cyclin A2 overexpression could have previously been overlooked due to a large amount of apoptosis in other cells with lower amounts of mRFP-cyclin A2, caused by longer times of DIAA incubation. The apoptotic cells could have triggered cell death in others that would have otherwise survived.

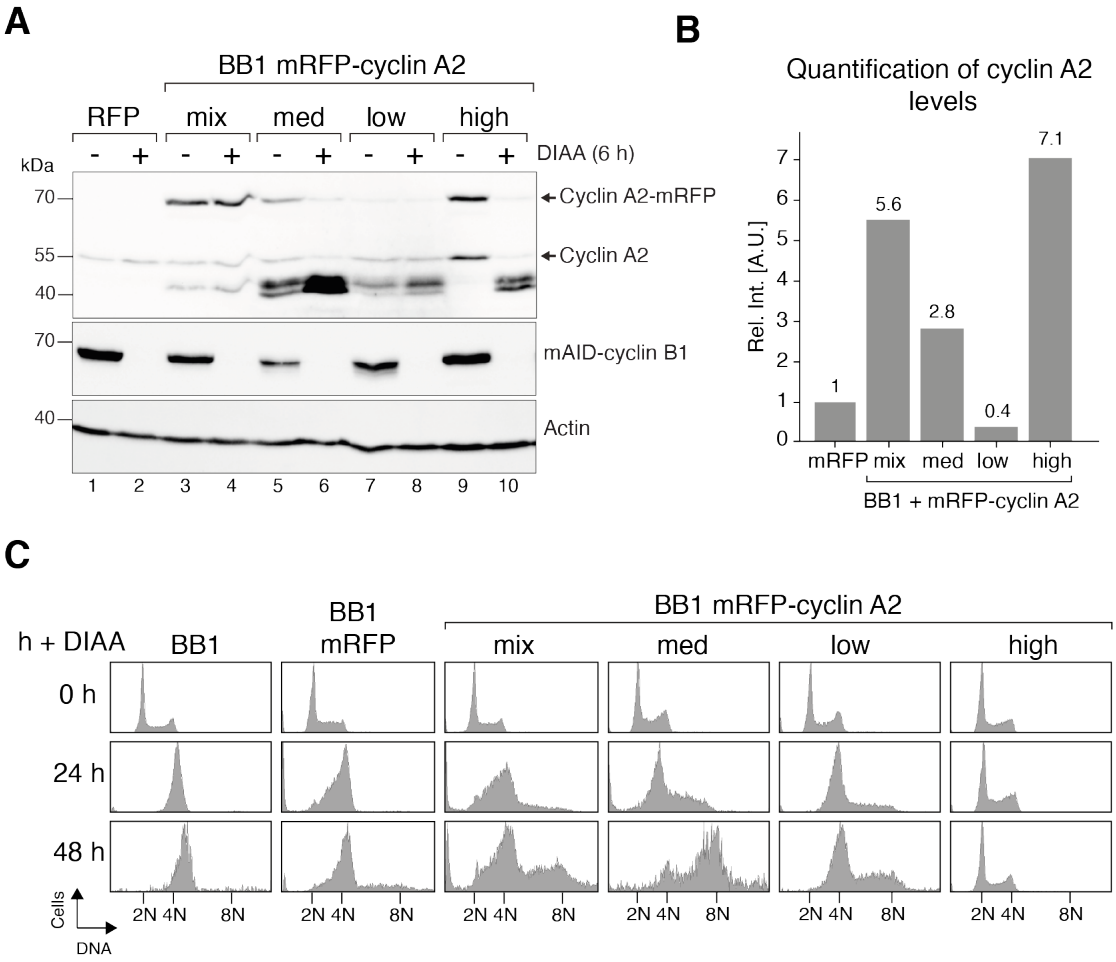

Figure 7.29: Establishing cell lines with different levels of mRFP-cyclin A2 overexpression. A: Western blot of three new clones of mRFP-cyclin A2 overexpression, named as low, med (medium) and high. Cells were treated with DIAA for 6 h prior to immunoblotting analyses. B: Quantification of mRFP-cyclin A2 signal as compared with the relative amount of endogenous cyclin A2 in untreated RFP cells. Only the top bands corresponding to mRFP-cyclin A2 were analysed. C: FACS analyses of clones in A, treated with DIAA as indicated. The BB1 parental cells are included as a control. A minimum of 10,000 cells were analysed for each condition and gated for the live population according to the amount of time of DIAA incubation, as described previously.

7.8.3.2 Live-cell imaging analyses confirmed that the three distinct phenotypes depend on the amount of mRFP-cyclin A2 expression

The above clones were assessed with live-cell imaging analyses and their phenotypes were classified as described previously (see Section 7.4.2). Cells were released from thymidine to ensure the analyses of the first mitosis post-cyclin B depletion. Figure 7.30 A shows the mitotic phenotype analyses of the LCI analyses. The cells expressing mRFP are included as a control.

Low levels of cyclin A2 are insufficient for NEBD in the absence of cyclin B The clone overexpressing low amounts of cyclin A2 (low, approximately 140%), is the most similar to the control cells. The majority of cells exit mitosis prior to NEBD (Figure 7.30 A). These cells also spent a significant amount of time in mitosis, similar to the control cells treated with DIAA (Figure 7.30 B). Observing the cells under the microscope revealed that they normally achieve a higher level of chromosome condensation than the BB1 cells with endogenous levels of cyclin A2.

Medium levels of cyclin A2 can promote NEBD, but not cell division, in cells lacking cyclin B Live-cell imaging analyses of the clone expressing medium amounts of mRFP-cyclin A2 (med, approximately 380% in total) demonstrated that the majority of cells that enter mitosis also progress past NEBD but are unable to successfully divide into two daughter cells, and are thus classified as post-NEBD exiting cells (Figure 7.30 A). Interestingly, these cells spent a much more uniform amount of time in mitosis, resembling the timing of an untreated population (Figure 7.30 B). The surviving cells apparently enter the next cell cycle, as observed by some cells re-entering mitosis towards the end of LCI analyses (data not shown), which likely leads to their accumulation as an 8N population observed with FACS analyses (Figure 7.29 C).

High levels of cyclin A2 lead to a successful mitosis in cells depleted of cyclin B Concomitant with the FACS analyses, the cells with high levels of cyclin A2 (high, approximately 810% in total) nearly completely rescue the mitotic exit as judged by the majority of cells exiting mitosis successfully (Figure 7.30 A). The amount of time these spend in mitosis is also much more similar to the control population that was untreated with DIAA, albeit it is still somewhat longer (Figure 7.30 B).

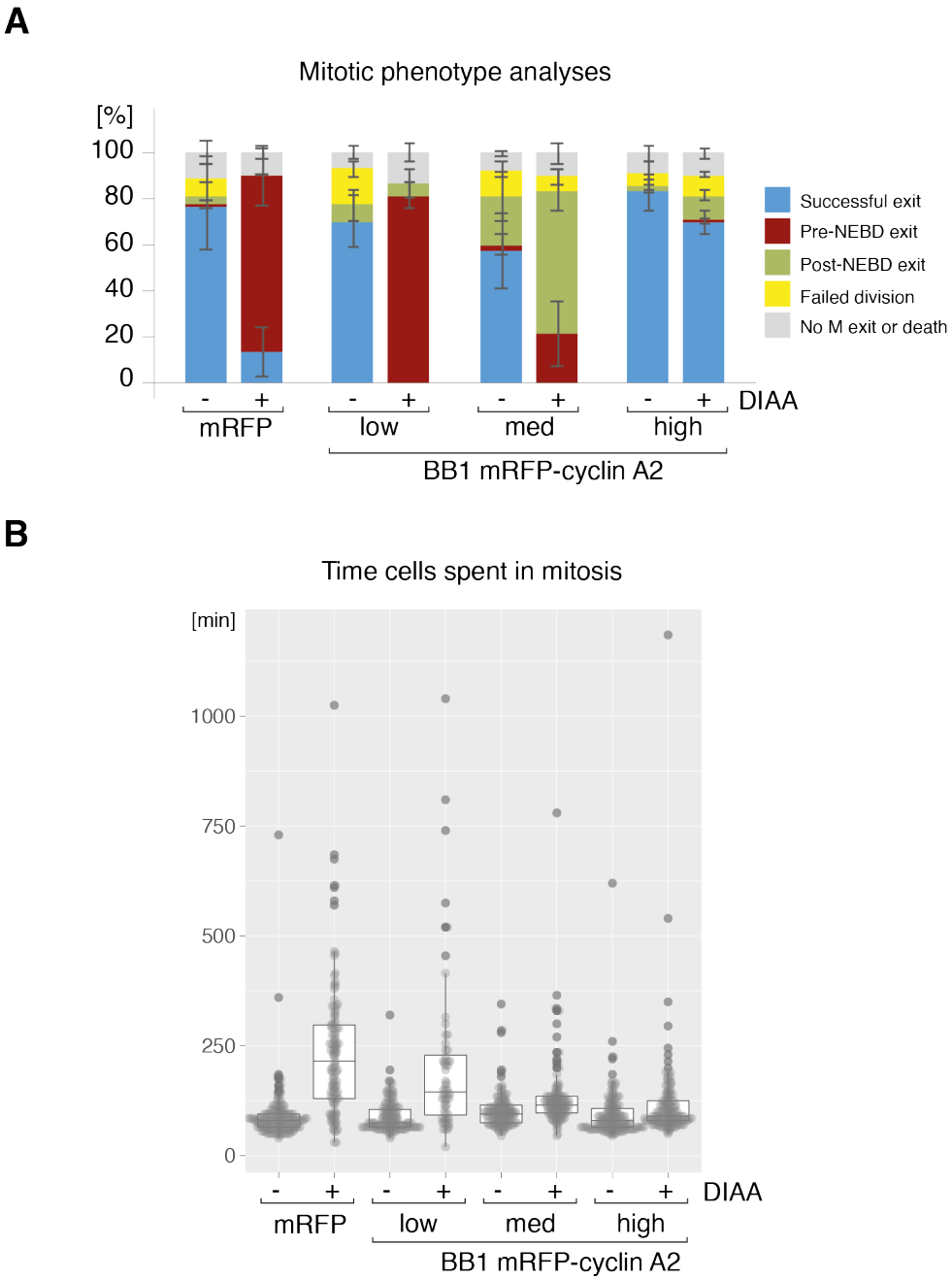

Figure 7.30: Live-cell imaging analyses of BB1mRFP-cyclin A2 clones. A: Mitotic phenotype analyses of the indicated cells. Cells were synchronised by a double thymidine block and treated or not with DIAA following the release from the second block. The cells were then imaged for 24 h, and their mitotic exit phenotypes were classified as described previously in Section 7.4.2. B: Analyses of time cells spent in mitosis, as previously (n > 18 per condition). Data in A and B were obtained from three sets of analyses, obtained from two biological repeats. Barplots and boxplots are as in Figure 7.3.

Overexpressing cyclin A2 does not promote a faster mitotic entry The mechanics of mitotic entry in these clones were investigated next. Since the cells were released from a double thymidine block, the mitotic entry mechanics were easily comparable. Previous data in this thesis showed that cyclin A2 alone triggers mitosis in RPE-1 cells, and also aids to the temporal control of mitotic entry in HeLa cells, as described in Chapter 4 Section 4.4.1. I hypothesised that higher levels of cyclin A2 could promote a premature mitotic entry in these cells, and possibly lead to eventual genomic instability, although this could not explain the successful mitotic exit of cells overexpressing high amounts of cyclin A2.

Mitotic entry mechanics of cells from a single live-cell imaging experiment were compared with a cumulative mitotic index. Cells separated into untreated (Figure 7.31 A) and treated with DIAA (Figure 7.31 B). No premature mitotic entry was observed in the cells overexpressing cyclin A2; instead there appears to be a slight delay in entry in both low and medium expressing clones.

Comparing the mitotic entry mechanics of cells lacking cyclin B, it is clear that cells with medium or high amounts of cyclin A2 enter mitosis as normal, whereas cells with low or endogenous amounts enter mitosis slower or not at all (Figure 7.31 B).

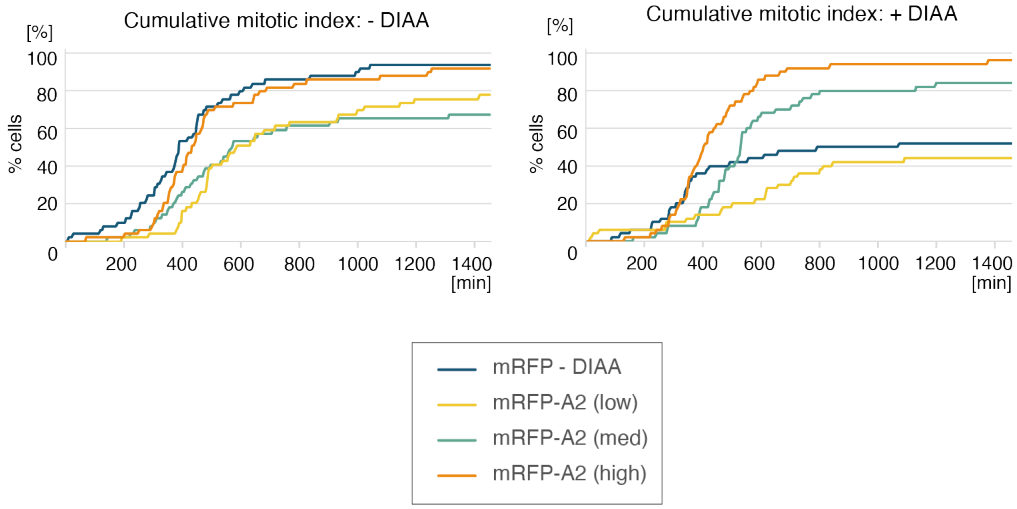

Figure 7.31: Mitotic entry comparison of BB1 cells overexpressing different amounts of mRFP-cyclin A2. A, B: Cumulative mitotic entry of cells that were synchronised by a double thymidine block and treated or not with DIAA following the release from the second block, imaged for 24 h. The graphs show percentage of total cells from a single experiment (n = 50).

7.8.3.3 Immunoblotting analyses reveal specific differences between the newly established cells overexpressing cyclin A2

Cells were synchronised by a double thymidine block and released between 8 and 14 h prior to harvesting. They were next subjected to immunoblotting analyses and blotted with the p-TPXK mitotic substrate antibody which was previously used to uncover the specific differences in mitotic substrate phosphorylations (Figure 7.10). The clones with low and medium amounts of cyclin A2 appear to slowly accumulate mitotic CDK1 substrate phosphorylations. There are also faint bands present at the two sizes that have previously been shown to be missing in BB1 + DIAA treated cells, at 34 and 50 kDa. There is no readily apparent differences between cells with low and medium expression of mRFP-cyclin A2, apart from an earlier presence of a faint 34 kDa band in the medium-expressing clone.

On the other hand, BB1 cells expressing high amounts of cyclin A2 are different in two ways: (i) they enter, as well as exit, mitosis sooner according to CDK substrate phosphorylations and dephosphorylations respectively, and (ii) the two specific bands are highly phosphorylated, similar to the untreated cells. These data further confirmed that cells with high levels of mRFP-cyclin A2 completely rescue cellular proliferation in the absence of cyclin B by promoting CDK's activity.

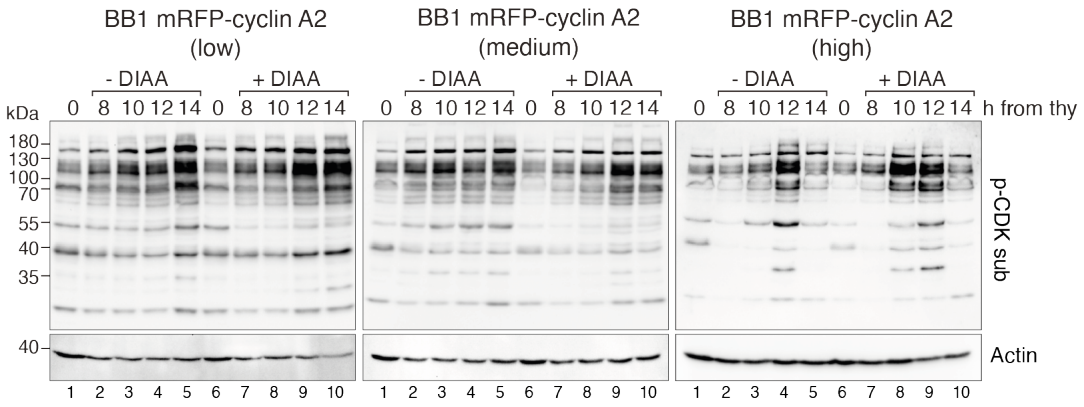

Figure 7.32: Cells with high levels of cyclin A2 completely restore all CDK substrate phosphorylations. Western blots of thymidine released cells as indicated. The phospho-CDK substrate antibody recognising a phosphorylated TPXK motif was used. Actin is the loading control.

7.8.3.4 Cells expressing high levels of cyclin A2 depend on CDK1 specifically for mitotic progression

As shown in Figure 7.31, cells overexpressing cyclin A2 do not enter mitosis prematurely, implying that there is a control over the timing of mitotic entry, regardless of cyclin B's presence. I next sought out to determine the mechanics of cell cycle progression in these cells.

I again utilised a CDK1 inhibitor RO3306, that was used previously in Section 7.6.1.1, which showed that asynchronous cells expressing high amounts of cyclin A2 arrest at G2 after 24 h of treatment, regardless of DIAA addition (Figure 7.33). This indicated that the cells rely specifically on CDK1 for their mitotic progression, however due to the possibility of CDK2 inhibition with RO3306, further work is required to determine the differences between the functions of the two cyclin-dependent kinases.

I next wondered whether these cells utilise both CDK2- and CDK1-cyclin A2 complexes (in the absence of cyclin B) to control the timing of their mitotic entry. To this end, I utilised immunoprecipitation analyses that will be shown in the next Section.

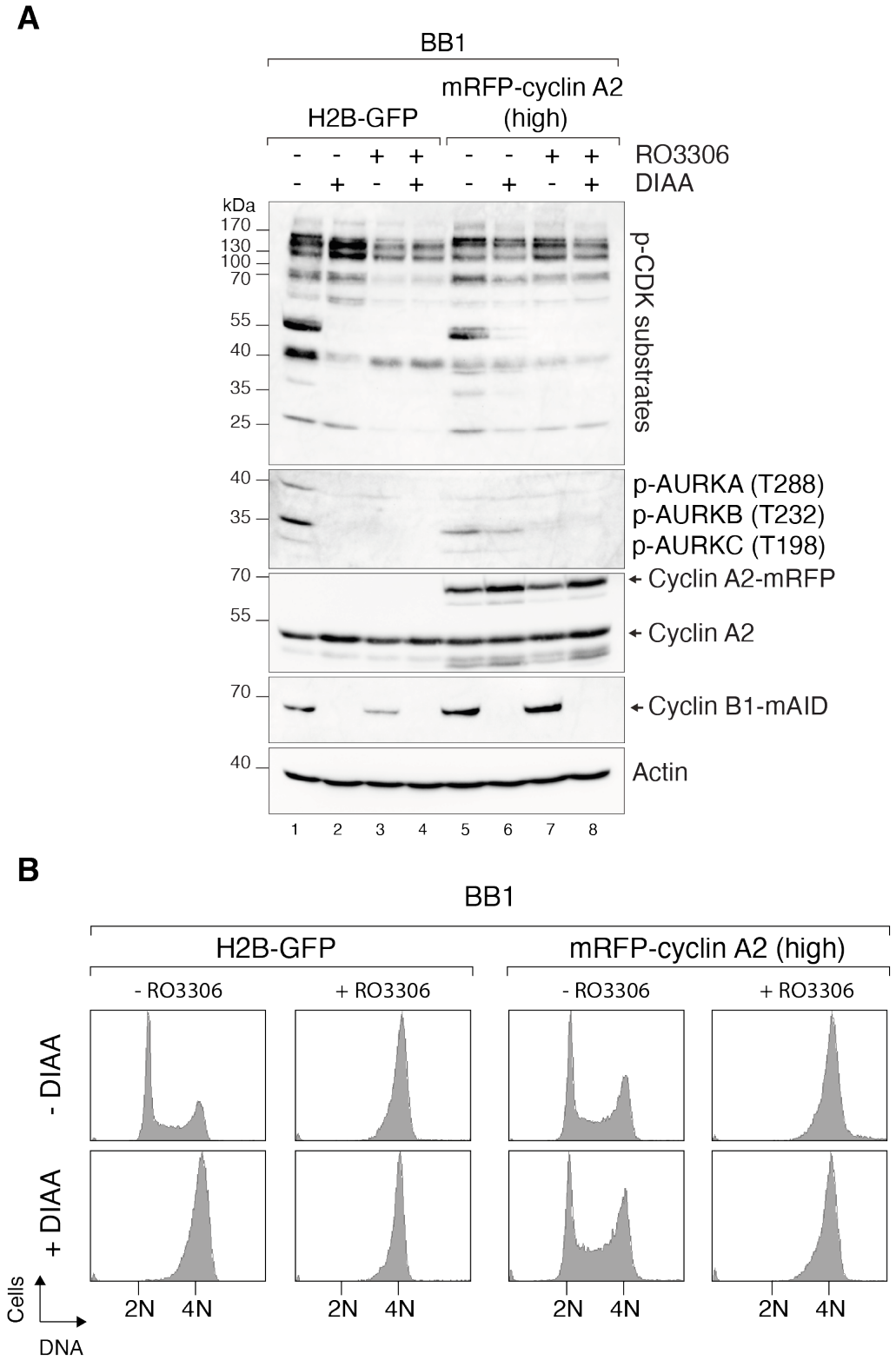

Figure 7.33: CDK1 is necessary for mitosis in cells with high levels of cyclin A2. A: Western blots analyses of asynchronous BB1 cells stably expressing H2B-GFP only (parental), or also expressing high levels of mRFP-cyclin A2 that were treated or not with DIAA and RO3306 for 24 h. The phospho-CDK substrate antibody recognising a phosphorylated TPXK motif was used. Additional antibodies were used as indicated on the blots. Actin is the loading control. B: FACS analyses of cells in A. A minimum of 20,000 cells were analysed and gated for the alive population.

Immunoprecipitation analyses confirmed that CDK1 controls mitotic entry I next synchronised BB1 cells expressing either H2B-GFP alone, or also mRFP-cyclin A2 (high amounts), with a double thymidine block and collected cell lysates at the indicated timepoints to capture their mitotic entry and progression.

Figure 7.34 shows cells that were untreated in A, and treated with DIAA in B. As previously, BB1 H2B-GFP cells show minimal amounts of the CDK1-cyclin A2 complex, which slightly increases upon DIAA treatment when cells enter the prophase-like arrest. On the other hand, cells expressing high levels of mRFP-cyclin A2 show a higher amount of CDK1-cyclin A2 complexes. Most strikingly, when these cells are treated with DIAA, an inhibitory phosphorylation on CDK1 Y15 is observable, which was normally undetectable in cells lacking cyclin B. This phosphorylation decreases as cells enter and progress through mitosis, implying that the control of mitotic entry is implemented similarly as in cells containing cyclin B, via CDK1 inhibition.

The above data imply that there is a trigger that promotes CDK1 activation at the appropriate time, and that CDK1-cyclin A2 in this condition is controlled similarly as CDK1-cyclin B during a normal cell cycle.

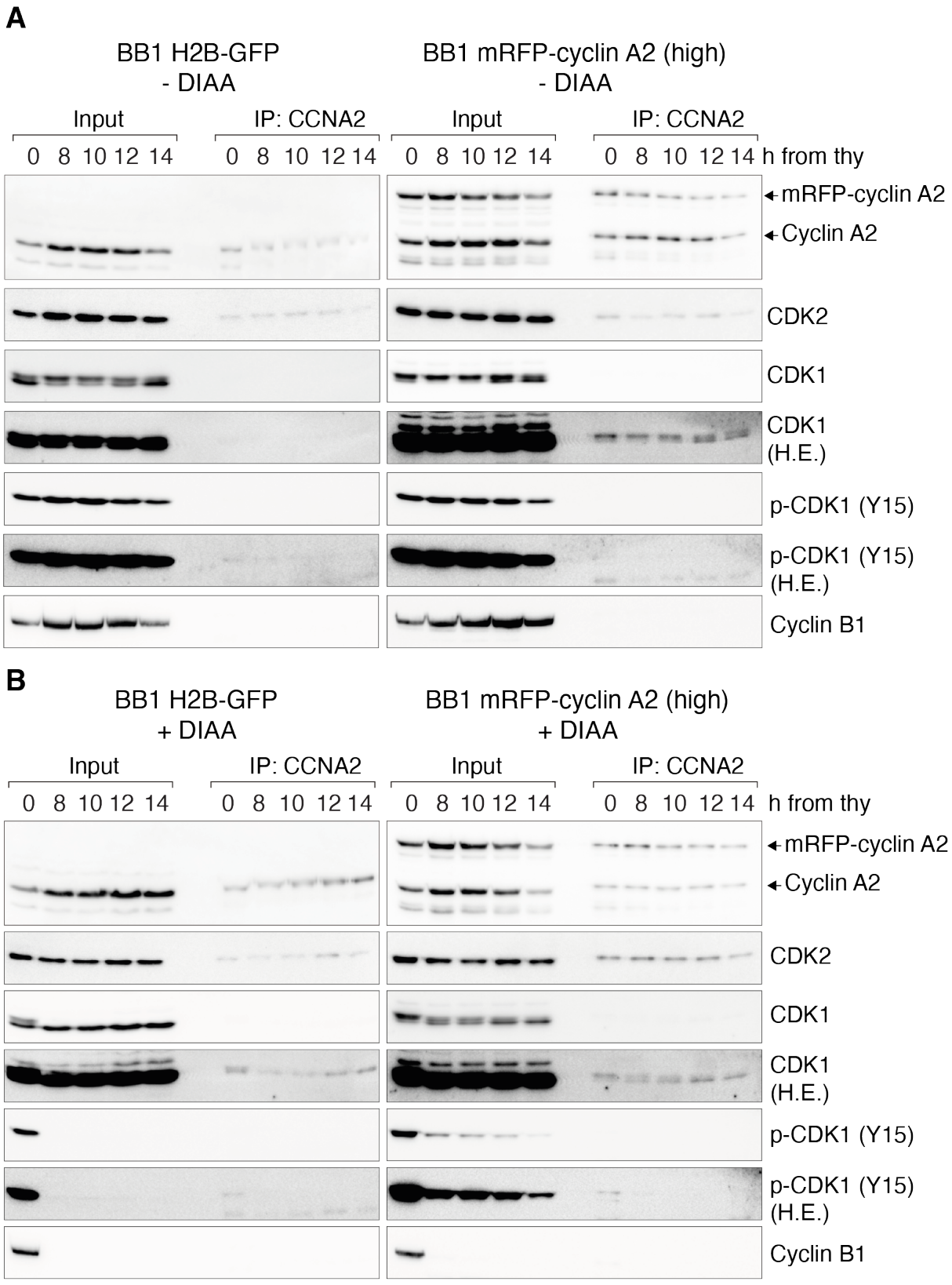

Figure 7.34: CDK1 is inhibited until mitosis in cells with high levels of cyclin A2. A: Western blots showing cells that were untreated with DIAA and synchronised with a double thymidine block. The cells were collected as indicated and subjected to immunoprecipitation with a cyclin A2 antibody. B: Same as in A, but cells were treated with DIAA.

7.9 Discussion

The definition of a prophase-like arrest HeLa cells lacking cyclin B were found to enter into a prophase-like arrest, which is somewhat similar to the antephase stage, described in Chapter 6, Discussion Section 6.5. Importantly, cells in antephase commonly attempted and failed cytokinesis, showing that they were indeed exiting from mitosis [425], whereas HeLa cells lacking cyclin B never attempted to divide into two cells, albeit some attempts of an uncondensed nuclear separation were observable. Additionally, a hallmark of antephase is chromosome condensation [425] – whereas clear chromosome-like structures were not readily observable in the prophase-like arrested cells, as also supported by the lack of phosphorylated histone H3. Further work is required to determine the point in mitosisn where cells enter a prophase-like stage or an antephase arrest, but it seems that the prophase-like arrest is somewhat earlier than antephase.

Cyclins A2 and B are redundant in HeLa cells This thesis has determined that the two mitotic cyclins, cyclin A2 and cyclin B, are able to fully compensate for each other in HeLa cells, depending on their protein levels. This is possible because cyclin A2 is able to bind both CDK2 and CDK1, thus contributing to mitotic entry and progression.

There are several possible explanations for the observable redundancy between cyclins A and B in HeLa cells: (i) cells normally have much higher levels of cyclin B, as compared with cyclin A2, and an eight-fold amount of cyclin A2 is more similar to the endogenous amount of cyclin B, (ii) cells still have some specificity towards cyclin B-mediated CDK1 phosphorylations, so only an overflow of cyclin A2 is able to compensate for these, or (iii) CDK2 preferentially binds with cyclin A2, so only overexpressing cyclin A2 resulted in a sufficient accumulation of a CDK1-cyclin activity that is required for mitotic progression.

Data shown in this Chapter supports the third possibility. Immunoblotting analyses have consistently shown a lack of p-Y15 on CDK1 in the absence of cyclin B, however, this phosphorylation is again observable when cyclin A2 is overexpressed, although at a lower level. Consistent with this, the p-Y15 is removed when cells enter and progress through mitosis. I show that the CDK1-cyclin A2 complexes that exist in these cells are controlled by the Y15 phosphorylation, explaining why no premature mitotic entry was triggered in cells overexpressing mRFP-cyclin A2.

These data imply that CDK2-cyclin A2, or an additional unknown trigger, could pro-

mote initial mitotic events and trigger the activation of CDK1-cyclin A2, thus contributing to the control of the cell cycle, and separating between activities required to trigger and progress through mitosis not by cyclin binding, but by the CDK that is activated.

Impaired degradation of cyclin A2 likely promotes the ability of cyclin A2 to carry out cyclin B's roles As mentioned above, a clear degradation of mRFP-cyclin A2 was undetectable with microscopy and immunoblotting analyses. This additional property likely contributed to the rescue of mitotic phenotypes observed in the absence of cyclin B, given that cyclin A2's degradation normally occurs in prometaphase [1].

Notably, overexpressing cyclin A2 was shown to promote the survival of some cancer cell lines [137] and it is tempting to speculate that these additional properties of cyclin A2, the impaired degradation and promoting mitotic events, could be one of the ways in which cancer cells increase their chances of survival and promote genomic instability. Further investigations with specific cancer cells, and research that determines the CDK that binds the majority of cyclin A2, will shed some more light on the mechanics of mitotic progression in HeLa, and other cancer cells.

Mitotic progression in HeLa cells depends on the quantity of specific cyclins As shown in this Chapter, HeLa cells require cyclin B to enter mitosis, but cyclin A2 may contribute to some mitotic events as noted by the delay in cells lacking cyclin A2 in Chapter 4. Figure 7.35 A shows that CDK1-cyclin B is mostly required for all mitotic substrate phosphorylations, but also indicates that there may be an additional unknown trigger that promotes the activation of CDK1-cyclin B complexes.

HeLa cells lacking cyclin B that express high amounts of cyclin A2 apparently rely mostly on CDK1-cyclin A2 activity to enter and progress through mitosis, although it is unclear whether CDK2-cyclin A2 or an additional outside trigger promotes these events. CDK1 control is implemented by WEE1 kinase family in these cells, as judged by the appearance of the p-CDK1 (Y15) in these cells (Figure 7.34). Further work is required to determine the difference in the function of CDK2 and CDK1 in these cells.

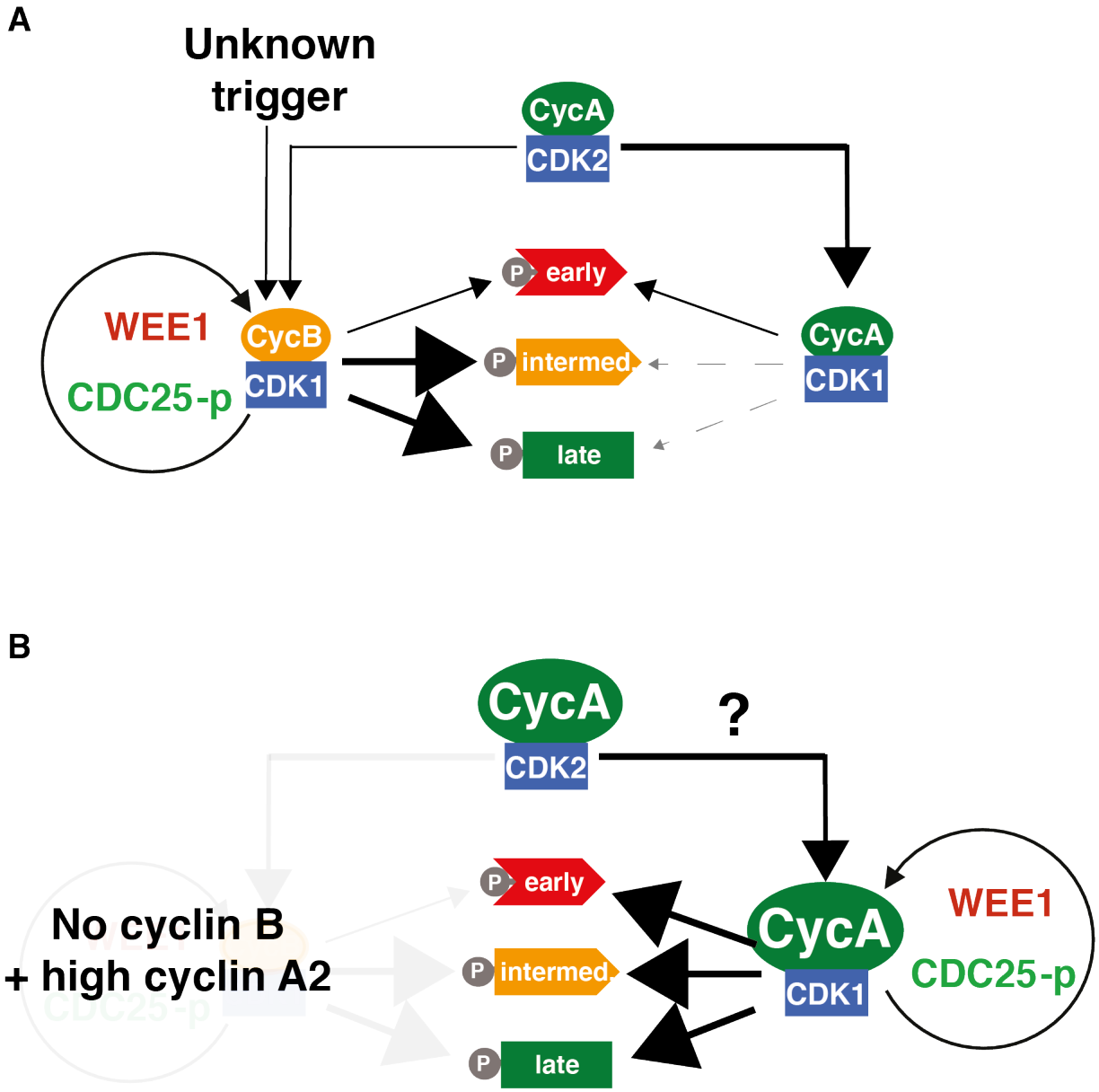

Figure 7.35: A: A model of the requirements for mitotic entry and progression in HeLa cells in normal conditions. B: A proposed model of mitotic substrate phosphorylation dependency in HeLa cells lacking cyclin B. The question mark indicates that the CDK2-cyclin A2 or an unknown trigger could activate the CDK1-cyclin A2 complexes.

Chapter 8

Discussion and future work

Re-defining the roles of key players in mitosis in human cells This thesis utilised novel degron tagging techniques to tackle some long-standing questions in the cell cycle field: why do human cells require two CDK-binding mitotic cyclins in mitosis, cyclins A2 and B (hereafter referred to as mitotic cyclins), and are their roles specific or redundant between each other? Previous studies have implied that the mitotic cyclin A2 promotes mitotic entry and controls the timing of mitotic progression by promoting nuclear envelope breakdown (NEBD) [16]. The same group later on showed that the second mitotic cyclin, cyclin B, has more important functions only after NEBD in mitosis, but, if localised to the nucleus, it could compensate for the loss of cyclin A2 in earlier stages to promote chromosome condensation and restore the time of mitotic progression [12]. Cyclin B is translocated into the nucleus only after prophase onset [13, 14], thus further supporting the redundancy of this cyclin to trigger mitotic events. Additional studies supported these claims by showing the necessity for cyclin A2 in mitotic entry [9, 52, 68], and the onset of mitosis in the absence of cyclin B [9, 11, 12, 16, 187]. Contrasting these findings are data from mouse embryos – Pines lab have shown that cyclin B is necessary for the onset of prometaphase, as judged by the lack of a key prometaphase marker, nuclear envelope breakdown (NEBD) [10]. The above data, apart from mouse embryo research, were mostly obtained with the use of RNA interference methods, which could lead to some inconsistencies due to an inefficient depletion of the targeted proteins.

This thesis aimed to elucidate the roles of mitotic cyclins in human cells with the use of novel degron tagging techniques, thus contributing to the understanding of mitotic entry and progression in human healthy and cancer cells. The roles of key cell cycle drivers were assessed in two well-established human cell lines, hTERT non-tumorigenic RPE-1 cells, and a cell line deriving from a cervical cancer termed HeLa.

Specifically, the findings in this thesis showed that the non-tumorigenic RPE-1 cells require specific mitotic activities to progress through different stages of mitosis, as shown in Chapters 4, 5 and 6. In contrast, this study also showed that the HeLa cancer cells rely on the total amount of the two mitotic CDK-binding cyclins as shown in Chapter 7. These data outlined how the cell cycle control of a specific cancer cell line could differ from healthy tissue. Additional analyses of other cancer types are crucial to determine whether the transition from requiring specific cyclins to a total cyclin presence is reserved within the researched cell line(s). Different types of cancer cells are not uniform in the expression

levels of cyclin A2, and both lower or higher expression could promote cancer proliferation [137, 172, 173]. Overexpression of cyclin B also implied a poorer outcome in some cancer types [264, 266–268]. Further studies could elucidate the common characteristics of some cancer types that share similarities in cyclin expression levels and thus help to predict better treatments and patient outcomes.

Further studies in RPE-1 cells There are still many unknowns surrounding cyclin specificity and redundancy in RPE-1 cells, which could be further investigated. These could be researched with a similar cyclin overexpression study that was conducted in Chapter 7 of this thesis using HeLa cells.

Preliminary observations by Dr. H. Hochegger showed that the overexpressed cyclin A2 could promote the metaphase plate alignment in RPE-1 cells (data not shown), but it is unclear whether cytokinesis was also restored. Further investigations determining the effects of cyclin A2's overexpression in cells lacking cyclin B are crucial to understand the control of mitotic progression in RPE-1 cells. While a level of specificity is implied, according to the data shown in Chapter 6 where specific mitotic players control separate stages of mitosis in RPE-1 cells, a possibility of cyclin redundancy has also been implied by overexpressing cyclin B in cells deficient in cyclin A in Chapter 4, Section 4.3.5. The redundancy between cyclins A and B in early stages fof mitosis is also supported by previous findings by Gong et al. [12].

Further studies in HeLa cells This work demonstrated that HeLa cells do not require cyclin A2 for proliferation (see Chapter 4, Section 4.4.1), whereas cyclin B is crucial for a successful mitosis (see Chapter 7). Additionally, a novel prophase-like stage was observed in HeLa cells lacking cyclin B, defined in Chapter 7, that was established and maintained by cyclin A2-CDK activity (see Chapter 7, Section 7.6.2). Overexpressing different levels of cyclin A2 in cells lacking cyclin B restored separate stages of mitosis in HeLa cells (see Chapter 7, Section 7.8.3). These data indicated that HeLa cells progress through mitosis in a dose-dependent manner of CDK-cyclin activity. This was supported by a complete rescue of specific CDK mitotic substrate phosphorylation sites, as shown by immunoblotting analyses in the same Section (Figure 7.32).

Previous research in HeLa cells did not predict these results, as noted in the above paragraphs. Instead, specific functions cyclin A2 in mitotic entry and for cyclin B in

later stages, following NEBD, were implied [9, 11, 12, 16, 187]. The majority of studies utilised RNA interference methods to assess cyclins' roles, which were likely incompletely depleting cyclin B and possibly also targeting other proteins (such as depleting cyclin A2 when targeting cyclin B in [440]). The increased efficiency of cyclin B's depletion using degron tagging methods and various levels of cyclin A2's overexpression were, to my knowledge, not analysed previously.

Future studies in HeLa cells could work to determine which are the essential CDK substrates that are not phosphorylated until high CDK-cyclin activity is present in the cells. Mass spectrometry analyses were planned to address this question, however due to the restrictions implemented by the COVID-19 lockdown, these samples could not be analysed in the given time. This work remains to be conducted in the Poon lab in the future, as proteomic analyses will provide crucial insight into which substrates are key for the mitotic progression in HeLa cells. This work could also complement the mass spectrometry analysis in RPE-1 cells, and a whole-scale comparison of both cell lines would be especially insightful to determine the differences between a qualitative – meaning specific CDK-cyclin complexes – and a quantitative requirement for CDK activity in mitotic progression of RPE-1 and HeLa cells, respectively. Analyses of additional cell lines are crucial to further determine the probability of cancer cells relying on cyclin quantity instead of cyclin specificity.

Further supporting the qualitative and quantitative models are findings that showed the effect of degradation of cyclin B in both of these cell lines. The work in Chapter 5 determined that only a subset of mitotic substrates are specifically affected by the loss of cyclin B in RPE-1 cells (Section 5.5.2), thus agreeing with the findings in HeLa cells, shown in Chapter 7, where only a few specific mitotic substrate phosphorylations were missing (Figure 7.10). However, cyclin B is apparently much less relevant for early mitotic events in RPE-1 cells, whereas HeLa cells arrest in a prophase-like stage in its absence. These findings complement a qualitative requirement for CDK-cyclin activity in RPE-1 cells, and a quantitative one in HeLa cells – substrate specificity allowed RPE-1 cells to progress through mitosis until cyclin B is specifically required, whereas the lack of cyclin activity to activate CDK in HeLa resulted in a prophase arrest.

Specific roles for cyclin A2 during mitosis Both RPE-1 and HeLa cells deficient in cyclin A2 spent significantly longer amounts of time in mitosis (see Chapter 4, Section

4.3.4.2 and Section 4.4.1), but the underlying cause has not been investigated in this study. Cyclin A2 has been implied in several mitotic roles thus far, especially in the regulation of kinetochore-microtubule attachments [50, 51] and PLK1 activation [51, 88], and the established degron tagging systems could further support the implied functions, or even help to uncover additional roles.

Antephase or prophase-steady state or prophase-like stage This thesis uncovered that both RPE-1 and HeLa cells arrest in an intermediate stage between G_2 and M, although the two stages are not the same based on some key characteristics such as the level of chromosome condensation and the temporal maintenance of the arrested stage – RPE-1 spent significantly less time in prophase-steady state, whereas HeLa stably arrested for up to 72 h, followed by apoptosis. Previous studies have already described an intermediate stage between G_2 and M and termed it antephase [418, 419, 425] and further work is required to investigate the similarities and differences between these stages..

Feringa et al. defined antephase by the appearance of chromosome condensation and cytokinesis of arrested cells [425], whereas the prophase-steady RPE-1 and prophase-like HeLa cells do not typically attempt to divide into two cells. Furthermore, chromosome condensation was not readily observable in HeLa cells. Instead, these cells rounded up and then slightly adhered back to the surface, while exhibiting some key differences in their morphological properties, shown in Chapter 7, Section 7.5.1.1. HeLa prophase-like cells were additionally characterised by a maintenance of a specific signature of mitotic CDK substrate phosphorylations where most of them were present, but some specific ones were missing (see Figure 7.32 in Chapter 7).

How does tumorigenesis influence cyclin dependency? As noted in RPE-1 cells, they require specific cyclins at different times in mitosis, albeit some redundancy was indicated above. It would be interesting to determine whether tumorigenesis promotes the change between cyclin specificity towards the quantity of CDK activity to drive cellular proliferation. The shift towards cyclin quantity over specificity in the HeLa cell line was difficult to approach methodologically, given their notorious genomic instability [398].

Future work could identify potential cancer types that rely on the quantity of cyclins for mitotic progression, and follow up with analyses of cancer tissues isolated from patients with different stages of tumour growth. These findings could define the hallmarks of

tumour development based on their cell cycle control, thereby greatly contributing to the understanding of cell cycle adaptation in cancer cells to ensure their own proliferation.

Testing the novel CDK2-cyclin A2 inhibitors is essential for future cancer therapy Further research could work towards eliminating the tumours to target with novel CDK-cyclin inhibitors as they are likely to compensate for their lack by other types of cyclins. The latter is showcased by HeLa cells and their resilience to the depletion of cyclin A2, indicating that the CDK2-cyclin A2 inhibitors that are currently under development [185] would be less effective to suppress their growth.

Additional analyses of this cell line using the novel inhibitors is of course essential to confirm this hypothesis. However, it is possible that CDK2-cyclin A2 and CDK1-cyclin A2 have specific roles in HeLa cells and the ablation of one complex could be sufficient to halt the cell cycle, thereby supporting the use of these inhibitors in clinical trials.

Conclusions Cyclin specificity during mitosis has been a long-standing question in the cell cycle field. This thesis has helped to uncover the fundamentals of mitotic progression and assign separate roles for cyclins, and other key mitotic players, in healthy cells. This study has also uncovered novel differences between two human cell line models: the need for specific CDK-cyclin activity in healthy cells, and the requirement for the absolute CDK activity, regardless of the specific mitotic CDK-binding cyclin, in a cancer cell line.

Further work is necessary to determine how cancer cells adapt their cell cycle to promote their own proliferation, and the use of degron tagging approaches, in combination with the novel sleeping beauty transposase system to overexpress other mitotic players, are attractive approaches to address additional long-standing questions in the field. The degron tags, together with with the sleeping beauty system, are part of a new chemical genetics toolbox that can be used for future research in human tissues.

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