

**DISTINCT FUNCTIONS FOR THE HIGHLY RELATED PP2A B55 REGULATORY
SUBUNITS (B α AND B δ) IN CELL CYCLE REGULATION AND TUMOURIGENESIS**

by

Dominik Sommerfeld

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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submitted by Dominik Sommerfeld in partial fulfillment of the requirements for
the degree of Doctor of Philosophy
in Experimental Medicine

Examining Committee:

Catherine Pallen, Ph.D.

Supervisor

Christopher Maxwell, Ph.D.

Supervisory Committee Member

Gregg Morin, Ph.D.

Supervisory Committee Member

Stephan Taubert, Ph.D.

University Examiner

Calvin Roskelley, Ph.D.

University Examiner

Additional Supervisory Committee Members:

Samuel Aparicio, Ph.D.

Supervisory Committee Member

Abstract

Progression through the phases of the cell cycle (G1, S, G2, and mitosis) is largely driven by the coordinated activities of protein kinases and phosphatases, which orchestrate the phase-specific phosphorylation of proteins. Protein Phosphatase 2A (PP2A) – a heterotrimeric holoenzyme complex composed of a scaffold (A), catalytic (C), and variable regulatory (B) subunit – has emerged as an essential cell cycle regulator. Specifically, PP2A complexes containing the B55 family of regulatory subunits (PP2A-B55) have been implicated in the control of various cell cycle phases. My studies investigated isoform-specific roles of the two highly related and abundantly expressed B55 subunits, B α and B δ , in the regulation of G1, S, G2 phase and mitotic progression.

Synchronization of HeLa cell populations at various stages of the cell cycle revealed that the B α and B δ subunits differentially regulate the kinetics of all cell cycle phases. Specifically, these highly related isoforms exert opposing effects on G1 phase progression, and B α but not B δ regulates progression through S phase. Furthermore, my studies demonstrate that the B α and B δ subunits play distinct roles in the control of mitotic exit progression, where B δ regulates early and B α mediates late mitotic exit events.

PP2A-B55 complexes are believed to negatively regulate entry into mitosis by antagonizing CDK1 activation and CDK1-mediated mitotic substrate phosphorylation. While I found that PP2A-B δ indeed antagonizes mitotic entry, my studies surprisingly revealed that PP2A-B α promotes proper mitotic entry and progression. Specifically, loss of B α resulted in the degradation of mitotic regulators, defective activation of CDK1 and other mitotic kinases, and the collapse of a pre-mitotic state during G2 phase.

Deregulation of CDK1 activation is associated with mitotic defects, reduplication of the genome, and genome instability in cancer cells. Therefore, I investigated whether loss of B α may

precipitate genome instability. Depletion of B α (but not B δ) increased the incidence of cytokinesis failure and genome reduplication, and lead to polyploidization.

PP2A activity is frequently attenuated in human tumours, with recent studies demonstrating loss of B α subunit expression in various cancers. My findings suggest that B α loss may support tumourigenesis by predisposing cells to polyploidization and genome instability.

Lay Summary

Cancer is the second leading cause of death worldwide. In Canada, nearly 1 in 2 people will be diagnosed during their lifetime and 1 in 4 will die from cancer. Cancer is a disease caused by the uncontrolled growth and division of cells. In normal conditions, cells divide in a highly controlled manner by following a coordinated, multistep process. A protein called PP2A plays an important role in controlling the cell division process at various steps, and its function and activity is often compromised in cancer. In this study, I investigated how PP2A regulates various stages of the cell division process, and how the loss of specific functions of PP2A may contribute to uncontrolled cell division and cancer.

Preface

I performed all of the experiments and data analyses presented herein. Except for a few sets of cell lysates that were prepared by A. Beigi, I also processed all samples for analysis. Dr. Catherine Pallen and I designed the study and experiments.

Parts of the work presented in Chapters 3 and 4 have been composed into a manuscript that will be submitted for publication in June 2018.

HeLa cells that stably express various fluorescent protein-tagged markers were provided to us by Dr. Christopher Maxwell's groups and were generated by the indicated labs and institutes (see section 2.1.1).

Cell cycle regulation in higher eukaryotes has been studied using various model systems, most notably including *Drosophila melanogaster* (fruit flies), *Xenopus laevis* (African clawed frog), and human immortalized and cancer cell lines (especially the HeLa cell line). Core cell cycle regulatory mechanisms are shared between these model systems, as are the names of key regulatory proteins. Please note that throughout this thesis, when referring to human proteins only, I have written abbreviated protein names in all CAPS. When referring to non-human (or *both* human *and* non-human) proteins, only the first letter of the abbreviated protein name is capitalized.

No ethics approval was required for the research presented herein.

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List of Symbols

α alpha

β beta

γ gamma

δ delta

ε epsilon

List of Abbreviations

APC/C	Anaphase promoting complex/cyclosome
BSA	Bovine serum albumin
CAK	CDK-activating kinase
Cdc20	Cell division cycle 20
Cdh1	Cdc20 Homolog 1
CDK	Cyclin-dependent kinase
CFF	Cleavage furrow formation
CKI	CDK inhibitor
CPC	Chromosome passenger complex
CRL	Cullin-RING ligase
Cul1	Cullin1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dThy	Double Thymidine block
EDTA	Ethylenediaminetetraacetic acid
eGFP-TUBA	eGFP-tagged α -Tubulin
ETP	Etoposide
FBS	Fetal bovine serum
Fbw7	F-box WD40 repeat-containing protein 7
FCM	Flow cytometry
FUCCI	Fluorescent ubiquitination-based cell cycle indicator
HAD	Haloacid dehalogenase

HCC	Hepatocellular carcinoma
HEK 293	Human Embryonic Kidney 293
HRP	Horseradish peroxidase
LC/MS	Liquid chromatography/Mass spectrometry
LCMT1	Leucine carboxyl methyl transferase 1
LOH	Loss of heterozygosity
MCC	Mitotic checkpoint complex
mCherry-H2B	mCherry-tagged Histone H2B
MEM	Minimum Essential Medium
mKO-Cdt1	Kusabira-Orange-tagged Cdt1
MLN	Neddylation Activating Enzyme inhibitor, MLN4924
MT	Microtubule
Na ₃ VO ₄	Sodium orthovanadate
NaCl	Sodium chloride
NaDOC	Sodium deoxycholate
NAE	Neddylation Activating Enzyme
NaF	Sodium fluoride
NEBD	Nuclear envelope breakdown
NIPA	Nuclear interaction partners of anaplastic lymphoma kinase
Noc	Nocodazole
NP-40	Nonidet-P40 or nonyl phenoxyethoxyethanol
NT	Non-targeting
PBS	Phosphate buffered saline
PBSBA	1%BSA in PBS

Pen/Strep	Penicillin and Streptomycin
PME-1	PP2A methyl esterase 1
PMSF	Phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride
PP	Protein phosphatase
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PPM	Metal-dependent protein phosphatases
PPP	Phosphoprotein phosphatase
pro-TAME	pro-tosyl-L-arginine methyl ester
PTP	Protein Tyrosine Phosphatases
PTPA	PP2A Activator
PVDF	Polyvinylidene fluoride
RING	Really Interesting New Gene
RNA	Ribonucleid acid
RO	CDK1 inhibitor, RO3306
SAC	Spindle assembly checkpoint
SCF	Skp1–Cul1–F-box protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Ser, S	Serine
si, siRNA	small-interfering RNA
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
Thr, T	Threonine

Tyr, Y	Tyrosine
UPP	Ubiquitin-Proteasome Pathway
UTR	Untranslated region
WB	Western blot
β -TrCP	β -transducin repeat-containing protein

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Last but certainly not least, I am forever grateful to my family and friends for their patience, understanding and support in good times and in bad times.

Dedication

This work is dedicated to my parents, Savitree and Dietmar Sommerfeld, for their unwavering support, immeasurable generosity and unconditional love. Without them none of this would have been possible.

Chapter 1: Introduction

1.1 The cell division cycle

'Omnis cellula e cellula' (all cells stem from cells) – this famous aphorism, popularized by Rudolf Virchow in 1855, encapsulates life's arguably most important process, the cell division cycle. The cell division cycle (or cell cycle) is a highly integrated, complex process that underlies the growth, development, repair and reproduction of living organisms in both health and disease. Its ultimate purpose is the faithful transmission of genetic information from a mother cell into two genetically identical daughter cells.

1.1.1 A general overview of the cell division cycle

The eukaryotic cell cycle is classically divided into four stages: G1/G₀, S, G2 and M (or mitosis) phases (Fig. 1.1) (Norbury and Nurse, 1992). Progression through the division cycle proceeds in a unidirectional, oscillatory manner, where DNA replication (S phase) is coupled with the segregation of duplicated chromosomes into genetically identical daughter cells (M phase or mitosis). These landmark events are separated by two gap phases (G1 and G2), during which cells prepare for S phase and mitosis, respectively, by accruing mass, duplicating cellular components (such as organelles), and organizing replicated chromosomes (Morgan, 2007; Schafer, 1998). Progression through G1 and G2 phase is regulated by complex signaling networks, commonly referred to as 'checkpoints', that ensure the metabolic and biosynthetic demands of cell division are met, and that replicated DNA is undamaged before proceeding with subsequent steps [reviewed in (Barnum and O'Connell, 2014; Hartwell and Weinert, 1989; Lukas et al., 2004)]. Based on cellular morphological attributes the cell cycle has also been subdivided into interphase (G1, S and G2 phases) and mitosis (Fig.1.1) (Morgan, 2007; Schafer, 1998). Mitosis is itself further subdivided into

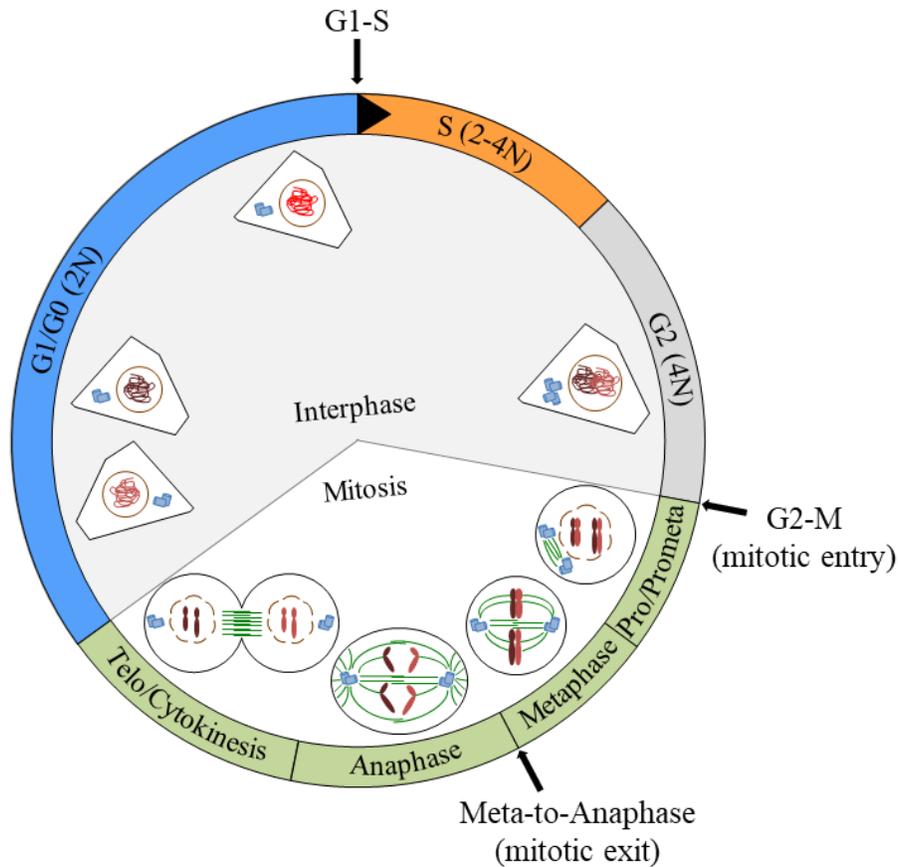


Figure 1.1: The phases of the cell division cycle

The eukaryotic cell cycle is classically divided into four stages: G1/G0 (blue), S (orange), G2 (gray) and M (or mitosis; green) phase. Each cycle is characterized by the duplication of the genome ($2N \rightarrow 4N$) during S phase, and the segregation of replicated chromosomes into genetically identical daughter cells during mitosis. These landmark events are separated by two gap phases (G1 and G2), during which cells ensure that the biosynthetic/metabolic demands of cell division are met, and that genomic DNA is undamaged. Mitosis is further divided into several stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.

Switch-like, irreversible transitions control the onset of S phase (G1-S), entry into mitosis (G2-M), and exit from mitosis (meta-to-anaphase) (indicated by black arrows).

Based on cellular morphological attributes the cell cycle has also been divided into interphase (G1, S, G2 phases, gray shaded area) and mitosis. Interphase cells are flattened with decondensed DNA, while mitotic cells are rounded with highly condensed chromosomes.

G0 denotes quiescence (i.e. cells that have exited the cell cycle). $2N$ indicates diploid and $4N$ indicates tetraploid (duplicated) genomic content. The black arrow head at G1-S transition indicates direction of cell cycle progression.

six stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Fig. 1.1) (Morgan, 2007). Entry into mitosis (or mitotic entry) occurs when cells transition from G2 phase into prophase, while mitotic exit begins with the transition from metaphase to anaphase when duplicated chromosomes start to segregate into the newly emerging daughter cells (Fig. 1.1, black arrows).

Switch-like transitions at the onset of S phase (G1-S), mitotic entry (G2-M) and mitotic exit (meta-to-anaphase) crucially separate the stages of the cell cycle (Fig. 1.1, black arrows), and ensure the unidirectional ordering of genome duplication and chromosome segregation (Fisher et al., 2012; Morgan, 2007). Strict ordering and alternation of S phase and mitosis is essential to safeguard genome integrity. While the cell cycle is controlled by a plethora of interlinked, complex regulatory mechanisms, it is universally recognized that reversible protein phosphorylation and targeted proteolysis play central roles in driving progression through the phases and establishing switch-like and irreversible transitions. Specifically, cyclin-dependent kinases (CDKs) and their counteracting protein phosphatases (PPs), and E3 ubiquitin ligase complexes control phase-specific protein phosphorylation cascades and proteolysis, respectively.

1.1.2 Cell cycle progression is regulated by reversible protein phosphorylation and targeted proteolysis

Progression through the cell cycle is associated with oscillations in the abundance and phosphorylation of distinct, phase-specific substrates, which execute defined functions during each stage [reviewed in (Nigg, 2001a; Poon, 2016; Teixeira and Reed, 2013)]. The ordered phosphorylation of these substrates is regulated by the carefully balanced activities of protein kinases and phosphatases, which are themselves controlled by phosphorylation and targeted proteolysis (Fisher et al., 2012; Nigg, 2001a; Novak et al., 2010a). Alterations in levels of cell cycle proteins result from phase-specific gene expression and targeted, ubiquitin-mediated proteolysis, processes

that are also subject to phosphorylation-dependent regulation [reviewed in (Poon, 2016; Teixeira and Reed, 2013)]. Indeed, phosphorylation-dependent and proteolytic pathways are critically interlinked, and their reciprocal regulation gives rise to the oscillatory behaviour of the cell cycle (Novak et al., 2007; Novak et al., 2010b; Yang and Ferrell, 2013). Here, I briefly introduce the major cell cycle kinases, phosphatases and ubiquitin ligases, before providing an overview of their specific, regulated activities in the different phases of the cell cycle.

1.1.2.1 Cyclin-dependent kinases in the driver's seat

The overwhelming majority of phosphorylation events associated with cell cycle progression occurs on serine (Ser, S) and threonine (Thr, T) residues (~85% and 15%, respectively), with less than 1% involving tyrosine (Tyr, Y) residues (Olsen et al., 2006; Olsen et al., 2010; Sharma et al., 2014). Therefore, progression through the cell cycle is guided by S/T kinases and phosphatases. While several kinases are critically involved in cell cycle regulation, particularly during mitosis, cyclin-dependent kinases (CDKs) are the main drivers of cell cycle phosphorylation [reviewed in (Morgan, 1997; Nigg, 2001a)]. As suggested by their name, CDKs require interaction with a cyclin subunit for activity. Initially discovered as proteins whose levels fluctuate in a cell cycle-dependent manner (Evans et al., 1983), cyclins are essential phase-specific activators of CDKs that also modulate substrate affinity and specificity (Bloom and Cross, 2007; Hunt, 1991). Indeed, it is through their association with different cyclin subunits that CDKs mediate stage-specific substrate phosphorylation (Hochegger et al., 2008; Koivomagi et al., 2011; Pagliuca et al., 2011). Eukaryotic genomes encode variable numbers of cyclins and CDKs (yeast: 15 cyclins, 16 CDKs; humans: 29 cyclins, 20 CDKs), reflecting differences in the complexity of cell cycle regulatory mechanisms (Malumbres, 2014). In yeast a single CDK (Cdc2 in *Schizosaccharomyces pombe* or fission yeast; Cdc28 in *Saccharomyces cerevisiae* or budding yeast) associates with a variety of phase-specific

cyclin subunits to mediate progression through the different phases (Bloom and Cross, 2007; Koivomagi et al., 2011; Nasmyth, 1996). In metazoans, however, different cell cycle phases are executed by distinct CDK-cyclin complexes where both the CDK and cyclin subunits vary. Importantly, mammalian cell cycle progression is predominantly driven by CDKs 1, 2, 4 and 6 and the D-, E-, A- and B-type cyclins [reviewed in (Hochegger et al., 2008; Malumbres and Barbacid, 2005; Morgan, 2007)].

The phase-specific activities of CDKs are subject to additional layers of regulation. CDKs contain both activating and inactivating phosphorylation sites, which must be phosphorylated and dephosphorylated, respectively, for full activation of the kinase complex [reviewed in (Morgan, 1997)]. The CDK-activating kinase (CAK) – a heterotrimer composed of CDK7, cyclin H and Mat1 – catalyzes the activating phosphorylation on a conserved threonine residue in the T-loop of all CDKs (T161 on CDK1) (Harper and Elledge, 1998; Russo et al., 1996). The phosphorylation of two inhibitory sites (T14/Y15 on CDK1) situated in the ATP-binding domain of the cell cycle-regulated CDKs is carried out by the Wee1 and Myt1 kinases, while the removal of these phosphates and activation of CDKs is mediated by Cdc25 family phosphatases [reviewed in (Boutros et al., 2007; Perry and Kornbluth, 2007)]. Furthermore, CDK inhibitory proteins (CKIs) can bind monomeric CDKs or CDK-cyclin heterodimers to suppress their activity, while small adaptor proteins of the highly conserved Cks family interact with CDK heterodimers to promote and specify substrate interactions [reviewed in (Lim and Kaldis, 2013; McGrath et al., 2013; Morgan, 1997; Pines, 1996)].

Together, these multilayered regulatory mechanisms give rise to highly tunable and responsive CDK-cyclin complexes and enable the integration and processing of intra- and extracellular signals that influence cell cycle progression. It is, therefore, no surprise that CDKs have earned the title of the “engines, clocks and microprocessors” of the cell cycle (Morgan, 1997).

1.1.2.2 Protein phosphatases: modulators of cell cycle phosphorylation

Site-specific phosphorylation of a protein is the result of a delicate balancing act between specialized protein kinase and phosphatase activities. However, because the complement of human genes encoding S/T kinases is approximately one order of magnitude larger than that of S/T phosphatases (428 vs. 40, respectively), protein phosphatases were long believed to be non-specific housekeeping enzymes that indiscriminately remove phosphates following the inactivation of a kinase (Manning et al., 2002; Moorhead et al., 2007). It is increasingly recognized, however, that protein phosphatases have exquisite substrate specificity and play highly specific roles in the cell [reviewed in (Moorhead et al., 2007; Virshup and Shenolikar, 2009)]. S/T phosphatases of the large phosphoprotein phosphatase (PPP) family – which notably includes PP1, PP2A, and PP6 – play essential roles in cell cycle regulation. Specifically, PP1 and PP2A exert pleiotropic effects on the cell cycle, in part by counteracting and regulating the activities of CDKs and other cell cycle kinases [reviewed in (Barr et al., 2011; Kolupaeva and Janssens, 2013; Stark et al., 2016; Wlodarchak and Xing, 2016)]. Briefly, PP1 and PP2A exist as holoenzyme complexes composed of scaffold, catalytic and regulatory subunits. The combinatorial interaction of a few catalytic subunit isoforms with a large array of regulatory subunits produces a rich diversity of highly specific protein phosphatase activities (Shi, 2009; Virshup and Shenolikar, 2009). PP1, for example, forms a heterodimer comprised of one of four catalytic subunit isoforms (PP1 α , β/δ , and γ) and a regulatory subunit that determines holoenzyme activity, subcellular localization, and substrate binding/specificity. More than 200 interacting partners have been identified for the PP1 catalytic subunit, potentially creating over 650 distinct holoenzymes with different functions [reviewed in (Bollen et al., 2010; Heroes et al., 2013; Peti et al., 2013)]. PP2A comprises a large family of heterotrimeric holoenzyme composed of a scaffold (A), catalytic (C), and variable regulatory (B) subunit. The structure and function of PP2A holoenzymes is introduced in great detail below. In addition to PP2A and PP1 holoenzymes,

other PPP family members (notably PP6), the Cdc14 and Cdc25 phosphatases of the Protein Tyrosine Phosphatase (PTP) superfamily, and Fcp1 of the haloacid dehalogenase (HAD) superfamily have been shown to play important roles in regulating the cell cycle [reviewed in (Boutros et al., 2007; Moorhead et al., 2007; Visconti et al., 2013)].

Akin to CDKs and other cell cycle proteins, the activities and functions of these phosphatases are also regulated by phosphorylation-dependent mechanisms throughout the cell cycle. Notably, these regulatory phosphorylations are carried out by CDKs and other cell cycle kinases and include direct phosphorylation of the catalytic or regulatory subunits (Kolupaeva et al., 2013; Kwon et al., 1997; Margolis et al., 2006; Visconti et al., 2012), and/or phosphorylation of small inhibitory proteins that act as competitive inhibitors of phosphatase activity (Gharbi-Ayachi et al., 2010; Leach et al., 2003; Mochida et al., 2010; Porter et al., 2013; Wang et al., 2008b; Wu et al., 2009).

Cell cycle kinases and phosphatases engage in tightly regulated networks characterized by reciprocal regulation. The carefully orchestrated interplay and integration of kinase and phosphatase activities plays a key role in the ordered progression through the phases and the switch-like transitions that generate directionality in a division cycle (Fisher et al., 2012; Novak et al., 2010a).

1.1.2.3 Cell cycle ubiquitin ligases and targeted proteolysis: insurance against reversibility

Regulated protein degradation constitutes a fundamental aspect of cell cycle control. The irreversible nature of proteolysis complements the intrinsically reversible regulation by phosphorylation and ensures directionality during cell cycle progression. Proteins are targeted for degradation by the 26S proteasome through ubiquitination, which is mediated by the highly coordinated and sequential actions of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligating enzyme (E3) [reviewed in (Teixeira and Reed, 2013)]. Importantly, the E3 ubiquitin ligases are responsible for recognizing, binding, and ubiquitinating

substrates. Two structurally related E3 ligases of the multisubunit cullin-RING ligase (CRL) family represent the key regulators of cell cycle-dependent proteolysis: the anaphase promoting complex/cyclosome (APC/C) and Skp1–cullin1–F-box (SCF) complexes [reviewed in (Mocciaro and Rape, 2012; Teixeira and Reed, 2013)]. The APC/C plays a particularly vital role in cell cycle regulation, and therefore, will be the focus of this overview. The APC/C is composed of 14 distinct protein subunits (Apc1-8, Apc10-13, and Apc15-16) and binds to one of two coactivator proteins, Cdc20 or Cdh1, in a cell cycle phase-dependent manner [reviewed in (Sivakumar and Gorbsky, 2015)]. In addition to stimulating APC/C activity, these coactivators mediate substrate recruitment and interaction (Glotzer et al., 1991; Kraft et al., 2005; Pflieger and Kirschner, 2000). APC/C complexes are active primarily during mitosis and G1 phase, where they target important cell cycle regulators for degradation, including mitotic cyclins and kinases, ensuring the irreversibility of mitotic exit and a stable G1 phase [reviewed in (Sullivan and Morgan, 2007)].

Post-translational modifications play complex but essential roles in the regulation of APC/C complex activity and substrate targeting. Phosphorylation-dependent regulation of APC/C activity is particularly complex, as some phosphorylations promote while others inhibit activity, in part, by modulating interactions with the coactivators. Furthermore, phosphorylation of the coactivators themselves differentially modulates their ability to bind the APC/C complex [reviewed in (Sivakumar and Gorbsky, 2015)]. Substrate recognition by the Cdc20 or Cdh1 coactivators is also influenced by phosphorylation; however, the underlying mechanisms are also shrouded in complexity as phosphorylation may enhance or reduce affinity for substrates [reviewed in (Sivakumar and Gorbsky, 2015)]. Importantly, the ubiquitin-proteasome pathway (UPP) and phosphorylation-dependent cell cycle control mechanisms are not only extensively interlinked but are also engaged in reciprocal and mutual regulatory relationships, which play an integral role in the irreversibility of cell cycle transitions.

1.1.3 G1 phase progression: control of the RB/E2F pathway by CDK-cyclin and PP1/PP2A

Commitment to a cell division cycle following mitogenic stimulation, or progression through G1 phase in actively cycling cells is regulated by an essential cell cycle checkpoint, called the restriction point (or R-point) (Pardee, 1974). The R-point is centered on the activity of the RB/E2F pathway, which regulates the expression of cell cycle-promoting genes [reviewed in (Foster et al., 2010; Malumbres and Barbacid, 2001)]. E2Fs, particularly activator E2Fs1-3, are transcription factors that induce the expression of genes involved in G1 progression, DNA replication and repair, and mitosis, notably including the genes that encode the E- and A-type cyclins, CDK2, DNA replication factors, and E2Fs themselves (Bracken et al., 2004; Polager et al., 2002). The retinoblastoma protein (pRB) and pRB-related p107 and p130 proteins (collectively referred to as the ‘pocket proteins’; herein interchangeably abbreviated as RB proteins) restrain progression through G1 phase by negatively regulating activator E2Fs. The activity of the RB/E2F pathway is largely controlled by multisite phosphorylation of the RB proteins. In their hypophosphorylated, active state RB proteins bind to and inhibit the E2F transcription factors [reviewed in (Giacinti and Giordano, 2006; Harbour and Dean, 2000)]. Following sufficient mitogenic stimulation, however, RB proteins are gradually inactivated through sequential phosphorylation by the CDK4/6-cyclin D and CDK2-cyclin E complexes during early-to-mid and mid-to-late G1 phase, respectively (Fig. 1.2A) [reviewed in (Ho and Dowdy, 2002; Lundberg and Weinberg, 1998; Sherr, 2000)]. Mitogens first induce the expression of D-type cyclins in a myc- and/or cJun-dependent manner (Bretones et al., 2015; Schreiber et al., 1999). As cyclin D accumulates, CDK4/6 complexes become increasingly activated and initiate low levels of RB phosphorylation, which are sufficient to induce the expression of E-type cyclins (Ho and Dowdy, 2002). Increasing levels of E-type cyclins subsequently lead to the activation of CDK2-cyclin E complexes, which hyperphosphorylate and inactivate the pocket proteins, resulting in the release of activator E2Fs and the full activation of their target genes

(Giacinti and Giordano, 2006; Ho and Dowdy, 2002; Lundberg and Weinberg, 1998). Full activation of E2Fs ultimately supports the sustained expression of genes required for DNA replication, including cyclin A, MCM2-7, PCNA, and DNA polymerases α and δ (Bracken et al., 2004).

The role of protein phosphatases in the regulation of G1 progression remains incompletely understood. However, it is widely recognized that CDK-mediated phosphorylation and inactivation of RB proteins is largely counteracted by PP1 and PP2A complexes (Fig. 1.2A), implicating them as negative regulators of G1 progression (Alberts et al., 1993b; Hirschi et al., 2010; Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2015). More precisely, PP1 directly interacts with pRB and mediates its abrupt dephosphorylation during exit from mitosis and early G1 phase, thereby activating the growth suppressive function of pRB (Grana, 2008; Ludlow et al., 1993; Vietri et al., 2006). PP2A holoenzymes appear to play a more prominent role in the dynamic regulation of pocket protein phosphorylation in response to signaling cues and throughout the cell cycle (Garriga et al., 2004; Kurimchak and Grana, 2012a, b, 2015). Accordingly, various PP2A heterotrimers have been implicated in the dephosphorylation of pRB, p107, and p130 in various cell types and conditions (Garriga et al., 2004; Jayadeva et al., 2010; Kolupaeva et al., 2013; Magenta et al., 2008; Voorhoeve et al., 1999). While these studies have demonstrated that PP1 and PP2A counteract the phosphorylation and inactivation of RB proteins, these activities have mostly been linked to cell cycle exit and quiescence, or G1 arrest (Berndt et al., 1997; Jayadeva et al., 2010; Kolupaeva et al., 2013). Therefore, it remains to be elucidated if and how these phosphatases dynamically modulate the kinetics of G1 progression.

Importantly, the phosphorylation status of RB proteins is controlled by the carefully balanced activities of CDK-cyclin complexes and PP1/PP2A holoenzymes. Therefore, disrupting this balance through the hyperactivation of CDK-cyclin complexes or inactivation of PP1/PP2A may lead to unrestrained cell proliferation through the constitutive inactivation of the RB proteins (Garriga et al.,

2004; Kolupaeva and Janssens, 2013; Kurimchak and Grana, 2012a). Correspondingly, the genes encoding the D- and E-type cyclins are commonly overexpressed or amplified in a variety of cancers [reviewed in (Hwang and Clurman, 2005; Sherr and Sicinski, 2018; Siu et al., 2012)]. Indeed, pRB is widely considered to be *the* prototypical tumour suppressor, and the components of the CDK/Rb/E2F pathway account for some of the most frequently altered genes across all cancers [reviewed in (Johnson, 2016; Malumbres and Barbacid, 2001; Nevins, 2001)].

1.1.4 Control of S phase progression by CDK-cyclin and PP1/PP2A

The error-free and complete duplication of the genome once per cell division is essential to the maintenance of genome integrity. To ensure the fidelity of the duplication process and restrict genome duplication to once per cell cycle, DNA replication is regulated by two sequential events: replication origin ‘licensing’ during the M-to-G1 transition and early G1 phase, and origin ‘firing’ at the G1-to-S transition [reviewed in (Fragkos et al., 2015; Woo and Poon, 2003)]. Replication licensing is characterized by the ordered assembly of multi-protein pre-replication complexes (pre-RCs) onto preferred sequence motifs in the genome (Cayrou et al., 2011; Delgado et al., 1998; Leonard and Mechali, 2013). This process involves the sequential and interdependent recruitment of origin recognition complex subunits (ORC1-6), Cdc6, Cdt1 (CDC10-dependent transcript 1), and the hexameric mini-chromosome maintenance complex (MCM2-7) onto thousands of distinct sites in the genome [reviewed in (Masai et al., 2010)]. At the G1-S transition, origin activation and firing require the phosphorylation-dependent formation of pre-initiation complexes (pre-IC) at licensed replication origins. CDK2-cyclin E and the Cdc7 kinase in complex with its activator, DBF4 (also known as DBF4-dependent kinase; DDK), play essential roles in directing the assembly of pre-ICs and subsequent replication origin firing (Fig. 1.2B) [reviewed in (Fragkos et al., 2015; Woo and Poon, 2003)]. The phosphorylation of numerous replication factors – notably MCM10, CDC45, the

RECQL4 DNA helicase, Treslin, DNA-topoisomerase 2-binding protein 1 (TOPBP1) and DNA polymerase ϵ (Pol ϵ) – promotes their loading onto licensed replication origins. Following pre-IC assembly, the phosphorylation of the MCM2-7 complex by these kinases triggers the unwinding of the DNA double helix and the recruitment and binding of additional replication factors, including replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and other DNA polymerases. This converts pre-ICs into functional replication forks, which drive the duplication of the genome [reviewed in (Fragkos et al., 2015)]. CDK2-cyclin E and DDK also phosphorylate licensing components to promote their disassociation from chromatin and their proteasomal degradation, thereby preventing relicensing and re-firing of replication origins and limiting replication to once per cell cycle [reviewed in (Fragkos et al., 2015; Woo and Poon, 2003)]. Once the initiation of DNA replication and the removal of licensing components have occurred, cyclin E is targeted for SCF^{Fbw7}-mediated ubiquitination and proteasomal degradation (Koepp et al., 2001; Won and Reed, 1996). At this point, CDK1/2-cyclin A complexes become the predominant kinase activity and promote the completion of genome duplication (Fig. 1.2B). Importantly, CDK1/2-cyclin A localizes to active replication forks, where it phosphorylates components of the replication machinery to ensure ordered and timely fork progression and genome replication [reviewed in (Woo and Poon, 2003)]. Therefore, the initiation and completion of DNA replication are largely driven by protein phosphorylation.

The role of protein phosphatases in the regulation of unperturbed DNA replication remains poorly understood. While PP1 negatively regulates initiation of DNA replication, PP2A appears to act as a positive regulator (Fig. 1.2B). Specifically, PP1 in conjunction with its regulatory protein, Rif1, has been shown to counteract DDK-dependent phosphorylations, thereby restraining replication initiation and progression [reviewed in (Stark et al., 2016)]. Conversely, PP2A activity is required for DNA replication initiation (Lin et al., 1998; Stark et al., 2016). The identity and precise roles of the specific PP2A heterotrimers remains largely unknown; however, it has been suggested that PP2A

positively regulates Treslin-dependent loading of CDC45 onto replication origins [reviewed in (Stark et al., 2016)]. More attention has been directed at understanding the roles for various phosphatases in DNA damage response pathways, where they have emerged as important modulators of DNA repair mechanisms, in part by preventing cell cycle progression in the presence of DNA damage [reviewed in (Lee and Chowdhury, 2011; Liu and Xu, 2011; Zheng et al., 2015)].

Importantly, the association of CDK-cyclin and other replication factors with replication origins and active replication forks is highly dynamic, and has been shown to be regulated by phosphorylation (Fragkos et al., 2015; Frouin et al., 2002; Henneke et al., 2003; Stark et al., 2016), suggesting that both the initiation and timely progression of DNA replication are controlled by a dynamic interplay between protein kinase and phosphatase activities. While PP2A positively regulates replication initiation, it remains largely unknown whether it is involved in modulating phosphorylation-dependent events during S phase progression.

1.1.5 The expression and accumulation of mitotic regulators during G2 phase is regulated by phosphorylation-dependent mechanisms

Progression through G2 phase is characterized by the accumulation of proteins and regulators required for the execution of mitosis. The expression of G2 and mitotic regulators is induced during S phase, peaks during G2 and M phase, and notably includes the genes encoding cyclin B1, CDK1, Cdc25, Plk1, and Aurora kinase A/B amongst others (Fig. 1.2C) (Liu et al., 2017; Whitfield et al., 2002). Akin to the gene expression programs that drive G1 and S phase progression, the transcription of G2/M genes is largely initiated and maintained by CDK-mediated phosphorylation (Bertoli et al., 2013; Fischer and Müller, 2017). Specifically, CDK2-cyclin E and CDK1/2-cyclin A complexes phosphorylate and activate a set of transcription factors, including B-Myb, FoxM1, NF-Y and NRF1,

which control the expression of G2/M genes (Fig. 1.2C) (Chae et al., 2004; Laoukili et al., 2008b; Wierstra and Alves, 2006; Ziebold et al., 1997). The correctly timed and sustained activation of G2/M gene expression is essential for mitotic progression, and deletion of these transcription factors or the disruption of their phosphorylation-dependent activation can lead to G2 arrest, mitotic defects and/or chromosome instability (Osterloh et al., 2007; Tarasov et al., 2008; Wolter et al., 2017; Wolter et al., 2012). For example, FoxM1 expression and activation by phosphorylation is required for proper entry and progression through mitosis (Fu et al., 2008; Laoukili et al., 2005; Wonsey and Follettie, 2005). The accumulation of G2/M regulators ensures that the biochemical setting of high kinase activity required for mitosis can be supported (Lindqvist et al., 2009). At the end of mitosis, G2/M transcription factors are degraded in an SCF- and APC/C-dependent manner to ensure that G2/M genes are no longer expressed and that exit from mitosis is irreversible (Charrasse et al., 2000; Laoukili et al., 2008a).

In addition to the accumulation of G2/M proteins, the phosphorylation-dependent signalling networks that control mitotic entry and progression are set up and primed during G2 phase. Specifically, CDK1/2-cyclin A complexes play a central role in driving cells through G2 phase and into mitosis by activating important mitotic regulators, including Plk1, CDK1-cyclin B1, and Cdc25C (Gheghiani et al., 2017; Mitra and Enders, 2004; Silva Cascales et al., 2017). One CDK1/2-cyclin A target of particular importance is Plk1, which acts as an essential driver of mitotic entry and progression by regulating the activity of other mitotic regulators, including Cdc25C, Wee1/Myt1, and CDK1-cyclin B1, and by coordinating essential mitotic events, such as mitotic spindle assembly (discussed below) [reviewed in (van Vugt and Medema, 2005)]. Furthermore, CDK1/2-cyclin A ensures that APC/C activity is inhibited until early mitosis to prevent premature degradation of mitotic regulators (Hein and Nilsson, 2016).

The role of protein phosphatases during the S-to-G2 transition and G2 progression is not well understood. However, it has become evident that the activities of G2/M kinases and opposing phosphatases have to be tightly regulated to ensure proper progression into and through mitosis, and that protein phosphatases play an active role in this process by setting activity thresholds for G2/M kinases (Domingo-Sananes et al., 2011; Fisher et al., 2012; Godfrey et al., 2017; Harvey et al., 2011; Krasinska et al., 2011).

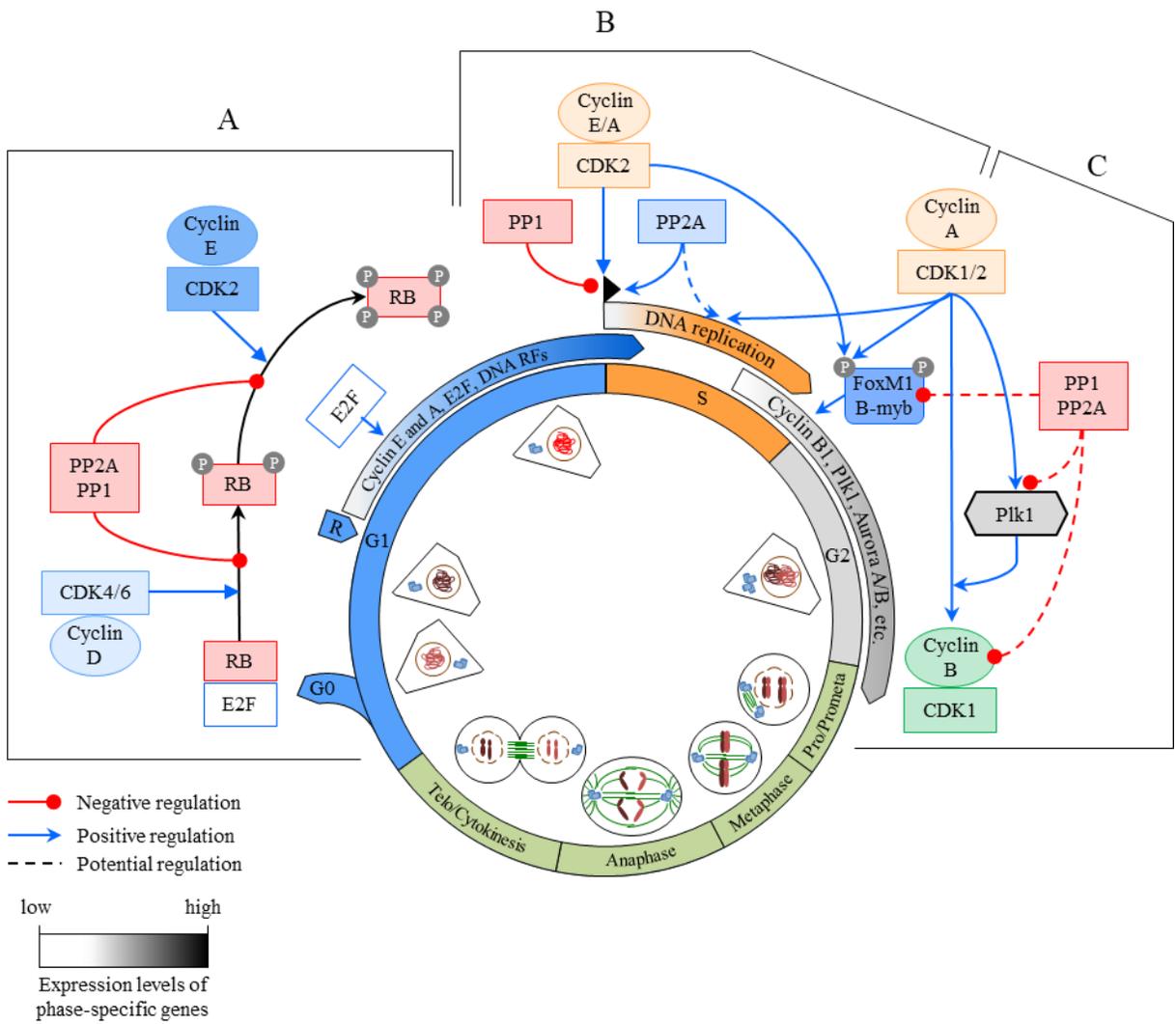


Figure 1.2: Major G1, S, and G2 phase events regulated by CDK-cyclin and PP1/PP2A

- A. Progression through G1 phase is regulated by the restriction point (R-point; R), which is centered on the activity of the RB/E2F pathway. In quiescent cells (G0) or early G1 phase, hypophosphorylated RB proteins (pRB, p107, p130) bind to and inhibit E2F transcription factors. As cells progress through G1 phase, CDK4/6-cyclin D complexes initiate the phosphorylation of RB proteins resulting in limited activation of E2F target genes, including cyclin E. Subsequent activation of CDK2-cyclin E complexes leads to hyperphosphorylation of RB proteins and full activation of E2F target genes, including E/A-type cyclins, E2F, and DNA replication factors (RFs). RB phosphorylation is antagonized by PP1 and PP2A complexes.
- B. At the G1-S transition, CDK2-cyclin E triggers replication origin firing and the initiation of genome duplication. Origin firing is antagonized by PP1, while PP2A positively regulates replication initiation and possibly progression. Following onset of DNA replication, CDK1/2-cyclin A regulates S phase progression.
- C. From late S and throughout G2 phase, CDK1/2-cyclin A complexes phosphorylate and activate regulators of G2/M gene expression programs, including FoxM1 and B-myb transcription factors. Notably, FoxM1/B-myb target genes include cyclin B1, Plk1, and Aurora A and B kinases, whose accumulation and activities are essential for the execution of mitosis. CDK1/2-cyclin A complexes also prime and set up phosphorylation-dependent mitotic entry circuits, by phosphorylating and activating Plk1. Plk1 and CDK1/2-cyclin A then participate in CDK1-cyclin B1 activation at the G2-M transition (mitotic entry) through the phosphorylation and activation of Cdc25C (not shown in diagram). Little is known about the roles of PP1 and PP2A in the regulation of G2/M gene expression programs, however, they have been implicated as negative regulators of FoxM1, Plk1 and CDK1-cyclin B1 activation.

1.1.6 Mitosis: key phosphorylation- and proteolysis-dependent signaling networks

In higher eukaryotes, mitosis proceeds in a highly ordered sequence of events that have been grouped into distinct phases based on cellular structural features: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Fig. 1.3). The major physical events that characterize prophase include: the reorganization of the actin and microtubule (MT) cytoskeleton to induce cell rounding and the opposite poleward movement of duplicated centrosomes, respectively (Lancaster and Baum, 2014); the fragmentation of the nuclear envelope, endoplasmic reticulum, and Golgi apparatus (Alvarez-Fernandez and Malumbres, 2014; Güttinger et al., 2009); and the compaction and cohesion of duplicated sister chromatids into the iconic X-shaped mitotic chromosomes (Antonin and Neumann, 2016). During prometaphase, the MT-based bipolar mitotic

spindle takes shape and captures chromosomes at their kinetochores, large multiprotein complexes that assemble on the centromeres of each sister chromatid. Through the highly dynamic nucleation, growth, and shrinkage of MTs the bipolar spindle coordinates the congression of captured chromosomes at the center of the cell, the metaphase plate (Cheeseman, 2014). Metaphase, the mid-point of mitosis, is characterized by the successful alignment of chromosomes at the metaphase plate and the bi-oriented (or amphitelic) attachment of opposing MTs to the kinetochores of conjoined sister chromatids (or sister kinetochores) (O'Connell and Khodjakov, 2007; Petry, 2016). Successful bi-oriented attachment of all sister kinetochores satisfies the major mitotic checkpoint, the spindle assembly checkpoint (SAC), and triggers the disengagement of sister chromatids and the metaphase-to-anaphase transition (or mitotic exit) (Musacchio and Salmon, 2007). During mitotic exit, sister chromatids are segregated towards opposite poles by shortening bipolar spindles and an elongating central spindle. This is accompanied by the ingression of the cell membrane at the center of the cell (or the cleavage plane), a process known as cleavage furrow formation (CFF). The assembly and activity of an actin-based contractile ring on the inside of the plasma membrane at the cell equator drives CFF, and ultimately the abscission (the physical separation) of the daughter cells during telophase and cytokinesis, respectively (Barr and Gruneberg, 2007; Normand and King, 2010; Sullivan and Morgan, 2007). As the newly emerging daughter cells form, the nuclear envelope and other membrane-bound organelles reassemble, chromosomes decondense, and the cells return to an interphase state.

The events of mitotic entry and exit are primarily driven by the phosphorylation and dephosphorylation of thousands of substrates, respectively (Dephoure et al., 2008; McCloy et al., 2015; Olsen et al., 2010; Rogers et al., 2015; Sharma et al., 2014). In addition to substrate dephosphorylation, APC/C-mediated targeted proteolysis also plays an essential role in triggering mitotic exit and ensuring its irreversibility (Novak et al., 2007; Sivakumar and Gorbsky, 2015).

Furthermore, the switch-like activation/inactivation of a few central kinases and phosphatases and the activation of the APC/C are necessary and sufficient to orchestrate these transitions and ensure the unidirectional progression of subsequent events (Hegar et al., 2016; Hutter et al., 2017; Novak et al., 2007; Novak et al., 2010b). The molecular mechanisms underlying mitotic progression are immensely complex and remain the subject of intense scrutiny, and only key aspects will be introduced.

Mitotic entry

In eukaryotes, entry into mitosis and progression to metaphase are primarily driven by the phosphorylation of thousands of proteins, most of which are phosphorylated at multiple sites, resulting in >32,000 mitotic phosphorylation events (Dephoure et al., 2008; Olsen et al., 2010; Sharma et al., 2014). This extensive network of phosphorylation events is coordinated by the activities of several kinases, most notably CDK1, Plk1, and Aurora A/B (Dulla et al., 2010; Kettenbach et al., 2011; Malik et al., 2009; Nigg, 2001b; Olsen et al., 2010; Petrone et al., 2016; Santamaria et al., 2011). CDK1 activity directly or indirectly drives the majority of this phosphorylation cascade, and its activation is absolutely required for mitotic entry and progression (Dephoure et al., 2008; Holt et al., 2009; Petrone et al., 2016; Sharma et al., 2014; Vassilev et al., 2006). Befittingly, entry into mitosis is controlled by two interlinked feedback loops: one centered on the activation of CDK1, and the other centered on the inactivation of CDK1-antagonizing phosphatases to enable the accumulation of mitotic substrate phosphorylation (Fig. 1.3) (Mochida et al., 2016). Briefly, in addition to its association with the mitotic cyclin, cyclin B1, and the activating phosphorylation at T161 (Draetta, 1993; Solomon et al., 1990), activation of CDK1 at the G2/M transition requires the dephosphorylation of its inhibitory T14/Y15 sites (Solomon et al., 1991). Phosphorylation at the T14/Y15 sites is controlled by the opposing activities of the inhibitory

Wee1/Myt1 kinases and the activating Cdc25C (and Cdc25B) phosphatase (Hoffmann et al., 1993; McGowan and Russell, 1993; Mueller et al., 1995). CDK1-cyclin B1—Wee1/Myt1 and CDK1-cyclin B1—Cdc25C form the core negative and positive feedback loops, respectively, to regulate CDK1 activation. That is, once activated, CDK1-cyclin B1 phosphorylates Wee1/Myt1 and Cdc25C, leading to their inactivation and activation, respectively. Therefore, the activation of a small pool of CDK1-cyclin B1 triggers an auto-amplification loop, in which CDK1 inactivates its inhibitor and activates its activator, promoting the activation of more CDK1-cyclin B1 complexes (Dunphy, 1994; Hoffmann et al., 1993; O'Farrell, 2001). These core feedback loops appear to regulate the activation of CDK1 in eukaryotic systems ranging from yeast to humans. However, in budding yeast Wee1/Myt1 and Cdc25 are known as Swe1 and Mih1, respectively.

Recent seminal works in *Xenopus laevis*, *Drosophila melanogaster* and mammalian cell lines (prominently HeLa cells), have demonstrated that inhibition of CDK1-counteracting phosphatases is also required for efficient mitotic entry [reviewed in (Bollen et al., 2009; Jeong and Yang, 2013; Mochida and Hunt, 2012)]. PP2A complexes containing members of the B55 family of regulatory subunits (discussed below), in particular, have emerged as key anti-mitotic phosphatases, and inhibition of PP2A-B55 is required for the activation of CDK1-cyclin B1 and mitotic substrate phosphorylation (Burgess et al., 2010; Mayer-Jaekel et al., 1994; Mochida et al., 2009). Inhibition of PP2A-B55 complexes is mediated by the Greatwall kinase (Gwl) and its human ortholog, MASTL (Burgess et al., 2010; Castilho et al., 2009; Mochida et al., 2010; Vigneron et al., 2009; Voets and Wolthuis, 2010; Yu et al., 2006). Specifically, Gwl/MASTL phosphorylates two small heat-stable proteins, Ensa and ARPP19, which function as potent competitive inhibitors of PP2A-B55 complexes by acting as exceedingly poor substrates (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Vigneron et al., 2009; Williams et al., 2014). This PP2A-B55 inhibitory pathway has been termed the PP2A-B55/Ensa/Gwl (or BEG) pathway (Cundell et al., 2013). The CDK1-Wee1/Myt1-

Cdc25 auto-amplification loop and the BEG pathway are interlinked at multiple points to generate a robust and highly sensitive mitotic entry regulatory circuit (Domingo-Sananes et al., 2011; Mochida et al., 2016). Importantly, Gwl/MASTL is itself activated by CDK1-mediated phosphorylation (Blake-Hodek et al., 2012; Vigneron et al., 2011). Furthermore, PP2A-B55 has been implicated as a negative regulator of Cdc25C and positive regulator of Wee1 activity (Clarke et al., 1993; Lucena et al., 2017; Zhao et al., 2008). CDK1-cyclin B1 is also able to directly phosphorylate ARPP19, thereby inhibiting PP2A-B55 in a Gwl-independent manner (Okumura et al., 2014). Therefore, the initial activation of a small pool of CDK1-cyclin B1 not only triggers the activation of an auto-amplification loop, but also a cascade of events that leads to the inactivation of CDK1-opposing phosphatase activities. Furthermore, CDK1-cyclin B1 also induces the inactivation of PP1, another well-established CDK1-counteracting phosphatase during mitosis. Specifically, CDK1 directly phosphorylates the PP1 catalytic subunit at its inhibitory T320 site (Kwon et al., 1997; Wu et al., 2009). The inactivation of the CDK1-counteracting phosphatase PP2A-B55 appears to be required for mitotic entry in most eukaryotic model systems (including fission yeast), with the notable exception of budding yeast. Strikingly, the budding yeast PP2A-B55 ortholog, PP2A-Cdc55, plays a rather divergent role in mitotic regulation, where it positively modulates entry into mitosis (Godfrey et al., 2017).

Interestingly, the current body of literature indicates that the activation of these interlinked regulatory loops inadvertently depends on the initial activation of a small pool of CDK1-cyclin B1. The molecular mechanisms that underlie this initial activation remain incompletely understood; however, it is widely accepted that this requires the balance of Wee1/Myt1 and Cdc25C activities to be tipped in favour of higher Cdc25C activity, and therefore, T14/Y15 dephosphorylation. However, the regulation of Wee1/Myt1 and Cdc25 activities is highly context-dependent, and

many kinases and phosphatases have been shown to target these regulators, including surveyors of the DNA damage response and replication completion [reviewed in (Lindqvist et al., 2009; Ma and Poon, 2011; O'Farrell, 2001)]. Nevertheless, CDK1/2-cyclin A and Plk1 have been implicated as key initiators of CDK1 activation, as alluded to above (Gheghiani et al., 2017; Ma and Poon, 2011; Mitra and Enders, 2004). CDK1/2-cyclin A likely plays a particularly important role, as it also critically regulates other mitotic entry events, including the coordination of nuclear envelope breakdown (NEBD) (De Boer et al., 2008; Fung et al., 2007; Furuno et al., 1999; Gong et al., 2007; Mitra and Enders, 2004).

Despite the lack of a detailed understanding of the initial trigger, it has become abundantly clear that the activation of these feedback loops requires the passing of a minimal CDK1-cyclin B1 activity threshold, which is set by the finely balanced and opposing activities of the component kinases and phosphatases (Domingo-Sananes et al., 2011; Hegarat et al., 2016; Mochida et al., 2016; Tuck et al., 2013). Importantly, the proper regulation and activation of CDK1-cyclin B1 is essential to the integrity of mitosis, as the disruption of the component activities perturbs normal mitotic entry and leads to a range of mitotic defects. Incomplete activation of CDK1-cyclin B1, for example, can precipitate centrosome fragmentation and/or premature cytokinesis in the presence of unsegregated chromosomes, leading to cytokinesis failure and a polyploid genome post-mitosis (McCloy et al., 2014; Vassilev et al., 2006). Similarly, loss of Gwl/MASTL in *Drosophila* and human cells causes multiple mitotic defects, including undercondensed chromosomes, defective chromosome segregation, cytokinesis failure, and mitotic collapse (Alvarez-Fernandez et al., 2013; Burgess et al., 2010; Cundell et al., 2013; Diril et al., 2016; Voets and Wolthuis, 2010; Yu et al., 2004). Therefore, deregulation of the mitotic entry circuitry may compromise the integrity of mitosis and the stability of the genome.

Mitotic progression

Following the initial activation of CDK1 and entry into mitosis, CDK1 activity continues to rise until shortly after nuclear envelope breakdown (NEBD), passing through various activity thresholds that are associated with distinct mitotic events (Akopyan et al., 2014; Gavet and Pines, 2010; Lindqvist et al., 2007). For example, cell rounding requires low levels of activity and occurs concurrently with initial cytoplasmic activation of CDK1-cyclin B1, while NEBD requires high levels of CDK1 activity (Gavet and Pines, 2010). In addition to direct action, CDK1-cyclin B1 also plays a role in increasing and directing the activity of other major mitotic kinases, including Plk1 and the Aurora kinases. Notably, CDK1 and Plk1 appear to engage in a positive feedback loop during mitotic entry, further amplifying each other's activities [reviewed in (Lindqvist et al., 2009; Ma and Poon, 2011)]. Furthermore, CDK1 targets Plk1 activity to specific cellular structures by creating docking sites for the Polo-box domain of Plk1. Indeed, Plk1 is targeted to hundreds of CDK1 substrates, including the mitotic spindle and kinetochores (Elia et al., 2003; Lindqvist et al., 2009). Accordingly, Plk1 is an essential regulator of mitotic spindle assembly and dynamics (Santamaria et al., 2011; Sumara et al., 2004; van Vugt et al., 2004). CDK1 also plays a role in targeting the chromosome passenger complex (CPC) to the kinetochores of mitotic chromosomes. The CPC is a multiprotein complex that contains Aurora kinase B as its enzymatically active subunit and plays a central role in modulating kinetochore-microtubule (KT-MT) attachments (Carmena et al., 2012; van der Horst and Lens, 2014). Both Plk1 and Aurora kinase B crucially regulate KT-MT attachments and drive chromosome congression from prometaphase to the meta-to-anaphase transition. KT-MT attachment is a highly dynamic process that depends on the carefully regulated interplay between the activities of Plk1, Aurora kinase B (AURKB), and various protein phosphatases, including PP1 and PP2A complexes containing members of the B56 family of regulatory subunits (discussed below)

(De Wulf et al., 2009; Funabiki and Wynne, 2013; Gelens et al., 2018; Wurzenberger and Gerlich, 2011). Another major G2 and mitotic kinase activity is represented by Aurora kinase A (AURKA). Aurora kinase A is active from late G2 phase and plays an essential role in the regulation of centrosome separation and the establishment of a bipolar mitotic spindle (Marumoto et al., 2005). The modulation of Aurora kinase A activity by counteracting phosphatases, including PP6, is also essential to the proper regulation of centrosome separation and spindle bipolarity, as inhibition of these phosphatases or hyperactivation of AURKA leads to severe centrosomal and mitotic spindle defects, including centrosome fragmentation and the formation of multipolar spindles. Therefore, progression through mitosis is not merely driven by the activities of mitotic kinases, but also requires an assortment of phosphatases that act in concert with kinases to carefully coordinate mitotic events (Barr et al., 2011; Bollen et al., 2009; Qian et al., 2013). While PP1 and PP2A-B55 complexes are largely inactivated during mitotic progression (until mitotic exit), other protein phosphatases play crucial roles in the regulation of mitotic events.

Mitotic exit

Mitotic exit is driven by the dephosphorylation of mitotic substrates and the degradation of G2/M regulators. Indeed, dephosphorylation and proteolysis are essential for mitotic exit, as inhibition of either prevents cells from progressing past metaphase (Skoufias et al., 2007). The initiation of mitotic exit requires the establishment of proper amphitelic KT-MT attachments at all sister chromatids and the concomitant silencing of the SAC. Importantly, preceding successful bi-oriented attachment of all sister kinetochores, the SAC largely prevents mitotic exit initiation by recruiting the APC/C coactivator, Cdc20, into an inhibitory complex with other SAC components, known as the mitotic checkpoint complex (MCC). Inactivation of the SAC disbands the MCC and

releases Cdc20, leading to the full activation of the APC/C (Musacchio, 2011; Sivakumar and Gorbsky, 2015). The activated APC/C-Cdc20 complex subsequently initiates mitotic exit by targeting two key substrates for proteasomal degradation, namely securin and cyclin B1. The degradation of securin results in the activation of the protease, separase, which cleaves centromeric cohesin linkages to abolish sister chromatid cohesion and enable chromosome segregation during anaphase (Peters, 2006; Sullivan and Morgan, 2007). The degradation of cyclin B1, on the other hand, triggers the inactivation of CDK1, a key event that enables the subsequent dephosphorylation of mitotic substrates (Sullivan and Morgan, 2007; Wurzenberger and Gerlich, 2011). Multiple protein phosphatases act in concert to coordinate substrate dephosphorylation throughout mitotic exit, most notably PP1 and PP2A-B55 complexes (Fig.1.3) (Hunt, 2013; Qian et al., 2013; Wurzenberger and Gerlich, 2011), and it has been suggested that these phosphatases may participate in a relay-like mechanism (Grallert et al., 2015). The drop in CDK1 activity at the onset of mitotic exit allows PP1 to reactivate itself through auto-dephosphorylation of the inhibitory T320 site, and the dephosphorylation of Inhibitor-1 (Wu et al., 2009). Activated PP1 subsequently dephosphorylates and inactivates Gwl/MASTL, thereby reducing Gwl/MASTL-mediated phosphorylation of Ensa/ARPP19 (Heim et al., 2015; Ma et al., 2016; Mochida, 2015; Rogers et al., 2016a). This tips the balance of activities in favour of PP2A-B55 complexes, which then dephosphorylate Ensa/ARPP19 to promote their own reactivation (Williams et al., 2014). PP2A-B55 also feeds back into this loop by further dephosphorylating and inactivating Gwl/MASTL (Hegar et al., 2014). Following reactivation, PP2A-B55 complexes contribute significantly to the bulk dephosphorylation of mitotic substrates and the completion of mitotic exit (Cundell et al., 2013; Cundell et al., 2016; Manchado et al., 2010; Schmitz et al., 2010). Importantly, the accurately timed dephosphorylation of distinct substrates plays a crucial role in ordering mitotic exit events, for example, ensuring that chromosome segregation precedes cytokinesis (discussed below) (Cundell et al., 2013; Cundell et al., 2016; Hein

et al., 2017; Schmitz et al., 2010). In addition to PP1 and PP2A-B55, Fcp1 has also been implicated as a CDK1-substrate phosphatase and may also play a role in activating APC/C-Cdc20 and dephosphorylating Gwl (Della Monica et al., 2015; Hegarat et al., 2014; Visconti et al., 2012)

Proper coordination of mitotic exit events not only relies on ordered substrate dephosphorylation, but also requires the regulated activities of mitotic kinases. Plk1 plays a vital role in ordering and driving mitotic exit events (Lens et al., 2010; Petronczki et al., 2008; van Vugt and Medema, 2005). For example, Plk1 regulates central spindle assembly and elongation, and this process involves cooperation between Plk1 and PP2A-B55 activities (Cundell et al., 2013; Hu et al., 2012). Furthermore, Plk1 critically regulates the initiation and completion of cytokinesis by coordinating the timely localization and activation of essential components of the central spindle, contractile ring, and abscission machinery (Bastos and Barr, 2010; Brennan et al., 2007; Burkard et al., 2007; Hu et al., 2012; Neef et al., 2003; Petronczki et al., 2007). As the enzymatically active subunit of the CPC, Aurora B kinase also plays an important role in the ordered execution of many mitotic exit events (Kitagawa and Lee, 2015). In addition to regulating KT-MT attachments during prometaphase and metaphase, the CPC is involved in a plethora of mitotic exit events. Following the meta-to-anaphase transition, CPCs translocate from chromosomes to the cell equator to promote the initiation and ingression of the cleavage furrow, and to the central spindle to control the timing of abscission and the completion of cytokinesis (Carmena et al., 2012; Kitagawa and Lee, 2015; van der Horst and Lens, 2014).

Finally, in addition to playing a central role in triggering the meta-to-anaphase transition, the APC/C also drives mitotic exit progression by targeting many mitotic regulators for proteasomal degradation. Importantly, the degradation of these regulators is required to ensure that mitotic exit is irreversible (Sivakumar and Gorbsky, 2015; Sullivan and Morgan, 2007). While the APC/C-Cdc20 complex is responsible for targeting early mitotic exit substrates, the APC/C-Cdh1 complex becomes

the predominant ubiquitin ligase during mid-to-late mitotic exit and throughout G1 phase (Sivakumar and Gorbsky, 2015). The switch between Cdc20 and Cdh1 binding to the APC/C complex occurs shortly following the meta-to-anaphase transition and is largely dependent on the dephosphorylation of Cdh1 and the APC/C subunit, Apc3 (Sivakumar and Gorbsky, 2015; Zhang et al., 2016). The APC/C-Cdh1 complex then targets many mitotic regulators, including Cdc20, FoxM1, Plk1, and the Aurora kinases, for degradation at various times during mitotic exit and progression into G1 phase (Garcia-Higuera et al., 2008; Laoukili et al., 2008a; Li and Zhang, 2009).

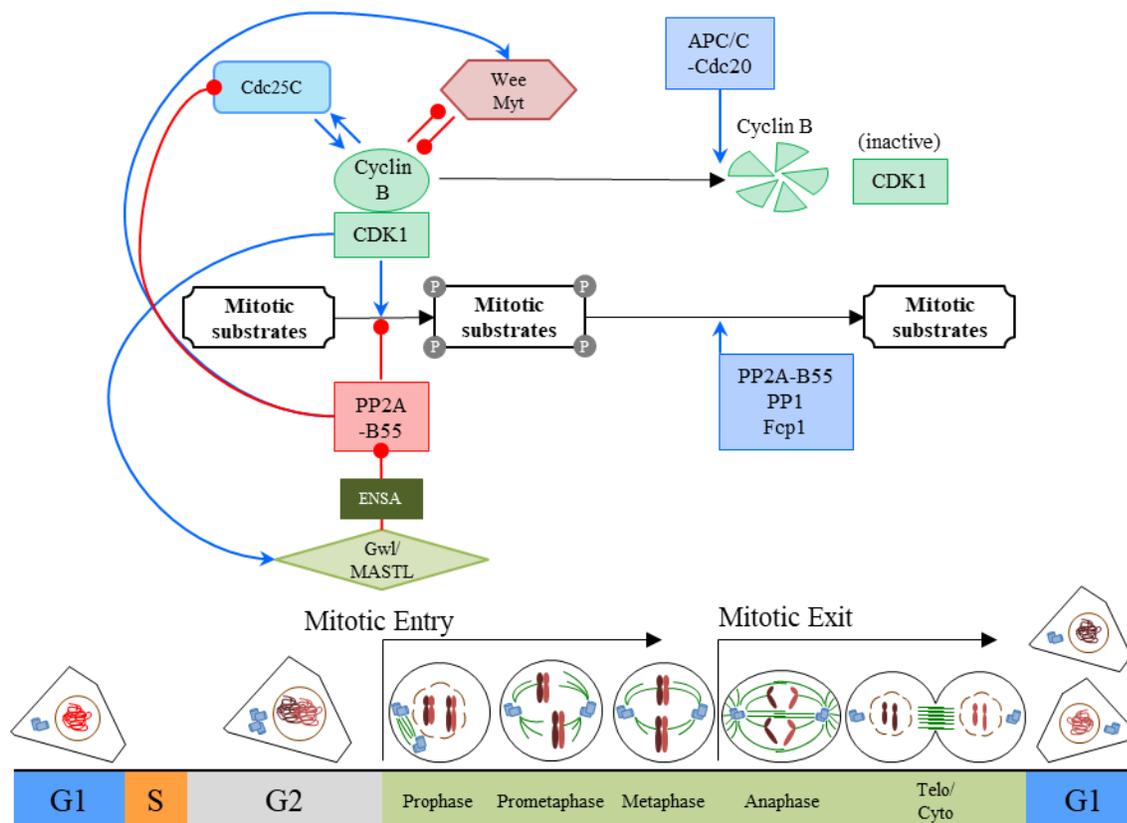


Figure 1.3: Regulation of mitotic entry and exit by CDK1-cyclin B1 and PP2A-B55

Entry into mitosis is regulated by two interlinked feedback loops centered on (i) the activation of CDK1-cyclin B1 and (ii) the inactivation of CDK1-antagonizing phosphatase, PP2A-B55.

- (i) CDK1-cyclin B1 engages in an auto-amplification loops, in which CDK1 phosphorylates and inactivates its repressor Wee1/Myt1 and phosphorylates and activates its activator Cdc25C.

- (ii) Activated CDK1 also phosphorylates and activates Greatwall/MASTL kinase (Gwl/MASTL), which phosphorylates Ensa/ARPP19 (referred to as ENSA) and turns it into a competitive inhibitor of PP2A-B55, thereby inhibiting the dephosphorylation of CDK1 mitotic substrates.

At the meta-to-anaphase transition (or mitotic exit), activation of the APC/C-Cdc20 complex leads to the degradation of cyclin B1 and the inactivation of CDK1. This results in the reactivation of PP2A-B55 and the dephosphorylation of mitotic substrates by PP2A-B55, PP1, and Fcp1.

1.2 Protein Phosphatase 2A: structure and function

Protein S/T phosphatases are grouped into three families based on their protein structures and catalytic mechanisms: the phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases (DxDxT) represented by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) (Shi, 2009). The PPPs comprise the largest family of S/T phosphatases and include PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7. Members of the PPP family exhibit one of the highest degrees of sequence conservation amongst all known enzymes across species, supporting a central role for these enzymes in cellular life (Brautigan, 2013; Cohen, 1997; Cohen et al., 1990; Orgad et al., 1990). PP2A is the most abundant member, accounting for up to 1% of total cellular protein depending on cell type and tissue of origin. Accordingly, PP2A (together with PP1) is responsible for the over 90% of all S/T phosphatase activity in the cell and is involved in the regulation of a myriad of cellular processes, including cell cycle regulation, cell growth, apoptosis, and metabolism [reviewed in (Eichhorn et al., 2009; Ruvolo, 2016; Van Hoof and Goris, 2003; Wlodarchak and Xing, 2016)]. Therefore, it comes as no surprise that the deregulation of PP2A activity is implicated in a variety of human diseases, including a wide range of cancers, Alzheimer's disease, and heart disease [reviewed in (Lubbers and Mohler, 2016; Ruvolo, 2016; Sontag and Sontag, 2014)]. The extensive involvement of PP2A in human pathophysiology has made it an increasingly attractive target for therapeutic intervention [reviewed in (Lambrecht et al., 2013; O'Connor et al., 2018; Voronkov et al., 2011)].

1.2.1 The PP2A holoenzyme: a structural and functional centipede

PP2A comprises a large family of multisubunit holoenzymes composed of a scaffold (A or PP2A_A), catalytic (C or PP2A_C), and regulatory (B) subunit (Fig. 1.4). The 65kDa scaffold and 36kDa catalytic subunits form an obligatory dimer, referred as the core enzyme (or PP2A_D), which binds a diverse array of regulatory subunits in a highly regulated and mutually exclusive manner to give rise to a plethora of distinct holoenzymes with specific cellular functions [reviewed in (Janssens and Goris, 2001; Mayer-Jaekel and Hemmings, 1994; Slupe et al., 2011)]. While PP2A complexes predominantly exist as heterotrimers containing a variable regulatory B subunit, approximately one third of PP2A is present in the dimeric core enzyme form (Kremmer et al., 1997). The scaffold and catalytic subunits each exist as two isoforms, α and β , encoded by distinct genes. Importantly, isoforms are defined as two or more distinct proteins that share a high degree of sequence identity. The scaffold subunit isoforms, A α and A β (encoded by the *PPP2RIA* and *PPP2R1B* genes, respectively), share 87% sequence identity. Despite the high degree of similarity, these isoforms exhibit differential binding to various regulatory subunits and are limited in their ability to functionally substitute for each other (Hemmings et al., 1990; Ruediger et al., 2001a; Zhou et al., 2003). Furthermore, the A α isoform is expressed far more abundantly in most metazoan adult tissues and is present in approximately 90% of PP2A complexes (Bosch et al., 1995; Hemmings et al., 1990; Hendrix et al., 1993b). The catalytic subunit isoforms, C α and C β , are encoded by the *PPP2CA* and *PPP2CB* genes, respectively, and exhibit 97% sequence identity. The α isoform is expressed at 10-fold higher levels and accounts for the vast majority of PP2A catalytic subunits in most cells (Khew-Goodall and Hemmings, 1988; Khew-Goodall et al., 1991; Stone et al., 1987). Due to their prevalence, it is generally supposed that most PP2A heterotrimers in the cell contain the α isoforms of the scaffold and catalytic subunits.

The B-type regulatory subunits are encoded by 15 genes in the human genome and belong to four distinct families: B/B55 (*PPP2R2* genes), B'/B56 (*PPP2R5* genes), B''/PR72 (*PPP2R3* genes), and B'''/Striatin (*PPP2R6* genes) (Fig. 1.4) [reviewed in (Eichhorn et al., 2009; Janssens et al., 2008)]. Alternate transcripts and spliceoforms exist for some of the subunits, ultimately giving rise to more than 23 distinct B-type subunit isoforms. In addition, the regulatory subunit isoforms exhibit distinct temporal, tissue-specific, and context-dependent expression and subcellular localization patterns, further increasing the complexity of PP2A biology [reviewed in (Eichhorn et al., 2009; Janssens and Goris, 2001; Sontag, 2001)]. The B-type subunits confer functional specificity to PP2A holoenzymes by regulating the activity, substrate recognition/recruitment and subcellular localization of the trimeric complexes [reviewed in (Janssens and Goris, 2001; Janssens et al., 2008; Slupe et al., 2011)]. Importantly, the different B-type families are structurally unrelated and contain distinct structural and biochemical features that underlie their diverse functions (Shi, 2009; Wlodarchak et al., 2013; Xu et al., 2008; Xu et al., 2006). Even within families, structural variation amongst isoforms likely accounts for differences in subcellular localization, protein-protein interactions, and therefore, function [reviewed in (Janssens and Goris, 2001; Janssens et al., 2008)].

B/B55 family

Higher eukaryote genomes contain four genes (α , β , γ , and δ) (Fig. 1.4) that yield six isoforms of B/B55 regulatory subunits (Eichhorn et al., 2009; Schmidt et al., 2002). This family exhibits the highest degree of overall sequence conservation amongst all B-type subunit families (>82% sequence identity within human family) (Fig. 1.5A). They are also remarkably conserved across species, exhibiting >50% sequence identity from yeast to human, and >72% identity across higher eukaryotes, including *Drosophila*, *Xenopus*, and humans (Fig. 1.5B and Table 1). While B α and B δ are expressed abundantly and ubiquitously, the B β and B γ subunits exhibit stricter temporal

and tissue-specific expression patterns [reviewed in (Eichhorn et al., 2009; Janssens and Goris, 2001)]. $B\gamma$ is almost exclusively restricted to the brain and testis, and its expression sharply increases after birth (Strack et al., 1998). The $B\beta$ subunit was initially believed to be largely restricted to the brain as well, however, more recently it has been shown to be expressed in a variety of cancer cell lines, including HeLa and breast cancer cell lines (Kolupaeva et al., 2013; Strack et al., 1998; Tan et al., 2014; Torres et al., 2010). The B/B55 subunits are composed of seven WD40 repeats, which form a β -propeller structure that is responsible for substrate interaction and recruitment (Xu et al., 2008). PP2A-B55 complexes, especially those containing the $B\alpha$ and $B\delta$ subunits, are involved in a broad range of cellular functions, but their roles in the regulation of mitosis have become a major point of focus (discussed below) [reviewed in (Eichhorn et al., 2009; Mochida and Hunt, 2012; Ruvolo, 2016)].

B'/B56 family

In mammals, the B'/B56 family is encoded by five genes (α , β , γ , δ , and ϵ) (Fig. 1.4) that produce eight distinct isoforms, which share a highly conserved central region (~80% identity) but contain variable N- and C-terminal regions that are thought to mediate isoform-specific subcellular localization and functions [reviewed in (Eichhorn et al., 2009; Janssens and Goris, 2001; Sontag, 2001)]. Members of this family are the most well-studied B-type subunits and, along with the B/B55 family, are the most abundantly and ubiquitously expressed in eukaryotes. B'/B56 subunits are composed of eight pseudo-HEAT repeats (huntingtin-elongation-A subunit-TOR), which form a structure of 18 stacked α -helices that provides several potential substrate interaction surfaces (Cho and Xu, 2007; Xu et al., 2006; Xu et al., 2009). Indeed, it has recently become evident that, in addition to a conserved binding pocket that recognizes a consensus sequence motif (LxxIxE) on their substrates, B56 subunits are also able to interact with substrates through other surfaces/sites (Hertz et

al., 2016; Xu et al., 2009). PP2A-B56 complexes are key regulators of a wide range of processes, including apoptosis (Janssens and Rebollo, 2012), development (Yang and Phiel, 2010), and mitosis/meiosis, with key functions in chromosome alignment and SAC signaling (Bollen et al., 2009; Funabiki and Wynne, 2013; Nijenhuis et al., 2014; Xu et al., 2014).

B''/PR72 family

The B''/PR72 subunit family is evolutionary more divergent and present only in vertebrates. Three genes (*PPP2R3A-C*) (Fig. 1.4) encode nine isoforms in mice, while the human genome only encodes two homologs of these genes (Hendrix et al., 1993a; Zwaenepoel et al., 2008). Compared to the B/B55 and B'/B56 families, the B'' subunit isoforms are less abundant and exhibit a more distinctive tissue distribution (Hendrix et al., 1993a; Sontag, 2001). Members of this family are uniquely distinguished by the presence of calcium-binding EFX domains and their activity is dependent on calcium (Ahn et al., 2007; Janssens et al., 2003). B''-containing PP2A heterotrimers have been shown to be involved in Wnt signaling (Creyghton et al., 2006), cell migration (Janssens et al., 2016), epidermal growth factor (EGF) signaling (Zwaenepoel et al., 2010), and cell cycle progression through roles in DNA replication (Davis et al., 2008; Wlodarchak et al., 2013; Yan et al., 2000) and pocket protein dephosphorylation (Magenta et al., 2008; Voorhoeve et al., 1999).

B'''/Striatin family

The B'''/Striatin subunits are encoded by three genes (*STRN*, *STRN3*, and *STRN4*) (Fig. 1.4) in mammalian genomes and represent the most recently discovered family of regulatory subunits (Moreno et al., 2000). While *STRN* and *STRN4* are highly enriched in neurons, *STRN3* is ubiquitously expressed in almost all tissues and exhibits cell cycle-dependent expression and nuclear localization during S and G2 phase (Muro et al., 1995; Shi et al., 2016). Striatins are large

multidomain proteins that bind the AC core dimer and act as scaffolds that recruit a myriad of other regulatory proteins, including kinases, to form supramolecular complexes known as striatin-interacting phosphatase and kinase (STRIPAK) complexes [reviewed in (Hwang and Pallas, 2014; Shi et al., 2016)]. STRIPAK complexes modulate a number of important signaling pathways and processes, including the HIPPO pathway [reviewed in (Hwang and Pallas, 2014; Shi et al., 2016)], and cell division, where they have been shown to play a role in cytokinesis (Hyodo et al., 2012).

The combinatorial interaction of the AC core enzyme with the diverse regulatory subunits has been suggested to yield in excess of 96 distinct PP2A holoenzymes (Janssens et al., 2008). Together with their temporal and tissue-specific expression, the variable subcellular localization of different isoforms, and the interaction with additional proteins, the immense complexity of PP2A-mediated biology becomes abundantly evident.

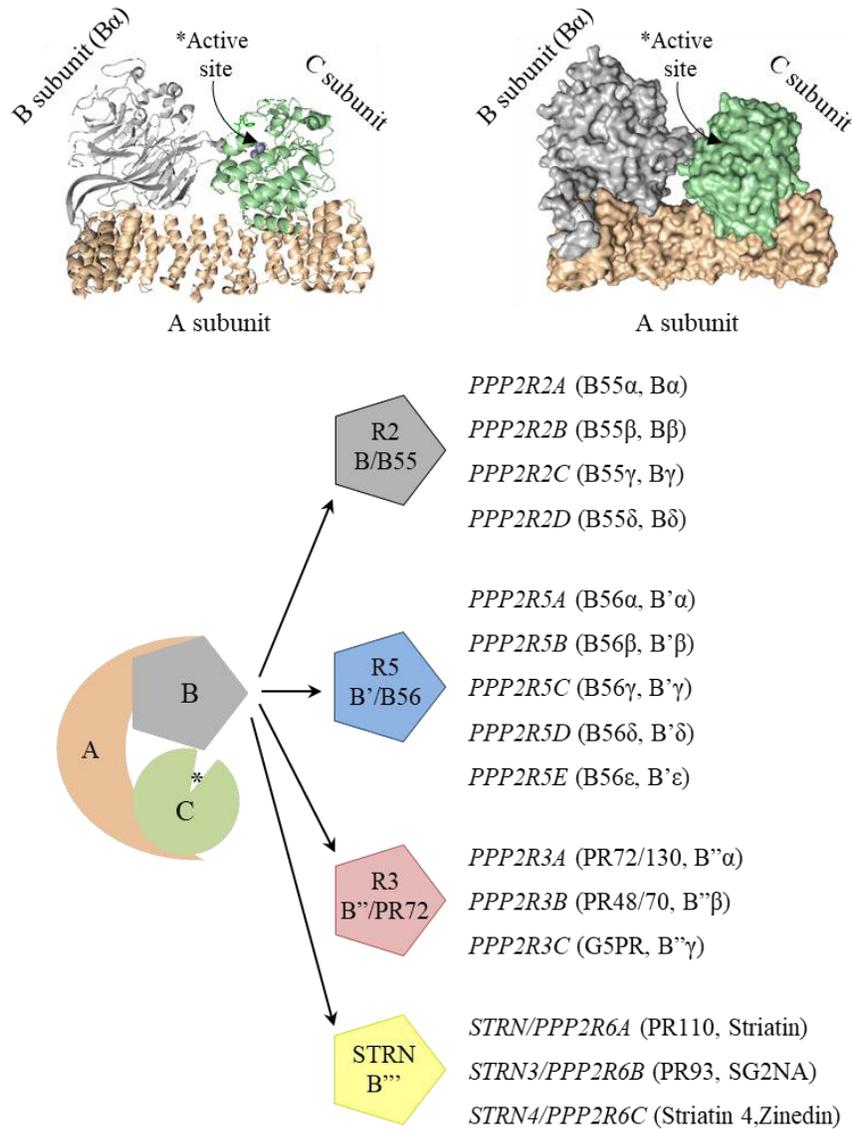


Figure 1.4: PP2A holoenzyme structure

The crystal structure (PDB: 3DW8 obtained from the RCSB Protein Data Bank; <http://www.rcsb.org/structure/3DW8>) of the PP2A-B55 α holoenzyme is shown (upper panels). The upper left panel illustrates the secondary structures of the scaffold A, catalytic C, regulatory B subunits, while the upper right panel shows the subunits' surface topology. The schematic in the lower panel depicts the PP2A holoenzyme, demonstrating the four B subunit families and their isoforms. Gene names are italicized, while protein isoform names are listed in parentheses.

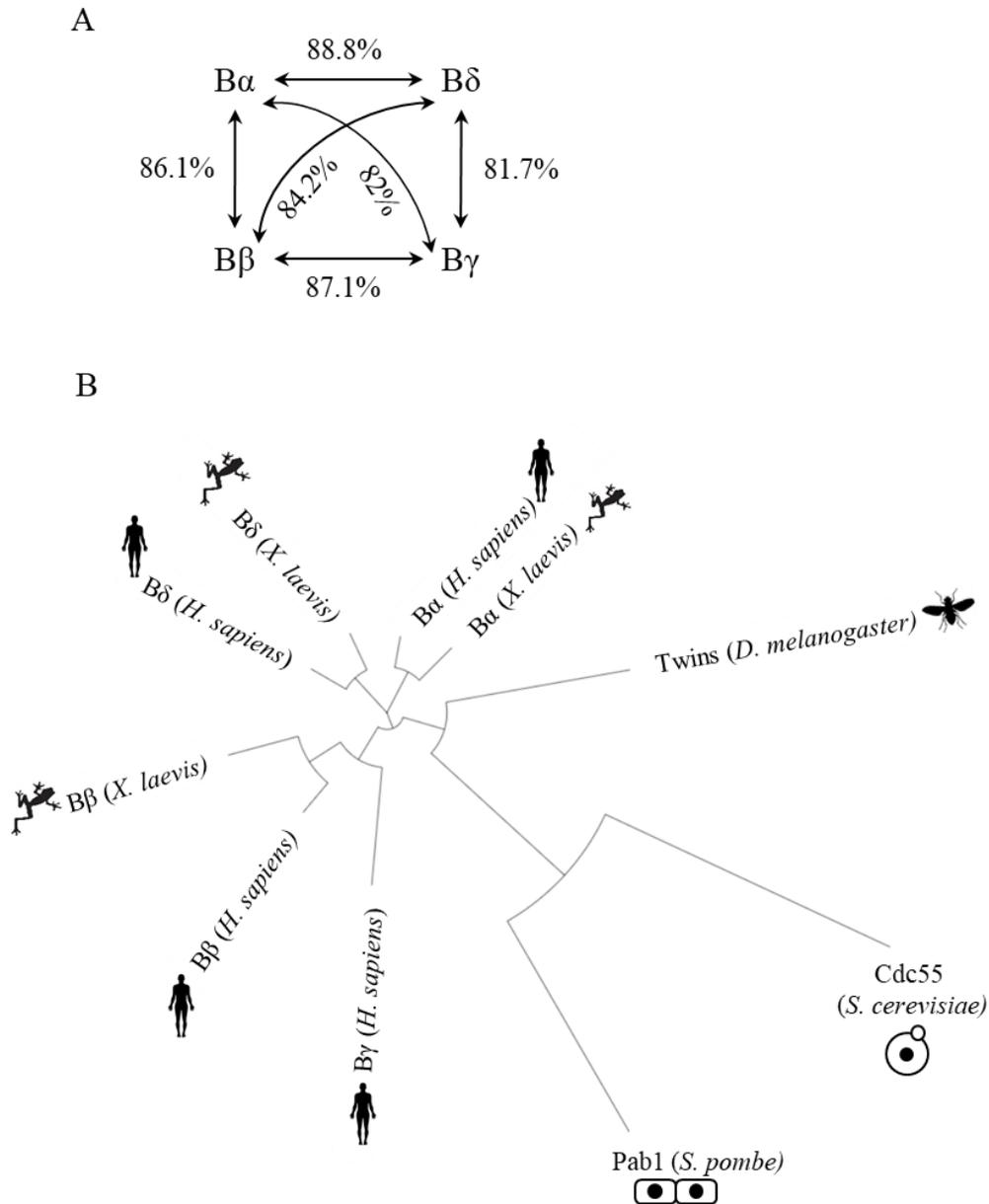


Figure 1.5: B55 subunits from yeast to humans

- A. The diagram shows the percent sequence identity between each isoform of the human B55 subunit family. The percent sequence identity values were generated by the ClustalOmega multiple sequence alignment tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), using protein sequences obtained from the UniProt Knowledgebase (<http://www.uniprot.org/>) as inputs.
- B. Shown is a phylogenetic tree of the B55 subunits in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, *X. laevis*, and *H. sapiens*. The phylogenetic tree was generated using the phyloT tree generator (<http://phyloT.biobyte.de/>) and is based on phylogram values obtained from Clustal Omega

protein sequence alignments. While budding/fission yeast and *Drosophila* only encode one B55 isoform, *Xenopus* and human cells encode three and four B55 isoform genes, respectively.

Table 1.1: Sequence identity matrix for B55 subunit from yeast to humans

The table shows the percent sequence identity between the B55 subunit isoforms from *Drosophila*, *Xenopus*, budding yeast, fission yeast and humans. The sequence identity matrix was generated using the Clustal Omega multiple sequence alignment tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and is based on protein sequences obtained from the UniProt Knowledgebase (<http://www.uniprot.org/>).

											
Species	B55 isoform	Twins	B δ	B δ	B α	B α	B γ	B β	B β	Cdc55	Pab1
<i>D. melanogaster</i>	Twins	100.0	77.2	77.1	78.3	77.4	72.6	75.8	73.0	54.2	56.1
<i>H. sapiens</i>	B δ	77.2	100.0	94.4	88.8	86.7	82.4	84.9	82.5	54.7	60.6
<i>X. laevis</i>	B δ	77.1	94.4	100.0	89.0	87.6	82.4	84.5	82.5	54.0	59.4
<i>H. sapiens</i>	B α	78.3	88.8	89.0	100.0	94.6	81.9	85.8	83.6	54.9	60.3
<i>X. laevis</i>	B α	77.4	86.7	87.6	94.6	100.0	81.1	84.4	82.4	54.2	60.8
<i>H. sapiens</i>	B γ	72.6	82.4	82.4	81.9	81.1	100.0	87.2	83.5	52.3	57.5
<i>H. sapiens</i>	B β	75.8	84.9	84.5	85.8	84.4	87.2	100.0	92.3	54.9	59.4
<i>X. laevis</i>	B β	73.0	82.5	82.5	83.6	82.4	83.5	92.3	100.0	53.8	58.0
<i>S. cerevisiae</i>	Cdc55	54.2	54.7	54.0	54.9	54.2	52.3	54.9	53.8	100.0	61.3
<i>S. pombe</i>	Pab1	56.1	60.6	59.4	60.3	60.8	57.5	59.4	58.0	61.3	100.0

1.2.2 The regulation of PP2A holoenzyme assembly

PP2A holoenzyme assembly is a highly complex and dynamic process that is tightly regulated by a variety of enzymes and interacting proteins and involves the post-translational modification of the catalytic and regulatory subunits [reviewed in (Janssens et al., 2008; Sents et al., 2013)]. The activation and construction of specific holoenzymes occurs in a tightly regulated, stepwise manner and involves at least five enzymes/interactors: $\alpha 4$, the scaffolding A subunit, PTPA (PP2A Activator), LCMT1 (leucine carboxyl methyl transferase 1), and PME-1 (PP2A methyl esterase 1) (Sents et al., 2013). Briefly, because free PP2A_C is inherently unspecific and poses a significant risk to the cell if its activity is not restrained and specified by interaction with other subunits, cells synthesize an inactive form of the catalytic subunit (Hombauer et al., 2007). Newly

translated, free catalytic subunits are bound by $\alpha 4$, which modulates C subunit stability and maintains a low-activity conformation (Kong et al., 2009; Sents et al., 2013). Binding of the scaffold A subunit displaces $\alpha 4$ and results in the formation of an inactive AC core dimer. At this point, some B-type subunits can be recruited to the AC core dimer to form heterotrimeric holoenzymes. Importantly, activation of PP2A catalytic activity requires the binding and action of PTPA, which induces an active conformation of PP2A_C. PTPA can bind and activate AC core dimers, B subunit-containing heterotrimers, as well as $\alpha 4$ -PP2A_C complexes [reviewed in (Janssens et al., 2008; Sents et al., 2013)].

The formation and activity of specific PP2A heterotrimers is tightly regulated by post-translational modifications of the catalytic subunit, specifically the reversible methylation or phosphorylation of its ultra-conserved C-terminal tail [reviewed in (Janssens et al., 2008; Sents et al., 2013)]. Reversible carboxymethylation of PP2A_C occurs on its Leu309 residue and is controlled by the opposing activities of the methyltransferase, LCMT1, and the methyltransferase, PME-1 (De Baere et al., 1999; Longin and Goris, 2006; Ogris et al., 1999). Methylation of Leu309 is specifically required for the association of PP2A_C with the B55 subunits, but not for the binding of the PP2A_A, B56, PR72, or Striatin subunits [reviewed in (Janssens et al., 2008)]. However, the assembly of B56-containing heterotrimers may be enhanced by Leu309 methylation (Cho and Xu, 2007; Xu et al., 2006). Similarly, the reversible phosphorylation of the Tyr307 and Thr304 residues in the PP2A_C C-terminal tail regulates the activity of specific PP2A holoenzymes. While Tyr307 phosphorylation prevents the association of B55 and B56 subunits (except for B56 δ , due to structural differences) with the AC core dimer, Thr304 phosphorylation selectively inhibits PP2A-B55 complex formation and activity (Chen et al., 1992; Janssens et al., 2008; Longin et al., 2007; Schmitz et al., 2010). Even though a few kinases have been shown to phosphorylate Tyr307 *in vitro*, including Src family kinases and growth factor receptor tyrosine kinases (Chen et al., 1992), the identity of the kinases

responsible for Tyr307 and Thr304 phosphorylation *in vivo* remains obscure. Dephosphorylation of these sites appears to be mediated by PP2A through autodephosphorylation (Chen et al., 1992; Guo and Damuni, 1993).

Holoenzyme assembly is also regulated by reversible phosphorylation of the B-type subunits. Phosphorylation-dependent regulation of B-type subunits appears to be rather complex and remains very poorly characterized. For example, phosphorylation of B56 subunits at different sites may alter substrate specificity, increase activity, or induce the assembly or disassembly of specific PP2A-B56 heterotrimers [reviewed in (Janssens and Goris, 2001; Janssens et al., 2008)]. Similarly, phosphorylation of B55 subunits at various sites can regulate their association with AC core dimers, and therefore, modulate the activity of PP2A-B55 complexes (Kolupaeva et al., 2013; Schmitz et al., 2010). Interestingly, the phosphorylation sites identified on B55 subunits (B55 α , specifically) appear to be regulated in a largely cell cycle-dependent manner, supporting an important role for PP2A-B55 complexes in cell cycle regulation (Kolupaeva et al., 2013; Schmitz et al., 2010). Furthermore, assembly of B55-containing PP2A heterotrimers seems to be the most tightly regulated (as compared to B56 and PR72 complexes), suggesting that strict control of their activities is of paramount importance to cellular health.

The diversity of potential PP2A heterotrimers and the multifaceted regulation of their assembly highlight the immense complexity that underlies the biology of PP2A holoenzyme composition and function. While substantial progress has been made in understanding the context-specific roles of various heterotrimers, it has also become abundantly evident that much remains to be explored. Importantly, deregulation of the enzymes that modulate PP2A holoenzyme assembly results in severe cellular defects that are associated with aberrant PP2A activity and function.

1.2.3 PP2A-B55: functions in interphase and mitosis

B55 subunits in the regulation of G1 and S phase progression

PP2A-B55 complexes have emerged as important regulators of cell cycle progression in most model systems, ranging from yeast to mammalian cell lines. During G1 phase, for example, PP2A-B α complexes have been shown to dephosphorylate and activate the p107 and p130 pocket proteins to induce cell cycle exit in human and rat chondrocytes and chondro-/osteosarcoma cells, implicating this heterotrimer as a negative regulator of G1 progression (Jayadeva et al., 2010; Kolupaeva et al., 2013; Kurimchak et al., 2013). Studies conducted in *Xenopus* oocyte cell-free extracts have identified B α -containing PP2A holoenzymes as positive regulators of the initiation and completion of DNA replication (Krasinska et al., 2011; Lin et al., 1998; Murphy and Michael, 2013; Wang et al., 2018). While the exact mechanisms underlying B α -mediated regulation of various aspects of DNA replication are unknown, a recent study identified numerous S phase-specific interactors of B α (and B β), including replication protein A and other components of the replication and DNA damage repair machinery, providing potential candidates for further investigation (Wang et al., 2018). The most well-studied cell cycle regulatory function of PP2A-B55 is its role as a regulator of mitosis, with increasing elucidation of its function in the complex regulatory circuitries that modulate mitotic entry and exit.

B55 subunits in mitotic entry regulation

As alluded to above, PP2A-B55 heterotrimers are major CDK1-antagonizing phosphatases, and proper regulation of their activity is required for mitotic progression. The anti-mitotic activity of PP2A-B55 complexes has long been recognized; however, the mechanistic details of their regulation and function have only emerged in recent years. Early studies investigating the regulation of CDK1

activation in *Xenopus* egg extracts identified the PP2A-B α complex as an anti-mitotic phosphatase whose activity was a major determinant of CDK1 activation dynamics (Cyert and Kirschner, 1988; Lee et al., 1991; Lee et al., 1994). Shortly thereafter, B β was also found to exhibit anti-mitotic activity, albeit when overexpressed (Iwashita et al., 1997). While Clarke *et al.* suggested that PP2A-mediated dephosphorylation and inactivation of Cdc25C may account for its CDK1-modulatory function in *Xenopus* extracts (Clarke et al., 1993), others proposed that PP2A-B55 anti-mitotic activity may stem from its ability to specifically dephosphorylate CDK1 substrates in vertebrate cell extracts and *Drosophila* models (Ferrigno et al., 1993; Mayer-Jaekel et al., 1994). Almost 15 years later, Mochida *et al.* demonstrated that depletion of the B δ subunit from cycling interphase *Xenopus* egg extracts led to premature entry into mitosis by lowering the CDK1 activity threshold required for the initiation of mitosis and the phosphorylation of CDK1 substrates. Therefore, it was proposed that PP2A-B δ restrains mitotic entry by counteracting CDK1-mediated phosphorylation, and that its inhibition is required for entry into mitosis (Mochida et al., 2009). Subsequent studies revealed that inhibition of PP2A-B55 is mediated by Gwl-phosphorylated Ensa/ARPP19, and that the BEG pathway comprises an essential component of the mitotic entry regulatory circuit (discussed in section 1.1.6; Mitotic entry) (Gharbi-Ayachi et al., 2010; Lorca et al., 2010; Mochida et al., 2010; Mochida et al., 2016). While this pathway was mostly characterized using *Xenopus* egg extracts, its prevalence has been confirmed in *Drosophila* and mammalian systems (Gharbi-Ayachi et al., 2010; Rangone et al., 2011). Furthermore, the PP2A-B55-mediated modulation of CDK1 activation through the regulation of Cdc25C (and Wee1/Myt1) has been supported by additional studies in *Xenopus* and fission yeast (Lucena et al., 2017; Zhao et al., 2008).

B55 subunits in mitotic exit regulation

A major focus of investigation in recent years has been the elucidation of B55-dependent functions during mitotic exit, especially in mammalian model systems (predominantly the HeLa cell line). Interestingly, the contribution of PP2A-B55 to mitotic substrate dephosphorylation varies in different systems. In cycling *Xenopus* egg extracts, for example, PP2A-B δ does not account for the bulk of mitotic substrate dephosphorylation, as its depletion from mitotic extracts does not significantly impede mitotic exit progression (Mochida et al., 2009). In contrast, the *Drosophila* B55 homolog, twins, plays a crucial role in mitotic exit progression and its depletion leads to aberrant chromosome segregation and defective CDK1-substrate dephosphorylation (Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993). Similarly, in mammalian model systems, the activities of B α - and B δ -containing PP2A complexes contribute significantly to CDK1-substrate dephosphorylation and the execution of specific mitotic exit events (Cundell et al., 2013; Cundell et al., 2016; Manchado et al., 2010; Schmitz et al., 2010). PP2A-B α , for example, was specifically shown to promote the reassembly of the Golgi apparatus during telophase through the dephosphorylation of the Golgi protein, GM130 (Lowe et al., 2000; Schmitz et al., 2010). While the identification of specific PP2A-B55 substrates has historically been slow, a recent phospho-proteomic study conducted by Cundell *et al.* successfully characterized numerous B55-specific substrates involved in various mitotic exit events. Notably, this study identified several mitotic and central spindle components (including the Aurora A coactivator, TPX2; the motor protein, KIF22; and the central spindle protein PRC1), and nuclear pore complex proteins (including nucleoporins NUP153, NUP107, NUP98) as important substrates of PP2A-B55 complexes, and demonstrated their involvement in the dynamics of mitotic/central spindle disassembly/assembly and nuclear pore reformation, respectively (Cundell et al., 2016). Importantly, this and other recent studies have shown that the kinetics of PP2A-B55 reactivation and substrate dephosphorylation play a central role in establishing an ordered

progression of mitotic exit events (Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017). Through mathematical modeling and experimental validation, it was first demonstrated that the precise timing of PP2A-B55 reactivation (under the control of the BEG pathway) after the meta-to-anaphase transition was essential to ensure the proper ordering of chromosome segregation and cytokinesis (Cundell et al., 2013). Subsequent studies revealed that PP2A-B55 complexes dephosphorylate different substrates (and even specific phosphorylation sites within the same protein) with distinct kinetics, leading to a highly ordered sequence of substrate dephosphorylation events that are associated with specific physical events during mitotic exit (Cundell et al., 2016; Hein et al., 2017). Strikingly, the kinetics, and therefore the order, of target site dephosphorylation are encoded into the substrates themselves. More precisely, B55 subunits preferentially target phosphorylation sites that are highly basic in character (Cundell et al., 2016), due to the acidic nature of a prominent substrate binding pocket on the B55 subunits (Xu et al., 2008). Therefore, highly basic sites are dephosphorylated early during mitotic exit, while a decreasing basic character dictates slower dephosphorylation kinetics and delayed dephosphorylation timing (Cundell et al., 2016; McCloy et al., 2015; Rogers et al., 2015). Furthermore, PP2A-B55 exhibits a marked preference for threonine over serine residues, and accordingly, threonine sites are dephosphorylated earlier during mitotic exit (Bouchoux and Uhlmann, 2011; Cundell et al., 2016; Hein et al., 2017; McCloy et al., 2015; Rogers et al., 2015). Therefore, PP2A-B55 complexes do not merely dephosphorylate mitotic substrates to drive mitotic exit progression, but they dictate the order of mitotic exit events by targeting substrates in a highly coordinated manner.

1.3 Deregulation of PP2A in cancer

PP2A is a well-established tumour suppressor and its importance in restraining aberrant cell growth and proliferation is epitomized by the myriad of mechanisms that deregulate its activity in a wide range of cancers. Over the past 30 years, a series of milestone discoveries have solidified the role of PP2A as an essential tumour suppressor [reviewed in (Janssens et al., 2005)]. Notably, several elegant biochemical and genetic studies have demonstrated that partial inhibition of PP2A is necessary and sufficient to transform immortalized cell lines (Arroyo and Hahn, 2005; Hahn et al., 1999; Rangarajan et al., 2004; Zhao et al., 2004). In recent years, detailed biochemical studies and the use of “Omics” approaches (including genomic, transcriptomic, and proteomic analyses) have led to the identification of a growing number of tumorigenic mechanisms that attenuate PP2A activity, including the overexpression of endogenous PP2A inhibitory proteins and genetic alterations of the scaffold and regulatory subunits. This has also led to a significant rise in the popularity of PP2A as a potential target for therapeutic intervention [reviewed in (O'Connor et al., 2018; Ruvolo, 2016; Sangodkar et al., 2016)].

1.3.1 Tumour-promoting toxins and viral oncoproteins target PP2A

The earliest clue to the tumour suppressive function of PP2A was provided by the discovery that tumour-promoting toxins, such as okadaic acid and calyculin A, potently inhibit the activity of PP2A (and other PPP family phosphatases) (Bialojan and Takai, 1988; Sukanuma et al., 1990; Sukanuma et al., 1988). Shortly thereafter, it was demonstrated that known oncoproteins encoded by various small DNA tumour viruses, including the polyomavirus small/middle T and SV40 small T antigens, target PP2A enzymes, and that their tumorigenic potential is dependent on their interaction with the AC core dimer (Campbell et al., 1995; Pallas et al., 1990). Importantly, these viral antigens induce cellular transformation in part by displacing specific B subunit isoforms from the AC core

dimer, leading to the loss of specific holoenzyme activities and the deregulation of their cellular targets (Arroyo and Hahn, 2005; Chen et al., 2007; Sablina et al., 2010; Westermarck and Hahn, 2008). The SV40 small T antigen, for example, displaces the B56 α , B56 γ and PR72 subunits, which results in the oncogenic activation of the Akt, c-Myc, and Wnt signaling pathways due the loss of negative regulation by these specific PP2A heterotrimers (Chen et al., 2004; Sablina et al., 2010). These studies demonstrated that disrupting the stoichiometry of B-type subunits, and therefore the composition and diversity of PP2A holoenzymes, can promote tumorigenesis by effectively abolishing the activities of specific PP2A heterotrimers.

1.3.2 Overexpression of endogenous cellular PP2A inhibitory proteins

In mechanisms analogous to those employed by viral oncoproteins, the endogenous cellular proteins Cancerous Inhibitor of PP2A (CIP2A) and Inhibitor 2 of PP2A (I2PP2A or SET) bind to and inhibit PP2A activity, and their overexpression plays a key role in cancer cell survival and therapeutic resistance (Khanna and Pimanda, 2016; Khanna et al., 2013; Li et al., 1996). CIP2A is overexpressed in almost all solid organ cancers and a variety of hematological cancers, where over 70% of patient tumour samples exhibit high expression levels [reviewed in (Khanna and Pimanda, 2016; Khanna et al., 2013)]. Furthermore, high CIP2A expression levels serve as a poor prognostic factor and are associated with chemotherapeutic drug resistance in several cancers (Choi et al., 2011; Khanna and Pimanda, 2016; Khanna et al., 2013). While the full extent of CIP2A-mediated PP2A inhibitory mechanisms remains largely unknown, CIP2A has been shown to inhibit the PP2A-dependent destabilization of c-Myc through direct interaction (with both PP2A and c-Myc), leading to the accumulation and oncogenic activation of c-Myc and its downstream targets (Junttila et al., 2007). I2PP2A, commonly known as SET, was first implicated in acute myeloid leukemogenesis through its role as an interactor and potent inhibitor of the PP2A catalytic subunit (Li et al., 1996).

SET overexpression has since been found to play a role in various hematological cancers, where it is associated with worse patient outcome/prognosis and drug resistance (Christensen et al., 2011; Cristobal et al., 2012; Perrotti and Neviani, 2006). High expression levels or increased activation of SET are also present in colorectal, breast and lung cancers [reviewed in (Perrotti and Neviani, 2013; Sangodkar et al., 2016)]. It remains unclear whether these endogenous inhibitory proteins target specific PP2A heterotrimers or whether they can act promiscuously. So far, only the B55 α and B56 β complexes have been implicated as targets of CIP2A regulation (Niemelä et al., 2012); however, an extensive investigation of the holoenzymes affected by these proteins remains to be conducted. Interestingly, overexpression of different endogenous inhibitors produces overlapping but distinct molecular defects and diseases, suggesting distinct specificities (Perrotti and Neviani, 2013).

1.3.3 Genetic aberrations and altered expression of multiple PP2A subunits in cancers

In addition to the deregulation of PP2A activity by exogenous and endogenous oncoproteins, genetic alterations affecting the different PP2A subunits are increasingly recognized to play key roles in the development and progression of many cancers [recently reviewed in (Ruvolo, 2016; Sangodkar et al., 2016)].

Scaffold A subunit mutations

The first genetic aberrations affecting PP2A subunits were identified in the scaffold subunit-encoding genes in breast, lung, colon carcinomas and melanoma (Calin et al., 2000; Wang et al., 1998). Since then, scores of different genetic aberrations affecting the *PPP2RIA* and *PPP2R1B* genes have been identified in a broad range of cancers, including breast, lung, melanoma, ovarian, endometrial, uterine, cervical, colon, and several leukemias [reviewed in (Ruvolo, 2016; Sangodkar et al., 2016)]. *PPP2RIA* aberrations are largely characterized by point mutations that result in the

substitution of highly conserved amino acids and disrupt regulatory and/or catalytic subunit binding (Calin et al., 2000; McConechy et al., 2011; Ruediger et al., 2001b; Ruediger et al., 2011). Substitution of Glu64 for Asp or Gly (E64D and E64G, respectively), for example, disrupts interaction of the A α subunit with members of the B56 subunit family, while binding of the catalytic and other B-type subunits remain unaffected (Ruediger et al., 2001b; Ruediger et al., 2011). Two other A α mutations, R418W and Δ 171–589, result in reduced binding to the catalytic and all B-type subunits tested (Ruediger et al., 2001b). Akin to the A α subunit, the A β -encoding gene also displays numerous point mutations that selectively disrupt its interaction with the catalytic and/or specific B subunits (most notably the PR72 subunits) (Calin et al., 2000; Ruediger et al., 2001a; Sablina et al., 2007; Tamaki et al., 2004; Wang et al., 1998). In addition to point mutations, *PPP2R1B* gene alterations also occur as homozygous deletions and exome deletions (Calin et al., 2000; Ruediger et al., 2001a; Sablina and Hahn, 2008; Tamaki et al., 2004; Wang et al., 1998). Notably, exome deletions yield aberrant transcripts and truncated A β subunits that are unable to bind the catalytic subunit (Calin et al., 2000; Ruediger et al., 2001a; Wang et al., 1998). Importantly, loss of heterozygosity (LOH) appears to be a key feature in the disruption of A β 's tumour suppressive function, as holoenzyme-altering point mutations or truncation mutants in one allele are frequently accompanied by the loss of the second allele (Sablina et al., 2007; Sablina and Hahn, 2008; Tamaki et al., 2004; Wang et al., 1998). Notably, genetic alterations affecting the A α subunit isoform typically only involve a single allele (Calin et al., 2000; Chen et al., 2005), underscoring the importance of maintaining minimal levels of PP2A activity for cell survival (Strack et al., 2004).

A common theme underlying these diverse types of mutations in the scaffold subunit isoforms is the alteration of cellular holoenzyme composition and the loss of specific holoenzyme activities. In the case of the A β subunit, the various types of mutations appear to render cells functionally null for this isoform. This suggests that complete loss of A β -containing holoenzymes is

required to abolish its tumour suppressive function (Sablina et al., 2007; Sablina and Hahn, 2008). In the case of the A α subunit, point mutations that disrupt the binding of the catalytic or specific B-type subunits effectively reduce the abundance and activity of select heterotrimers, resulting in the activation of the pathways they restrain. Therefore, A α subunit mutations produce a state of haploinsufficiency that reduces the activity of PP2A holoenzymes and impedes their ability to suppress growth and proliferation. Indeed, detailed biochemical studies have demonstrated that A α mutations induce a haploinsufficient state that is capable of transforming immortalized cells (Chen et al., 2005). In agreement with this notion, some cancers that do not contain scaffold subunit mutations exhibit markedly reduced expression of the A subunit (Chen et al., 1992; Chen et al., 2007; Colella et al., 2001).

Regulatory B subunit mutations, deletions and altered expression

Cancer-associated genetic alterations in the B subunits are generally less common, but their incidence and relevance to tumorigenesis is becoming increasingly evident. Analogous to the A subunits, point mutations in the gene encoding the B56 γ subunit (*PPP2R5C*) have been identified in melanomas and lung carcinomas (Nobumori et al., 2013; Shouse et al., 2010). Consistent with the theme of specific holoenzyme disruption, Nobumori *et al.* demonstrated that the tumorigenic point mutations in the *PPP2R5C* gene abolish B56 γ interaction with either the scaffolding or the catalytic subunit (Nobumori et al., 2013). Far more common than holoenzyme-disrupting point mutations are genetic alterations that affect the expression levels, and therefore the stoichiometry, of specific B subunits. Members of the B55 family of regulatory subunits exhibit decreased expression in a variety of cancers, and this occurs through several distinct mechanisms. Expression of the B β -encoding gene (*PPP2R2B*), for example, is suppressed by hypermethylation in colorectal and breast carcinomas (Kurimchak and Grana, 2012a; Muggerud et al., 2010; Tan et al., 2010). Remarkably, epigenetic

silencing of *PPP2R2B* occurs in >90% of human colorectal cancer. Furthermore, loss of B β expression plays a vital role in enabling MYC-mediated rapamycin resistance, as ectopic re-expression of B β effectively re-sensitizes colorectal cancer cells to rapamycin-induced senescence (Tan et al., 2010). In addition to epigenetic silencing mechanisms, miRNA-mediated suppression of the B γ subunit (*PPP2R2C*) plays a driving role in ovarian and prostate carcinomas, and is highly correlated with metastasis and prostate cancer specific mortality (Bluemn et al., 2013; Spencer et al., 2012; Wu et al., 2016). Similarly, B δ subunit expression is significantly downregulated by miR-133b in hepatocellular carcinomas (HCC), and B δ re-expression (or miR-133b depletion) counteracts CDK1-driven hyperproliferation and increases the sensitivity of HCC cells to cisplatin treatment (Zhuang et al., 2016).

Strikingly, B α subunit expression is reduced in a wider range of cancers (as compared to other B55 subunits) by a multitude of mechanisms. Heterozygous (and homozygous) deletions of the B α -encoding gene (*PPP2R2A*) are associated with significantly reduced or ablated expression levels and have been reported in leukemias (Mosca et al., 2013), prostate (Cheng et al., 2011; Liu et al., 2008), colorectal (Cristobal et al., 2014), and luminal B breast carcinomas (Curtis et al., 2012). Importantly, Curtis *et al.* identified loss of the *PPP2R2A* gene as a potential driver of the oncogenic phenotype in highly mitotic estrogen receptor (ER)-positive luminal B breast tumours (Curtis et al., 2012). B α -targeting miRNA's have been identified as important drivers in lung (Liu et al., 2010) and hematological cancers (Ruvolo, 2015; Ruvolo et al., 2014). In acute myeloid leukemia, it was specifically shown that strongly reduced expression of the B α subunit, and hence loss of PP2A-B α activity, is associated with increased T308 phosphorylation of AKT and shorter duration of complete remission in patients (Ruvolo et al., 2011; Ruvolo et al., 2014). PP2A-B α dephosphorylates T308 on AKT to attenuate Akt-mediated growth signaling (Kuo et al., 2008).

Catalytic C subunit alterations

Mutations in the genes encoding the catalytic subunit isoforms have not been reported (Ruvolo, 2016). However, deletions of the *PPP2CB*-containing region on the 5q chromosome have been identified in myelodysplastic syndrome and breast cancer (Curtis et al., 2012; Sallman et al., 2014). Furthermore, markedly reduced expression of the C α isoform has been reported in androgen-insensitive prostate cancer and acute myeloid leukemia (AML) (Ramaswamy et al., 2015; Singh et al., 2008). PP2A catalytic activity is unequivocally required for cell viability, as global inhibition of PP2A activity or depletion of both catalytic subunit isoforms is lethal in all organisms tested (Gotz et al., 1998; Sneddon et al., 1990; Strack et al., 2004). Therefore, it is unsurprising that C subunit aberrations are exceedingly rare.

These studies underscore the important tumour suppressive functions of PP2A and strongly suggest that disruption of specific PP2A heterotrimers contributes to tumorigenesis in a wide variety of cancers. Furthermore, they support the notion that the properly balanced stoichiometry of regulatory B-subunits is of paramount importance to cellular homeostasis and health.

1.3.4 PP2A-targeted therapies: reactivation of tumour suppressor activity

The reactivation of silenced or inactivated tumour suppressors in the treatment of cancer has become the subject of much interest in recent years [reviewed in (Guo et al., 2014; Merkel et al., 2017)]. In particular, because the partial inactivation of PP2A activity is implicated in the majority of cancers and contributes to treatment resistance, its reactivation has become a popular and viable strategy to supplement or improve the treatment of diverse cancers (O'Connor et al., 2018; Perrotti and Neviani, 2013; Ruvolo, 2016; Sangodkar et al., 2016).

While a variety of PP2A-activating therapeutic approaches are being explored, the endogenous inhibitory proteins, CIP2A and SET, have emerged as the most promising target

candidates. Due to their susceptibility to chemical inhibitors, CIP2A and SET are also the most viable targets for therapeutic strategies aimed at the reactivation of PP2A (O'Connor et al., 2018; Perrotti and Neviani, 2013; Sangodkar et al., 2016). FTY720, for example, binds to and potently inhibits SET, leading to the reactivation of PP2A and the induction of apoptosis and/or the inhibition of growth-promoting pathways (including the ERK and PI3K/AKT pathways) [reviewed in (Cristobal et al., 2016; O'Connor et al., 2018; Perrotti and Neviani, 2013; White et al., 2016)]. Similarly, Celastrol is an anticancer compound that binds to CIP2A and promotes its ubiquitin-dependent degradation, thereby relieving CIP2A-mediated inhibition of PP2A (Liu et al., 2014; O'Connor et al., 2018). Several other compounds that inhibit CIP2A or SET either directly or indirectly exist, many of which have already been approved for use in a variety of cancers and other diseases (O'Connor et al., 2018; Perrotti and Neviani, 2013; Sangodkar et al., 2016). Importantly however, it will be necessary to gain a better understanding of the PP2A activities that are suppressed by CIP2A and SET to be able to properly evaluate the safety and utility of these reactivation therapies.

1.4 Outstanding questions

Studies in various model systems have implicated the PP2A-B55 heterotrimers as important regulators of cell cycle progression. While their roles in mitotic control are being elucidated in great detail, little is known about their involvement in the dynamic regulation of interphase progression. Notably, PP2A complexes have been proposed to dynamically modulate RB protein phosphorylation throughout the cell cycle (Garriga et al., 2004); however, the identity of the regulatory subunits involved and their effects on the kinetics of cell cycle progression are unclear. Even though PP2A-B α has been shown to dephosphorylate and activate the RB proteins p107 and p130 during G1 phase, this induces cell cycle exit (Jayadeva et al., 2010; Kolupaeva et al., 2013). Therefore, it remains to be

elucidated whether PP2A-B α (and other B55 isoforms) dynamically modulate RB protein phosphorylation, and therefore the kinetics of G1 phase progression. Similarly, while PP2A-B α heterotrimers have been shown to positively regulate the initiation of DNA replication in *Xenopus* (Lin et al., 1998; Murphy and Michael, 2013; Wang et al., 2018), it remains to be determined whether this, and other PP2A-B55 complexes, are involved in the dynamic control of replication initiation and progression in mammalian cells.

Importantly, investigations into B55 isoform-specific functions in cell cycle regulation are almost completely lacking. In model systems that express only one B55 isoform, such as *Drosophila* (Twins) and budding (Cdc55) or fission yeast (Pab1) (Fig. 1.5B), this may not pose a problem. However, in the *Xenopus* and mammalian systems, which encode three and four isoforms (Fig. 1.5B), respectively, isoform-specific activities may play important roles. This is especially relevant to the mammalian B α and B δ subunits, as they are both abundantly and ubiquitously expressed (Janssens and Goris, 2001; Sontag, 2001). Alas, due to their high degree of sequence identity (88.8%; Fig. 1.5A), it is frequently assumed that these isoforms perform redundant functions in the cell. This supposition may be misguided, as the B α and B δ isoforms not only perform distinct functions but may exert opposing effects on the same pathway (Batut et al., 2008). Consequently, an investigation of B α - and B δ -specific functions in cell cycle regulation may be warranted. This is particularly applicable to B55-dependent roles in mitotic regulation. While substantial progress has been made in elucidating the involvement of PP2A-B55 complexes in mitotic exit progression, it is important to point out that the relevant studies depleted either both B α and B δ , or all B55 subunit isoforms from their experimental systems (Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017), thereby precluding the identification of potentially disparate functions of individual isoforms. Indeed, experiments conducted by Manchado *et al.* showed that the B α and B δ subunits do not contribute equally to CDK1-substrate dephosphorylation during mitotic exit in mouse embryonic fibroblasts

(Manchado et al., 2010). Similarly, it not known whether the B α and B δ subunits are equally involved in G2 phase and mitotic entry regulation in mammalian cells. In cycling *Xenopus* egg extracts, the B δ subunit appears to represent the predominant anti-mitotic B55 isoform during entry into mitosis. However, the authors suggested that this effect was due to its greatly increased abundance as compared to the B α and B β isoforms in their experiments (Mochida et al., 2009). Therefore, in a system where both B α and B δ are abundantly expressed, it would be interesting to investigate potentially distinct functions. Furthermore, expression of the B α isoform is specifically deregulated in a variety of cancers, while the B δ isoform has only been reported to be downregulated in HCC cells (described in section 1.3.3). This suggests that the B α subunit may play tumour suppressive roles that are specific to this isoform and not shared by the B δ isoform.

Taken together, the most pressing unanswered questions concern the role of PP2A-B55 complexes in the dynamic regulation of interphase progression, the investigation of potential isoform-specific functions for the B α and B δ subunits in mammalian mitotic regulation, and the basis for the selective enrichment of B α -targeting genetic aberrations in various cancers.

1.5 Hypothesis and aims of the study

1.5.1 Summary of rationale

PP2A-B55 heterotrimers have been implicated as central regulators of cell cycle progression in various species, with conserved functions in mitotic regulation across higher eukaryotes (*Drosophila* to humans). Interestingly, PP2A-B55 activity is reduced in a variety of cancers through CIP2A-mediated inhibition or decreased/ablated expression of specific B55 isoforms, with B α being the most frequently affected isoform. Furthermore, the assembly (and activity) of B55-containing holoenzymes appears to be the most strictly regulated across all B subunit families and is influenced by the methylation and phosphorylation status of the catalytic subunit as well as the cell cycle-

dependent phosphorylation status of the B55 (B α specifically) subunits themselves. This further supports the important cell cycle regulatory role of these PP2A holoenzymes.

Taken together, these observations suggest that PP2A-B55 heterotrimers, especially PP2A-B α , represent important tumour suppressors and that loss of their activity may contribute to the development and/or progression of tumourigenesis through the deregulation of the cell cycle. Furthermore, it has been suggested that the highly abundant B α and B δ isoforms perform distinct functions in the cell. However, this has yet to be investigated, especially with respect to the functions of PP2A-B55 heterotrimers during mammalian mitosis.

1.5.2 Hypothesis and aims of this study

Hypothesis: The PP2A B α and B δ regulatory subunits play distinct roles in the regulation of progression through the different phases of the mammalian cell cycle. Furthermore, the deregulation of B α -specific cell cycle regulatory functions contributes to tumourigenesis.

Aims

1. Determine whether the B α and B δ subunits differentially regulate progression through the different cell cycle phases.
2. Determine whether the B α and B δ subunits play distinct roles during mitotic entry and exit, and investigate the mechanisms underlying their potentially different functions.
3. Determine whether loss of B α or B δ contribute to tumourigenesis by undermining genome stability.

These aims were investigated using the HeLa cell line as a model system, and various cell cycle synchronization techniques were employed to elucidate phase-specific functions of the B α and B δ subunits.

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.1 Maintenance of HeLa cells

HeLa cells were purchased from American Type Culture Collection (ATCC) and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml Penicillin and 100 µg/ml Streptomycin (PenStrep, Gibco) (hereafter also referred to as 'complete growth medium'). HeLa cells that stably express eGFP-tagged α -Tubulin (eGFP-TUBA) and mCherry-tagged Histone H2B (mCherry-H2B) (Barr laboratory, University of Oxford) were cultured in complete growth medium supplemented with 0.3 µg/ml puromycin (Sigma) and 0.5 µg/ml blasticidin S (Sigma). HeLa cells that stably express Kusabira-Orange-tagged Cdt1 (mKO-Cdt1) and Azami-Green-tagged Geminin (AG-Geminin), also referred to as the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system (RIKEN BioResourceCenter, Japan), were cultured in complete growth medium. HeLa cells that stably express mCherry-tagged cyclin B1 (RIKEN BioResourceCenter, Japan) were grown in complete growth medium supplemented with 200 µg/ml hygromycin B (Sigma). All cell lines were grown at 37°C in a humidified, 5% (v/v) CO₂ incubator.

2.1.2 Maintenance of HEK 293 cells

Human embryonic kidney 293 (HEK 293) cells were purchased from American Type Culture Collection (ATCC) and cultured in Minimum Essential Medium (MEM, Gibco) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. Cells were grown at 37°C in a humidified, 5% (v/v) CO₂ incubator.

2.1.3 Transfection (siRNA)

Small interfering RNA sequences are listed in Table 2.1. Cationic lipid-based reverse transfections were performed, where cells, medium and the transfection mixture were concurrently seeded into 100 mm cell culture plates as described below. To transfect cell lines, siRNA was incubated in 1.25 ml of Opti-MEM™ (Gibco) for 5 minutes at room temperature. In parallel, 15 µl of Lipofectamine® RNAiMAX transfection reagent was also incubated in 1.25 ml of Opti-MEM™. Following the 5 minutes incubation period, the two mixtures were combined (collectively referred to as the ‘transfection mixture’) and incubated for 20 minutes at room temperature to allow for the formation of siRNA-lipid complexes. During this incubation period, cells that had been grown to 70-80% confluency were trypsinized, counted, and seeded at 30% into 100 mm cell culture plates, and complete growth medium was added to a volume of 7.5 ml. After the 20-minute incubation period, the transfection mixture (2.5 ml) was added drop-wise to the cells. Cells were allowed to adhere and incubate with the transfection mixture for 8 hours, after which cells were placed in 7 ml of complete growth medium overnight. Between 20-24 hours post transfection, cells were trypsinized, counted and seeded for analyses as described below. Time-point collections or live cell imaging were performed between 72-96 hours post transfection.

B α -targeting siRNA consisted of a pool of three siRNAs (16.67 nM each), while B δ - and non-targeting siRNA pools contained four distinct siRNAs each (12.5 nM each) (see Table 2.1). For single-target depletion (B α or B δ) and the non-targeting control, final concentrations of 50 nM siRNA were used, while 50 nM of each siRNA were used for B α + δ co-depletion.

2.1.4 Cell cycle synchronization

Various chemical and mechanical methods were used to induce synchrony at different stages of the cell cycle and are described separately below. Unless otherwise indicated, cells were seeded

for time-point analyses (one plate per time-point) or live cell imaging before synchronization procedures were initiated. Specifically, cells were trypsinized, counted and seeded 8-12 hours before synchronization was initiated (corresponding to 28-36 hours post transfection).

For synchronization at the G1/S boundary, cells were subjected to a double thymidine block (dT_{hy}). Treatment of cells with high concentrations of thymidine interferes with deoxynucleotide biosynthetic pathways and leads to DNA replication arrest (Ma and Poon, 2017). Cells were treated with 2 mM thymidine (Sigma) for 17 hours, released into drug-free medium for 9 hours, and then incubated with 2 mM thymidine for another 17 hours. Following the second thymidine incubation, cells were released into drug-free complete growth medium and either (i) collected and processed at indicated time-points or (ii) imaged by time-lapse microscopy in accordance with each experiment.

For mitotic exit assays, cells were synchronized in prometaphase by a combination of chemical and mechanical techniques. Cells were first treated with 2 mM thymidine for 20 hours, released into drug-free medium for 2 hours, and then treated with 100 ng/ml nocodazole (Sigma) for 12 hours. Nocodazole interferes with the polymerization of microtubules, thereby preventing mitotic spindle assembly and arresting cells in prometaphase of mitosis (Zieve et al., 1980). Following treatment with nocodazole, prometaphase-arrested cells were isolated by mechanical shake-off, washed once with pre-warmed phosphate buffered saline (PBS) and twice with complete growth medium, before being seeded into cell culture plates for subsequent time-point collections.

To synchronize cell populations at the G2/M boundary, cells were treated with 2mM thymidine for 20 hours, released into drug-free medium for 3 hours, and then incubated with 10 μ M RO3306 for 17 hours. RO3306 is a small molecule that specifically and reversibly inhibits CDK1 activity, thereby preventing entry into mitosis. For experiments that investigated the subsequent progression through mitosis and G1 phase, cells were released into drug-free medium following RO3306 treatment and collected at designated time-points. For experiments that investigated protein

degradation during RO3306-mediated synchronization, cells were additionally treated with 10 μ M MG132 (Selleck Chemicals), 30 μ M pro-TAME (Boston Biochem), 100 or 500 nM MLN4924 (Selleck Chemicals), or dimethyl sulfoxide (DMSO, Sigma) as a vehicle control at 9 hours of RO3306 treatment and collected at designated time-points. MG132, pro-TAME, and MLN4924 reversibly interfere with ubiquitin-dependent proteolysis via distinct mechanisms. While MG132 directly inhibits the proteolytic activity of the 20S proteasome, TAME (the metabolized form of the prodrug pro-TAME) and MLN4924 respectively inhibit APC/C and SCF ligase-mediated ubiquitination (Goldberg, 2012; Soucy et al., 2009; Zeng et al., 2010).

To induce a prolonged G2 phase arrest, asynchronously cycling cell populations were treated with 1 μ M etoposide (Selleck Chemicals) for 12, 24, or 48 hours and collected at indicated times. Etoposide is a topoisomerase II inhibitor that induces double-stranded DNA breaks, resulting in the activation of the DNA damage checkpoint and arrest in G2 phase (Lock and Ross, 1990).

Cells collected at designated time-points were processed for analysis by flow cytometry (FCM) and/or Western blotting (WB) in accordance with each experiment. In experiments where each time-point was analyzed by both flow cytometry and Western blotting, cells were trypsinized and resuspended, and an approximately equal number of cells per sample and time-point was counted out ($0.25\text{-}0.5 \times 10^6$ cells/sample) and processed for flow cytometry as described below. The remaining cells in each sample were washed once with PBS, pelleted by centrifugation (1200 rpm for 4 minutes), and stored at -80°C until they were processed for analysis by Western blotting as described below.

2.2 Flow cytometry

Cells collected for analysis by flow cytometry were washed once with PBS, fixed in ice-cold 70% ethanol, and stored at -20°C overnight or until they were processed for analysis. All flow cytometry-based analyses were performed on ethanol-fixed cells.

Relative DNA content analysis was used to determine the distribution of unsynchronized or synchronized cell populations in the G1, S, and G2/M phases. Ethanol-fixed cells were washed three times with ice-cold 1% BSA in PBS (hereafter referred to as PBSBA) and resuspended at a concentration of 1×10^6 cells/ml in PBSBA containing 40 µg/ml Ribonuclease A (RNase A, Sigma) and 30 µg/ml Propidium Iodide (PI, Sigma). Cells were then incubated at room temperature and protected from light for 30 minutes before being analyzed. HeLa cells that stably express eGFP-TUBA and mCherry-H2B were stained with 10 µg/ml Hoechst-33342 (Life Technologies) instead of PI due to the extensively overlapping emission spectra of mCherry and PI. In addition to serving as a stand-alone marker for cell cycle phase distribution, PI staining (and relative DNA content analysis) was also used as a base stain in the presence of phase-specific markers.

To determine the proportion of a cell population that is actively undergoing DNA replication, cells were pulsed with 10 µM 5-ethynyl-2'-deoxyuridine (EdU) for 1 hour prior to being collected and fixed. Following an overnight (or longer) fixation period, cells were processed and probed for EdU incorporation using the Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Life Technologies) in accordance with the manufacturer's instructions. Prior to analysis on the flow cytometer, cells were also stained for DNA content (as described above).

Mitotic populations were specifically identified by (i) performing a bivariate analysis of cyclin B1 expression and histone H3 phosphorylation levels as previously described (Taylor, 2004), or (ii) by analyzing the levels of the mitotic phosphoepitope MPM2. In both approaches, fixed cells were washed once with PBS and permeabilized using 0.25% Triton X-100 in PBS for 15 minutes on

ice. Each sample was subsequently incubated with (i) 500 ng of AlexaFluor®488-conjugated anti-cyclinB1 and 750 ng of anti-pS10H3 antibodies, or with (ii) 500 ng of anti-MPM2 antibody for 3 hours at room temperature and protected from light. Cells were then washed with PBSBA and stained with (i) Alexa Fluor® 647-conjugated goat anti-rabbit secondary antibody, or with (ii) Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody for 30 minutes at room temperature and protected from light. Finally, cells were washed in PBSBA, stained for DNA content (as described above) and analyzed. Detailed information regarding the primary and secondary antibodies used to detect phase-specific makers are listed in Tables 2.2 and 2.3, respectively.

All samples were analyzed using the BD LSRFortessa™ X-20 cell analyzer (BD Bioscience) or the BD FACSCanto™ flow cytometer (BD Bioscience). Resulting data were processed and analyzed using the flow cytometry data software FlowJo (Tree Star, Inc., Ashland, Oregon, USA) versions 9.6.5 or 10.0.1.

2.3 Protein extraction and Western blot analysis

Protein extracts were prepared by lysing cells in RIPA Buffer (50 mM Tris-Cl, pH7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% NaDOC, 0.1% SDS, 50 mM NaF, supplemented with freshly added 0.5 mM Na₃VO₄, 2 mM PMSF, 10 µg/ml leupeptin, and 4 µg/ml aprotinin) on ice for 20 minutes. Cell lysates were subsequently clarified by centrifugation at 16000 X g for 15 minutes at 4°C and protein concentration was determined using the colorimetric Bradford Protein Assays (Bradford, 1976). Protein concentrations were normalized across samples to 1 mg/ml and mixed with SDS sample buffer. Protein extracts were resolved by SDS-PAGE (10% acrylamide for 150-30kDa proteins and 12% acrylamide for proteins <25kDa) and transferred onto PVDF membranes (Immobilon-P, Millipore). Membranes were probed with the indicated primary antibodies, which

were then detected by HRP (horseradish peroxidase)-conjugated secondary antibodies (Sigma and BioRad), enhanced chemiluminescence (homemade reagent), and exposure to X-ray films (Diamed).

To quantify changes in protein expression or phosphorylation levels observed on Western blots, X-ray films were scanned as TIFF files, and a densitometric analysis of protein bands was performed using the Quantity One® 1-D Analysis Software (Bio-Rad). Densitometric measurements are generally expressed as ratios that are based on a common control sample included in each Western blot.

2.4 Live cell imaging

Cells were seeded into 96 well plates (Corning) 24 hours post transfection, and in accordance with each experiment, were either allowed to grow asynchronously for 36-48 hours, or were synchronized to the G1/S or G2/M boundary before imaging was initiated. For live cell imaging, plates were placed in a 37°C and 5% CO₂ environmental chamber (ImageXpress Micro XL), and images were taken using a 40X 0.75 NA dry objective with the MetaXpress 5.0.2.0 software (Molecular Devices Inc.) on the ImageXpress Micro XL epifluorescence microscope (Molecular Devices Inc.). Images were taken every 10, 15, or 20 minutes, as indicated in each figure, using 2x2 binned resolution, with 25% of full lamp intensity per channel. Time-lapse images were analyzed using the MetaXpress 5.0.2.0 software. Specifically, changes in the signal intensity and signal intensity standard deviation were measured for the indicated colours (red, green, and transmitted light) and graphed for each cell that was analyzed. These measurements were taken for every frame captured during the imaging time-course.

2.5 Sequence alignments and identity matrices

The amino acid sequences for the B regulatory subunit isoforms from *Homo sapiens* (humans), *Xenopus laevis* (African clawed frog), *Drosophila melanogaster* (Fruit fly), *Saccharomyces cerevisiae* (budding yeast), and *Schizosaccharomyces pombe* (fission yeast) were obtained from the UniProt online database (The UniProt Consortium). The sequence alignments and identity matrices were generated using the online Multiple Sequence Alignment tool Clustal Omega (EMBL-EBI, Hinxton).

2.6 Reagents and antibodies

Reagents (listed in Table 2.4) and primary and secondary antibodies (listed in Tables 2.2 and 2.3, respectively) were used as indicated. Primary antibodies were diluted in PBS containing 0.1% Tween20 and 3% BSA, except for MPM2, which was diluted in PBS containing 0.1% Tween20 and 5% skim milk. Secondary antibodies for Western blotting were diluted in PBS containing 0.1% Tween20, while those for flow cytometry were diluted in PBSBA.

2.7 Statistics

Data are shown as the mean \pm standard deviation (s.d.) or as the mean \pm standard error of mean (s.e.m.) as indicated in each figure. Statistical analyses were performed on data sets containing three or more experimental replicates as indicated in each figure. Data were analyzed by two-way ANOVA, as each experiment encompassed at least four distinct treatments (non-targeting or NT, B α -, B δ , and B α + δ -targeting siRNA). Multiple comparisons of means were made, where the mean of each treatment was only compared to control (i.e. siNT vs. siB α , siNT vs. siB δ , siNT vs. siB α + δ). The results were considered significant at P<0.05 as indicated in each figure.

Table 2.1 siRNA sequences

siRNA	Target Sequence	Product Information
siB α #1	5' GAAAUUACAGACAGGAGUU 3'	siGENOME Human PPP2R2A (5520) siRNA – Set of 4 Upgrade (Dharmacon, Catalog # MU-004824-01)
siB α #2	5' UAUCAAGCCUGCCAAUAUG 3'	
siB α #3	5' UAUGAUGACUAGAGACUAU 3'	
siB α #4	5' GCAGAUGAUUUGCGGAUUA 3'	
siB δ #1	5' UCGGAUAGCGCCAUCAUGA 3'	siGENOME Human PPP2R2D (55844) siRNA – Set of 4 Upgrade (Dharmacon, Catalog # MU-032298-00)
siB δ #2	5' GAGACUACCCUGUCGGUGAA 3'	
siB δ #3	5' UCAGGAUGUUUGAUAGAGA 3'	
siB δ #4	5' GAGAACGACUGCAUCUUUG 3'	
Non-targeting Pool #2	Proprietary	siGENOME Non-targeting siRNA Pool #2 (Dharmacon, Catalog # D-001206-14)
Non-targeting Pool	5' UGGUUUACAUGUCGACUAA 3' 5' UGGUUUACAUGUUGUGUGA 3' 5' UGGUUUACAUGUUUUCUGA 3' 5' UGGUUUACAUGUUUCCUA 3'	ON-TARGETplus Non-targeting Pool (Dharmacon, Catalog # D-001810-10)

Table 2.2 Primary antibody dilutions

Antibody	Company	Catalog #	Host	Dilution
Actin	Sigma	A2066	Rb	WB: 1:4000
Apc3 (Cdc27)	BD	610455	Ms	WB: 1:500
Aurora A	Abcam	ab12875	Rb	WB: 1:500
Aurora A pT288	Cell Signaling	3079	Rb	WB: 1:1000
Cdc20 (p55 CDC)	Santa Cruz	sc-13162	Ms	WB: 1:500
Cdc25C	Santa Cruz	sc-13138	Ms	WB: 1:500
Cdh1 (Frz1)	Abcam	ab118939	Rb	WB: 1:500
CDK1 pT14/Y15	Santa Cruz	sc-12340-R	Rb	WB: 1:500
Chk1	Cell Signaling	2360	Ms	WB: 1:1000
Chk1 pS345	Cell Signaling	2348	Rb	WB: 1:1000
Cullin 1	Santa Cruz	sc-17775	Ms	WB: 1:1000
Cyclin A2	Cell Signaling	4656	Ms	WB: 1:1000
Cyclin B1	Cell Signaling	4138	Rb	WB: 1:1000
Cyclin B1 (488 conjugated)	Santa Cruz	sc-245 AF488	Ms	FCM: 1:60
Cyclin D1	Cell Signaling	2922	Rb	WB: 1:1000
Cyclin D3	Cell Signaling	2936	Ms	WB: 1:1000
Cyclin E1	Cell Signaling	4129	Ms	WB: 1:1000
Cyclin E2	Cell Signaling	4132	Rb	WB: 1:1000
Emi1	Santa Cruz	sc-365212	Ms	WB: 1:500
FoxM1	Cell Signaling	5436	Rb	WB: 1:1000
MASTL	Cell Signaling	12069	Rb	WB: 1:1000
MPM2	Millipore	05-368	Ms	WB: 1:1000, FCM: 1:200
Myt1	ThermoFisher	PA5-17665	Rb	WB: 1:1000
NEDD 8	Cell Signaling	2754	Rb	WB: 1:1000
NIPA	Abcam	ab1776847	Rb	WB: 1:2000
NIPA pS354	Abcam	ab63557	Rb	WB: 1:1000
Plk1	Cell Signaling	4513	Rb	WB: 1:1000
Plk1 pT210	Cell Signaling	5472	Rb	WB: 1:1000
PP2A B α	LifeSpan Biosciences	LS-C26216	Ms	WB: 1:4000
PP2A B δ	Genetex	GTX116609	Rb	WB: 1:4000
PRC1 pT481	Abcam	ab62366	Rb	WB: 1:500
pS10H3	Santa Cruz	sc-8656-R	Rb	WB: 1:500, FCM: 1:40
Securin (PTTG1)	Santa Cruz	sc-56207	Ms	WB: 1:500
γ -H2AX	ABM	YO11268	Rb	WB: 1:1000

Table 2.3 Secondary antibody dilutions

Antibody	Company	Catalog #	Host	Dilution
Anti-Mouse IgG (whole molecule)-Peroxidase	Sigma	A4416	Gt	WB (2°): 1:3000
Anti-Rabbit IgG (H+L)-HRP Conjugate	BioRad	170-6515	Gt	WB (2°): 1:4000
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Invitrogen	A-21244	Gt	FCM (2°): 1:200
Goat anti-Mouse IgG, IgM (H+L) Secondary Antibody, Alexa Fluor 488	Invitrogen	A-10680	Gt	FCM (2°): 1:200

Table 2.4 List of reagents

Reagent	Company	Catalog #	Stock concentration	Working concentration
Blasticidin S	Sigma	15205	1 mg/ml	0.5 µg/ml
BSA	Sigma	A3912		
Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	LifeTechnologies	C10419		
DMEM, high-glucose	HyClone	SH30243.01		
DMSO	Sigma	D4540		
Etoposide	Selleck Chemicals	S1225	10 mM	1 or 100 µM
FBS	Gibco	12483-020		
Hoechst-33342	LifeTechnologies	H3570	10 mg/ml	10 µg/ml
Hygromycin B	Invitrogen	10687-010	50 mg/ml	200 µg/ml
Lipofectamine® RNAiMAX	Invitrogen	13778150		
MEM	Gibco	11090-081		
MG132	Selleck Chemicals	S2619	10 mM	10 µM
MLN4924 (Pevonedistat)	Selleck Chemicals	S7109	1 mM	as indicated
Nocodazole	Sigma	M1404	5 mg/ml	100 ng/ml
Opti-MEM™	Gibco	31985070		
PBS	HyClone	SH30028.02		
PenStrep	Gibco	15140122	10,000 U/ml Pen 10,000 µg/ml Strep	100 U/ml Pen 100 µg/ml Strep
Propidium Iodide (PI)	Sigma	P4170	1 mg/ml	30 µg/ml
pro-TAME	Boston Biochem	I-440	20 mM	30 µM
Puromycin	Sigma	P8833	1 mg/ml	0.3 µg/ml
Ribonuclease A from bovine pancreas	Sigma	R6513	10 mg/ml	40 µg/ml
RO3306	Sigma	SML-0569	20 mM	10 µM
Thymidine	Sigma	T1895	1 M	2 mM

Chapter 3: A general characterization and overview of the effects of loss of B α and B δ on the kinetics of S-to-G2, G2/M, Mitotic exit and G1-phase progression

3.1 Rationale and Hypothesis

Inquiries into the cell cycle regulatory roles of PP2A-B55 complexes in various model systems have primarily focused on entry into and exit from mitosis (Burgess et al., 2010; Cundell et al., 2013; Juanes et al., 2013; Manchado et al., 2010; Mayer-Jaekel et al., 1993; Mochida et al., 2009). With mitosis in the spotlight, little attention has been paid to potential roles for PP2A-B55 complexes in modulating progression through the G1, S, and G2 phases. Some evidence, however, suggests that PP2A-B α is involved in the control of S phase progression (Krasinska et al., 2011; Lin et al., 1998; Murphy and Michael, 2013; Wang et al., 2018), and G2 phase progression in response to DNA damage in cycling *Xenopus* extracts (Wang et al., 2015). Furthermore, B α -containing PP2A complexes have also been shown to regulate pocket protein phosphorylation, specifically p107 and p130, in mammalian cell lines (Jayadeva et al., 2010; Kolupaeva et al., 2013; Kurimchak et al., 2013), suggesting a role in the regulation of G0/G1 progression. In mammalian systems, much of this work has taken the approach of depleting both B α and B δ , or all B55-type subunits (B α , β , γ , and δ) (Burgess et al., 2010; Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017; Manchado et al., 2010). While this strategy has revealed functions that may be specific to two or more isoforms, or common to all B55-type subunits, it precludes the identification of isoform-specific functions. Importantly, distinct and even opposing functions of the highly related B α and B δ subunits have been described (Batut et al., 2008), warranting further examination of their individual roles in cell cycle regulation. Therefore, I investigated the specific roles of the B α and B δ subunits in the PP2A-mediated regulation of S-to-G2, G2/M and G1 phase progression. The experiments described in this chapter aim to provide a general characterization and overview of the cell cycle phase-specific functions of the B α and B δ subunits.

3.2 Results

3.2.1 Loss of B α has no effect on the cell cycle distribution of asynchronously cycling cell populations

The B α subunit is the most studied member of the B55 family, as well as the most commonly deregulated B55 isoform in human cancers. Therefore, my pilot experiments focused on examining the cell cycle-regulatory role of the B α subunit, and siRNA-mediated depletion of the B δ subunit was not included. To examine the effects of B α loss, HeLa cells were treated with all or none of four siRNA duplexes targeting different sequences in the 5' untranslated region (UTR) of B α mRNA. Protein levels of B α , as measured by quantitative Western blot analysis, were reduced by 81-91% by siRNA duplexes #2, #3, or #4, but remained unaffected by siRNA #1, as compared to control cells (Fig. 3.1A). A pool of siRNA #2, #3, and #4 (hereafter referred to as 'siB α ') was used for all subsequent experiments and compared to treatments with a non-targeting siRNA control.

Investigations into the role of the PP2A-B α holoenzyme in cell cycle regulation have mostly focused on specific cell cycle phases, and it has not been explored whether depletion of the B α subunit acutely disrupts cell cycle progression and leads to arrest in one or more cell cycle phases. Therefore, I first set out to determine whether transient ablation of B α might affect cell cycle distribution in asynchronously cycling cell populations. Compared to untreated and control (non-targeting siRNA-treated, NT) HeLa cell populations, unsynchronized cell cycle distribution, as measured by relative DNA content analysis, was not affected by depletion of B α (Fig. 3.1B and C). The absence of an overt effect indicates that B α -deficiency does not result in arrest or severely impaired progression through the G1, S, and G2/M cell cycle phases. Furthermore, this suggests that phase-specific B α -dependent effects may be subtle and only become apparent in synchronously cycling cell populations.

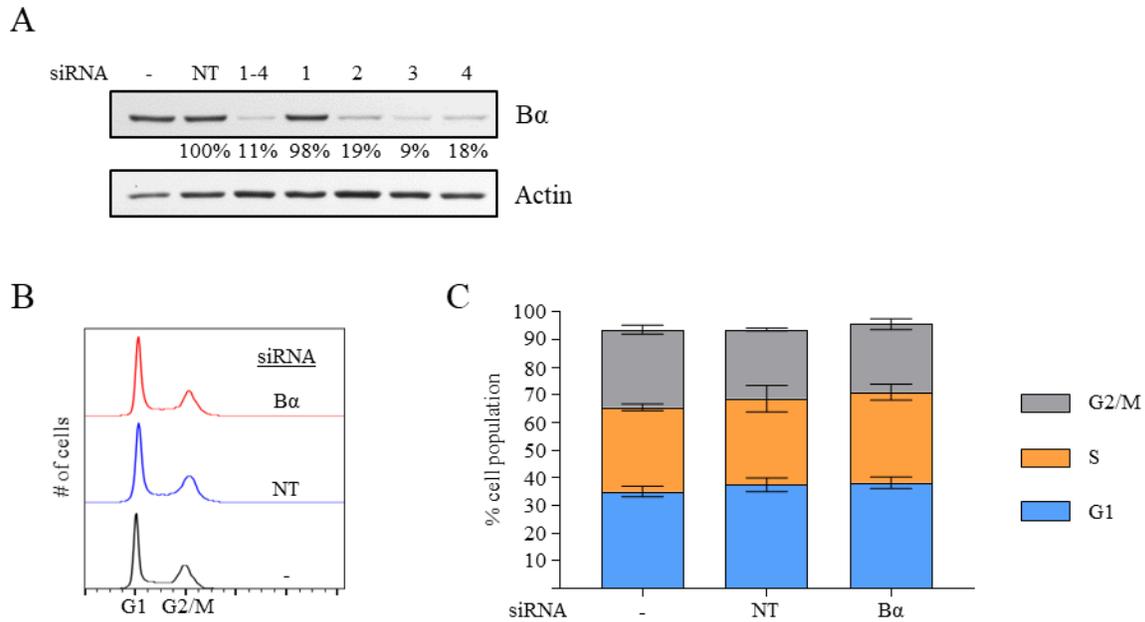


Figure 3.1: Loss of B α has no effect on cell cycle distribution of asynchronously cycling cell populations

- A. HeLa cells were treated with 50 nM non-targeting (NT) siRNA or with four (#1-4) siRNA duplexes together or alone targeting B α . B α protein levels were measured by quantitative Western blotting at 72 h following transfection. Percent values indicate levels of B α expression standardized to the non-targeting control. Equal loading was confirmed by probing for Actin. Subsequent experiments utilized a pool of B α -targeting siRNA #2, #3, and #4 at a final concentration of 50 nM.
- B. Representative DNA content histograms from untreated (-), non-targeting control siRNA- (NT) and B α -targeting siRNA-treated, unsynchronized HeLa cell populations at 72 h following transfection. To obtain DNA content histograms, cells were stained with 30 μ g/mL propidium iodide for 30 min and analyzed by flow cytometry.
- C. Distribution of unsynchronized HeLa cell populations in G1, S, and G2/M phases. The percent cell population in each phase was calculated from DNA content histograms using the Watson Pragmatic Cell Cycle Module in FlowJo v9.6.5. Data are representative of four independent experiments (n=4, mean \pm s.d.). No significant differences were found between treatments.

3.2.2 Preamble: an overview of cell cycle synchronization techniques used to investigate phase-specific B55-dependent effects

Cell cycle regulatory events most often occur in a phase-dependent manner, and examination of the underlying biochemical mechanisms invariably requires cell-cycle synchronization of cell populations (Banfalvi, 2011; Davis et al., 2001; Ma and Poon, 2017). As suggested above (end of section 3.2.1), the elucidation of phase-specific B55-dependent effects may require the investigation of synchronously cycling cell populations. Therefore, HeLa cell populations were synchronized in their cell cycle progression for the ensuing exploration of the regulatory roles of the PP2A-B55 complexes.

To study the phase-specific effects of B55 subunit depletion, various methodologies were employed to synchronize cell populations at distinct cell cycle phase transitions. For the investigation of progression through S phase, G2 phase and mitosis cell populations were synchronized at the G1/S phase transition using the double thymidine blocking method (see section 2.1.4) (Fig. 3.2A). To examine B55-dependent effects on the progression from the meta-to-anaphase transition (mitotic exit) into G1 phase, cell populations were synchronized to prometaphase using consecutive thymidine and nocodazole blocks (see section 2.1.4) (Fig. 3.2B). I also investigated the effects of B55 subunit depletion on the progression through mitosis and G1 phase (and into S phase) by synchronizing cell populations to the G2/M boundary using consecutive thymidine and RO3306-mediated blocks (see section 2.1.4) (Fig. 3.2C). The use of various synchronization methodologies revealed distinct roles for the highly related B α and B δ subunits in the regulation of progression through the different cell cycle phases. These are discussed in detail in the subsequent sections.

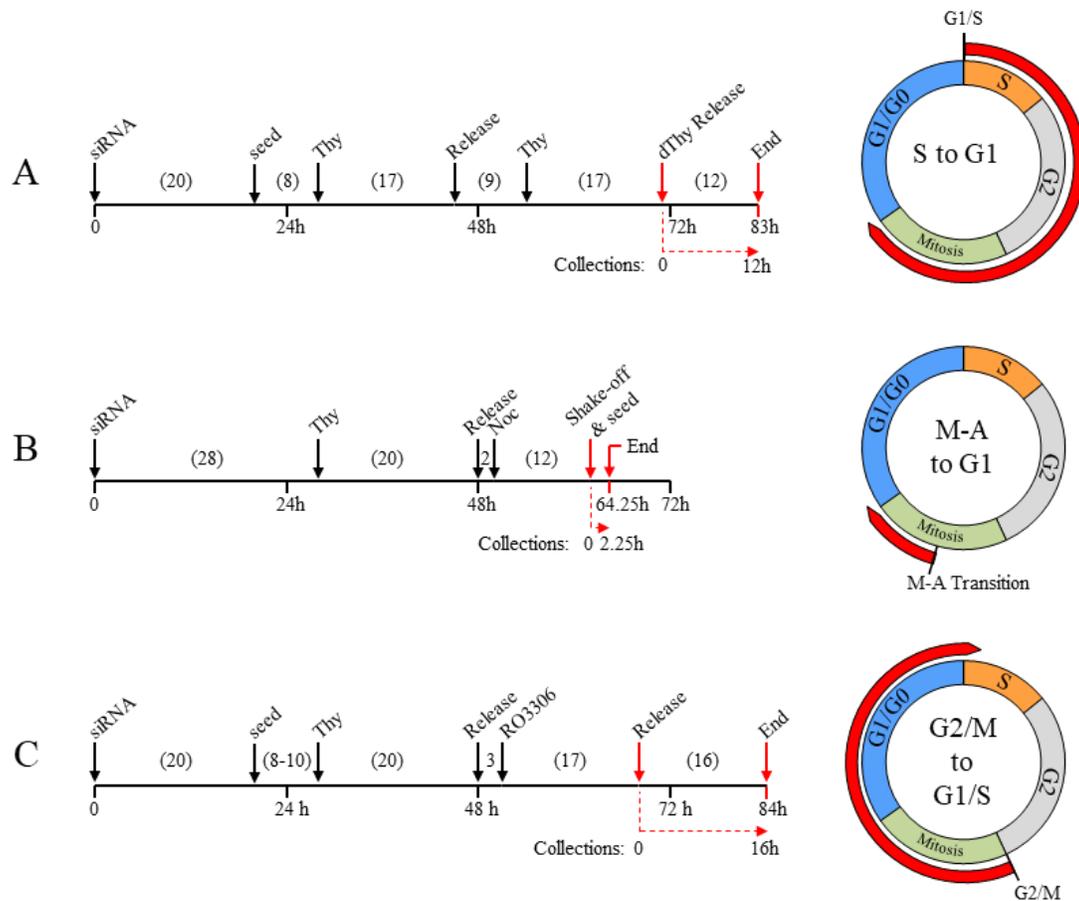


Figure 3.2: An overview of cell cycle synchronization techniques used to investigate phase-specific B55-dependent effects

To investigate the effects of B55 subunit depletion on the progression of HeLa cell populations through the different cell cycle phases, control cells (non-targeting siRNA, siNT) and cells depleted of B α , B δ , or both (B α + δ) using isoform-specific siRNA duplexes were synchronized to distinct cell cycle phase transitions using various methodologies. The diagrams illustrate the experiment timeline for each type of cell cycle synchronization, starting at the time of siRNA transfection until the end of the sample collection. The synchronization procedures are also described in section 2.1.4. The numbers in the parentheses indicate the hours between each step of the experiment. The red arrows (solid and dashed) delineate the start and end of the period during which samples were collected for analysis. The cell cycle diagrams on the right-hand side depict the phases that were examined using the respective synchronization methodologies.

- A. Double thymidine (dThy) blocking method. HeLa cell populations were synchronized at the G1/S transition using consecutive thymidine (Thy) treatments as depicted. Samples were collected as cell populations progressed through S, G2 and M (or mitosis) phase.
- B. Mitotic exit assay. HeLa cell populations were synchronized to prometaphase of mitosis using consecutive thymidine (Thy) and nocodazole (Noc) treatments. Following the isolation of mitotic (prometaphase) cells by mechanical shake-off, cells were seeded for subsequent timepoint

collections. Samples were collected as cells progressed from prometaphase through mitosis and into G1 phase.

- C. Analysis of G1 progression. HeLa cell populations were synchronized at the G2/M boundary using consecutive thymidine (Thy) and RO3306 treatments. Samples were collected as cell populations progressed from the G2/M boundary through mitosis, G1 phase and into S phase.

3.2.3 Establishing the synchronized S-to-G2/M and G2/M-to-G1 progression timeline

Synchronization at the G1/S-boundary using the double thymidine block method (see Materials and Methods, section 2.1.3) provides a convenient and non-toxic means to investigate mechanisms that control the kinetics of S-to-G2, G2-to-Mitosis, and mitotic progression. Using relative DNA content analysis, an examination of the 24h-progression timeline of HeLa cells showed that cell populations undergo one full cycle (from G1/S-release to G1/S-transition in the next division cycle) within approximately 12h (Fig. 3.3A). Cells enter another round of division, initiating DNA replication, and attaining as much as 60% S phase synchrony by 18h; however, after this point, the populations rapidly lose synchrony by 24h (Fig. 3.3A).

A more in-depth examination of the S-to-G2/M and G2/M-to-G1 timeline confirmed that cell populations complete transition into G2 phase by 6h after release (Fig. 3.3B). Between 7.5-11h all cells undergo mitosis, and completely progress into G1 phase by 12h (Fig. 3.3B). At 9.5h, approximately half of the cell population is actively undergoing mitosis (intersection of G2/M and G1 populations, Fig. 3.3B), a point I hereafter frequently refer to as peak mitosis. Treatment of cell populations with non-targeting siRNA did not affect the kinetics of cell cycle progression, as compared to untreated control cells (Fig. 3.3B).

To investigate potentially distinct roles for the B α and B δ subunits in modulating the kinetics of S-to-G2, G2-to-M and mitotic progression, my initial experiments focused on the established timeline as outlined in Fig. 3.3B and C.

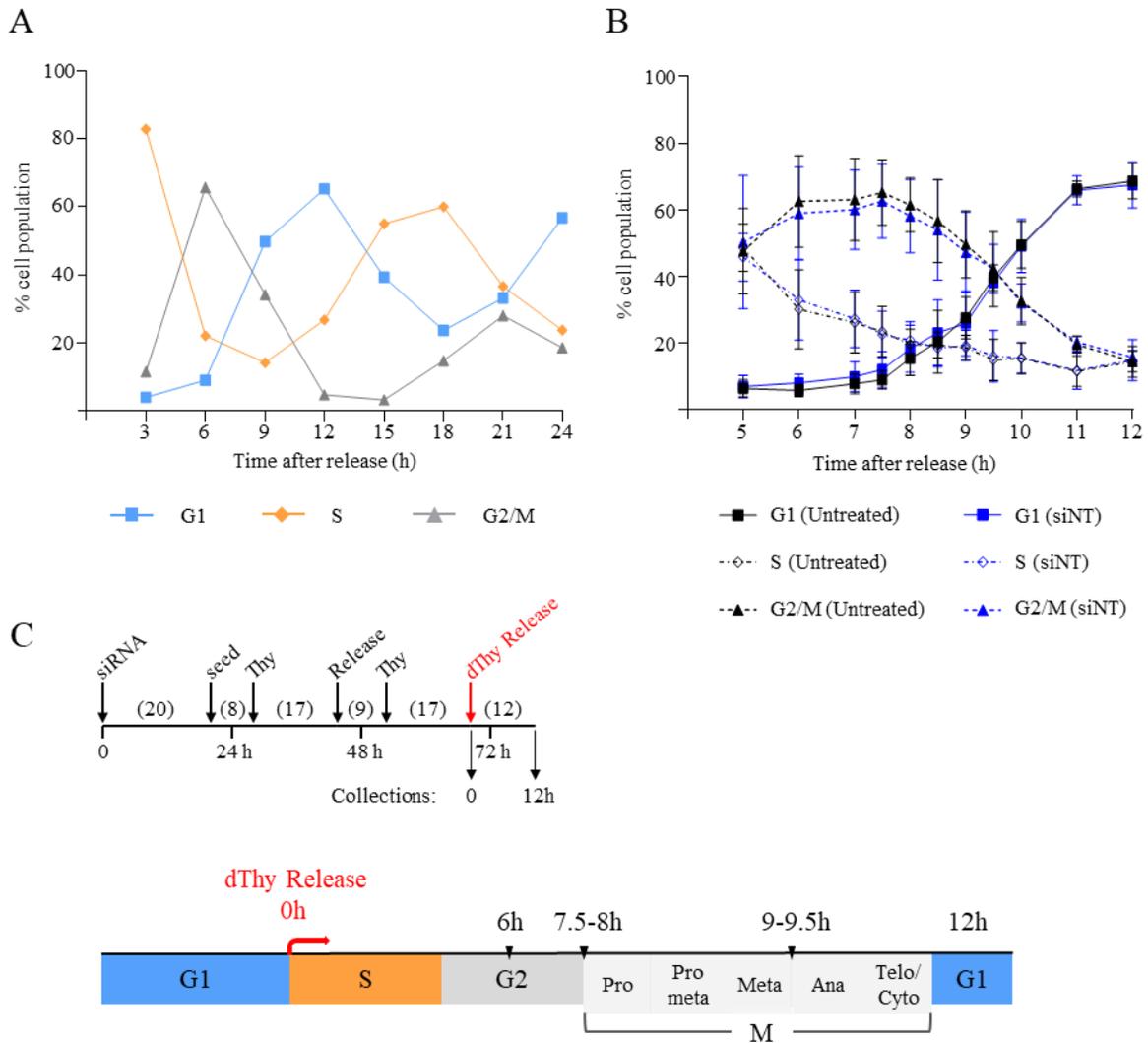


Figure 3.3: Establishing the synchronized S-to-G2/M and G2/M-to-G1 timeline

- A. Untreated HeLa cells were synchronized at the G1/S boundary using the double thymidine blocking method. Following release, cells were collected every 3 h for 24 h and processed for relative DNA content analysis by flow cytometry. The percent cell population in G1, S, and G2/M phases at each timepoint was calculated from resulting DNA content histograms using the Watson Pragmatic Cell Cycle Module in FlowJo v9.6.5.
- B. Untreated and non-targeting siRNA-treated (NT) cells were synchronized, collected and processed as in (A) at the indicated timepoints. Data are representative of five independent experiments (n=5, mean \pm s.d.).
- C. Upper panel: The diagram illustrates the timeline of the experiment, from the time of siRNA transfection until the end of sample collection. Cell populations are collected between 71 and 83 h

post-transfection. The number in parentheses indicates the hours between different steps of the experiment.

Lower panel: Diagram of the timeline of cell cycle phase progression following synchronization of cell populations at the G1/S boundary based on data as in (B).

3.2.4 Loss of B α or B δ differentially alters the timing and duration of G2/M progression

Following the initial examination of B α -dependent effects on asynchronous cell cycle distribution (section 3.2.1), my subsequent key aim was to determine whether the PP2A-B α and -B δ complexes play distinct roles in the regulation of S-to-G2/M and G2/M-to-G1 (or mitotic) progression. To this end, I investigated the general effects of B α and B δ subunit depletion on the kinetics of S-to-G2/M-to-G1 progression using relative DNA content analysis. B α subunit protein levels were depleted as described above (Fig. 3.1A). To transiently reduce B δ expression levels, a pool of four separate siRNA duplexes targeting the 5' UTR of B δ mRNA was used. The expression of the B α and B δ subunits, when targeted separately, was efficiently reduced throughout the duration of the time-course experiments (Fig. 3.4A). Furthermore, depletion of the B α subunit did not affect the expression level of B δ , and vice versa (data not shown). When these subunits were targeted for depletion together, their expression levels were also markedly reduced, albeit at a slightly lower efficiency as compared to individual depletions (Fig. 3.4A).

G1/S-synchronized control HeLa cells and cells depleted of the B α and/or B δ subunits were released into the cell cycle, and progression was monitored by flow cytometry. In all treatments, more than 50% of the cell population entered G2/M phase by 6 h post-release, and by 8.5 h approximately half of the control population had undergone mitosis as demonstrated by the shift from G2/M to an equivalent population of G1 cells (Fig. 3.4B). Depletion of either or both B55 subunits delayed progression through G2/M, as an equivalent distribution of cells in G2/M and G1 was attained later at about 9.5 h in B α -depleted cells and at 9 h in B δ -depleted cells, while cells with co-depleted B α + δ subunits appeared kinetically most similar to cells lacking B δ (Fig. 3.4B).

In multiple experiments, relative DNA content analysis clearly revealed B55-dependent effects on G2/M progression, with B α depletion resulting in the most pronounced shift in timing, and with B δ and B $\alpha+\delta$ depletion producing intermediate and similar phenotypes (Fig. 3.4C). Comparisons of the kinetics of control cells with each cycling B55-deficient population (Fig. 3.4D) showed that B α -depleted cells exhibited significantly slowed S-to-G2/M transition (see 5 h timepoint), attaining peak G2/M considerably later than control cells (at ~8 h post-release compared to ~6 h for control cells). Furthermore, higher G2/M phase populations were present from 8-11 h post-release (Fig. 3.4D, left panel). Less pronounced delays in attaining peak G2/M were apparent in cell populations depleted of B δ or B $\alpha+\delta$ (at 6-7 h post-release), but significantly higher G2/M populations of these cells persisted from 8.5-11 h post-release (Fig. 3.4D, middle and right panels). Despite these delays, all the B55 subunit-depleted cells completed the transition back to G1 phase by 12 h after release.

Altogether, these results indicate that B α - and, to a lesser degree, B δ -deficient cells experience a slower transition from S to G2/M phase and may also progress more slowly through G2/M. Specifically, while the depletion of B δ and B $\alpha+\delta$ only mildly delays S-to-G2/M progression (and G2/M peak attainment), their depletion has a much more pronounced effect on G2/M-to-G1 progression. Furthermore, the more severe impact on the overall S-to-G2/M-to-G1 progression timeline of B α depletion compared to the loss of B δ suggests that these highly related PP2A subunits may play overlapping but non-redundant roles in regulating S-to-G2/M and G2/M-to-G1 progression.

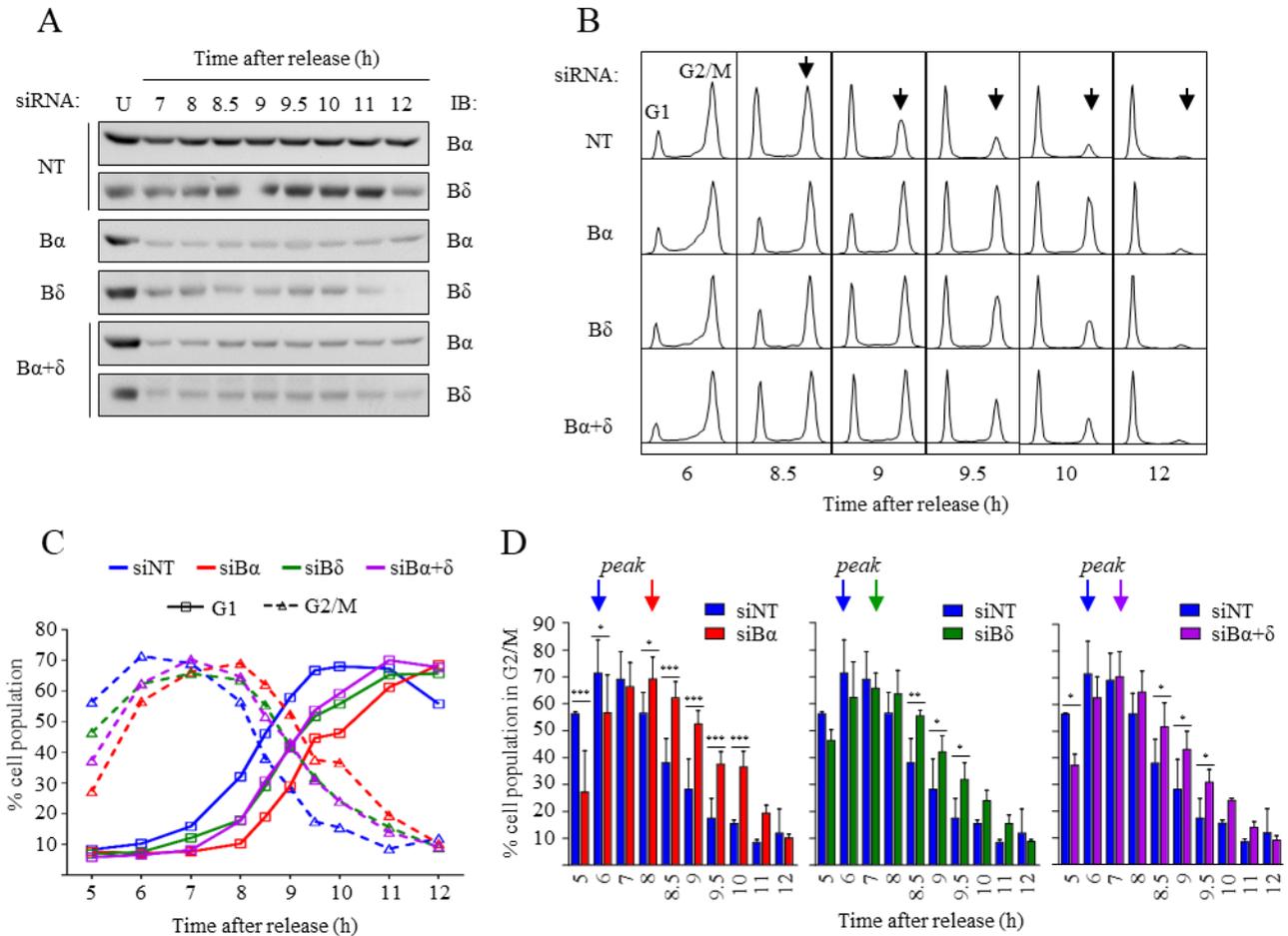


Figure 3.4: Loss of B α and δ subunits alters the timing and kinetics of G2/M progression

Control cells (non-targeting siRNA, siNT) and cells depleted of B α (siB α), B δ (siB δ), or both (siB α + δ) using isoform-specific siRNA duplexes were synchronized at the G1/S phase transition using a double thymidine block, then released into the cell cycle and collected at the indicated times post-release.

- Cell lysates were probed (IB) for B α and B δ subunits to determine siRNA-mediated depletion throughout the course of the experiment. Lysate prepared from a single unsynchronized cell population (U) was used as a standardized control for protein expression levels across different gels.
- Relative DNA content in synchronously cycling cell populations was determined using propidium iodide staining and flow cytometry. The resulting DNA content histograms show the distribution of cell populations in G1 and G2/M phases at selected times post-release.
- The time-dependent cycling of G1 (solid lines) and G2/M (dotted lines) control and B55-depleted cell populations was determined and averaged from four independent experiments. Percentage values of G1 and G2/M populations were determined using Watson-Pragmatic Cell Cycle Module in FlowJo v9.6.5 (n=4).

- D. The percent cells in G2/M phase at the indicated times post-release was calculated as the average value of data from four independent experiments as in (C) and is shown as a comparison between the control cells and each indicated B55-depleted population (n=4, mean \pm s.d., * P <0.05, ** P <0.01, *** P <0.001, ANOVA).

3.2.5 B55-dependent alterations of S phase progression

Initial relative DNA content analysis indicated a predominantly B α -dependent, slowed S-to-G2 transition (Fig. 3.4C, 5 to 7h following release). This observation prompted me to investigate whether this effect may be due to hindered cycling through S phase. Using EdU-incorporation to detect cells actively undergoing DNA replication (Fig. 3.5A), I confirmed that loss of B α in single and co-depleted (B α + δ) populations indeed resulted in notably impeded progression through S phase (Fig. 3.5A and B). B α and B α + δ -depleted cells progressed out of S phase between 5-6h after release from G1/S arrest, as compared to control cells, which started exiting S phase between 3-4 hours following release (Fig. 3.5B and C, left and right panels). Ablation of B δ resulted in a less pronounced delay with cells initiating progression out of S phase between 4 and 5h after release (Fig. 3.5C, middle panel). Progression through S phase may be hindered by the activity of an intra-S phase checkpoint that is triggered in response to DNA damage incurred during genome duplication (Iyer and Rhind, 2017). Therefore, I examined the phosphorylation-dependent activation of Checkpoint Kinase 1 (Chk1) and Histone H2A (H2AX), two well-established markers of the DNA damage checkpoint. Depletion of B55 subunits did not result in the phosphorylation of Chk1 or H2AX (Fig. 3.5D), indicating that the observed, B α -dependent postponement in S phase progression was not due to DNA damage checkpoint activation.

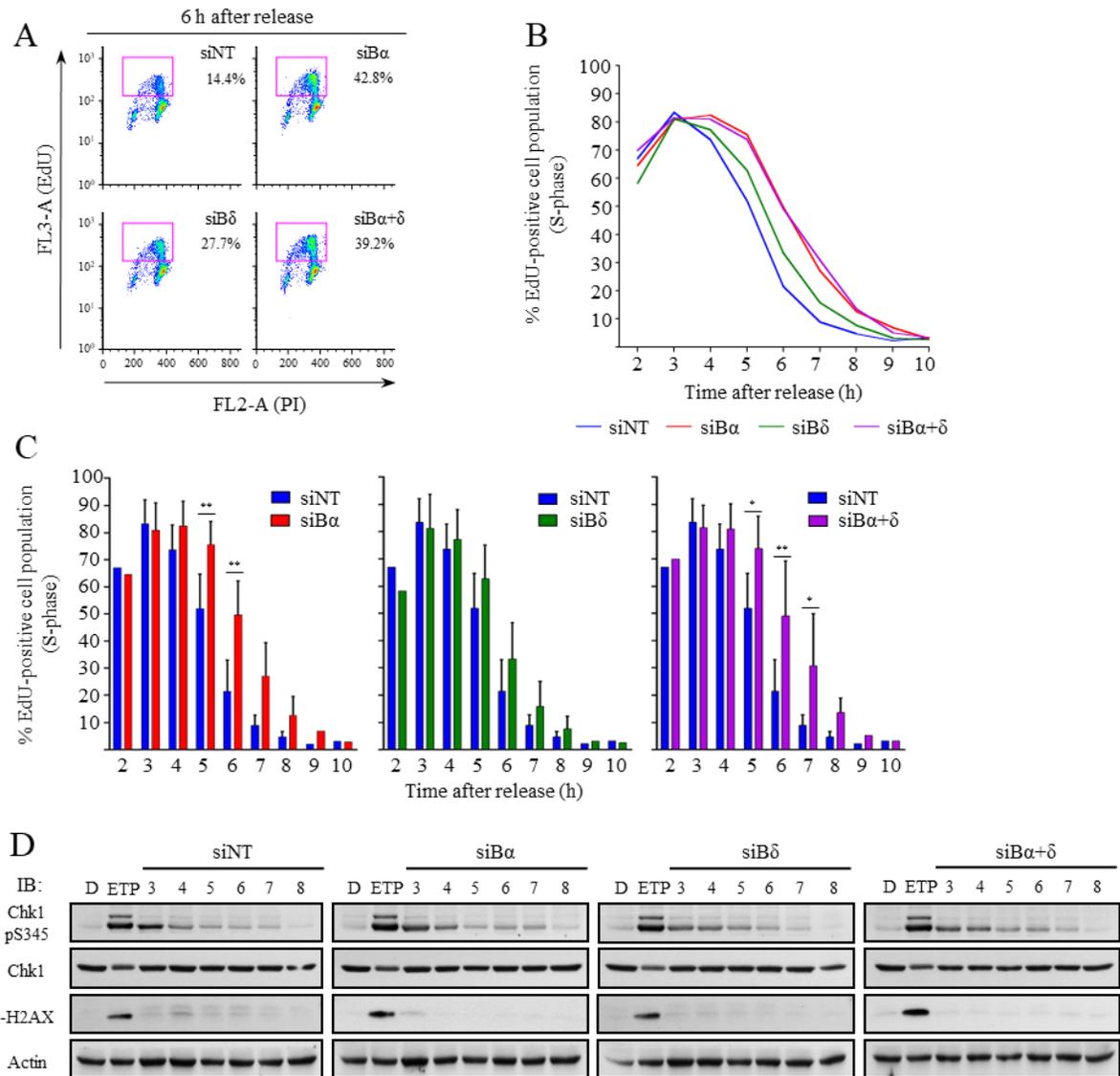


Figure 3.5: B55-dependent alterations of S-phase progression

Control cells and B55 subunit-depleted cell populations were synchronized at the G1/S phase transition using a double thymidine block. Following release into S-phase, cells were pulsed with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU) for 1 h prior to being collected at the indicated timepoints.

- EdU-incorporation was measured by flow cytometry. A bivariate plot of cells labeled with EdU (FL3-A) and propidium iodide (FL2-A) at 6 h after release is shown. The percent values indicate the proportion of the EdU-positive, S-phase population.
- The percent cells in S-phase at the indicated timepoints was calculated as the average value of data from four independent experiments and is shown as a comparison between all siRNA treatments (siNT, siB α , siB δ , siB α + δ) (n=4, mean).

- C. The data as in (B) are shown as a comparison between the control cells and each indicated B55-depleted population (n=4, mean \pm s.d., * P <0.05, ** P <0.01, ANOVA).
- D. Cell lysates from synchronized cell populations were probed for DNA damage checkpoint activation markers Chk1 (phosphorylated at Serine 345 and total protein) and γ -H2AX, and actin (loading control). All Western blots included two samples of lysate from double thymidine synchronized cell populations that were released for 30 min, treated with 100 μ M Etoposide (ETP) or DMSO vehicle control (D) for 2 h, and collected immediately after treatment. These samples serve as positive and negative controls for DNA damage checkpoint activation, respectively.

Therefore, these data point to a previously undescribed role for the B α subunit in the regulation of S phase progression in G1/S-synchronized mammalian cells. Furthermore, this suggests that the B α -dependent shift in the timing of G2/M progression described above (Fig. 3.4C) may be due to slowed completion of DNA replication. The differences in B α - and B δ -depletion phenotypes also support the idea that these subunits play distinct rather than redundant roles in cell cycle regulation. Interestingly, the B α -like S phase delay seen in B α + δ -depleted populations does not manifest in an equivalent postponement in mitotic initiation. While this phenotype is somewhat puzzling, it is consistent and representative of four independent experiments and may hint at a complex relationship between the functions of the B α and B δ subunits. Alternatively, the variable siB α + δ kinetic phenotypes may be the result of reduced depletion efficiency in co-depleted cell populations. This is discussed further in section 3.4.

3.2.6 B α and B δ subunits differentially regulate the timing and duration of mitosis

Analysis of relative DNA content in cell populations limits the determination of cell cycle distribution to G1, S, and G2/M phases, and precludes explicit identification of mitotic populations. Therefore, to more precisely determine the effects of B α - and/or B δ -depletion on progression from the G1/S boundary into and through mitosis, I measured the expression of two widely used mitotic markers, cyclin B1 and Serine10-phosphorylated histone H3 (pS10H3). Cyclin B1 expression

increases during G2, peaks at metaphase, and its degradation is triggered at the meta-to-anaphase transition (Clute and Pines, 1999; Sherwood et al., 1994). Bulk phosphorylation of histone H3 on Serine 10, an indicator of chromosome condensation, occurs during prophase and reaches maximal levels in metaphase, while its dephosphorylation starts during anaphase and concludes in telophase (Hendzel et al., 1997). Bivariate analysis of these markers by flow cytometry (Fig. 3.6A) revealed that loss of B α noticeably delays initiation of mitosis over the 6-8 h period post-release, and results in a shift of peak mitotic populations to 9 h post-release compared to 8 h in control populations (Fig. 3.6B, left bar graph). However, the ablation of B δ or B $\alpha+\delta$ does not delay the initiation of mitosis but extends the presence of peak mitotic populations by 1h (Fig. 3.6B, middle and right bar graphs). Immunoblotting for pS10H3 in lysates from G1/S-synchronized cell populations confirmed the altered phosphorylation kinetics of H3 in B55-depleted cells (Fig. 3.6C). Therefore, compared to the kinetics of control populations, loss of the B α subunit leads to a shift of mitotic timing, while depletion of B δ or B $\alpha+\delta$ results in prolonged mitotic duration (Fig. 3.6D, both panels).

Extending my previous observations of distinct B α - and B δ -dependent effects in G2/M progression based on relative DNA content analysis (Fig. 3.4B-D), this subsequent investigation of mitotic kinetics elucidated that in synchronously cycling cell populations (released from G1/S arrest), B α and B δ play different roles in regulating the kinetics of mitosis. Furthermore, supporting the similarity between the B δ - and B $\alpha+\delta$ -depletion phenotypes described in Figure 3.4, loss of B $\alpha+\delta$ also results in prolonged rather than delayed mitosis.

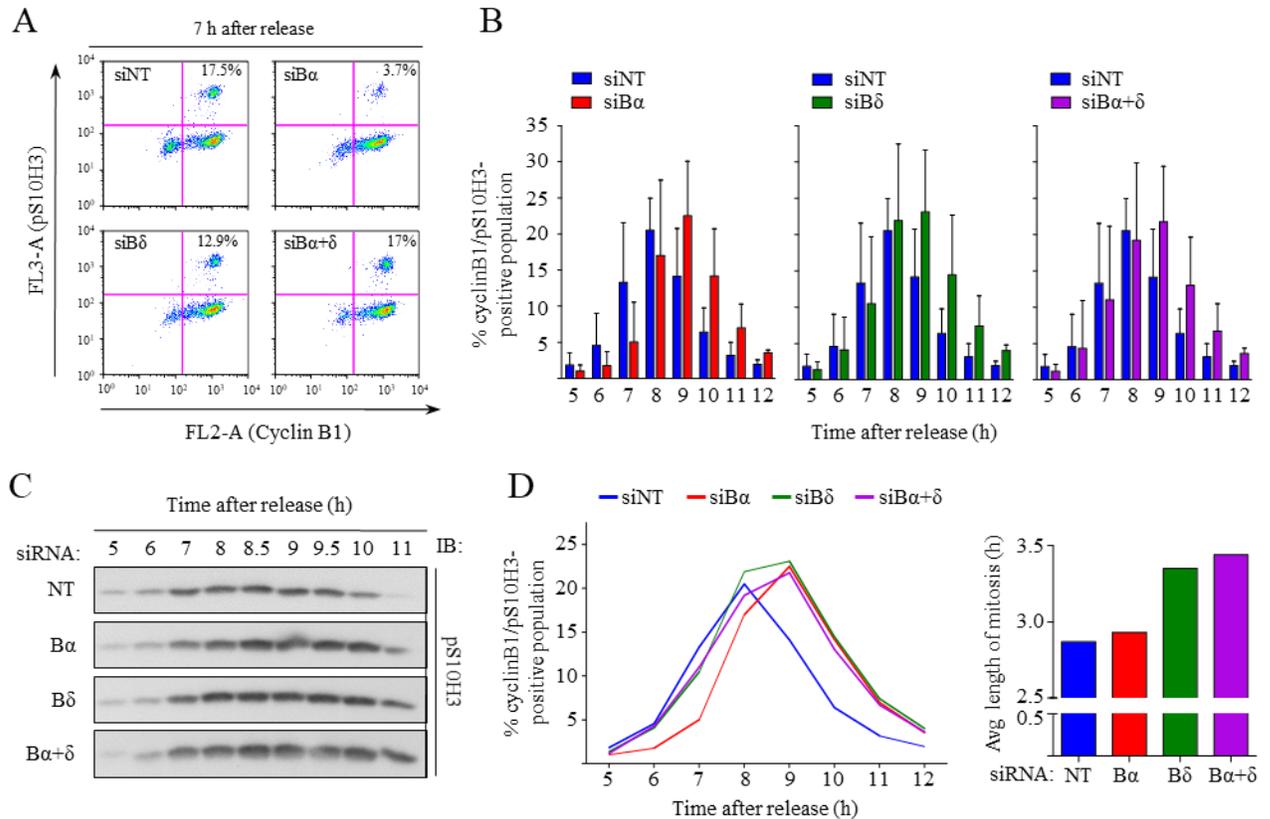


Figure 3.6: B α and B δ subunits differentially regulate timing and duration of mitosis

Control and B55 subunit-depleted cell populations were synchronized using a double thymidine block, collected at the indicated timepoints, and processed for analysis by flow cytometry and Western blotting.

- Samples were analyzed for cyclin B1 expression (FL2-A) and histone H3 Ser10 phosphorylation (FL3-A) by flow cytometry. Bivariate dot blots representative of samples collected 7 h after release are shown. Mitotic cell populations are positive for both cyclin B1 and pS10H3 (top right corner of each plot). The percent values indicate the proportion of mitotic cells.
- The percent mitotic cells at the indicated times post-release was calculated as the average value of data from four independent experiments ($n=4$, mean \pm s.d., no statistical significance, ANOVA).
- Samples were collected at indicated timepoints, and cell lysates probed (IB) for pS10H3 as shown. This blot is representative of results from four independent experiments.
- Progression of control and B55-depleted cell populations into and through mitosis was determined and averaged from four independent experiments (left panel) ($n=4$). The data are the same as graphed in (B). The column graph (right panel) shows the length of mitosis, defined as the time during which greater than or equal to half of the control or B55-depleted mitotic populations exhibited positivity for cyclin B1/pS10H3. The 'length of mitosis' values underlying the column graph (right panel) were derived from measurements performed directly on the graph

in the left panel. This graph largely serves as a representation of the length of mitosis as defined above and does not reflect independent measurements.

3.2.7 Loss of B55 subunits alters the activation kinetics of mitotic regulators

In higher eukaryotes the timing of mitosis is tightly controlled by the activities of the Cdc25C phosphatase and the Myt1/Wee1 kinases, which regulate the activation of the main mitotic driver, the CDK1-cyclin B1 complex (Fig. 3.7A). I examined whether the B55-dependent alterations in mitotic timing and duration were associated with changes in the kinetics of activation/inactivation of these key mitotic regulators. As introduced above (see section 1.1.6 Mitotic entry), the activities of Cdc25C, Myt1/Wee1 and CDK1 are regulated by phosphorylation. Therefore, to investigate the kinetics of activation/inactivation of these regulators, cell lysates from the cycling, G1/S-synchronized HeLa cell populations were processed for Western blot analysis and probed for changes in the phosphorylation of CDC25C, MYT1, and CDK1.

In agreement with the B α -dependent delay in G2/M progression (Fig. 3.4C-D) and mitotic initiation (Fig. 3.6B-D), the hyperphosphorylation and activation of CDC25C, detected by an increased signal of a higher molecular weight band (indicated by the black arrow head), is delayed in B α -depleted cells (Fig. 3.7B). Loss of B δ , however, does not affect the phosphorylation-dependent activation of CDC25C, as B δ -depleted cells exhibit control-like CDC25C phosphorylation kinetics (Fig. 3.7C). Similarly, depletion of B α + δ results in a B δ -like (or control-like) kinetic phenotype of CDC25C phosphorylation and activation (Fig. 3.7B), echoing the G2/M progression and mitotic initiation kinetics described above (Fig. 3.4C-D and Fig. 3.6B-D, respectively).

Hyperphosphorylation of MYT1 is associated with the inhibition of its kinase activity and is required for the timely activation of CDK1 (Booher et al., 1997; Mueller et al., 1995). Compared to control populations, ablation of B α , but not B δ or B α + δ , delays the inhibitory phosphorylation of the

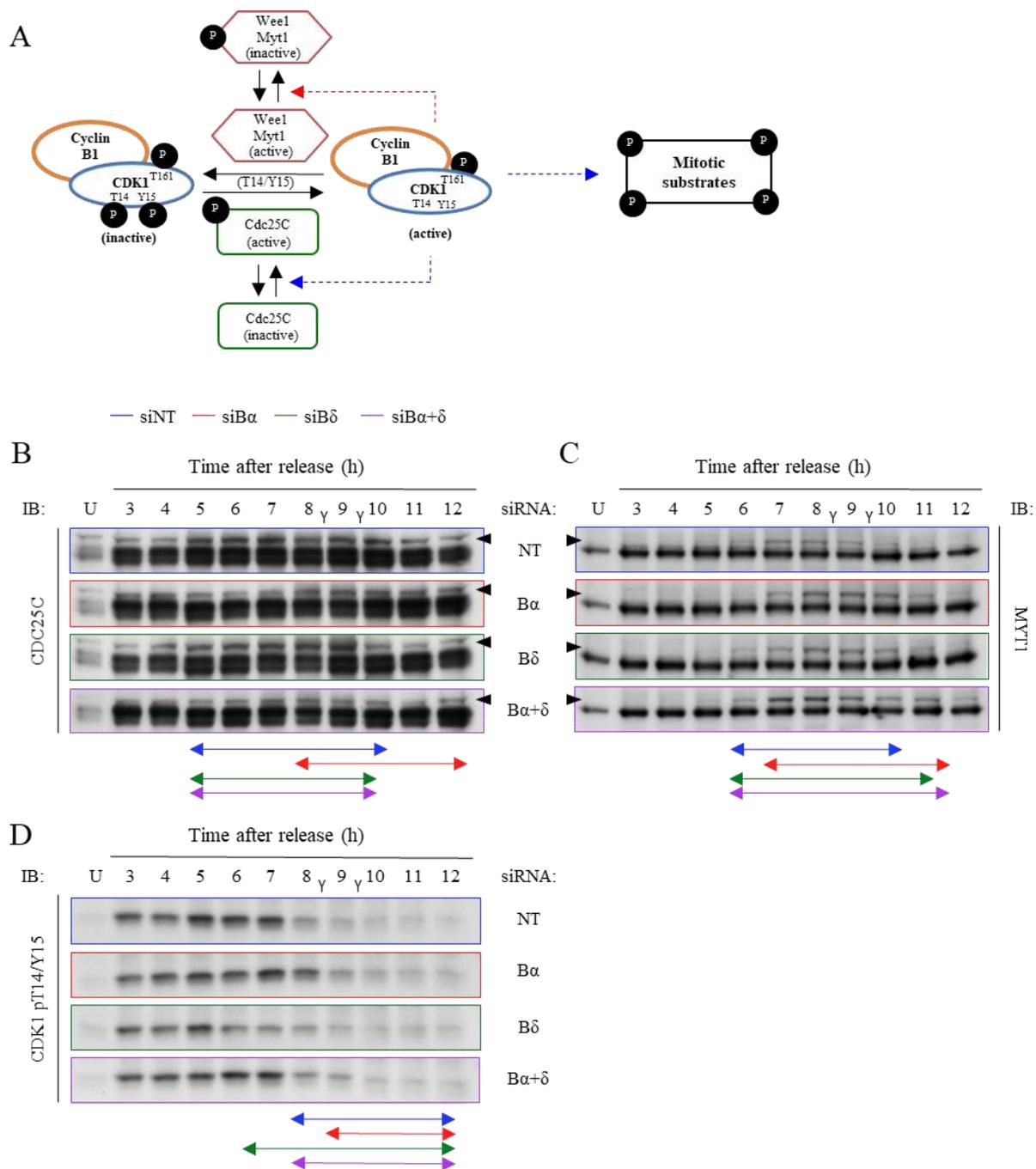


Figure 3.7: Loss of B55 subunits alters activation kinetics of mitotic regulators

Control and B55 subunit-depleted cell populations were synchronized using a double thymidine block, collected at the indicated timepoints, and cell lysates were processed for Western blot analysis. Blots are representative of results from three independent experiments (n=3). Lysates were obtained from the same samples that were analyzed by flow cytometry as shown in Figure 3.4.

- A. Diagram depicting the core components of the positive feedback loop that activates CDK1-cyclin B1, thereby initiating mitosis. Black arrows indicate changes in phosphorylation status, blue dotted arrows indicate activating phosphorylation, and the red dotted arrow indicates inactivating phosphorylation.
- B. Western blot of CDC25C total protein. The hyperphosphorylated isoform of CDC25C (higher molecular weight band indicated by black arrowhead) represents phosphorylation-dependent activation of CDC25C.
- C. Western blot of MYT1. The hyperphosphorylated isoform of MYT1 (higher molecular weight band indicated by black arrowhead) represents phosphorylation-dependent inactivation of MYT1.
- D. Western blot of CDK1 inhibitory phosphorylation sites T14/Y15. Disappearance of signal indicates dephosphorylation and indicates activation of CDK1-cyclin B1 complex.

Lysate prepared from a single unsynchronized cell population (U) was used as a standardized control for protein expression and phosphorylation levels. Coloured arrows below each set of Western blots indicate approximate timing and duration of phosphorylation, assessed qualitatively. Western blots were cropped and stitched to remove half-hour timepoints (8.5 and 9.5 h), indicated by Y.

MYT1 kinase (Fig. 3.7C; 7h post-release in $B\alpha$ - vs 6h in $B\delta/B\alpha+\delta$ -depleted and control populations).

This delay in MYT1 inhibition is also consistent with the $B\alpha$ -dependent delay in G2/M progression and mitotic initiation (Fig. 3.4C-D and Fig. 3.6B-D, respectively). It has been postulated that dephosphorylation and reactivation of MYT1 is required for full inactivation of CDK1 during mitotic exit and for the completion of mitosis (Potapova et al., 2011; Ruiz et al., 2010). In support of this hypothesis, I observed prolonged phosphorylation of MYT1 (Fig. 3.7C) concurrent with extended mitotic duration (Fig. 3.6D) in the absence of $B\delta$ (in single and co-depleted conditions).

Changes in the activation kinetics of CDC25C and MYT1 were associated with altered timing of the activating T14/Y15 dephosphorylation of CDK1 (Fig. 3.7D). Dephosphorylation and activation of CDK1 was delayed in the absence of $B\alpha$ but remained largely unaffected by the ablation of $B\delta$ (in single and co-depleted cells) as compared to control cells (Fig. 3.7D).

These findings implicate $B\alpha$ in the regulation of CDC25C phosphorylation, and both $B\alpha$ and $B\delta$ as modulators of MYT1 phosphorylation, particularly in regulating its dephosphorylation as cells leave mitosis. Thus, in accordance with their distinct roles in regulating G2/M and mitotic kinetics,

the B α and B δ subunits appear to differentially modulate the activation/inactivation of mitotic regulators.

3.2.8 B δ regulates the timing of mitotic exit

The observation that the initiation of mitosis is not delayed but that its duration is extended in B δ -deficient cell populations suggests that these cells may be hindered in their ability to progress through mitosis. Therefore, to determine whether prolonged mitotic duration in the absence of B δ (Fig. 3.6D, left panel) is due to aberrations in mitotic progression, I investigated the effect of B55 subunit deficiency in mitotic exit assays. Control and B55-depleted HeLa cell populations were arrested in prometaphase using consecutive thymidine and nocodazole treatments and, following release, progression through G2/M into G1 phase was measured by relative DNA content analysis (Fig. 3.8A, left panel). The onset of mitotic exit occurs at the metaphase-to-anaphase transition, when bipolar attachment of all sister chromatids satisfies the SAC and triggers chromosome segregation (see section 1.1.6 Mitotic exit). Importantly, the mitotic exit assay described below measures the progression from prometaphase (at the time of release) to the completion of cytokinesis, when daughter cells are detected as individual cells with G1 DNA content by flow cytometry. Therefore, this assay does not directly measure the duration of mitotic exit per se; however, for the sake of simplicity I will use the term ‘mitotic exit’ to indicate the progression from prometaphase to G1 phase.

Mitotic exit is delayed in the absence of B δ (Fig. 3.8A, 60-75 min compared to 45-60 min in control and siB α /siB α + δ populations), with marked differences between control and siB δ G2/M populations occurring from 75 to 120 minutes after release (Fig. 3.8A and B, middle panel). B α deficiency results in only a small lag in exit timing (Fig 3.8A and B, left panel).

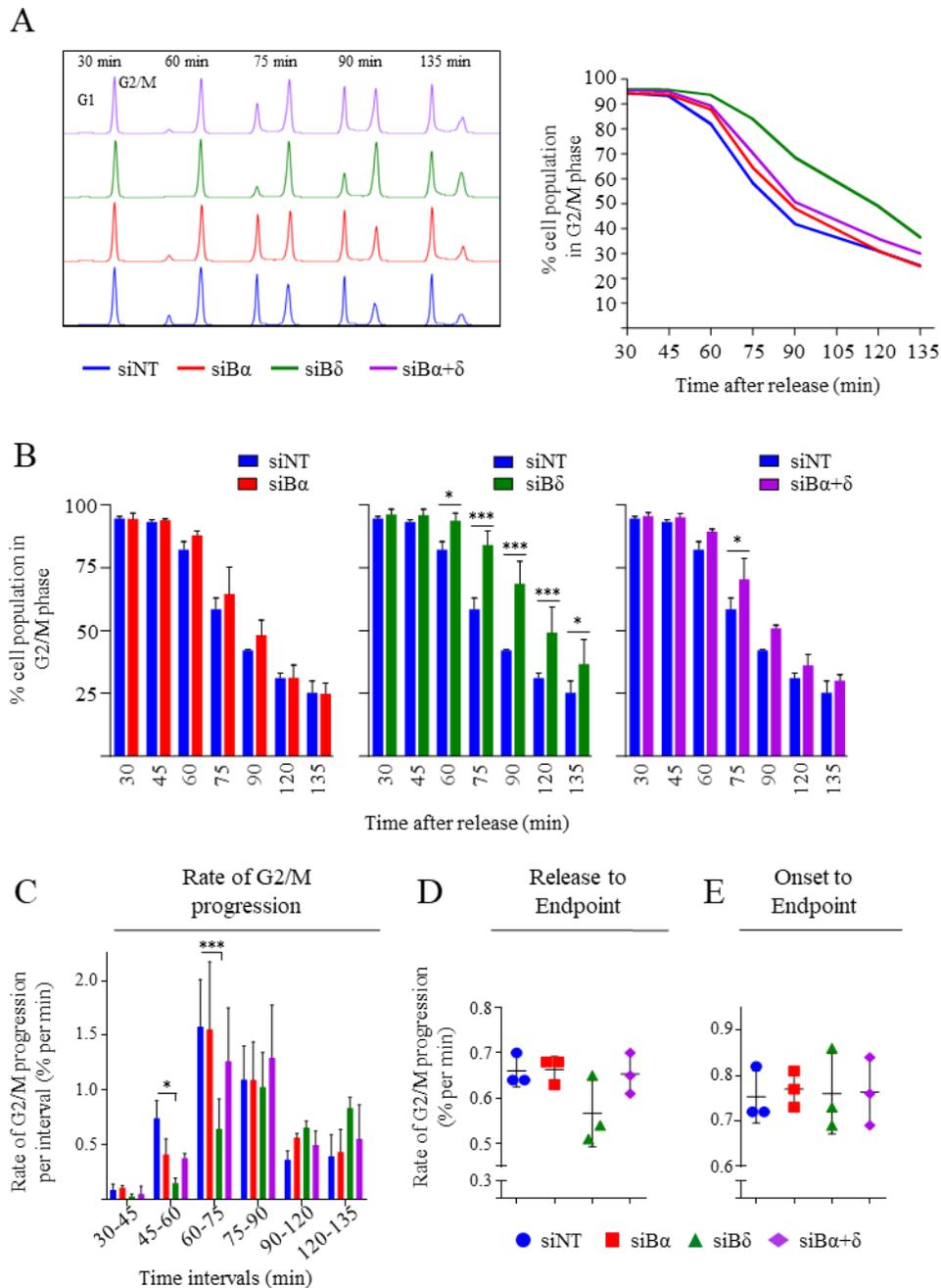


Figure 3.8: B δ regulates timing of mitotic exit onset

Control and B55 subunit-depleted cell populations were arrested in prometaphase using consecutive thymidine and nocodazole blocks (as described in section 2.1.4). Following release into mitosis, cells populations were collected at the indicated timepoints and analyzed by flow cytometry.

- A. Relative DNA content analysis by flow cytometry was used to assess mitotic exit progression. Comparative DNA content histograms from selected timepoints are shown (left panel). Transition out of G2/M was measured and averaged from three independent experiments. Percent values for

G2/M populations (right panel) were calculated from DNA content histograms (n=3) using the Watson Pragmatic Cell Cycle Module FlowJo v9.6.5.

- B. Data as in (A) are shown as a comparison between the control cells and each indicated B55-depleted population (n=3, mean \pm s.d., * P <0.05, *** P <0.001, ANOVA).
- C. The rate of G2/M progression, expressed as percent per minute (% per min), was calculated from data as in (A), and graphed for each time interval measured during the time-course experiment (n=3, mean \pm s.d., * P <0.05, *** P <0.001, ANOVA).
- D. Rate of G2/M progression (% per min) was calculated from the time of release (from prometaphase arrest) to the experiment endpoint (n=3, mean \pm s.d.).
- E. Rate of G2/M progression (% per min) was calculated from the time of mitotic exit onset, defined here as the last timepoint preceding a decrease in % G2/M cells, to the experiment endpoint, defined as t=135 min post-release (n=3, mean \pm s.d.).

Measurements of G2/M kinetics showed that B α -depleted cells exhibit a slowed rate of progression from 45 to 60 min (0.41% \pm 0.14 and 0.38% \pm 0.04 per minute in siB α and siB α + δ cells, respectively, compared to 0.74% \pm 0.16 per minute in control cells), but attain control-like kinetics during the remainder of mitotic exit (Fig 3.8C). In the absence of B δ , however, the rate of progression is notably reduced during 30 min to 75 min post-release (0.74% \pm 0.16 vs. 0.15% \pm 0.05 from 45-60 min and 1.58% \pm 0.43 vs. 0.64% \pm 0.28 per minute from 60-75 min for control vs. siB δ cells, respectively). Due to this initial delay of onset timing, B δ -depleted cells also exhibit an overall reduced rate of progression from the time of release (30 min) to the endpoint (135 min) of the experiment (0.57% for siB δ cells compared to 0.66%, 0.66%, and 0.65% per minute for control, siB α and siB α + δ cells, respectively) (Fig. 3.8D). However, once B δ -deficient cells initiate mitotic exit, they proceed at a rate similar to that of control cells (0.76%, 0.77%, 0.77% and 0.76% per minute, respectively, for control, siB α , siB δ , and siB α + δ cells) (Fig. 3.8E). Unexpectedly, siB α + δ -treated cells exhibit a kinetic phenotype more akin to B α -depleted cells in these mitotic exit assays (Fig. 3.8A-E). This is discussed further in section 3.4.

The kinetic analysis of mitotic exit progression showed that B δ plays a more prominent role than B α in regulating mitotic exit timing, which may underlie prolonged mitotic duration observed in the B δ -deficient cells.

3.2.9 B δ -dependent mitotic exit delay is associated with altered APC/C activation and cyclin B1 degradation kinetics

To verify that the observed kinetic effects of B55 ablation are reflected in the molecular events underlying mitotic exit, I examined the phosphorylation and expression of APC3 and cyclin B1, respectively. APC3, a component of the APC/C ubiquitin ligase complex, is dephosphorylated during mitotic exit, and accordingly, undergoes a distinctive mobility shift on SDS-PAGE (Manchado et al., 2010; Mochida et al., 2009; Sivakumar and Gorbsky, 2015). Quantitative Western blotting of APC3 in cell lysates from prometaphase-synchronized HeLa cell populations confirmed that depletion of B δ results in a pronounced delay in APC3 dephosphorylation (Fig. 3.9A). Marked dephosphorylation of APC3 occurs between 60 and 75 min after release in siB δ populations, as compared to between 45 and 60 minutes in control cells (Fig.3.9A, see bottom graph). Lack of B α only mildly delays APC3 dephosphorylation (Fig. 3.9A). Interestingly, knocking down both B α and B δ yields APC3 dephosphorylation kinetics that are more similar to those observed in B α -deficient populations (Fig. 3.9A).

Degradation of cyclin B1 is a pivotal event marking the onset of mitotic exit. Using a HeLa cell line that stably expresses mCherry-tagged cyclin B1, cyclin degradation was measured by flow cytometry (Fig. 3.9B). Kinetic phenotypes similar to those observed for G2/M progression (Fig. 3.8A) and APC3 dephosphorylation (Fig. 3.9A) were also detected for cyclin B1 degradation (Fig. 3.8B).

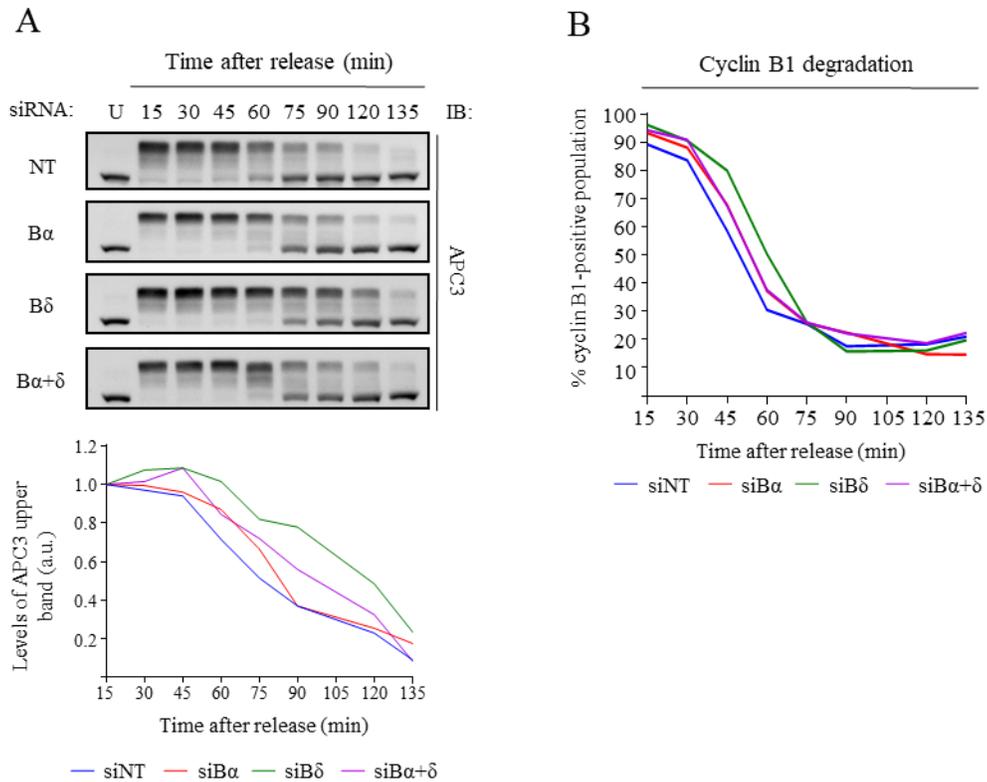


Figure 3.9: B δ -dependent mitotic exit delay is associated with altered APC/C activity and cyclin B1 degradation kinetics

Control and B55 siRNA-treated cell populations were arrested in prometaphase, and following release into mitosis, cells populations were collected at the indicated timepoints and analyzed by Western blotting and flow cytometry.

- Western blot demonstrating phosphorylation-dependent mobility shift of APC3 during mitotic exit. Cell lysates were prepared from the same cell populations as in Fig. 3.8 (upper panel). Signal intensity of the upper band was quantified from three independent experiments, normalized to the first timepoint (15 min), and graphed to illustrate the kinetics of APC3 dephosphorylation (lower panel) (n=3, mean).
- Cyclin B1 expression levels were measured in HeLa cells stably expressing mCherry-tagged cyclin B1. Degradation kinetics of cyclin B1 were calculated and graphed as the average from three independent experiments (n=3, mean).

Previous studies have established a role for B55 subunits as positive regulators of mitotic exit (Cundell et al., 2013; Cundell et al., 2016; Mayer-Jaekel et al., 1994; Mayer-Jaekel et al., 1993), with

some findings specifically implicating B α as the prominent player (Lowe et al., 2000; Schmitz et al., 2010). My findings indicate that B δ acts as the more prominent mediator of B55-dependent events during mitotic exit. Specifically, these data indicate that B δ may play a positive role in triggering exit from mitosis. Furthermore, these observations support the hypothesis that prolonged mitosis in the absence of B δ is due to delayed mitotic exit progression.

3.2.10 B55-deficiency leads to prolonged metaphase duration

My analyses of B55-dependent effects on mitotic kinetics in synchronized HeLa cell populations revealed that B α regulates the initiation of mitosis (Fig. 3.6), while B δ controls mitotic duration (Fig. 3.6), possibly through a role in modulating mitotic exit timing (Fig. 3.8 and 3.9). To investigate more closely whether depletion of these B55 subunits alters the kinetics of individual mitotic phases, I used time-lapse microscopy to follow G1/S-synchronized HeLa cells that stably express eGFP-tagged α -tubulin (eGFP-TUBA) and mCherry-tagged Histone H2B (mCherry-H2B) as they progress through mitosis (Fig. 3.10A). B55 subunit depletion does not affect the duration of prophase and prometaphase, as chromosomes condensed and aligned at the metaphase plate of all cells within 30 minutes (Fig. 3.10A and B). However, B α -, B δ -, and co-depleted cells remained in metaphase longer (to around at least 40 min compared to 30 min for control cells), delaying the onset of chromosome segregation (Fig. 3.10A). Analysis of chromosome condensation dynamics, as measured by changes in fluorescence intensity associated with the condensation and relaxation of chromosomes, supported observations of prolonged mitotic duration (Fig. 3.10B).

Notably, this single-cell, live imaging analysis of the duration of the different mitotic phases suggests that depletion of B α also prolongs mitosis. This is in apparent conflict with previous data, which demonstrate that depletion of B δ (in single and co-depleted cell populations), but not B α , extends mitotic duration. Potential factors underlying this discrepancy are discussed in section 3.4.

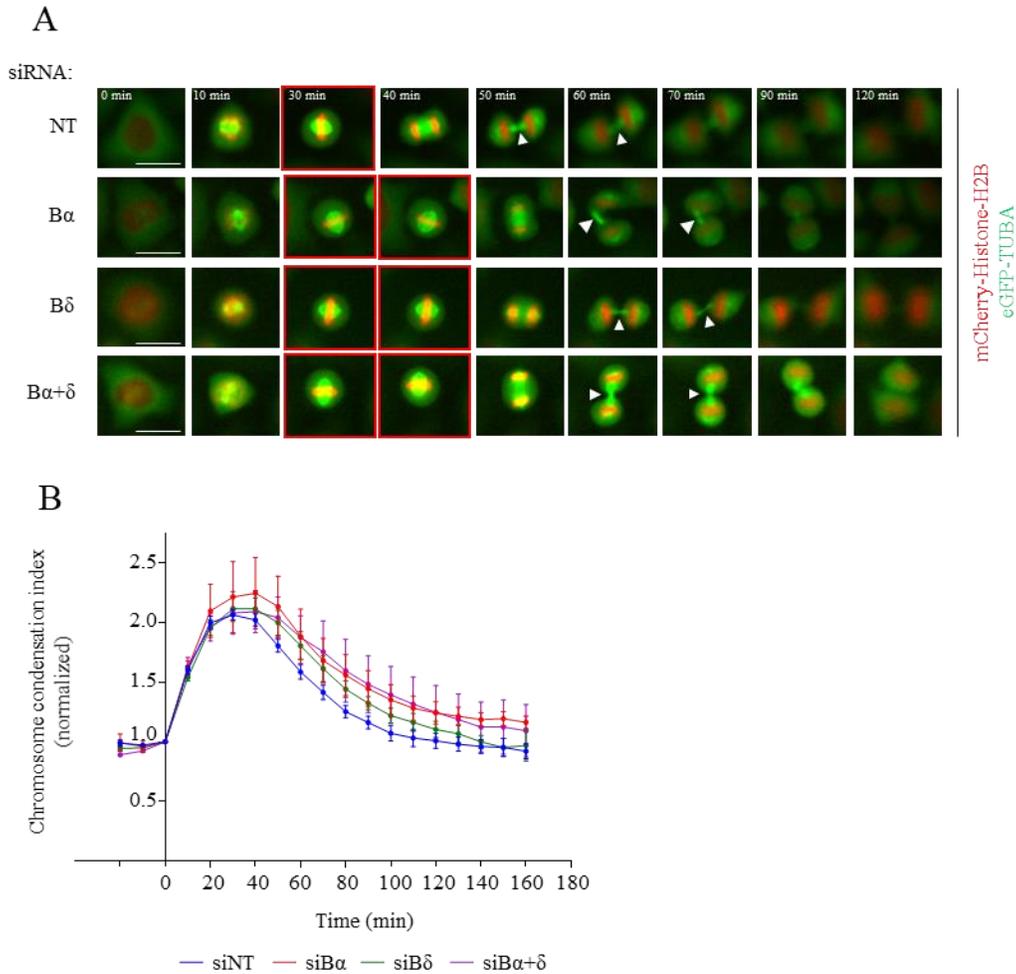


Figure 3.10: B55-deficiency leads to prolonged metaphase duration

- A. HeLa cells expressing eGFP-TUBA and mCherry-H2B were treated with non-targeting siRNA or siRNA targeting B α and/or B δ , and were synchronized at the G1/S boundary using a double thymidine block. Following release, cells were allowed to progress through S and into G2 phase for 5 hours before being imaged by time-lapse microscopy as they progressed through mitosis. Images were taken every 10 min. The red frame denotes cells in metaphase and the white arrowheads indicate the central spindle. Scale bars=25 μ m.
- B. Chromosome condensation kinetics were determined from videos that were generated by concatenating images from each timepoint. The chromosome condensation index measures changes in mCherry fluorescence intensity associated with chromosome compaction and relaxation. The last frame before cells rounded up and entered mitosis was defined as timepoint 0. (n=3 independent experiments, mean \pm s.e.m., 25-30 cells per experiment were measured for a total of 75-90 cells per treatment).

3.2.11 $B\alpha$ but not $B\delta$ regulates post-mitotic events

The mitotic exit events that have previously been shown to be specifically regulated by PP2A- $B\alpha$, including the reassembly of the Golgi apparatus and nuclear envelope, are restricted to the late stages of mitosis and post-mitosis (Cundell et al., 2013; Lowe et al., 2000; Schmitz et al., 2010). Together with my findings, this raises the possibility that $B\delta$ regulates mitotic exit timing and progression, while $B\alpha$ plays a more prominent role in late and post-mitotic stages. To determine whether this may be the case, I examined the kinetics of post mitotic cell-spreading using time-lapse microscopy (Fig. 3.11A). During mitotic entry, cells largely detach from the substratum and round up to provide the appropriate cellular morphology for cell division. Following cytokinesis, cells reattach and spread out as they transition out of mitosis into G1 phase. Using transmitted light imaging, I measured the kinetics of these morphological changes (Fig. 3.11B). Control cells start spreading out immediately following cytokinesis, with one or both daughter cells fully reattaching and flattening to an interphase morphology by 70-90 min (Fig. 3.11A). Depletion of $B\delta$ causes a modest delay in post-mitotic spreading, which may be a consequence of prolonged mitotic duration rather than slowed reattachment. However, in the absence of $B\alpha$ (in single and co-depleted contexts) cells exhibit markedly impaired cell spreading and often remain in a rounded state past 90 min (Fig. 3.11B).

These findings support the notion that the $B\delta$ and $B\alpha$ subunits differentially regulate the molecular events that drive mitotic exit and suggest that they may act in a chronological hierarchy where $B\delta$ activity precedes that of $B\alpha$.

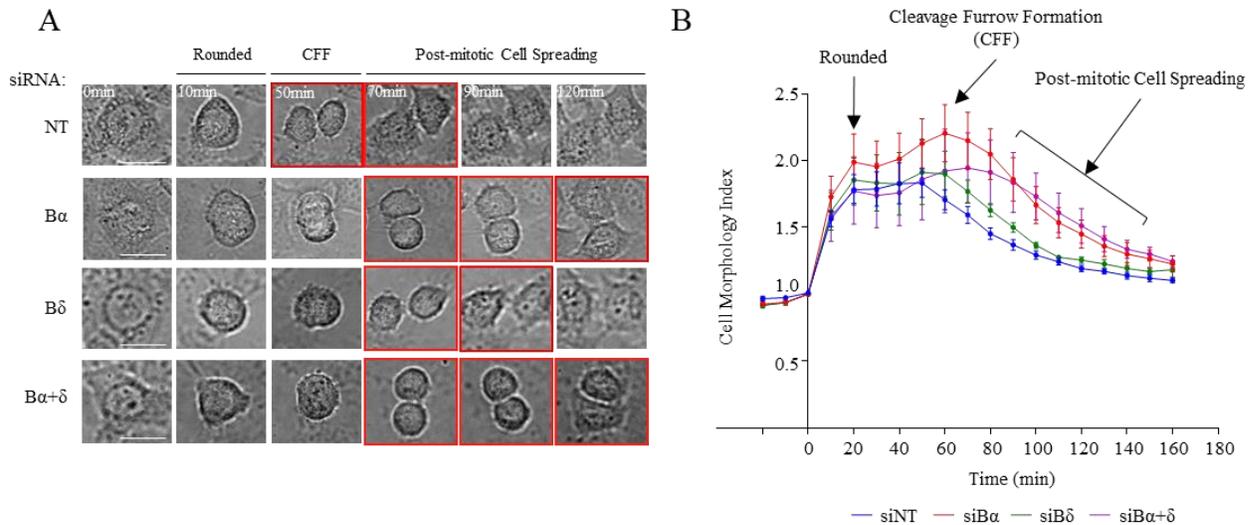


Figure 3.11: B α but not B δ regulates late and post-mitotic events

- A. HeLa cells expressing eGFP-TUBA and mCherry-H2B were treated with control or B55-targeting siRNA, synchronized at the G1/S boundary, and followed through mitosis by time-lapse microscopy. Data shown are from the same experiments as in Fig. 3.9A. The transmitted light channel was used to visualize the morphological changes associated with mitosis, including cell rounding, cleavage furrow formation (CFF) and post-mitotic cell spreading. The red frame denotes cells actively undergoing post-mitotic cell spreading. Scale bar=25 μ m.
- B. Morphological changes were quantified using a Cell Morphology Index, which measures changes in the standard deviation of transmitted light signal intensity within a defined area. The last frame before cells rounded up and entered mitosis was defined as timepoint 0 min. Shown is the mean \pm s.e.m. (n=3 independent experiments, 25-30 cells per treatment per experiment were measured for a total of 75-90 cells).

3.2.12 B55-dependent effects on M-to-G1 and G1 phase progression

Aberrations in mitotic exit kinetics and the underlying molecular events are likely to impact transition into G1 phase and may carry over into the subsequent round of cell division. Therefore, I investigated whether B55 subunit depletion affects progression into and through G1 phase. G1/S-synchronized cell populations gradually lose cell cycle synchrony following one round of mitosis (Fig. 3.3A), hindering the examination of B55-dependent effects on G1 phase progression. Inhibition of CDK1 activity using the small molecule inhibitor RO3306 effectively synchronizes cell populations at the G2/M border (Vassilev, 2006), and enables the examination of M-to-G1 and G1

phase kinetics (Fig. 3.12A). Following release from consecutive thymidine and RO3306 treatments, HeLa cell populations rapidly enter mitosis within the first hour, complete transition into G1 phase by 4 hours, and initiate DNA replication for the next division cycle between 9 and 12 hours post-release (Fig. 3.12A). Depletion of B α , but not B δ , delays entry into mitosis and markedly impairs progression into G1 phase (Fig. 3.12B). In control and B δ -deficient cell populations, the majority of cells (defined as more than two-thirds of the cycling population) complete transition into G1 phase by 4 hours after release ($3.7\text{h} \pm 0.3$ and $3.8\text{h} \pm 0.8$, respectively) (Fig. 3.12B-D), while populations lacking B α attain an equivalent proportion of G1 cells by 5 hours ($5\text{h} \pm 1.0$ and $5.2\text{h} \pm 0.3$, for single and co-depletion, respectively) (Fig. 3.12B-D). Furthermore, G1 phase duration appears to be shortened in the absence of B α , as siB α populations initiate S phase at the same time as control cells (between 11 and 12 hours) albeit at a slowed rate (Fig. 3.12B and C, left and right panels). In contrast, ablation of B δ extends the length of G1 phase by at least one hour, as these cells transition into G1 phase with control-like kinetics but do not initiate DNA replication until 12-14h post-release (Fig. 3.12B and C, middle panel). B δ -deficient cells also attain a higher G1 peak population compared to control cells (Fig. 3.12B and C, middle panel). Strikingly, in the absence of B α , 15-20% fewer cells ultimately transition into G1 phase as compared to control and siB δ cells (Fig. 3.12B; see 9h post-release). This phenotype is explored further in Chapter 4.

These results support a role for B α as a positive regulator of G1 progression (entry into G1) and a negative regulator of G1/S transition, as B α -depleted cell populations exhibit a markedly slowed progression into G1 phase but transition into S phase at the same time as control cells. In contrast, B δ may act as a negative regulator of G1 phase progression and positive regulator of G1/S transition. Therefore, these highly related B55 subunit isoforms appear to exert opposing regulatory effects on the kinetics of G1 phase.

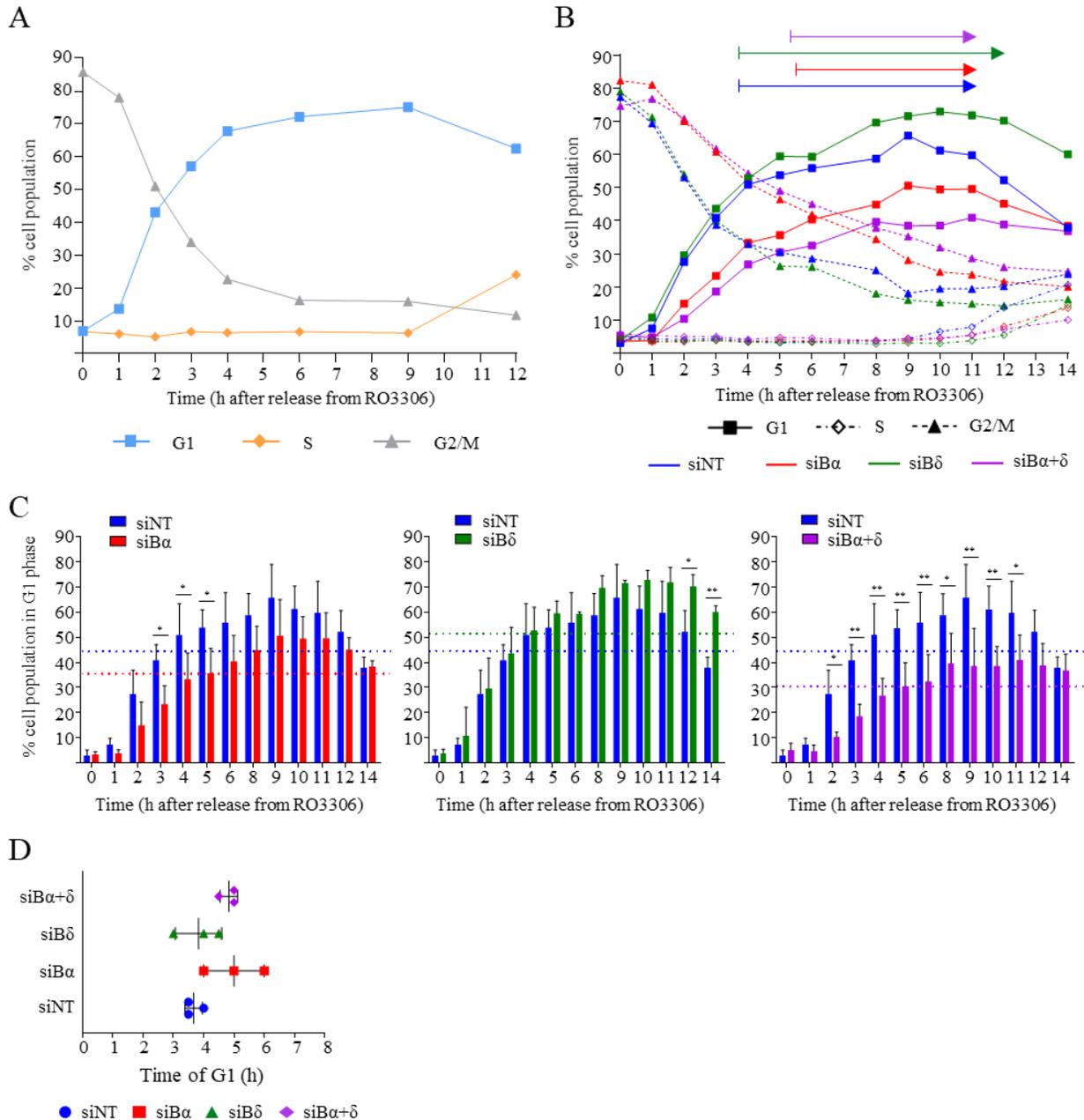


Figure 3.12: B55-dependent effects on M-to-G1 and G1 phase progression

HeLa cells were treated with control siRNA or siRNA duplexes targeting B α and/or B δ , and synchronized at the G2/M border by consecutive treatments with thymidine (2 mM for 20 h) and CDK1-specific inhibitor, RO3306 (10 μ M for 17 h). Following release, cells were collected at the indicated timepoints and analyzed by flowcytometry and Western blotting (n=3).

- A. Relative DNA content was analyzed by flow cytometry and used to establish the timeline of M-to-G1 and G1-to-S phase progression in untreated HeLa cell populations (n=1).
- B. The kinetics of M-to-G1 and G1 phase progression were measured using DNA content analysis. A comparison of all siRNA treatments is shown. The percent cell population in each phase at each timepoint was calculated from DNA content histograms using the Watson Pragmatic Cell Cycle Module in FlowJo v10.0.1. Coloured arrows denote the approximate length of G1 phase, which is delineated at the start by the time at which more than two-thirds of the cell population has entered G1 and at the end by the last timepoint that precedes progression into S phase (n=3, mean).
- C. The percent cells in G1 phase at the indicated timepoints post-release was calculated as the average value of data from three independent experiments as in (A) and is shown as a comparison between the control cells and the indicated B55-depleted population (n=3, mean \pm s.d., * P <0.05, ** P <0.01, ANOVA). The dotted lines indicate the threshold above which more than two-thirds of the cycling population have transitioned into G1 phase.
- D. The timepoints at which two-thirds of the cell population has transitioned into G1 phase (time of G1) is graphed (n=3, mean \pm s.d.).

3.2.13 B55-dependent effects on G1 phase progression are associated with altered G1/S cyclin expression

Progression through G1 phase and initiation of DNA replication are largely driven by the activities of the CDK4/6-cyclin D and CDK2-cyclin E complexes, respectively. During early to mid-G1 phase, the expression of the D-type cyclins increases, enabling the activation of CDK4/6, while late G1 phase and transition into S phase are characterized by rising levels of the E-type cyclins and activation of CDK2 (Ho and Dowdy, 2002; Hochegger et al., 2008; Sherr, 1996). The B55-dependent alterations in G1 and G1-to-S phase transition observed above (Fig. 3.12) prompted me to investigate whether these kinetic changes are associated with deregulated D-type and E-type cyclin expression. Western blot analysis of cyclin D3 expression in G2/M-synchronized cell populations revealed that loss of B α leads to deregulated cyclin levels. In control and B δ -depleted cells, cyclin D3 is rapidly degraded within 1-2 hours post-release (Fig. 3.13A) as cells enter and progress through mitosis (Fig. 3.12B). Ablation of B α impairs cyclin D3 degradation and overall higher levels of this cyclin remain

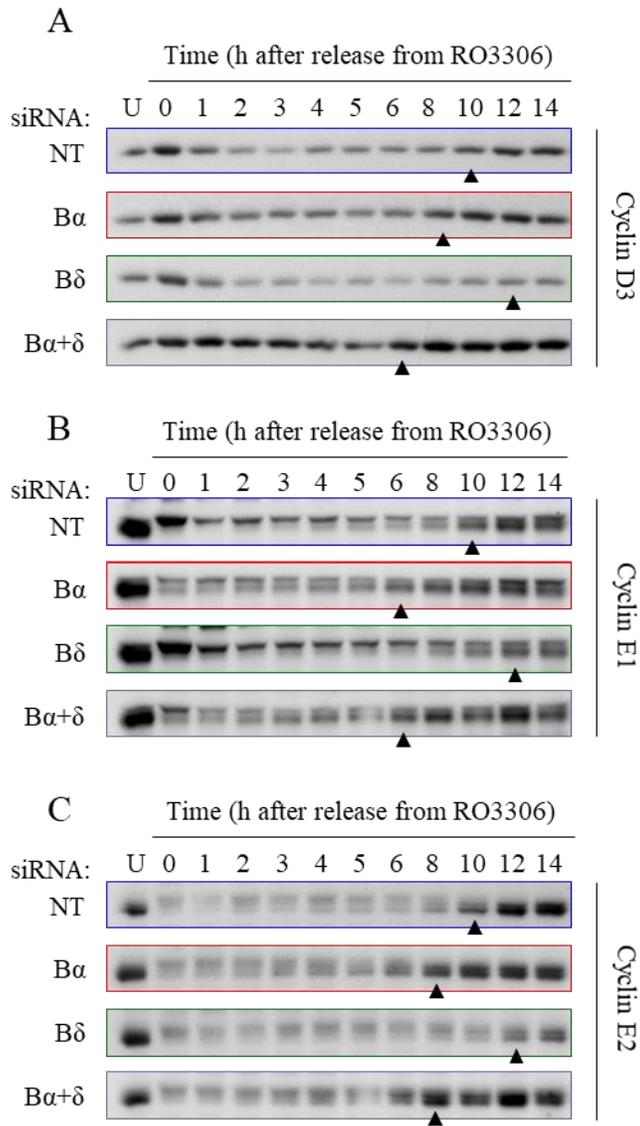


Figure 3.13: B55-dependent effects on G1 phase progression are associated with altered G1/S cyclin expression

- Western blot of cyclin D3. Cell lysates from G2/M-synchronized cell populations (as in Fig. 3.11B) were probed for cyclin D3 levels. Black arrowheads indicate the timepoint at which expression levels increase.
- Western blot of cyclin E1. Cell lysates from G2/M-synchronized cell populations as in (A) were probed for cyclin E1 levels. Black arrowheads indicate the timepoint at which expression levels increase.
- Western blot of cyclin E2. Cell lysates from G2/M-synchronized cell populations as in (A) were probed for cyclin E2 levels. Black arrowheads indicate timepoint at which expression levels increase.

throughout G1 progression (Fig. 3.13A). Interestingly, co-depletion of B α and δ severely obstructs and delays cyclin D3 degradation (Fig. 3.13A). In accordance with the observed delay in G1-to-S phase transition, cells lacking B δ exhibit reduced cyclin D3 levels and delayed initiation of cyclin re-expression during G1 phase (Fig. 3.13A), as compared to control cells. Similarly, cyclin E1 and E2 expression levels are differentially altered in the absence of the B55 subunits. During M-to-G1 progression, siB δ cells demonstrate control like cyclin E degradation kinetics, while cyclin E re-expression during late G1 and G1-to-S phase is delayed by at least 2 hours (Fig. 3.13B and C). On the contrary, depletion of B α appears to induce premature cyclin E expression, as protein levels of both cyclin E1 and E2 begin to increase at least 2 hours before expression is observed in control cells (Fig. 3.13B and C). Strikingly, cyclin E1 levels are markedly reduced at the time of release from the RO3306 block (Fig. 3.13B, timepoint 0 h).

Together, these results demonstrate that the B α and B δ subunits differentially regulate G1 phase progression and G1/S phase transition and show that this is associated with B55-dependent altered regulation of D-type and E-type cyclin expression.

3.2.14 B α and B δ play differential roles in regulating multiple cell cycle phases

The FUCCI (fluorescent ubiquitination-based cell-cycle indicator) system provides a powerful tool for the investigation of cell cycle phase kinetics in real time using live-cell microscopy (Sakaue-Sawano et al., 2008). This system exploits the phase-dependent and inversely oscillating expression of red-emitting Kusabira Orange-tagged Cdt1 (mKO-Cdt1) and green-emitting Azami Green-tagged Geminin (AG-Geminin) to enable the visualization of the cell cycle phases. Starting in early G1 phase and continuing until the G1/S phase transition, cells express and accumulate mKO-Cdt1, causing them to emit red fluorescence during G1 progression (Fig. 3.14A). During the G1/S transition and early S phase, AG-Geminin expression increases, leading cells to fluoresce yellow

(Fig. 3.14A). As AG-Geminin accumulates, it promotes the degradation of mKO-Cdt1 during early S phase, giving rise to green-emitting cells throughout the remainder of S phase, G2 phase and into mitosis (Fig. 3.14A). AG-geminin is then degraded following the metaphase-to-anaphase transition as cells exit mitosis (McGarry and Kirschner, 1998; Sakaue-Sawano et al., 2008). Notably, in my time-lapse imaging experiments complete loss of green fluorescence was consistently observed at the time cells underwent cytokinesis. Therefore, cells emit no colour as they transition from cytokinesis (or late mitosis) into G1 phase (M-to-G1), until mKO-Cdt1 expression is initiated during early G1 phase (Fig 3.14A).

I investigated the effect of B55 subunit depletion on cell cycle phase kinetics in unsynchronized, cycling HeLa-FUCCI cells using time-lapse microscopy. Loss of B α extends the duration of M-to-G1 phase (colourless) by more than 1h compared to control and B δ -depleted cells (Fig 3.14B). Together with the slowed cell spreading following cytokinesis that I observed in cells lacking B α (section 3.2.11), these results implicate a role for B α in promoting timely recovery from mitosis, and subsequent cell cycle progression. Co-depletion of B α + δ results in an intermediate phenotype, with these cells exhibiting a mild lengthening of the M-to-G1 phase compared to control cells (Fig. 3.14B). The length of G1 phase was extended in the absence of B δ (~1.7 h) (Fig. 3.14C). B δ appears to play a more prominent role in controlling G1 phase length, as the B α + δ co-depletion phenotype resembled B δ ablation. Interestingly, cells lacking both B55 subunits mildly lag (>30min) in early S phase as compared to control and B α or B δ -depleted cells (Fig 3.14D), while progression through the remainder of S- and G2-phase was not notably affected by B55 ablation (Fig. 3.14E). In agreement with my previous observations, the loss of B δ or B α + δ , but not B α , prolonged mitotic duration (Fig. 3.14F). This analysis of overall cell cycle phase kinetics in unsynchronized HeLa cell populations reaffirmed previous phase-specific findings and demonstrated that B α and B δ subunits differentially regulate progression through specific phases.

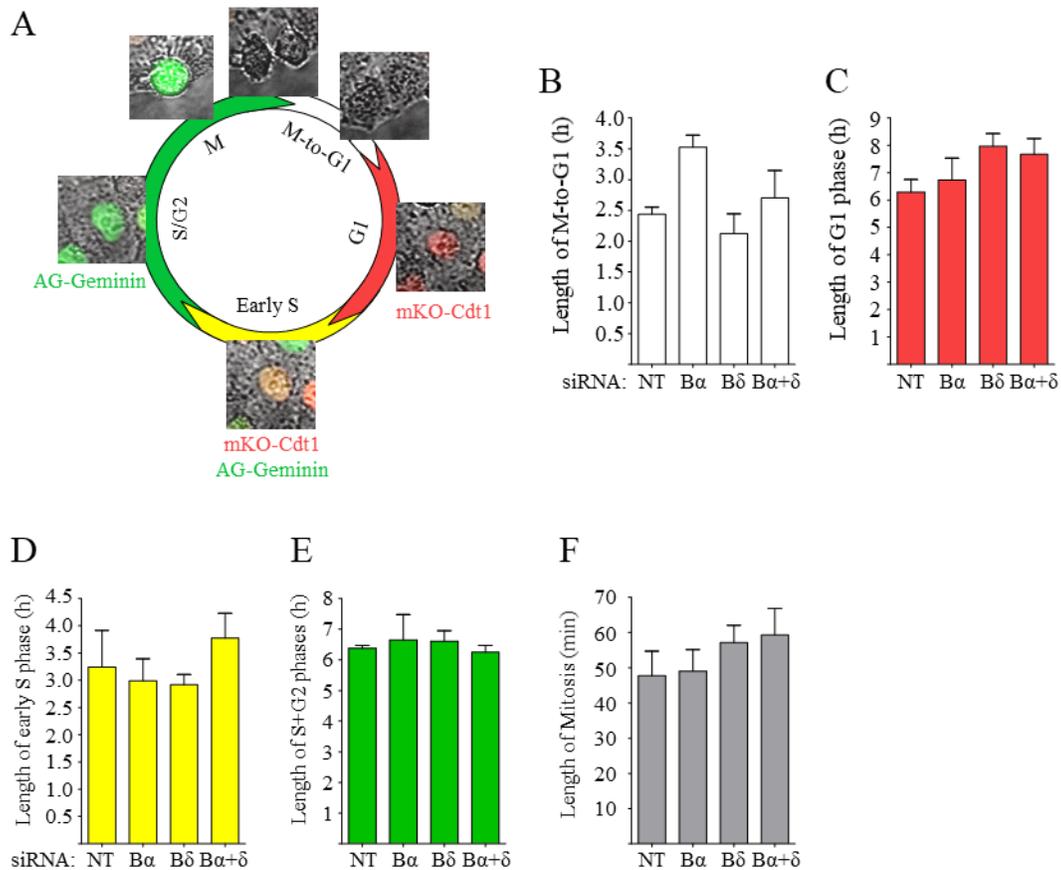


Figure 3.14: $B\alpha$ and $B\delta$ play differential roles in regulating multiple cell cycle phases

Unsynchronized, cycling HeLa-FUCCI cells were treated with control siRNA or siRNA duplexes targeting $B\alpha$ and/or $B\delta$ subunits and followed through complete cell cycles by time-lapse microscopy. Following cytokinesis of a mother cell, emerging individual cells were monitored as they progressed through the M-to-G1, G1, G1/S-to-early S and S-G2-M phases. For each cell, the red and green fluorescence signal intensities were measured at every frame throughout the division process. Specific red and green signal intensity thresholds were then used to define the colourless (M-to-G1), red (G1), yellow (G1/S-to-early S), and green (S-G2-M phases) phases. Images were taken every 15 min for 24 h. Shown are the means \pm s.d. calculated from 10-15 cells per experiment. The data are representative of three independent experiments (for a total of 30-45 cell per treatment).

- Diagram illustrating cell cycle phase-dependent changes as defined by the expression of coloured marker proteins. Cells emit no fluorescence during the transition from late mitosis (cytokinesis) to G1 phase (M-to-G1), fluoresce red during G1 progression, yellow during the G1/S transition and early S phase, and green during S, G2 phase and mitosis.
- Length of M-to-G1 transition based on measurements of the duration of the colourless phase. Importantly, cells exhibited complete loss of colour at the time of cytokinesis. Therefore, the colourless phase measures the duration from cytokinesis to early G1 phase, when mKO-Cdt1 expression has surpassed a specific threshold.

- C. Length of G1 phase based on measurements of the duration of the mKO-Cdt1-expressing, red-emitting phase.
- D. Length of early S phase based on measurements of the duration of where both mKO-Cdt1 and AG-Geminin are expressed.
- E. Length of S-G2/M phase based on measurements of the duration of the AG-Geminin expressing phase.
- F. Length of mitosis as measured from the time of cell rounding (mitotic entry) to cleavage furrow formation/cytokinesis.

3.3 Key Findings

1. Loss of the B α subunit delays S-to-G2 phase transition, providing support for a role as a positive regulator of S phase progression. Prolonged S phase in B α -deficient populations precipitates a delay in timing of mitosis.
2. PP2A-B δ positively regulates mitotic exit timing. Depletion of B δ delays mitotic exit progression, which leads to prolonged duration of mitosis.
3. PP2A-B α positively regulates late mitotic exit events and M-to-G1 transition. Cells depleted of B α exhibit markedly slowed post-mitotic cell spreading and delayed expression of the G1-specific marker, Cdt1.
4. Loss of B α shortens the duration of G1 phase and results in premature expression of G1/S cyclins, supporting a role as a negative regulator of G1 and G1-to-S progression.
5. In the absence of B δ , G1-to-S phase transition and G1/S cyclin expression is delayed, suggesting that PP2A-B δ positively regulates the initiation of DNA replication.
6. PP2A-B α and -B δ exhibit distinct roles in the regulation of mitotic progression and opposing roles in the control of G1 phase duration.

3.4 Discussion

The involvement of PP2A-B55 complexes in the control of mitosis in *Xenopus*, *Drosophila* and mammalian cell cycle models has been investigated extensively in recent years. Of these model systems, only *Xenopus* and mammals encode more than one isoform of the B55-type subunits. It has been well established that the B δ isoform is the primary mediator of PP2A-dependent regulation of mitotic entry and exit in *Xenopus* (Castilho et al., 2009; Mochida et al., 2009). In mammalian systems, however, the contribution of individual B55 subunits to mitotic regulation, especially of the abundantly expressed B α and B δ isoforms, remains unclear. Similarly, the roles of these two subunits in the control of mammalian G1 and S phase progression are poorly understood. The data presented in this chapter provide a general characterization and overview of B55 isoform-specific effects on the kinetics of the different cell cycle phases. I provide evidence that the human B α and B δ isoforms play distinct roles in the regulation of interphase and mitotic progression. Potential mechanisms underlying distinct B α - and B δ -dependent roles are discussed in accordance with each cell cycle phase. Importantly, the mechanisms suggested below are largely speculative and intended to inform potential future investigations.

Potential sources of discrepancies in cell cycle phase-specific phenotypes

Before delving into the potential mechanisms that may underlie the B55 isoform-specific cell cycle phase phenotypes, it is important to point out and discuss phenotypic discrepancies across different experiments. Most notably, my live imaging analysis of the duration of the mitotic phases (Fig. 3.10) showed that depletion of B α (as well as B δ and B α + δ) leads to a prolonged arrest in metaphase and mitosis. This observation is conflict with other data presented herein, which demonstrates that loss of B α delays the initiation of mitosis (Fig. 3.6 and 3.7) but does not prolong mitotic duration (Fig. 3.6) or mitotic exit progression (Fig. 3.8 and 3.9). Several factors may lie at the

root of this discrepancy. First, these experiments made use of rather distinct markers to measure the duration and kinetics of mitosis. For the live imaging analysis, I used morphological markers, namely the compaction and relaxation of chromosomes, to assess the duration of mitotic phases (Fig. 3.10). In the other experiments, however, I examined specific molecular markers associated with mitosis (Fig. 3.6) or relative DNA content (Fig. 3.8). Importantly, while the molecular markers used to investigate mitotic duration (cyclin B1 and pS10H3, Fig. 3.6) are associated with mitosis, their combined use does not correlate directly with chromosome morphology. Therefore, measurements of chromosome condensation and cyclinB1/pS10H3 positivity may reflect different aspects of the mitotic process, and therefore yield distinct kinetic phenotypes. Secondly, the live imaging analysis was based on single cell measurements (Fig. 3.10), while other experiments examined cell populations (Fig. 3.6 and 3.8). It is conceivable that kinetic phenotypes differ between measurements performed on individual cells as compared to heterogenous cell populations. To this point, the data resolution of single cell live imaging is arguably much lower than that of a cell population analyzed by flow cytometry. Specifically, live imaging analyses only included 75-90 cells in total (across three independent experiments) as compared to thirty to forty thousand cells for the flow cytometric analysis. Finally, it is conceivable that the time resolution of the live imaging experiments was insufficient. In my experiments, cells were imaged every 10 minutes, while previous studies that have investigated mitotic kinetics using time-lapse imaging captured images at one- to two-minute intervals to reveal B55-dependent changes (Cundell et al., 2013; Schmitz et al., 2010).

The B55 isoform-specific kinetic phenotypes of various cell cycle phases also differed between synchronized and unsynchronized cell populations (Fig. 3.14). I propose that this variability may be rooted in differences between the biochemical settings generated by distinct synchronization methods and the arguably more native cellular state in unsynchronized cells. Additional insight into the roles of the B α and B δ subunits in the regulation of different cell cycle phases could be gained by

performing more measurements on unsynchronized cell populations, as the cell cycle machinery in these cells would be unaffected by forced arrest in one or more phases. Under these conditions, the direct effect of B55 subunit depletion on uninterrupted cell cycle progression could be examined. To this end, HeLa cells expressing the FUCCI system (Fig. 3.14) could be sorted into G1 (red), early S (yellow), and S-G2-M (green) populations using fluorescence activated cell sorting (FACS), followed by biochemical studies on the isolated cell cycle-phase specific populations.

Another notable discrepancy in mitotic kinetic phenotypes was observed in cell populations synchronized to the G2/M boundary using the CDK1-specific inhibitor RO3306 (Fig. 3.12). In contrast to G1/S- and prometaphase-synchronized cell populations (Fig. 3.6 and 3.8, respectively), G2/M-synchronized populations exhibited impaired progression through G2/M in the absence of B α (and B α + δ) but control-like kinetics in the cells lacking B δ (Fig. 3.12B). While this discrepancy may appear jarring at first, the underlying mechanism is elucidated in **Chapter 4**. Briefly, in siB δ populations prolonged arrest at the G2/M border induced by RO3306 treatment results in the hyperphosphorylation (and hyperactivation) of mitotic regulators and substrates, which may ‘override’ or mask the mitotic duration delays observed in G1/S- and prometaphase-synchronized cell populations (see **Chapter 4**). Furthermore, assessing mitotic kinetics using relative DNA content only may not reveal somewhat subtle kinetic alterations in mitosis. Conversely, ablation of B α in G2/M-synchronized cells leads to a collapse of a pre-mitotic state and the dephosphorylation of mitotic regulators and substrates, which precipitates severe mitotic defects (see **Chapter 4**). Taken together, G2/M synchronization using RO3306 results in B55 isoform-specific pre-mitotic abnormalities that significantly impact mitotic progression. Therefore, the mitotic kinetic phenotypes observed in RO3306-synchronized cell populations should not be directly compared to G1/S- or prometaphase-synchronized populations.

Finally, the variable kinetic phenotypes of the B α + δ co-depleted populations presents another inconsistency to be addressed. Interestingly, while co-depleted populations appear to differentially ‘mimic’ the kinetic phenotypes of siB α or siB δ populations, they also exhibit intermediate phenotypes depending on the assay performed and the cell cycle phase investigated. While this variable behaviour may point to a complex functional interaction between these B55 subunit isoforms, it may also be the result of variable and incomplete depletion efficiencies of each isoform. As measurements of the protein levels of the B α and B δ isoforms were not performed for every single experiment, it is difficult to determine the impact of variable protein depletion efficiencies.

Importantly, these proposed underlying factors are largely speculative and represent tentative explanations for the discrepancies observed.

Mitotic entry regulation by PP2A-B α and -B δ

Entry into mitosis requires the inhibition of PP2A-B55 in order to allow the accumulation of CDK1-phosphorylated mitotic substrates. Experiments conducted primarily in *Xenopus* and *Drosophila* have demonstrated that inhibition of PP2A-B55 by the Gwl-Ensa/ARPP19 pathway is required for timely mitotic entry (Castilho et al., 2009; Gharbi-Ayachi et al., 2010; Lorca et al., 2010; Mochida et al., 2010; Rangone et al., 2011), and that depletion of the B55 subunit (B δ in *Xenopus* and Twins in *Drosophila*) results in premature entry into mitosis (Mochida et al., 2009). Similarly, the human Gwl ortholog, MASTL, promotes mitotic entry by negatively regulating PP2A (Burgess et al., 2010; Manchado et al., 2010; Voets and Wolthuis, 2010), and inhibition of PP2A by okadaic acid or depletion of all B55 isoforms (α , β , γ , δ) in G1/S-synchronized HeLa cells mildly accelerates entry into mitosis (Burgess et al., 2010; Cundell et al., 2013). Somewhat surprisingly, I did not observe premature mitotic entry in G1/S-synchronized HeLa cells that were transiently depleted of B α and/or B δ . Instead, entry into mitosis proceeded normally in B δ -deficient cell populations, and was delayed

in the absence of B α . The shift in mitotic timing of siB α populations is likely a consequence of slowed progression through S phase (discussed below), rather than defective entry into mitosis. However, simultaneous depletion of both B α and δ rescued the S-phase-dependent delay in mitotic timing, indicating that these cells exhibit a shortened G2 phase duration/accelerated entry into mitosis. In light of previous findings, my results suggest that all B55-type subunits may be involved in mammalian mitotic entry regulation, as depletion of either one of the two most abundant isoforms (or both in combination) is insufficient to produce premature mitotic entry. In support of this, early studies in *Xenopus* demonstrated that the B α and B β subunits also antagonize mitotic progression (Cyert and Kirschner, 1988; Iwashita et al., 1997; Lee et al., 1991; Lee et al., 1994), and it has been suggested that the relative abundance of the B55 isoforms determines their contribution to mitotic regulation (Mochida et al., 2009). I investigate and discuss B55-dependent control of mitotic entry in greater depth in Chapter 4.

Mitotic exit: a temporal hierarchy of PP2A-B α and -B δ actions?

The events that characterize exit from mitosis (including chromosome segregation and decondensation, mitotic spindle breakdown, and reassembly of the nuclear envelope and Golgi apparatus) must occur in an exquisitely regulated temporal sequence to ensure viable, genetically identical daughter cells. As one of the major CDK1-antagonizing phosphatases, the PP2A-B55 heterotrimer plays an essential role in regulating the kinetics of these events (Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017; Manchado et al., 2010; Schmitz et al., 2010). PP2A-B55 reactivation during mitotic exit occurs progressively under the control of the BEG (B55/Ensa/Gwl) pathway, and is associated with a temporal order of PP2A-B55 substrate dephosphorylation (Cundell et al., 2013; Cundell et al., 2016). A series of landmark publications has shown that this temporal order is determined by two main factors: the intrinsic preference of B55 for phosphothreonine (pT)

residues (Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017; Rogers et al., 2015) and the biochemical properties of its target sites, specifically the presence of a bipartite polybasic recognition determinant (BPR) (Cundell et al., 2016). Therefore, as B55 activity passes through a series of thresholds during mitotic exit progression, mitotic proteins are selectively dephosphorylated based on their affinity and competence as B55 substrates. My findings add to the current model of B55-regulated mitotic exit progression by implicating temporally distinct roles for the B α and B δ subunits. Hein *et al.* recently showed that PP2A-B α / δ heterotrimers promote the activation of the APC/C-CDC20 complex during early mitotic exit (at the Metaphase-to-Anaphase transition) by dephosphorylating CDC20 and APC3 (Hein et al., 2017). In similar experiments, I provide evidence that early APC3 dephosphorylation is specifically regulated by B δ and not B α . Loss of B α had little effect on APC3 dephosphorylation kinetics and mitotic exit progression. Together, these findings suggest that B δ reactivation/activity may precede that of B α and may initiate and drive early mitotic exit events. In line with this notion, others have shown that late exit and post-mitotic events, including reassembly of the nuclear envelope and Golgi apparatus (Cundell et al., 2016; Lowe et al., 2000; Schmitz et al., 2010) and mitotic/central spindle breakdown (Cundell et al., 2013), are specifically regulated by B α . Similarly, I found that B α , but not B δ , controls post-mitotic cell spreading and M-to-G1 progression.

These findings raise the questions as to what may underlie temporal differences in the reactivation of B δ and B α ? Ensa/Arpp19 is both an inhibitor and substrate of PP2A-B55, and during mitotic exit the B55 heterotrimer relieves its own inhibition by dephosphorylating Ensa/Arpp19 (Williams et al., 2014). B δ appears to dephosphorylate Ensa/Arpp19 more rapidly and efficiently (Williams et al., 2014), which may enable PP2A-B δ to reactivate itself at lower total PP2A-B55 activity thresholds, and this provides a potential mechanism for the sequential reactivation of the B55 isoforms. This would permit B δ to regulate early mitotic exit events, including APC3 and CDC20

dephosphorylation during the meta-to-anaphase transition, and at least in part could explain the extended mitotic duration in the absence of B δ . Additional *in vitro* binding and dephosphorylation assays are needed to confirm differences in the reactivation kinetics of individual B55 isoforms. Nevertheless, despite the high overall sequence identity (88.8%) and the conserved nature of a major, acidic substrate-binding surface on the B α and B δ subunits (Cundell et al., 2016; Xu et al., 2008), it is plausible that amino acid substitutions at another enzyme-substrate interface may account for isoform-specific differences in substrate binding affinity and dephosphorylation kinetics. One such site may be the interface between the B55 and C subunits of the PP2A trimer, which forms contacts with bound Ensa/Arpp19 (Cundell et al., 2016; Mochida, 2014). Interestingly, the region in B55 subunits that interacts with the C subunit houses a few isoform-specific amino acid substitutions, including bulky aromatic amino acids that may exert structural effects (Xu et al., 2008). A more detailed understanding of the structural underpinnings of Ensa-B55 binding is needed to further investigate whether B α and B δ bind Ensa with different affinities, and whether this affects the respective dephosphorylation and reactivation rates. Further potential differences in the regulation of the B α and B δ subunits are discussed in **Chapter 6**.

B55-dependent regulation of S phase kinetics: to BEG and/or not to BEG

The BEG pathway has also been implicated in the regulation of S phase progression. Specifically, Ensa promotes the initiation of DNA replication by stabilizing Treslin, a component of replication forks and DNA replication origins (Charrasse et al., 2017). Depletion of either Gwl or Ensa results in decreased Treslin phosphorylation, which leads to Treslin degradation, reduced replication fork density, and intermediate to pronounced S phase delays (Charrasse et al., 2017). It is believed that the phosphatase-inhibiting activity of Ensa plays a central role in this pathway; however, neither direct involvement of PP2A-B55 nor its inhibition has been demonstrated

(Charrasse et al., 2017). Interestingly, I found that depletion of B55 subunits, especially B α , also causes a delay in S phase progression. Intuitively, if inhibition of PP2A-B55 activity by Gwl/Ensa was principally responsible for Ensa-mediated S phase progression, then depletion of B55 should precipitate accelerated DNA replication, as increased phosphorylation of Treslin has been shown to accelerate replication initiation and S phase progression (Sansam et al., 2015). Therefore, my results suggest that rather than interacting in the conventional, antagonistic manner dictated by the BEG pathway, B55 and Gwl/Ensa both appear to positively regulate S phase progression. Proper subcellular localization of Gwl is essential to its function, and is regulated by phosphorylation (Alvarez-Fernandez et al., 2013; Wang et al., 2013a; Wang et al., 2016; Yamamoto et al., 2014). During interphase, Gwl is localized to the nucleus. As cells enter mitosis (but before nuclear envelope breakdown), CDK1-cyclin B1 phosphorylates Gwl in its nuclear localization signal (NLS) sequence to induce its export from the nucleus. At the end of mitosis (during cytokinesis), PP2A-B55 dephosphorylates Gwl to ensure its nuclear localization during interphase (Wang et al., 2016). Interestingly, PP2A-B55 is mostly cytoplasmic throughout the cell cycle (Santos et al., 2012; Wang et al., 2013a; Wang et al., 2014b). I propose that PP2A-B55 may positively regulate S phase progression by ensuring that Gwl is dephosphorylated and remains nuclear where it can phosphorylate Ensa and promote Treslin stabilization. This can be tested by examining Gwl localization during S phase in a B55-depleted context. Furthermore, because Gwl is dephosphorylated during cytokinesis (i.e. late mitotic exit) (Wang et al., 2016), PP2A-B α complexes may play a more prominent role, accounting for the greater B α -dependent S phase delay.

Alternatively, a BEG-independent role for PP2A-B55 in S phase regulation has previously been demonstrated. Studies in *Xenopus* egg extracts revealed that PP2A complexes containing B α , and to a far lesser extent, B δ , are important DNA replication initiation factors, and are absolutely required for DNA replication (Krasinska et al., 2011; Murphy and Michael, 2013). Murphy and

Michael specifically show that depletion of B α significantly extends S phase duration (Murphy and Michael, 2013). The ATM and ATR kinases have been shown to negatively regulate DNA replication origin firing in *Xenopus* in the absence of DNA damage (Shechter et al., 2004). Murphy and Michael suggest that PP2A-B α opposes DNA damage-independent, ATR-mediated attenuation of DNA replication origin firing (Murphy and Michael, 2013). Interestingly, in human lung cancer cells lines, PP2A-B α negatively regulates ATM by dephosphorylating its regulatory Ser1981 site, and depletion of B α was associated with increased ATM phosphorylation and G1/S phase arrest, albeit in the presence of DNA damage (Kalev et al., 2012). It is noteworthy, however, that I was not able to detect B α -dependent changes in ATM phosphorylation in G1/S-synchronized HeLa cell populations (data not shown).

G1 phase progression: B α pumps the (G1) brakes, B δ pushes the (G1/S) boundary

Progression through G1 and into S phase is driven by the activities of CDK4/6-cyclin D and CDK2-cyclin E complexes, which are respectively activated during mid and late G1 phase by the induction and accumulation of D-type and E-type cyclin expression (Sherr, 1993, 2000). My findings indicate that PP2A-B α may be involved in modulating the timely expression of these G1 cyclins. I propose two possible, sequential mechanisms by which PP2A-B α could regulate their expression. Firstly, during M-to-G1 transition, the transcription factor c-Jun is phosphorylated at Ser63 and Ser73, leading to its association with CBP (CREB binding protein) (Bannister et al., 1995) and the initiation of cyclin D1 and D3 expression (Tsuchiya et al., 2007; Wang et al., 1996; Wisdom et al., 1999). Phosphorylation of c-Jun, and therefore cyclin D expression, is opposed by PP2A; however, the specific B subunit involved remains unknown (Al-Murrani et al., 1999; Alberts et al., 1993a; Tsuchiya et al., 2007). Considering the central role that PP2A-B α plays in late mitosis and transition into G1 phase, it is plausible that B α represents this elusive B regulatory subunit. Secondly, during

mid to late G1 phase, the expression of a plethora of cell cycle-promoting genes, including cyclin D and E, is controlled by the activity of the RB/E2F pathway. From late mitosis to early/mid G1 phase, hypophosphorylated RB proteins (including pRb, p107 and p130) bind to and inhibit E2F transcription factors and repress the activation of E2F target genes [reviewed in (Dick and Rubin, 2013; Macdonald and Dick, 2012)]. Phosphorylation and inactivation of the RB proteins in mid G1 is controlled by the opposing actions of cyclin D-CDK4/6 and PP2A [reviewed in (Kurimchak and Grana, 2012b, 2015)]. Indeed, hitherto unidentified PP2A complexes have been shown to dynamically regulate RB phosphorylation throughout the cell cycle (Garriga et al., 2004). While PP2A-B α is known to associate with and dephosphorylate p107 to promote G1 arrest and cell cycle exit (Jayadeva et al., 2010; Kolupaeva et al., 2013; Kurimchak et al., 2013), my results suggest that B α -containing complexes may also be involved in the dynamic regulation of RB phosphorylation during G1 phase. Therefore, loss of B α may lead to premature cyclin D and E expression due to accelerated RB hyperphosphorylation and activation of E2F target gene expression. In light of the premature cyclin D3 expression I observed in B α -depleted cells, and the requirement of cyclin D-CDK4/6 activity for E2F-mediated cyclin D and E gene expression in mid-to-late G1 phase, it seems more likely that B α regulates early G1 phase gene transcription through c-Jun. However, this needs to be investigated further, and it is possible that B α contributes to both mechanisms.

PP2A-B δ has not been shown to regulate retinoblastoma protein phosphorylation and my results indeed suggest that B δ positively regulates G1/S progression, as its depletion leads to prolonged G1 phase/postponed G1-to-S transition. Interestingly, an investigation of genes that are upregulated during early G1 identified the B δ -encoding gene (*PPP2R2D*) (Fukuoka et al., 2013). *PPP2R2D* gene expression increases toward mid G1, suggesting that it may play a role in mid to late G1 events. PP2A-B δ has recently been shown to inhibit AMPK activity (Joseph et al., 2015), a known regulator of mTORC1 (Gwinn et al., 2008). Importantly, passage through late G1 phase has

been proposed to be regulated by a checkpoint centered on mTORC1 activity [reviewed in (Foster et al., 2010; Shackelford and Shaw, 2009)], suggesting that B δ may modulate late G1 progression by promoting mTORC1 activity via repression of AMPK. However, the proposed mTORC1-mediated late G1 checkpoint is largely under metabolic control and may not be relevant to cell cycle regulation in nutrient-/growth factor-rich conditions, or cancer cell cycles.

In addition to the potential regulation of a late G1 nutrient checkpoint by a PP2A-B δ /AMPK/mTORC1 axis, recent studies have also implicated the BEG pathway in a mechanism coupling nutrient sensing/cell growth to cell division in budding yeast. Specifically, the BEG has been shown to mediate communication between the TOR and CDK-cyclin pathways (Chica et al., 2016; Perez-Hidalgo and Moreno, 2017). It was demonstrated that nutrient-stimulated TORC1 activity results in the inactivation of *Rim15* (Gwl/MASTL) and *Igo1/Igo2* (Ensa), and the activation of PP2A-Cdc55 (B55). PP2A-Cdc55, in turn, dephosphorylates the CDK inhibitory protein, *Sic1* (p27Kip1), leading to its degradation and the subsequent activation of CDK-cyclin. Therefore, TORC1-mediated activation of PP2A-Cdc55 promotes CDK-cyclin activation and cell cycle progression. In the absence of TORC1 activity, however, an active *Rim15 – Igo1/2* axis inhibits PP2A-Cdc55, leading to the accumulation of *Sic1*, the inhibition of CDK-cyclin and G1 arrest. In this context, it is conceivable that loss of PP2A-Cdc55 activity, for example through the depletion of Cdc55, could also promote *Sic1* stabilization and G1 phase arrest. Considering that the BEG pathway is highly conserved in evolution (Labandera et al., 2015), it is possible that a similar mechanism exists in higher eukaryotes, where PP2A-B55 positively regulates a G1 nutrient checkpoint and loss of B55 activity promotes the stabilization of p27Kip1, inhibition of CDK-cyclin and G1 arrest.

In summary, my results demonstrate that the highly related B α and B δ subunits play overlapping as well as distinct roles in the different phases of the cell cycle. While entry into mitosis is principally regulated by one B55 isoform in *Xenopus* (B δ), I show that several B55 subunits are likely involved in mammalian systems, a distinction that has not been made before (Fig. 3.15). Furthermore, I provide evidence for temporally separate roles of the B α and B δ subunits during exit from mitosis, where B δ appears to regulate early mitotic exit events while B α regulates late mitotic exit/post-mitotic events (Fig. 3.15 B) and suggest that this may be due to differences in reactivation kinetics. These findings indicate that the temporal regulation of mitotic exit events by PP2A-B55 may be even more intricate, adding another layer of complexity to the current model of B55-regulated mitotic exit kinetics. Regulation of S phase progression by the BEG pathway has recently been shown. I demonstrate B α -dependent effects in S phase but propose that rather than acting in the established antagonistic manner, Gwl/ENSA and B α may cooperate to positively regulate DNA replication (Fig. 3.15A). Finally, my findings demonstrate distinct roles for the B α and B δ subunits in the regulation of G1 phase kinetics. B α appears to negatively regulate G1 progression by modulating the timely expression of G1 cyclins during early to mid G1 phase, while B δ may positively regulate late G1/G1-S transition (Fig. 3.15C and D). This work provides the basis for further investigation of potentially novel functions for these PP2A-B55 complexes in multiple cell cycle phases.

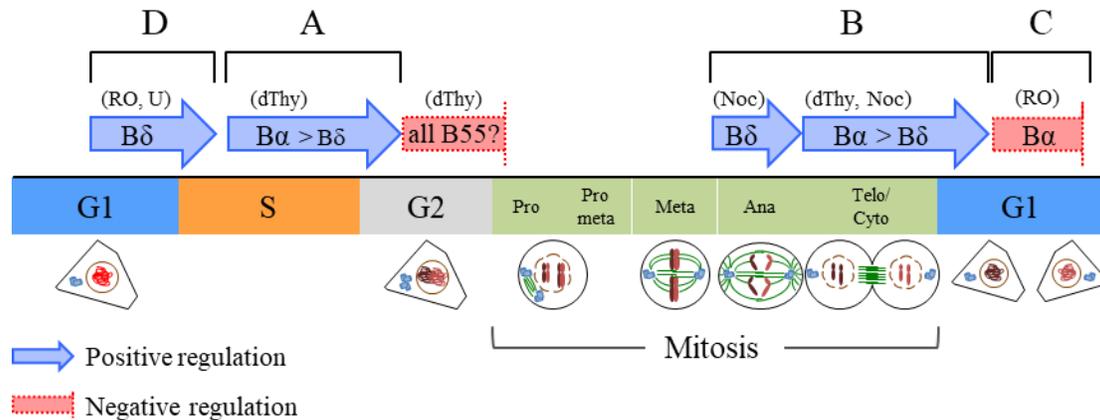


Figure 3.15: Overview of B55-regulated cell cycle progression

Using various cell cycle synchronization techniques, the experiments in **Chapter 3** identified previously undescribed roles and expanded upon established activities of the B α and B δ subunits in cell cycle regulation.

The abbreviations enclosed by parentheses indicate the method of synchronization that was used to reveal the respective regulatory effect. dThy = double thymidine block, synchronization at G1/S boundary; Etp = Etoposide treatment (48 h), G2 arrest; RO = consecutive single thymidine and RO3306 blocks, synchronization at G2/M boundary; Noc = consecutive single thymidine and nocodazole treatments, arrest in Prometaphase; U = unsynchronized. The ‘greater than’ symbol (>) denotes apparent importance of the subunits in a given process.

- A. B α positively regulates timely progression through S and into G2 phase. PP2A-B δ may also play a role, albeit much less prominent.
- B. PP2A-B δ appears to regulate early mitotic exit events, including mitotic exit initiation. PP2A-B α plays a more prominent role during the later stages of mitotic exit and the transition into G1 phase.
- C. B α restrains progression through G1 phase.
- D. B δ may positively regulate G1-to-S phase progression, possibly through regulatory effects on a late G1 checkpoint.

Chapter 4: A novel role for PP2A-B α (but not B δ) in regulating G2 and mitotic regulators

4.1 Rationale and Hypothesis

Based on genetic and biochemical evidence from an extensive body of work, it is almost universally accepted that PP2A-B55 heterotrimers act as negative regulators of the mitotic state, and this is largely ascribed to their function as major CDK1-antagonizing phosphatases. Much of the experimental work that directly demonstrates B55-dependent dephosphorylation of CDK1 substrates stems from studies of mitotic exit regulation in various higher eukaryotic model systems, including mammalian cell lines (Cundell et al., 2013; Cundell et al., 2016; Ferrigno et al., 1993; Hegarat et al., 2014; Manchado et al., 2010; Mayer-Jaekel et al., 1994; Mochida et al., 2009; Vigneron et al., 2009). Evidence for its role in the G2-to-M transition/mitotic entry, however, is largely indirect, focuses on MASTL/Gwl/Ensa-mediated inhibition of B55 complexes, and is derived from work in non-mammalian eukaryotes (mostly *Xenopus*). Furthermore, due to the high level of sequence identity between the B55 subunits, and the existence of fewer isoforms in non-mammalian model systems, it is widely assumed that these subunits perform analogous, if not redundant, functions. In **Chapter 3**, I demonstrated functionally distinct roles for the B α and B δ subunits in the regulation of different cell cycle phases. Furthermore, I demonstrated a B α -dependent postponement in mitotic timing. Although this was interpreted as a consequence of slowed transition through S phase, rather than defective mitotic entry regulation (section 3.2.5 and 3.2.6), later findings in G2/M-synchronized HeLa cell populations revealed delayed initiation of mitosis and markedly impaired progression through G2/M in cells lacking B α (but not B δ) (section 3.2.12). Together, these observations strongly suggest that B α may indeed play an unexpected role as a positive regulator of mitosis. This prompted me to more closely examine the effects of B55 depletion on the transition from G2 phase into mitosis.

4.2 Results

4.2.1 Loss of B α causes defective mitotic entry and reduced mitoses in G2/M-synchronized HeLa cells

To investigate more closely whether PP2A-B α may positively regulate entry into mitosis, HeLa cell populations were synchronized at the G2/M border using the CDK1-specific inhibitor, RO3306 (Fig. 4.1A). An examination of mitotic progression by relative DNA content analysis confirmed that loss of B α (in single and co-depleted conditions) results in delayed entry into mitosis compared to control or B δ -depleted populations (Fig. 4.1B). Control and siB δ -treated cells start progressing out of G2/M during the first hour following release from the G2/M block (Fig. 4.1B). In the absence of B α , however, cells only initiate this progression between 1-2 hours after release. Furthermore, these cell populations exhibit diminished progression through G2/M, as approximately 30% of siB α (and siB α + δ) treated cells undergo mitosis by 5hr after release, compared to 50% of NT and siB δ populations (Fig. 4.1B).

To more accurately assess mitotic populations, I analyzed the levels of the mitotic marker MPM2 by flow cytometry. MPM2 represents a consensus epitope that is found on more than 40 mitotic proteins, including many important mitotic regulators and effectors, and is targeted and phosphorylated by numerous kinases (Albert et al., 2004; Jacobberger et al., 2008; Yaffe et al., 1997). Ablation of B α reduces the mitotic index of G2/M-synchronized cell populations by 60% on average (Fig. 4.1C), and only approximately half as many cells enter and undergo mitosis compared to control cells (Fig. 4.1C, see MPM2-peak at 60 min in siB α vs. 40 min in siNT). B α -deficient cells also exhibit slowed kinetics of mitotic progression, as peak MPM2-positivity occurs later and decreases more slowly (Fig. 4.1C). On the contrary, loss of B δ results in an increased mitotic index following release at the G2/M border, and approximately 25% more cells enter mitosis as compared to control populations, as estimated from the approximately 25% more cells exhibiting MPM2-

positivity (Fig. 4.1C). Dephosphorylation of MPM2 also occurs at a diminished rate, with greater mitotic populations remaining at later time points (Fig. 4.1C). Furthermore, an analysis by time-lapse microscopy of RO3306-synchronized HeLa cells expressing eGFP-TUBA/mCherry-H2B showed that depletion of B α , but not B δ , significantly increased the proportion of cells that did not enter or undergo mitosis (Fig. 4.1D).

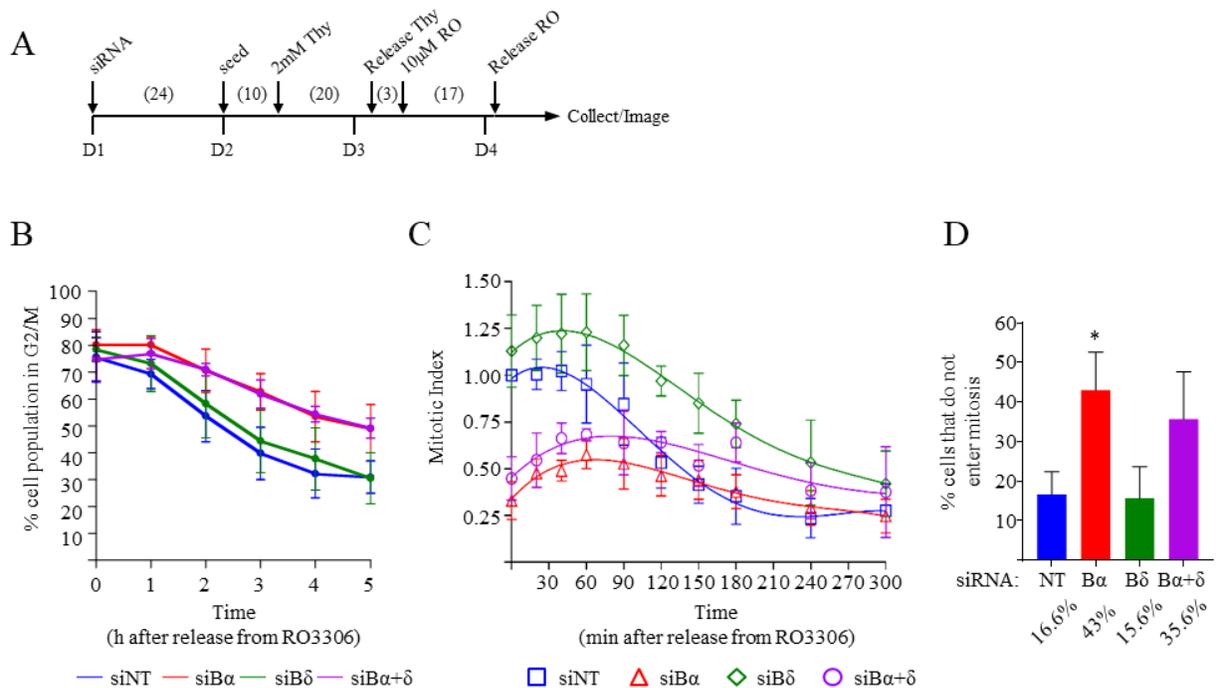


Figure 4.1: Loss of B α causes defective mitotic entry and reduced mitoses in G2/M-synchronized

HeLa cells

Control (non-targeting, NT), B α -, and/or B δ -depleted HeLa cells were synchronized at the G2/M boundary by consecutive thymidine and RO3306 treatments. Following release from the RO3306 block, cells were either collected at the indicated timepoints and analyzed by flow cytometry (B and C) or imaged by time-lapse microscopy for 20 h (D).

- A. The diagram represents the timeline of the experiment (described in section 2.1.4 Cell cycle synchronization). Briefly, cells were transfected with siRNA duplexes and allowed to grow for 24 h. Cells were then seeded into multiple cell culture dishes (one dish for each timepoint) and allowed to attach for 10 h before the synchronization procedure was initiated by the addition of thymidine (2 mM). After 20 h, thymidine was washed out, and cells were released into drug-free

medium for 3h before RO3306 (10 μ M) was added. After 17 h of RO3306 treatment, cells were washed, released into drug-free medium, and collected at the indicated timepoints. D1-4 denotes time in days; numbers in parentheses indicate time in hours inbetween steps; Thy=thymidine; RO=RO3306.

- B. Samples collected at the indicated timepoints were processed for relative DNA content analysis by flow cytometry. The percent cell population in G2/M phase at each timepoint was calculated from DNA content histograms using the Watson Pragmatic Cell Cycle Module in FlowJo v10.0.1. Data are representative of four independent experiments (n=4, mean \pm s.d.)
- C. Samples collected at the indicated timepoints were fixed and stained with anti-MPM2 antibody to specifically identify mitotic populations by flow cytometry. Data were collected as percent MPM2-positive population and converted to a “Mitotic Index” by standardizing percent values to the control in each experiment. Data are representative of four independent experiments (n=4, mean \pm s.d.).
- D. HeLa cells stably expressing eGFP-TUBA and mCherry-H2B were treated with control (NT) or B55 isoform-specific siRNA duplexes, and were synchronized at the G2/M boundary as described in (A). For analysis by time-lapse microscopy, cells were seeded into 96-well plates, and following release from RO3306 treatment were placed in a humidified imaging chamber (37°C, 5% CO₂). Images were taken every 15 min for 24 h. Entry into mitosis was defined as the rounding-up of cells. Therefore, “cells that do not enter mitosis” were defined as cells that stayed attached and retained an interphase morphology throughout the duration of imaging. Data are representative of three independent experiments, where 150-200 cells were assessed per experiment and per siRNA treatment (n=3 experiments, n=150-200 cells per siRNA treatment per experiment, mean \pm s.d., * P <0.05, p =0.068 for NT vs. B α + δ , ANOVA).

These results show that in RO3306-synchronized cell populations, B α is required for promoting efficient mitotic entry and may play a role in priming G2-phase cells for mitosis. In contrast, B δ appears to antagonize mitotic entry, as its depletion increases the mitotic index of G2/M-synchronized cell populations. This supports the notion that B δ represents the predominant B55 isoform that negatively regulates mitosis, as suggested by others (Mochida et al., 2009). However, in the discussion of my results in **Chapter 3**, I propose that all B55 subunits play a role in negatively regulating entry into mitosis. This may seem in conflict to the data presented here and is discussed in more detail below (section 4.4). Despite the distinct mitotic entry phenotypes, the slowed rates of MPM2 dephosphorylation observed in both B α - and B δ -deficient populations support previous findings that both PP2A-B55 heterotrimers remove mitotic phosphorylations during exit from mitosis

(Castilho et al., 2009; Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017; Manchado et al., 2010).

My findings not only hint at a novel role for the B α subunit as a positive regulator of mitosis (or “mitotic readiness”), but also provide further evidence for distinct functions of these highly related B55-type subunits.

4.2.2 B α -dependent mitotic entry defects are associated with reduced phosphorylation of CDC25C and MYT1

Entry into mitosis in higher eukaryotes is regulated by a phosphorylation-dependent feedback loop centered around CDK1, Cdc25C and Wee1/Myt1. To determine whether the observed mitotic entry defects were due to reduced activity of this feedback loop, I examined the phosphorylation-dependent activation of the CDK1-activating phosphatase, CDC25C, and inactivation of the CDK1-inactivating kinase, MYT1. I used Western blotting to quantify the relative abundance of phosphorylated, higher molecular weight forms of CDC25C and MYT1 over the course of 5 hours following release from a G2/M block (Fig. 4.2A and B, arrow heads). Loss of B α noticeably reduces phosphorylation (and activation) of CDC25C. Indeed, the hyperphosphorylated CDC25C isoform is barely detectable in B α -depleted cells (single and co-depletion) (Fig. 4.2A). In line with the increase in mitotic populations observed above (Fig. 4.1C), ablation of B δ augments and prolongs CDC25C phosphorylation (Fig. 4.2A). Similarly, MYT1 phosphorylation (and inactivation) is strongly diminished in the absence of B α but enhanced by depletion of B δ (Fig. 4.2B). The kinetics of phosphorylation of CDC25C and MYT1 very closely match those of MPM2-positivity (Fig. 4.1C vs. Fig. 4.2A and B, lower panels). This strongly suggests that the mitotic entry phenotype in siB α populations is due to defective activation/inactivation of CDK1 regulators, and therefore, of CDK1 itself.

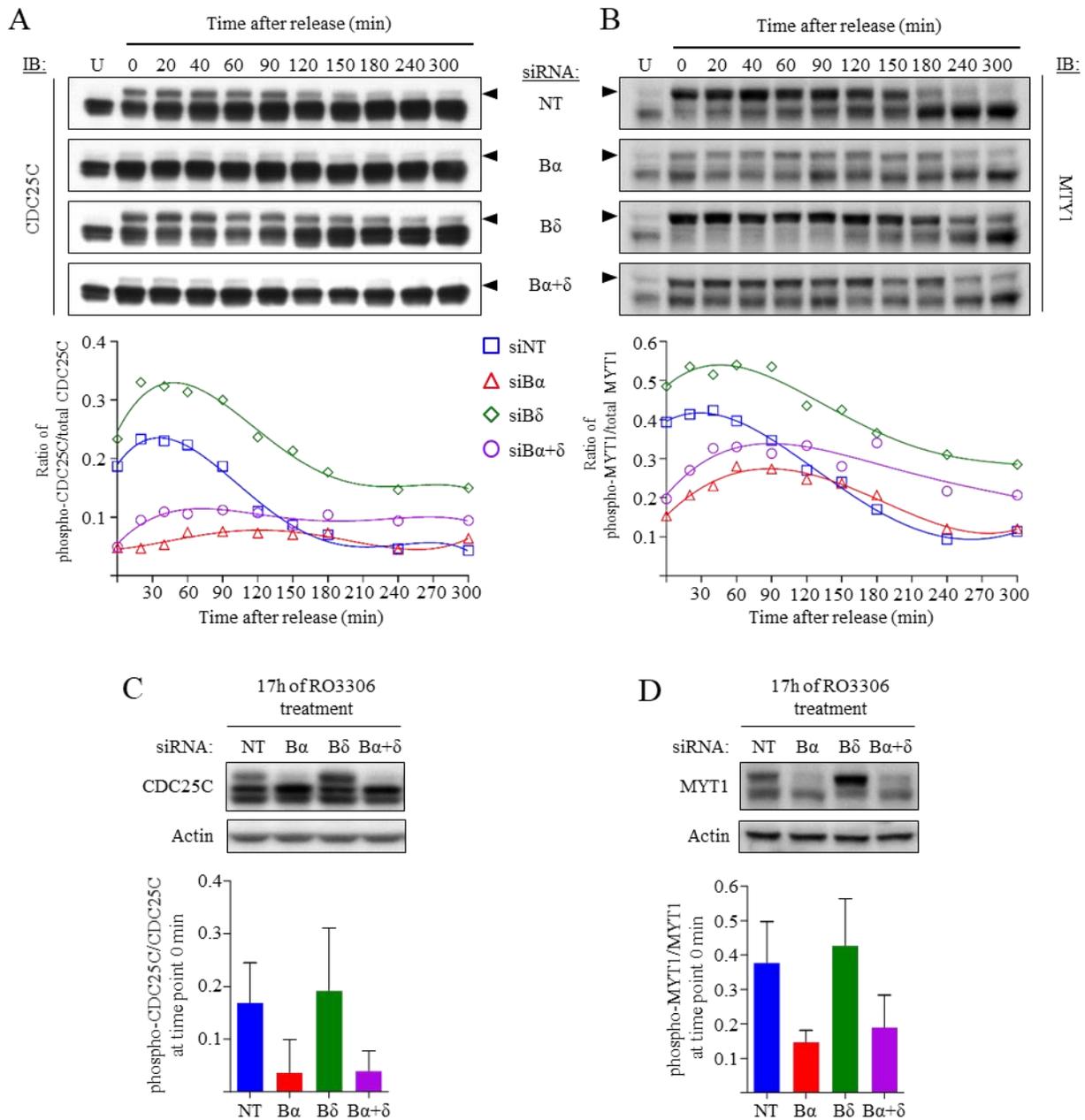


Figure 4.2: B α -dependent mitotic entry defects are associated with reduced phosphorylation of CDC25C and MYT1

Control, B α -, and/or B δ -depleted HeLa cells were synchronized at the G2/M boundary as described for Figure 4.1A. Following release from the RO3306 block, cells were collected at the indicated timepoints and processed for analysis by quantitative Western blotting. Cell lysates were obtained from the same samples as in Fig. 4.1C. During the collection procedure, samples were split and processed separately for analysis by flow cytometry (Fig. 4.1C) and Western blotting.

- A. Cell lysates were immunoblotted for CDC25C. Upper panel: phosphorylation-dependent activation of CDC25C results in the appearance of a band with slower mobility that is indicated by the black arrow head. As cells progress through and out of mitosis, CDC25C is dephosphorylated, leading to the gradual disappearance of the slower mobility form. A lysate prepared from a single unsynchronized cell population (U) was used as a standardized control for protein expression and phosphorylation levels, and was included in all gels. Blots are representative of three independent experiments. Lower panel: the “ratio phospho-CDC25C/total CDC25C” was calculated by dividing the signal intensity of the upper slower mobility band by the combined signal intensities of the upper and lower bands. Graphed values at each timepoint represent the mean from three independent experiments. A line of best fit was generated using GraphPad Prism v7.03 and is based on a non-linear, fourth-order polynomial fit.
- B. B α -depleted cells exhibit strongly reduced phosphorylation-dependent inactivation of MYT1. Cell lysates were immunoblotted for MYT1. Upper and lower panels as in (A). Note: phosphorylation of MYT1 results in inactivation, rather than activation as for CDC25C.
- C. A side-by-side comparison of CDC25C phosphorylation status at the time of release from RO3306 block (i.e. after 17 h of RO3306 treatment). The blot is representative of six independent experiments. The ratio of phosphorylated CDC25C (upper band) over total CDC25C (all bands) was calculated from six independent experiments and graphed as the mean \pm s.d.
- D. A side-by-side comparison of MYT1 phosphorylation status as in (C).

To confirm that the observed differences in phosphorylation are not due to variations between individual Western blots, I performed a side-by-side comparison of CDC25C and MYT1 at the time of release from the G2/M block. The results confirm that depletion of B α (single or co-depletion) impairs, while loss of B δ enhances, the phosphorylation and activation or inactivation of CDC25C and MYT1, respectively (Fig. 4.2C and D). Interestingly, B α -deficiency had a greater effect on the phosphorylation of CDC25C than MYT1 (Fig. 4.2A-D). This is discussed further below.

Here, I demonstrate that the observed B α - and B δ -dependent mitotic entry effects are associated with inefficient and enhanced phosphorylation of core mitotic entry regulators, respectively. Furthermore, these findings provide a molecular basis for the contrasting roles that the B α and B δ subunits appear to play in the regulation of mitotic entry, and show that B α supports activation of the CDK1 auto-amplification loop, whereas B δ opposes its activity.

4.2.3 $B\alpha$ -dependent mitotic entry defects may be due to premature proteasome-dependent degradation of cyclin B1

Control and $B\delta$ -depleted cells rapidly enter mitosis following relief of RO3306-mediated CDK1 inhibition. This is consistent with the hyperphosphorylated state of CDC25C, MYT1, and MPM2, and indicates that these cells are primed for entry into mitosis and that mitotic progression is only hindered by chemical inhibition of CDK1. Notably, loss of $B\alpha$ not only impairs CDC25C and MYT1 phosphorylation at the G2/M border but following wash-out of RO3306 and de-repression of CDK1 activity, their phosphorylation does not recover (Fig 4.2A and B). This strongly suggests that the activity of the CDK1/CDC25C/MYT1 feedback loop is disrupted, and that CDK1 is not being activated despite removal of RO3306. In addition to being regulated by phosphorylation, CDK1 must bind its mitotic partner, cyclin B1, to become activated. Therefore, it is possible that loss of $B\alpha$ affects cyclin B1-mediated activation of CDK1. Indeed, ablation of $B\alpha$, but not $B\delta$, significantly reduces cyclin B1 levels at the time of release from G2/M arrest (Fig. 4.3A). Cell cycle phase-specific fluctuations in cyclin levels are controlled by periodic upregulation of gene expression and protein degradation by the ubiquitin-proteasome pathway. To determine whether the $B\alpha$ -dependent decrease in cyclin B1 levels is due to untimely protein degradation or reduced gene expression, I probed cyclin B1 protein levels at various time-points throughout the RO3306-mediated synchronization process in the presence or absence of the proteasome inhibitor, MG132. Expression of cyclin B1 increases from 0 to 12 hours of RO3306 treatment, irrespective of siRNA treatment (Fig. 4.3B). However, in the absence of $B\alpha$, cyclin B1 is degraded between 12h to 17h in a proteasome-dependent manner (Fig. 4.3B). Inhibition of the proteasome in $B\alpha$ -deficient cells restores cyclin B1 from ~35% to ~80% of control levels (Fig. 4.3A vs. C). Ablation of the $B\delta$ subunit has no effect on cyclin B1 levels (Fig. 4.3A and B), indicating that PP2A may positively regulate cyclin B1 stability via the ubiquitin-proteasome pathway in a $B\alpha$ -specific manner.

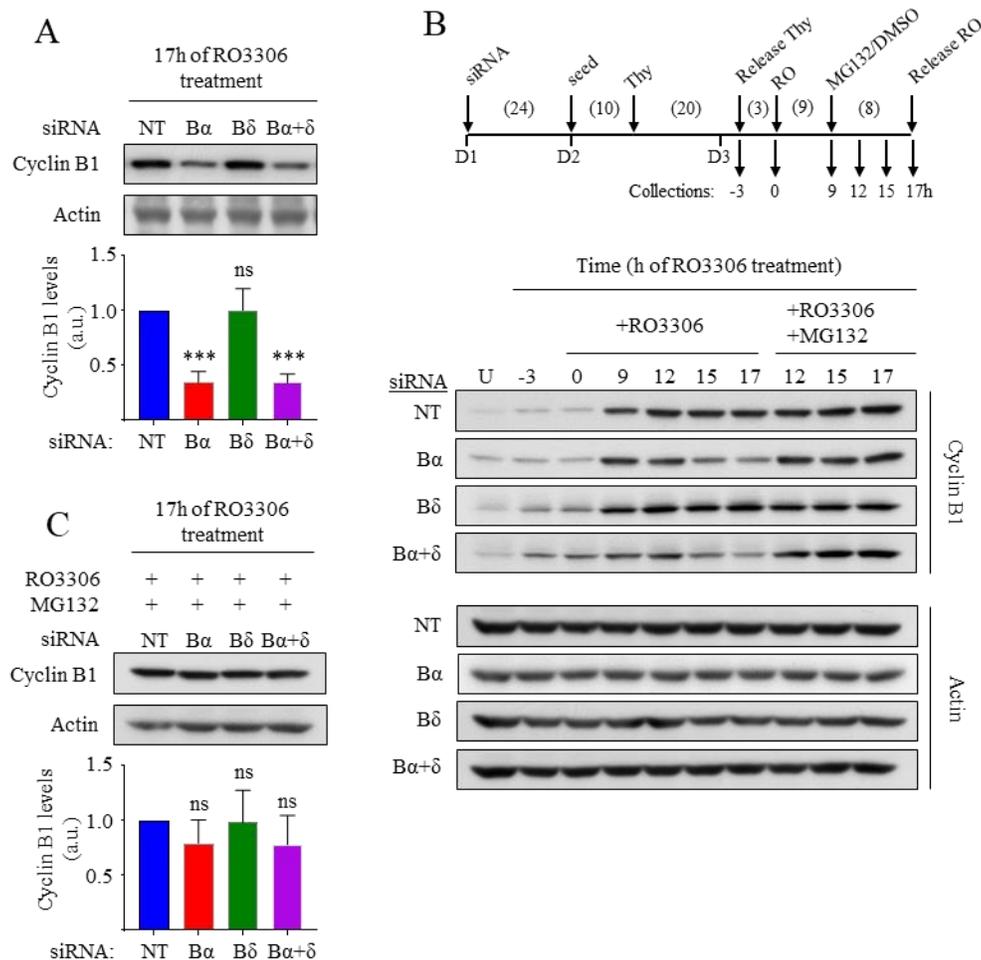


Figure 4.3: B α -dependent mitotic entry defects may be due to premature proteasome-dependent degradation of cyclin B1

Control, B α -, and/or B δ -depleted HeLa cell populations were synchronized at the G2/M boundary as described in Figure 4.1A.

- A side-by-side comparison of cyclin B1 expression demonstrates reduced levels in B α -deficient cells. At the time of release from RO3306 treatment (i.e. after 17 h of RO3306 treatment), cells were collected and processed for Western blotting. Cell lysates were immunoblotted for cyclin B1. Cyclin B1 levels were normalized to actin (loading control), and their abundance relative to control levels was calculated and graphed. The blots and data are representative of nine independent experiments (n=9, mean \pm s.d., *** P < 0.001, ns=not significant, ANOVA).
- B α -dependent reduction in cyclin B1 levels is rescued by proteasome inhibition. HeLa cell populations were synchronized at the G2/M boundary in the presence or absence of the proteasome inhibitor, MG132 (10 μ M), and cyclin B1 levels were determined by Western blotting (lower panels). The upper schematic depicts the timeline of the experiment. Briefly,

rather than collecting cells following release from RO3306, samples were collected at the indicated timepoints during the RO3306-synchronization process. As depicted, cell populations were treated with MG132 or DMSO (vehicle control) at 9 h of RO3306 treatment, and collected thereafter at 12, 15, and 17 h. Since cells were collected during the synchronization process, release from RO3306 (“Release RO”) did not occur, and its inclusion in the diagram only serves as a reference for comparison to previous experiment timelines.

A lysate prepared from a single unsynchronized cell population (U) was used as a standardized control for protein expression and phosphorylation levels, and was included in all gels. Blots are representative of three independent experiments.

- C. A side-by-side comparison of cyclin B1 expression levels in the presence of MG132 at 17 h of RO3306 treatment. Cyclin B1 levels were normalized and calculated as in (A). The blots are representative of three independent experiments, and the graph shows the mean \pm s.d. (n=3, ns=not significantly different from control (NT), ANOVA).

Proteins can be targeted for ubiquitination and proteasomal degradation by sequence-specific phosphorylation. This raises the possibility that PP2A-B α may directly interact with and dephosphorylate cyclin B1 during G2 phase, preventing its ubiquitination and degradation. To test this, I immunoprecipitated cyclin B1 from RO3306-treated cells and examined its phosphorylation, ubiquitination and binding to B α . I found no evidence of increased cyclin B1 phosphorylation or ubiquitination in the absence of B α , nor could I detect an interaction between cyclin B1 and B α (data not shown).

Loss of B α not only results in strongly reduced phosphorylation of CDC25C/MYT1, but also induces precocious cyclin B1 degradation. This significantly disrupts the regulatory circuitry that controls mitotic entry, and culminates in defective entry into mitosis in B α -deficient cells. These findings place PP2A-B α upstream of CDC25C and MYT1 phosphorylation, and implicate a role in the regulation of cyclin B1 stability.

4.2.4 S-G2 and G2-M cyclins and regulators, but not G1/S cyclins, are degraded in a B α - and proteasome-dependent manner

The observed defects in MPM2 and CDC25C/MYT1 phosphorylation and the destabilization of cyclin B1 suggest that the activities of G2 regulators that operate upstream of CDK1/CDC25C/MYT1 are impaired. Therefore, I examined whether the ablation of B α affects the phosphorylation and stability of additional G2 and mitotic regulators. PLK1 and CDK1/2-cyclin A are crucial upstream regulators of G2 progression and mitotic entry, and CDK1/2-cyclin A complexes have specifically been shown to regulate the activation of CDK1 and CDC25C (Bruinsma et al., 2015; Gheghiani et al., 2017; Gong et al., 2007; Mitra and Enders, 2004; Silva Cascales et al., 2017). As observed with cyclin B1, the levels of PLK1 and cyclin A2 are significantly reduced in B α -deficient cells, and can be rescued by proteasome inhibition (Fig. 4.4A). In addition to decreased protein levels, PLK1 activation, as measured by the level of Thr210 phosphorylation, is also significantly attenuated in the absence of B α (Fig 4.4B). Similarly, the protein and phosphorylation levels of Aurora kinase A (AURKA), which phosphorylates and activates PLK1 during the transition into mitosis (Bruinsma et al., 2014; Macurek et al., 2008), are also markedly diminished in B α -deficient cells (Fig. 4.4C). Consistent with the disrupted phosphorylation of other, more upstream mitotic entry regulators, MASTL also exhibits decreased phosphorylation in the absence of B α (Fig. 4.4C). Therefore, depletion of B α causes widespread defects in the stabilization and activation of G2 and mitotic entry regulators. These findings place PP2A-B α upstream of CDK1/2-cyclin A2, AURKA, and PLK1 activation, and implicate its involvement in the regulation of protein degradation. This raises the question of whether PP2A-B α may more generally regulate the stability of other cyclins and cell cycle regulators. Interestingly, loss of B α also leads to proteasome-dependent degradation of cyclin E1 (Fig. 4.4D), but not of the G1 cyclins D1 and D3 (Fig. 4.4E). In fact, cyclin D1 expression consistently appears to increase in the absence of B α (Fig. 4.4E).

described for the experiment in Figure 4.3B. Samples were collected at the time of RO3306 addition (0 h) and release (17 h of RO3306 treatment) during the synchronization process, and processed for analysis by quantitative Western blotting to determine the levels and phosphorylation of various S, G2/M, and G1 regulators.

- A. PLK1 and cyclin A2 are degraded in a $B\alpha$ - and proteasome-dependent manner. Upper panels: cell lysates at 0 h and 17 h of RO3306 treatment in the presence and absence of MG132 were immunoblotted for cyclin A2 and PLK1. Lower panels: Cyclin A2 and PLK1 levels were normalized to actin (loading control), and their abundance was calculated and graphed relative to control levels. Cyclin A2 (- MG132) blots and data are representative of six independent experiments ($n=6$, mean \pm s.d., *** $P<0.001$, ANOVA). PLK1 (- MG132) blots and data are representative of nine independent experiments ($n=9$, mean \pm s.d., ** $P<0.01$, *** $P<0.001$, ANOVA). Cyclin A2 and PLK1 (+ MG132) blots and data are representative of three independent experiments ($n=3$, mean \pm s.d.).
- B. PLK1 activating Thr210-phosphorylation is reduced in $B\alpha$ - and $B\alpha+\delta$ -deficient cells. Left panel: Cell lysates were collected at 17 h of RO3306 treatment, processed for Western blotting, and immunoblotted for PLK1 and PLK1 pT210. Blots were first probed for PLK1 pT210, then stripped and re-probed for PLK1. Right panel: PLK1 levels were normalized to actin, and the PLK1 Thr210 phosphorylation was calculated by dividing the signal intensity of PLK1 pT210 by the normalized signal intensity of PLK1. Blots and data are representative of eight independent experiments ($n=8$, mean \pm s.d., * $P<0.05$, ANOVA).
- C. Aurora kinase A (AURKA) protein levels and activating Thr288-phosphorylation are reduced, and MASTL phosphorylation is lost, in the absence of $B\alpha$. Cell lysates were collected at 17 h of RO3306 treatment and immunoblotted for AURKA pT288, AURKA total protein, and MASTL total protein. The blots are representative of three independent experiments.
- D. Cyclin E1 is degraded in a $B\alpha$ - and proteasome-dependent manner. Cell lysates were collected at 17 h of RO3306 treatment in the presence and absence of MG132 and immunoblotted for cyclin E1. Blots are representative of three independent experiments.
- E. The G1 cyclins, cyclin D1 and D3, are not degraded in the absence of $B\alpha$. Samples were collected at 0 h and 17 h of RO3306 treatment, and were immunoblotted for cyclin D1 and D3. Blots are representative of six independent experiments.

Altogether, PP2A- $B\alpha$ seems to play a role in stabilizing S, G2 and mitotic cyclins and regulators, which suggests that it positively modulates late S-to-early G2 events that are vital to the proper activation of G2/M drivers.

4.2.5 Prolonged arrest in G2 triggers dephosphorylation and degradation of G2 and mitotic regulators

As described in section 4.2.3, expression of cyclin B1 increased from the time of release from the initial thymidine block (-3h of RO3306 treatment) to 12h of RO3306 treatment, and its B α -dependent degradation occurs between 12 to 17h of RO3306 treatment (Fig. 4.3B). A concomitant analysis of relative DNA content shows that following release from the thymidine-induced G1/S phase block, the majority of the cycling cell populations enters G2 phase by 9 hours and completely arrests in G2 by 15 hours of RO3306 treatment (Fig. 4.5A). Therefore, cyclin B1 degradation is initiated in G2-arrested cells. Similarly, initiation of MPM2, CDC25C, MYT1, and MASTL phosphorylation coincides with the accumulation of cell populations in G2 phase at 9h, irrespective of siRNA treatment (Fig. 4.5B). In the absence of B α , however, MPM2, CDC25C, MYT1, and MASTL are dephosphorylated after 12h, while the phosphorylation levels of these proteins remain steady or increase until 17h in control and B δ -depleted cells, respectively (Fig 4.5B).

These findings demonstrate that loss of B α does not impair the initial expression and phosphorylation of S-G2-M cyclins and regulators, but that depletion of B α impedes G2 progression to result in the maintenance of a prolonged G2 state. Thus, PP2A-B α appears to play a role in facilitating progression by either preserving the activity of an unknown G2 kinase, or inhibiting a G2/M-antagonizing phosphatase. In either instance, my findings provide evidence for a novel role of PP2A-B α as a positive regulator of the maintenance of and progression through G2 phase.

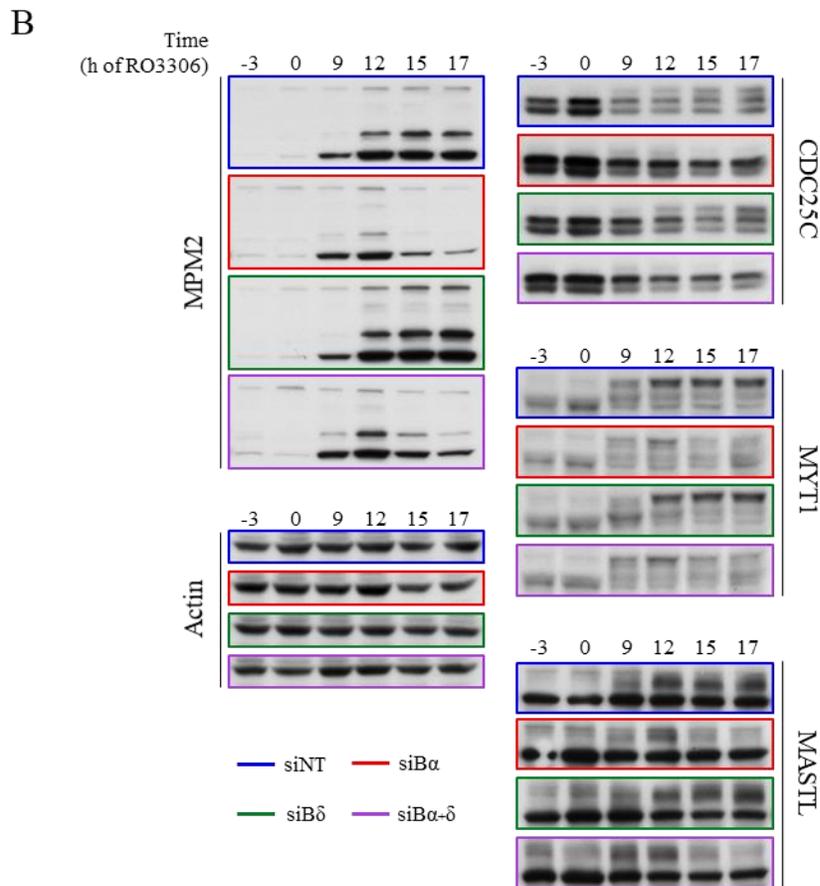
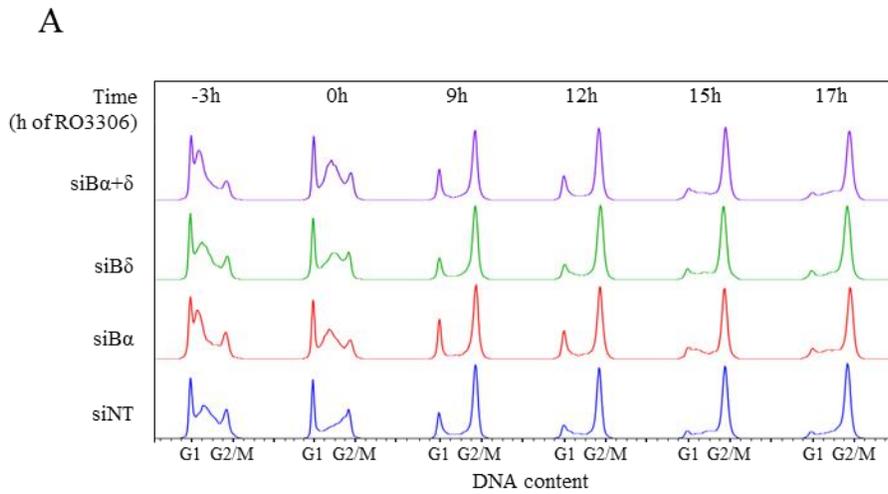


Figure 4.5: Prolonged arrest in G2 triggers dephosphorylation and degradation of G2 and mitotic regulators

HeLa cell populations were treated with control, B α -, and/or B δ -targeting siRNA duplexes and synchronized at the G2/M border, and samples were collected at the indicated timepoints during the

RO3306-synchronization procedure as shown in the schematic of Fig. 4.3B. The samples were processed for analysis by flow cytometry (A) and Western blotting (B).

- A. Relative DNA content analysis by flow cytometry. Shown are the DNA content histograms of control and B55-depleted cell populations at the indicated timepoints during the RO3306-mediated synchronization process. The data are representative of three independent experiments. Loss of B α results in a delay in S-to-G2 transition.
- B. Samples as in (A) were analyzed by Western blotting to determine the changes in phosphorylation of MPM2, CDC25C, MYT1, and MASTL as cell populations accumulate and arrest in G2 phase. B α -depleted cells exhibit initial phosphorylation of these proteins, but are unable to sustain this phosphorylation at 15 and 17 h of RO3306 treatment. Actin serves as the loading control.

4.2.6 Degradation of cyclin B1 is specific to RO3306-mediated G2 arrest

I next investigated whether the destabilization of cyclin B1 is a general phenomenon that occurs as a consequence of prolonged G2 arrest in the absence of B α . To test this, cell populations were treated with a low dose of the DNA damaging agent, etoposide, to induce activation of the DNA damage checkpoint and prevent progression into mitosis. After 12h of etoposide treatment cells largely accumulate in S and G2 phase, and by 24h cell populations completely arrest in G2 phase (Fig. 4.6A). Cyclin B1 degradation was not observed, even at 48h of etoposide treatment (Fig. 4.6B). Instead, cyclin B1 levels gradually increased over the course of the experiment. Similarly, cells that are arrested in prometaphase using the microtubule destabilizer, nocodazole, did not exhibit cyclin B1 destabilization in any of the siRNA treated cells (Fig. 4.6C). Therefore, B α -dependent degradation of cyclin B1, and likely other S-G2-M cyclins and regulators, is not a general result of prolonged arrest in G2 or prometaphase, but represents a phenomenon specific to RO3306-mediated G2 arrest.

These observations raise another intriguing point. Since RO3306 specifically inhibits CDK1 at the concentration used in these experiments, my findings implicate CDK1 activity in the stabilization and phosphorylation of S/G2/M cyclins, regulators, and substrates. In addition to

interacting with cyclin B1 to drive mitosis, CDK1 also forms active complexes with other cyclins, including A-type and E-type cyclins, to promote progression through S and G2 phase (Aleem et al., 2005; Katsuno et al., 2009; Koseoglu et al., 2008; Satyanarayana and Kaldis, 2009). Therefore, in the context of these experiments, the B α -dependent maintenance of S/G2/M regulator stability and phosphorylation is intrinsically linked to the activity of CDK1. Furthermore, because these effects are specific to B α deficiency, and are not observed in control or B δ -depleted conditions, these data hint at a hitherto unidentified pathway in which CDK1 and PP2A-B α cooperate to positively modulate the stability and phosphorylation of cell cycle regulators. This is explored in greater detail below.

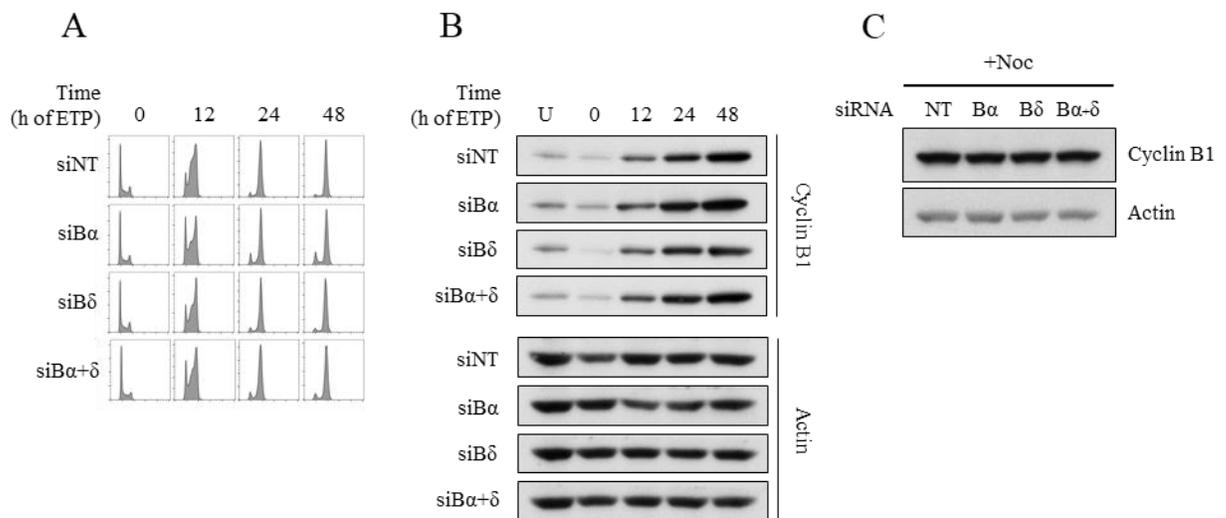


Figure 4.6: Degradation of cyclin B1 is specific to RO3306-mediated G2 arrest

Control, B α -, and/or B δ -depleted HeLa cell populations were synchronized in G2 phase or early mitosis (Prometaphase) and analyzed for cyclin B1 expression by Western blotting.

- A. A low dose of etoposide (ETP) induces G2 arrest within 24 h, irrespective of siRNA treatment. HeLa cell populations were treated with a low dose of etoposide (1 μ M) for 0, 12, 24, and 48 h to gradually induce G2 arrest. The cell cycle distribution in G1, S and G2 phase was determined by relative DNA content analysis using flow cytometry. Shown are the resulting DNA content histograms.

- B. Cyclin B1 levels increase as cell populations accumulate in G2 phase, irrespective of siRNA treatment. Samples as in (A) were processed for analysis by Western blotting, and were probed for cyclin B1. Actin serves as the loading control.
- C. Cyclin B1 levels in Prometaphase are unaffected by loss of B α . HeLa cell populations were arrested in Prometaphase of mitosis by consecutive thymidine (2 mM) and nocodazole (100 ng/ml) treatments. Following release from nocodazole, cells were collected by mitotic shake-off and processed for Western blot analysis. Cell lysates were immunoblotted for cyclin B1 and actin.

4.2.7 Depletion of B α in RO3306-synchronized cell populations promotes precocious activation of the APC/C

Ubiquitin-dependent proteolysis of mitotic regulatory proteins is largely controlled by the APC/C. When bound to its coactivator, CDC20, the APC/C regulates degradation-dependent events during early mitosis and at the onset of mitotic exit. Amongst many other proteins, the APC/C-CDC20 complex targets cyclin A2 during pro-/prometaphase, and cyclin B1 at the meta-to-anaphase transition. During late mitotic exit and into G1 phase, the APC/C acts in conjunction with its other co-activator, CDH1, to target PLK1 and AURKA, amongst other proteins [reviewed in (Sivakumar and Gorbsky, 2015)]. My results show that cyclin B1, cyclin A2, and PLK1 are degraded in a proteasome-dependent manner in G2-arrested, B α -depleted cell populations (Fig 4.3 and 4.4). This raises the possibility that the APC/C is activated precociously in these cells, leading to premature ubiquitination and degradation of its substrates. Providing further support for this untimely activation, securin, a prototypical mitotic substrate of APC/C-CDC20, is also degraded by the proteasome in the absence of B α during RO3306-induced G2 arrest (Fig. 4.7A).

The activity of APC/C (in complex with either CDC20 or CDH1) can be repressed by the small molecule inhibitor, tosyl-L-arginine methyl ester (TAME) (Zeng et al., 2010). Therefore, to determine whether the APC/C is indeed activated prematurely and contributes to mistimed degradation, cell populations undergoing RO3306-mediated synchronization were treated with proTAME, a cell-permeable prodrug form of TAME (Zeng et al., 2010). Addition of proTAME at 9h

of RO3306 treatment was able to largely rescue degradation of cyclin B1, cyclin A2, PLK1, and securin (Fig. 4.7B). Quantification of Western blots shows that inhibition of the APC/C is able to restore cyclin B1 expression in B α -depleted populations (single and co-depletion) to 70-75% of control levels (Fig. 4.7C). The extent of TAME-mediated rescue falls just short of that observed in MG132-treated B α -deficient cell populations (69.7% vs 79.3%, respectively; Fig. 4.2C vs 4.7C). This suggests that erroneous activation of the APC/C is responsible for the majority of proteasome-dependent degradation of these proteins. Remarkably, treatment with proTAME appears to completely rescue MPM2 phosphorylation in RO3306-synchronized cell populations (Fig. 4.7D).

While cyclin B1 and cyclin A2 are primarily substrates of APC/C-CDC20, PLK1 is targeted by APC/C-CDH1. This raises the question as which to complex is responsible for the untimely degradation of these substrates. To investigate this, I examined the expression and phosphorylation of various regulators and components of the APC/C. Under unperturbed conditions, premature APC/C activation during G2 phase is prevented by binding of the inhibitory protein, EMI1. Therefore, it is plausible that deregulation of EMI1 may underlie APC/C activation. However, EMI1 levels are not altered in any RO3306-treated cell populations (Fig. 4.7E), indicating that mistimed APC/C activation occurs by a mechanism that circumvents EMI1-mediated APC/C inhibition. Interestingly, CDC20, but not CDH1, also appears to be targeted for degradation (Fig. 4.7E), and this is mitigated by inhibition of the APC/C (Fig. 4.7F). Because CDC20 is able to autoubiquitinate but is also targeted by CDH1, it remains unclear which of these co-activators is responsible for APC/C activation in the context of these experiments. Binding of CDC20 and CDH1 to the APC/C complex is regulated, in part, by the phosphorylation of the APC3 subunit. Phosphorylation of APC3 is required for activation by CDC20. In contrast, CDH1 can bind APC/C complexes containing unphosphorylated APC3. Interestingly, APC3 is dephosphorylated in the absence of B α , suggesting that it is unlikely that CDC20 can bind and activate the APC/C (Fig. 4.7E). Together, the observed degradation of CDC20

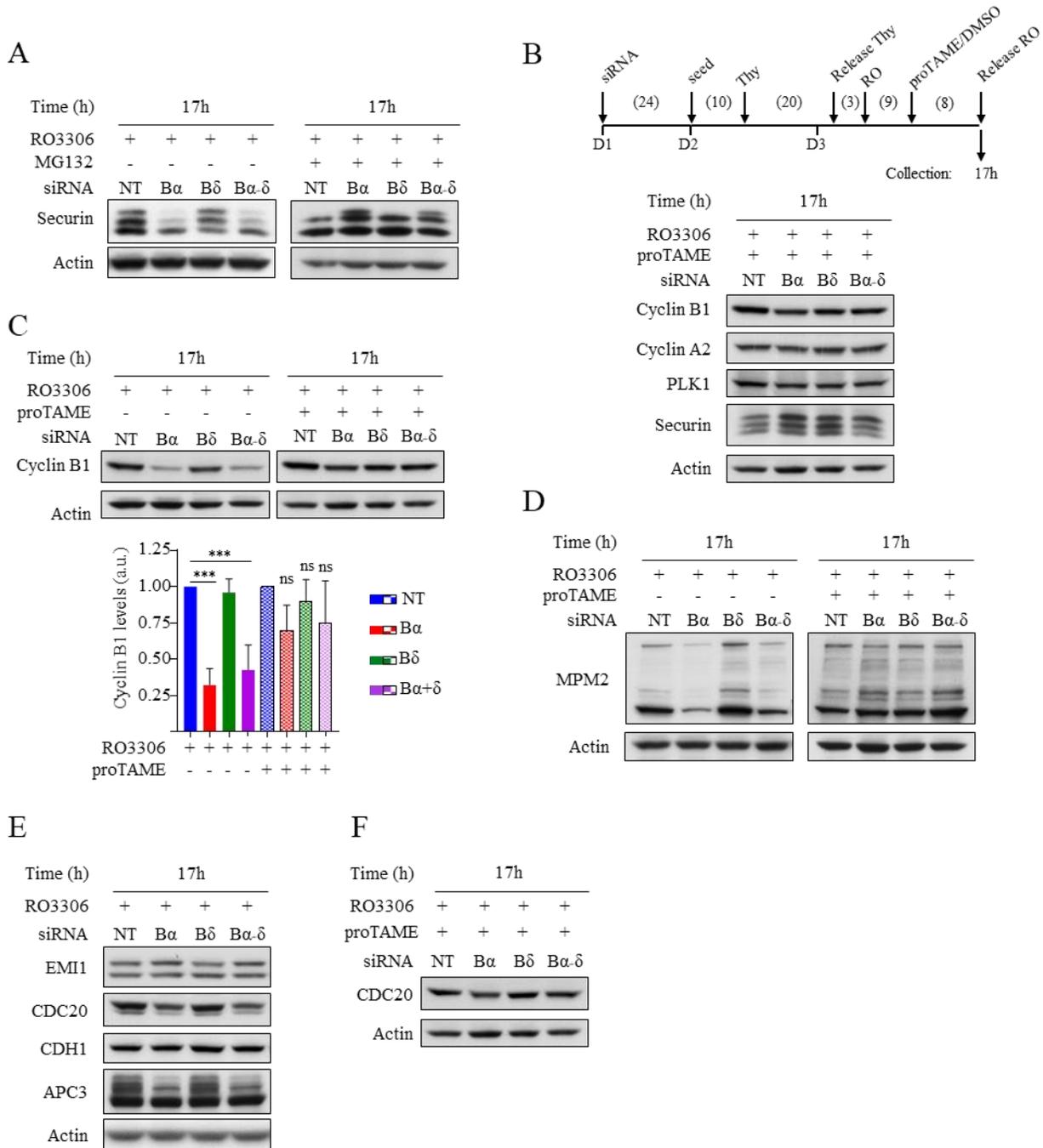


Figure 4.7: Depletion of B α in RO3306-synchronized cell populations promotes precocious activation of the APC/C

HeLa cell populations were treated with control, B α -, and/or B δ -targeting siRNA duplexes and synchronized at the G2/M border. Following release, cells were treated with RO3306 in the presence or absence of (A) the proteasome inhibitor, MG132 (10 μ M), or (B-F) the APC/C ubiquitin ligase inhibitor,

tosyl-L-arginine methyl ester (TAME; 30 μ M). Samples were collected at 17 h of RO3306 treatment, and processed for Western blot analysis.

- A. Securin, a prototypical APC/C substrate, is degraded by the proteasome in the absence of B α . Cell populations were treated with RO3306 and/or MG132 as shown in the timeline in Fig. 4.3B, and lysates were analyzed for securin expression levels. Blots are representative of three independent experiments.
- B. B α -dependent degradation of cyclin B1, cyclin A2, PLK1 and securin is largely rescued by APC/C inhibition. The top diagram shows the timeline of HeLa cell population treatment. Briefly, cells were synchronized at the G2/M boundary, released, and treated with RO3306 for 17 h before collection. At 9 h after the addition of RO3306, the APC/C inhibitor, proTAME (30 μ M) or DMSO (vehicle control) was added to the media. The cyclin B1, cyclin A2, PLK1, and securin levels were determined by Western blotting of cell lysates (lower panels). Blots are representative of three independent experiments.
- C. Quantification of the rescue of cyclin B1 levels by treatment with APC/C inhibitor, proTAME. Cell lysates from populations treated with RO3306 and/or proTAME were immunoblotted for cyclin B1 (upper panel). Cyclin B1 levels were normalized to actin, and their amount relative to control levels was measured and graphed (lower panel). Blots and data are representative of three independent experiments (n=3, mean \pm s.d., *** P <0.001, ANOVA).
- D. Inhibition of APC/C results in complete rescue of MPM2 phosphorylation. Samples as in (C) were immunoblotted for MPM2 phospho-epitopes. Blots are representative of three independent experiments.
- E. CDC20 is partially degraded and APC3 is dephosphorylated in the absence of B α . Cell lysates from populations treated with RO3306 only (17 h) were immunoblotted for the APC/C inhibitory protein EMI1, the APC/C co-activators CDC20 and CDH1, and the APC/C subunit APC3. Blots are representative of three independent experiments.
- F. APC/C inhibition largely rescues CDC20 degradation. Samples as in (B) were probed for CDC20 expression levels. Blots are representative of three independent experiments.

and the dephosphorylated state of APC3 strongly suggest that the proteolysis of cyclin B1, cyclin A2 and PLK1 is mediated by the APC/C-CDH1 complex.

These results demonstrate that the APC/C is activated precociously in B α -deficient, RO3306-synchronized cells, and that this activation is likely mediated by CDH1. This precipitates the untimely degradation of essential G2/M regulators, including cyclin B1, cyclin A2 and PLK1, leading to attenuated kinase activities, reduced phosphorylation of G2/M substrates, and defective entry into mitosis. Therefore, PP2A-B α appears to positively regulate the stability of G2/M

regulators by modulating APC/C activation. Possible mechanisms underlying this function of B α are discussed below.

4.2.8 SCF Ubiquitin Ligases are not involved in premature degradation of mitotic cyclins and regulators

The SCF (Skp1–Cul1–F-box protein) ubiquitin ligase complex represents another prominent ubiquitin ligase that regulates cell cycle progression via targeted degradation of key cell cycle proteins. Through its interaction with different F-box proteins, the SCF complex ensures timely progression through S and G2 phase and into mitosis by targeting important regulatory proteins, including cyclins and CKIs, for degradation. In complex with the NIPA F-box protein, for example, the SCF ligase targets cyclin B1 to the proteasome to modulate its accumulation during G2 phase, and to prevent premature activation of CDK1 and mitotic entry (Bassermann et al., 2007; Bassermann et al., 2005a; Bassermann et al., 2005b).

In the experiments described in section 4.2.7, inhibition of the APC/C did not fully prevent proteasome-dependent degradation of cyclin B1, as approximately 10% of cyclin B1 degradation was not accounted for by the APC/C (Fig. 4.3C vs. 4.7C). While this may be the result of incomplete inhibition, it also raises the possibility that the SCF^{NIPA} complex may contribute to cyclin B1 proteolysis. Phosphorylation of NIPA at several residues, including Ser354, by Erk2 and CDK1 at the G2/M transition induces its dissociation from cyclin B1, terminating the ubiquitination process, and resulting in the stabilization of cyclin B1 (Bassermann et al., 2007; Illert et al., 2012). Therefore, a quick way to test for a potential role of SCF^{NIPA} in B α -dependent cyclin B1 degradation is to examine its phosphorylation status. Interestingly, phosphorylation of Ser354 was reduced in the absence of B α (Fig. 4.8A). Furthermore, NIPA exhibited a reduced phosphorylation-dependent mobility shift on SDS-PAGE (Fig. 4.8A, black arrow head), indicating that NIPA is less

phosphorylated in B α -deficient cells, and thus may remain bound to cyclin B1 to promote its degradation. To further investigate the involvement of SCF complexes in the proteolysis of G2/M regulators, I examined the protein levels of cyclin B1, cyclin A2, and PLK1 in the presence or absence of MLN4924, an indirect inhibitor of SCF ligase-mediated ubiquitination (Soucy et al., 2009). Briefly, assembly of the SCF ligase complex requires neddylation of the Cullin1 scaffolding protein, and it has been shown that MLN4924-mediated inhibition of the Neddylation Activating Enzyme (NAE) prevents Cullin1 neddylation and activation of the SCF ligase complex. Therefore, ubiquitination and degradation of SCF ligase substrates can be blocked by NAE inhibition. MLN4924 inhibits Cullin1 neddylation in a dose-dependent manner, with marked reductions in neddylation occurring at concentrations $\geq 0.1 \mu\text{M}$ (Fig. 4.8B). Previous studies have used concentrations of $0.5 \mu\text{M}$ MLN4924 to inhibit SCF ubiquitin ligase activity (Charrasse et al., 2017). In my experiments I used 0.1 and $0.5 \mu\text{M}$ MLN4924 to examine SCF ligase involvement.

Treatment of RO3306-synchronized cell populations with 0.1 or $0.5 \mu\text{M}$ MLN4924 did not appear to rescue degradation of cyclin B1, cyclin A2 or PLK1 (Fig. 4.8C). In fact, inhibition of NAE, and ostensibly SCF ligase activity, impaired expression of these G2/M regulators in a dose-dependent manner (Fig. 4.8C), irrespective of siRNA treatment. However, even at the attenuated expression levels following MLN4924 treatment, B α -dependent degradation of cyclin B1, cyclin A2 and PLK1 was not prevented (Fig. 4.8C).

These findings suggest that SCF ubiquitin ligase complexes are unlikely to contribute to the degradation of G2/M regulators in B α -depleted, G2/M-synchronized cell populations. Furthermore, NAE activity and/or SCF ligase-mediated protein turnover appears to be crucial to proper expression of G2/M regulators. Thus, the reduced phosphorylation of NIPA observed upon B α depletion (Fig. 4.8A) may be a downstream consequence of the widespread reduction in G2/M protein phosphorylation, rather than reflecting a specific involvement of SCF^{NIPA} in cyclin B1 proteolysis. It

is important to point out, however, that the reduced overall expression of these markers in the presence of MLN4924 impedes the ability to draw steadfast conclusions about the role of SCF ligase complexes in the $B\alpha$ -dependent degradation phenotype.

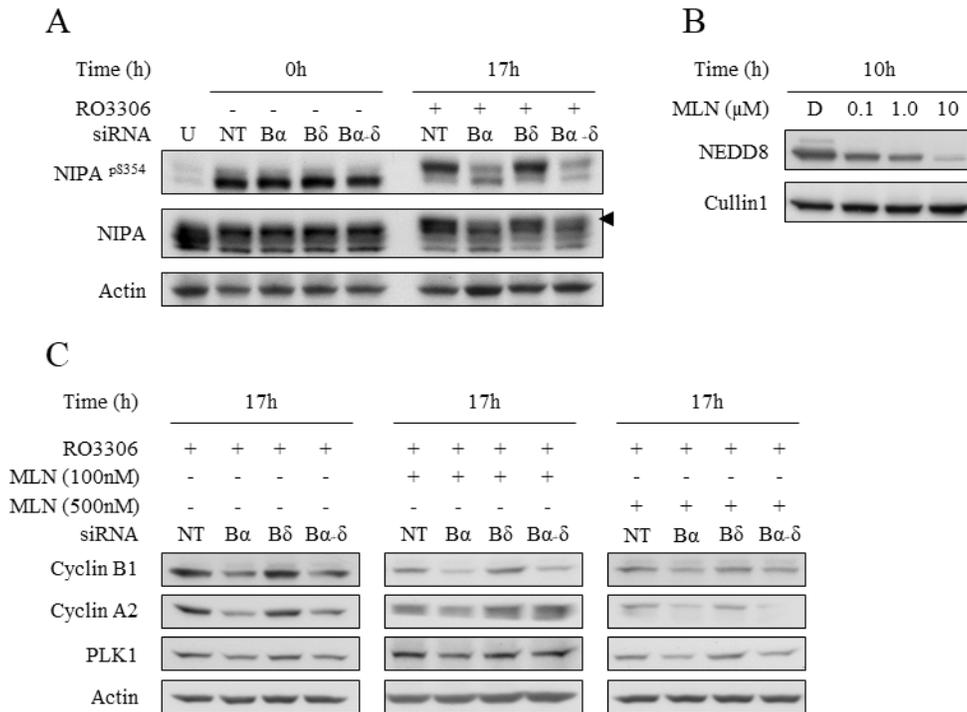


Figure 4.8: SCF Ubiquitin Ligases are not involved in premature degradation of mitotic cyclins and regulators

- A.** Phosphorylation of the F-box protein NIPA is reduced in the absence of $B\alpha$. HeLa cell populations were treated with control, $B\alpha$ -, and/or $B\delta$ -targeting siRNA duplexes and synchronized at the G2/M border. After release, and according to the timeline shown in Fig. 4.3B, RO3306 was added. Samples were collected at the time of RO3306 addition (0 h) and release (17 h of RO3306 treatment) during the synchronization process and processed for Western blot analysis. Cell lysates were probed for NIPA Ser354 phosphorylation, total NIPA, and actin. The black arrow head indicates a phosphorylation-dependent form of NIPA with reduced mobility. Blots are representative of three independent experiments.
- B.** The neddylation inhibitor MLN4924 effectively inhibits Cullin-1 neddylation. Unsynchronized HeLa cell populations were treated with DMSO or 0.1, 1.0, and 10 μ M MLN4924 for 10 h, and were processed for Western blot analysis. Cell lysates were probed for NEDD8 and Cullin1. Blots were first probed for NEDD8, then stripped and re-probed for Cullin1. Blots are representative of three independent experiments.

- C. MLN4924-mediated inhibition of SCF ubiquitin ligases does not prevent the degradation of G2/M regulators. Control, B α -, and/or B δ -depleted HeLa cell populations were synchronized and treated as in (A), and 9 h after the addition of RO3306 either control DMSO (left panel) or 100 or 500 nM MLN4924 (middle and right panels) was added to the media. Samples were collected at 17 h of RO3306 treatment, processed for analysis by Western blotting, and probed for cyclin B1, cyclin A2, and PLK1. Actin serves as the loading control. Blots are representative of three independent experiments.

4.2.9 Reduced phosphorylation and activation of FOXM1 may contribute to reduced levels of mitotic regulators at G2/M border

Orderly progression through the cell cycle depends on the periodic activation of phase-specific gene clusters, which are regulated by a plethora of transcription factors (Whitfield et al., 2002). The FOXM1 transcription factor plays an essential role in promoting progression through G2 phase and mitosis by activating the expression of cyclin B1 and PLK1, among many other important G2/M regulators. Indeed, inactivation or loss of FOXM1 leads to pleiotropic mitotic defects, including severely delayed entry into mitosis (Laoukili et al., 2005; Wang et al., 2005; Wonsey and Follettie, 2005). FOXM1 is itself activated during late S phase by cyclin E-CDK2 and cyclin A-CDK1/2 (Wierstra and Alves, 2006). Specifically, phosphorylation by cyclin A-CDK1/2 has been shown to relieve an autoinhibitory intramolecular interaction between the N- and C-termini of the FOXM1 protein (Laoukili et al., 2008b). The experimental results described in the preceding sections of this chapter illustrate that loss of B α results in both the untimely degradation and dephosphorylation of many S-to-G2 and G2/M regulators, including cyclin E1, cyclin A2, PLK1, CDC25C, MYT1 and NIPA. This prompted me to determine how widespread these alterations were, and whether transcription factors were also affected. I found that loss of B α in G2/M-synchronized cell populations not only leads to dephosphorylation of FOXM1, but also triggers its degradation (Fig. 4.9). This observation further supports a role for APC/C-CDH1 in the degradation of G2/M regulators, as FOXM1 represents another prominent substrate of the APC/C-CDH1 complex during

mitotic exit (Laoukili et al., 2008a). This finding also demonstrates that depletion of B α has far-reaching effects on G2/M regulation, disrupting not only the stability and phosphorylation of proteins but also their transcriptional regulation.

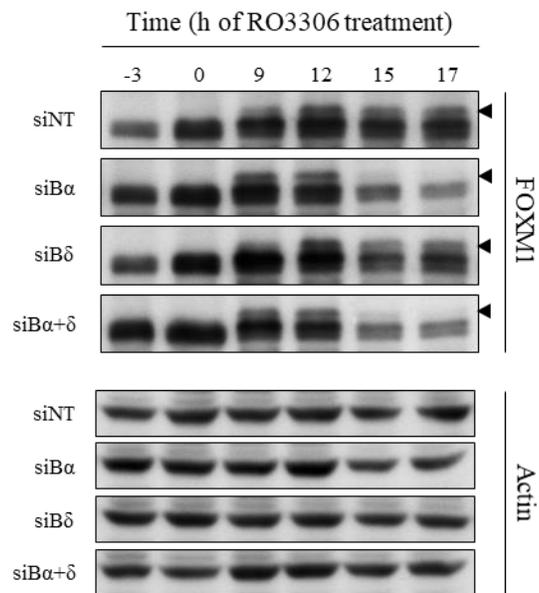


Figure 4.9: FOXM1 is degraded and dephosphorylated upon depletion of B α

HeLa cell populations treated with control, B α -, and/or B δ -targeting siRNA duplexes were synchronized at to the G2/M border. Samples were collected at the indicated timepoints during the RO3306-synchronization procedure (as described in Fig. 4.3B), were processed for Western blot analysis, and immunoblotted for FOXM1 (black arrowheads indicate a phosphorylated, slow mobility form of FOXM1). Actin serves as the loading control. Blots are representative of three independent experiments.

4.3 Key Findings

1. Loss of B δ results in increased mitotic index and prolonged phosphorylation of mitotic substrates.
2. PP2A-B δ represents the prototypical, mitosis-antagonizing B55 subunit, and may directly dephosphorylate CDC25C and MYT1.
3. Loss of B α severely impairs the ability of cell populations to enter and progress through mitosis. This is due to the widespread dephosphorylation and proteasome-dependent degradation of mitotic regulators and substrates during G2 phase.
4. B α -dependent degradation of G2/M regulators is specific to the context of RO3306-mediated CDK1 inhibition.
5. Degradation of S/G2 and G2/M regulators is due to precocious activation of APC/C-CDH1, and independent of SCF ubiquitin ligase activity.
6. B α -dependent effects are far-reaching, affecting the G2/M transcription factor, FOXM1.

4.4 Discussion

PP2A-B55 has long been understood to antagonize entry into mitosis by opposing CDK1 activation and mitotic substrate phosphorylation (Clarke et al., 1993; Cyert and Kirschner, 1988; Felix et al., 1990; Ferrigno et al., 1993; Kinoshita et al., 1993; Lee et al., 1991; Lee et al., 1994; Mayer-Jaekel et al., 1994; Solomon et al., 1991). In model systems that contain a single B55 isoform, such as *Drosophila* (*twins*) and fission (*pab1*) or budding (*Cdc55*) yeast, delineation of its involvement has been relatively straightforward. However, in higher eukaryotes, including *Xenopus* and mammalian systems, which encode multiple B55 isoforms, the function of individual family members in mitotic regulation remains poorly defined. In fact, over the last three decades, there have

been inconsistencies in attributing B55-dependent activities to the various isoforms in *Xenopus*. Early studies identify the B α and B β isoforms as important negative regulators of mitosis (Iwashita et al., 1997; Lee et al., 1991; Lee et al., 1994), while more recent studies maintain that the B δ subunit plays a predominant role, in part due to its greater abundance relative to other isoforms (Gharbi-Ayachi et al., 2010; Mochida et al., 2009; Mochida et al., 2010). Investigations of B55 isoform-specific roles in mitotic entry regulation are completely lacking in mammalian models. Here, I provide strong evidence for remarkably distinct functions for the ubiquitous B α and B δ subunits in the regulation of G2 and mitotic entry progression. As an antagonist of CDK1 activation, PP2A-B55 has been proposed to directly regulate Cdc25C and Wee1/Myt1 phosphorylation in yeast and *Xenopus* (Clarke et al., 1993; Kinoshita et al., 1993; Lee et al., 1991; Lee et al., 1994; Mochida et al., 2009; Mochida et al., 2010; Vigneron et al., 2009; Wicky et al., 2011; Zhao et al., 2008). In mammalian cell lines, a direct role for B55 subunits in the regulation of CDC25C and WEE1/MYT1 remains experimentally undefined, and is largely assumed. Here, I demonstrate B55 isoform-specific regulation of CDC25C and MYT1 phosphorylation in HeLa cells. My findings indicate that B δ , but not B α , may mediate dephosphorylation of CDC25C and MYT1 in human cell lines. Compared to control cells, depletion of B δ results in a global increase in CDC25C and MYT1 phosphorylation at mitotic entry and throughout mitotic progression (Fig. 4.2). In contrast, their phosphorylation is significantly reduced at mitotic entry in absence of B α , making a direct phosphatase-substrate interaction highly unlikely. During mitotic exit, however, MYT1 may be a target of both B α - and B δ -mediated dephosphorylation, and may represent a more prominent substrate than CDC25C, as depletion of either subunit leads to slowed kinetics of dephosphorylation of MYT1 but not CDC25C (Fig. 4.2B). Similar observations were made in G1/S-synchronized populations, where depletion of B α and/or B δ results in sustained MYT1 phosphorylation (Fig. 3.6). Dephosphorylation and reactivation of MYT1 during mitotic exit is required for proper completion of mitosis, including the reassembly of the

Golgi and ER (Nakajima et al., 2008; Potapova et al., 2009). Therefore, slowed MYT1 dephosphorylation may underlie prolonged mitosis in B δ -deficient cell populations (Fig. 3.5), and impaired late mitotic events in B α -depleted cells (Fig. 3.10) (Schmitz et al., 2010).

Proper mitotic entry timing is regulated by a tightly controlled balance of kinase and phosphatase activities (Domingo-Sananes et al., 2011). During late S and G2 phase, the activities of multiple phosphatases (including PP2A and PP1) counteract and limit the activation and activity of G2 and mitotic kinases (including CDK1/2-cyclin A, Plk1, Aurora kinase A). This tug-of-war between kinases and phosphatases sets a threshold of kinase activities and substrate phosphorylation that prevents the premature activation of CDK1-cyclin B1 and entry into mitosis. As cells progress through G2 phase, kinase activities rise as a result of the increasing expression and activation of G2/M kinases, and through their engagement in positive feedback loops [reviewed in (Lindqvist et al., 2009)]. Furthermore, kinases and phosphatases interact in feed-forward loops, in which the activation of kinases leads to the inactivation of phosphatases (Domingo-Sananes et al., 2011; Hegarat et al., 2016; Mochida et al., 2016), tipping the balance toward increasing kinase activities and substrate phosphorylation. This culminates in the passing of the pre-mitotic kinase activity/phosphorylation threshold and results in the switch-like activation of CDK1-cyclin B1 through the dephosphorylation of its inhibitory sites, and the initiation of mitosis (Domingo-Sananes et al., 2011; Hegarat et al., 2016; Hutter et al., 2017; Mochida et al., 2016; Santos et al., 2012). Therefore, entry into mitosis is crucially regulated by the opposing activities of kinases and phosphatases and can only occur when kinases surpass an activity threshold that tips the balance toward full kinase activation/phosphatase inactivation and substrate phosphorylation.

An interesting observation that emerged early on during my investigation of B55-dependent mitotic entry effects was that numerous mitotic entry regulators and mitotic epitopes (MPM2) were highly phosphorylated in control and B δ -deficient populations at the time of release from the

RO3306-mediated G2/M block (Fig. 4.1B; 4.2C and D; 4.4B and C). This has several interesting implications and consequences. Firstly, the significant presence of MPM2 phosphorylation, which is held to be specific to mitosis (Davis et al., 1983; Ding et al., 1997; Tapia et al., 2006), is taking place in the absence of CDK1 activity, and therefore, before the initiation of mitosis. The significance of this is not entirely clear, but it strongly suggests that G2 and mitotic kinase activity is high, and that these cells have initiated much of the phosphorylation cascade associated with mitotic entry. While Plk1 and Aurora A have been shown to generate MPM2-reactive phospho-epitopes during mitosis (Kumagai and Dunphy, 1996; Oshimori et al., 2006), the extent of MPM2 epitope phosphorylation indicates that other proline-directed kinases may also be involved. Potential candidates include CDK2 (likely in association with A-type cyclins), mitogen activated protein kinases (MAPKs), and casein kinases (CKs) (Escargueil et al., 2000; Lu et al., 2002; Whitmarsh and Davis, 1996; Yde et al., 2008). It follows that these kinases may be responsible for a more significant proportion of pre- and early mitotic phosphorylation than previously believed. Secondly, hyperphosphorylation of MASTL implies that this kinase is primed for activity (if not fully activated) during RO3306-mediated G2 arrest. Phosphorylation and activation of MASTL is believed to be mediated primarily by CDK1 during entry into mitosis (Blake-Hodek et al., 2012; Vigneron et al., 2011; Vigneron et al., 2016; Yu et al., 2006). Plk1 has also been shown to phosphorylate Gwl/MASTL; however, this appears to be restricted to a DNA damage checkpoint recovery pathway (Peng et al., 2011a). Therefore, my results indicate that other kinases are not only able to phosphorylate MASTL but may play a larger role than previously anticipated. Thirdly, despite the phosphorylation of mitotic substrates (MPM2) and the phosphorylation-dependent activation of CDC25C, PLK1, AURKA, and MASTL, cells do not exhibit NEBD, chromosome condensation, centrosome separation, or mitotic spindle formation, which is in accord with the universally accepted view that these latter events depend on CDK1 activity/activation [reviewed in (Malumbres and Barbacid, 2005; Nigg, 1993)]. Together, these

observations show that during RO3306-mediated G2/M arrest in control and B δ -deficient cells, kinase activities rise to (or beyond) the threshold required for mitotic entry (culminating in the dephosphorylation of CDK1 inhibitory sites (data not shown)), that phosphatases are likely inactivated, and that mitotic entry is only prevented by chemical inhibition of CDK1.

Interestingly, loss of B α interferes with this phosphorylation cascade, and results in the degradation of essential G2 and mitotic regulators at the G2/M border. Relative DNA content analysis demonstrates that cells undergoing RO3306-mediated synchronization exhibit delayed S-to-G2 phase progression in the absence of B α (Fig. 4.5). This raises the possibility that these B α -dependent effects may be due to aberrations that affect late S phase events, including the initiation of gene expression programs that drive progression through G2 phase and into mitosis. However, an examination of cyclin B1 expression (Fig. 4.3), and of the phosphorylation of MPM2, CDC25C, MYT1, and MASTL throughout the synchronization timeline (Fig. 4.5) shows that the gene expression and phosphorylation programs associated with G2/M progression are initiated normally in the absence of B α . Instead, B α -depleted cells fail to maintain protein phosphorylation and exhibit widespread dephosphorylation of G2/M substrates and regulators, as well as activation of the APC/C and degradation of APC/C target proteins. With respect to protein phosphorylation, this suggests that B α may play a role in sustaining the activity of one or more kinases, and/or the inhibition of one or more protein phosphatases (discussed below). While the degradation of cyclin A2, PLK1, and AURKA and the concomitant reduction in their kinase activities may partially account for the loss of phosphorylation, the scope of the observed dephosphorylation also suggests that the activity of unchecked phosphatases may be at work. Interestingly, the extensive dephosphorylation and degradation of mitotic regulators, including the APC/C components, APC3 and CDC20, is reminiscent of the events that characterize mitotic exit. Furthermore, cyclin B1 degradation is not observed in etoposide-mediated G2 arrest, which implies that CDK1 inhibition, another essential

feature of mitotic exit initiation, is requisite to the collapse of the pre-mitotic state. Together, these observations lead me to propose that in the absence of CDK1 activity, loss of B α destabilizes the pre-mitotic state, and triggers a mitotic exit-like process that is accompanied by the activation of protein phosphatases and the APC/C.

This raises the question as to which phosphatases may be activated during the B α -dependent pre-mitotic collapse. Notably, my results indicate that PP2A-B δ is not involved in this mitotic exit-like process because co-depletion of B α and B δ results in the same dephosphorylation and degradation phenotypes seen in cells that are depleted of B α alone. Considering the molecular resemblance to mitotic exit, it is likely that other major mitotic exit phosphatases are reactivated to effect the pre-mitotic collapse. In addition to PP2A-B55, PP1 represents another essential phosphatase that is reactivated during mitotic exit to mediate dephosphorylation of mitotic substrates (Wu et al., 2009). As CDK1 directly phosphorylates and inactivates PP1 (Wu et al., 2009), it is plausible that in the prolonged absence of CDK1 activity, site-specific PP1 inhibition is compromised, enabling PP1 to be reactivated more readily. Fcp1 is another protein phosphatase that has recently been identified as an important mitotic regulator (Son and Osmani, 2009; Visconti et al., 2012). Indeed, activation of Fcp1 is one of the earliest events during mitotic exit initiation, and is required for the de-repression of PP2A-B55 (Della Monica et al., 2015; Hegarat et al., 2014; Visconti et al., 2012). Strikingly, Fcp1 appears to trigger mitotic exit initiation by dephosphorylating Cdc20 and the deubiquitinating peptidase USP44, thereby activating the APC/C (Visconti et al., 2012). It has been postulated that Fcp1 acts as an essential liaison between the dephosphorylation and ubiquitination events to drive exit from mitosis (Visconti et al., 2012). Although the precise mechanisms that regulate Fcp1 activity during mitotic exit are still unknown, it has been shown that Fcp1 activity is regulated by phosphorylation (Friedl et al., 2003). More pertinently, CDK1 has been implicated in the repression of Fcp1, as RO3306-mediated inhibition of CDK1 triggers the activation

of Fcp1 and results in the dephosphorylation of MPM2 phospho-epitopes and other Fcp1 substrates (Visconti et al., 2012). Interestingly, it has also been shown that prolonged CDK1 inhibition by RO3306 results in APC/C activation; however, the underlying mechanism remains unknown (Ma et al., 2009). It is conceivable that Fcp1 plays an integral role in this mechanism, and further investigation is warranted. While the widespread dephosphorylation of mitotic substrates and regulators during the pre-mitotic collapse suggests that mitotic exit phosphatases may be at work, the dephosphorylation of MASTL may provide particularly relevant insight into the identity of these phosphatases. Specifically, MASTL dephosphorylation during mitotic exit is believed to be regulated by only a few phosphatases. Importantly, in addition to PP2A-B55, these phosphatases are PP1 and Fcp1 (Della Monica et al., 2015; Hegarat et al., 2014; Heim et al., 2015; Ma et al., 2016; Rogers et al., 2016a). Taken together, prolonged CDK1 inhibition during the RO3306-mediated G2 arrest may result in the reactivation of PP1 and Fcp1, thereby leading to the widespread dephosphorylation of mitotic substrates and regulators, as well as the activation of the APC/C.

While prolonged inhibition of CDK1 activity may play an important role in the reactivation of mitotic exit phosphatases and the APC/C, it remains to be explained why this mitotic exit-like process only occurs in the $B\alpha$ -deficient setting. I believe that the answer to this lies in the stabilization of the high kinase activity observed in control and in $B\delta$ -depleted cells.

As described above, the increasing activity of CDK2-cyclin A, Plk1, Aurora A, and other kinases during G2 phase culminates in the passing of a kinase activity threshold that leads to full activation of CDK1, inactivation of CDK1-antagonizing phosphatases, and subsequent phosphorylation of mitotic substrates. Importantly, it is widely held that once this threshold is surpassed, the maintenance of high kinase activities and substrate phosphorylation is dependent on the activity of CDK1-cyclin B1 [reviewed in (Hegarat et al., 2016)]. My results indicate that cells are able to surpass this threshold and sustain high kinase activity/substrate phosphorylation in the absence of

CDK1 activity, suggesting that an overall high kinase activity is able to compensate for the lack of CDK1. Interestingly, my findings strongly suggest that in the absence of CDK1 activity, the stability of high kinase activity/substrate phosphorylation is dependent on PP2A-B α . Intuitively, the depletion of a major antagonizing phosphatase should not only lower the kinase activity threshold required for mitotic entry, but also facilitate the maintenance of high kinase activity and substrate phosphorylation. This appears to be case for PP2A-B δ , as depletion of the B δ subunit consistently results in increased phosphorylation of mitotic substrates and entry regulators as compared to control. On the contrary, depletion of B α destabilizes the phosphorylation circuitry and tips the balance towards decreased kinase and increased phosphatase activity. This observation suggests that PP2A-B α either plays a role in sustaining the activity of one or more kinases, and/or the inhibition of one or more protein phosphatases. While it is possible that PP2A-B α negatively modulates the activity of other phosphatases, such regulatory interactions have not been reported. In fact, mounting evidence suggests that mitotic phosphatases directly or indirectly activate, rather than inactivate, each other (Della Monica et al., 2015; Grallert et al., 2015; Hegarat et al., 2014; Heim et al., 2015; Ma et al., 2016; Mochida, 2015; Visconti et al., 2012). Therefore, it is more likely that PP2A-B α positively regulates the activation/activity of another protein kinase that contributes to attainment of the high kinase activity and entry into mitosis. CK2 represents an appealing candidate, as several lines of evidence demonstrate specific roles for CK2 in mitotic entry and progression (Escargueil et al., 2000; Krek et al., 1992; Peng et al., 2011b; Schwindling et al., 2004; Theis-Febvre et al., 2003; Yde et al., 2008; Yu et al., 1991). Importantly, CK2 positively regulates CDK1 activation at the G2/M transition by directly phosphorylating Cdc25C and Wee1, thereby modulating their respective subcellular localization/activation and degradation (Guerra et al., 2003; Noll et al., 2006; Schwindling et al., 2004; Theis-Febvre et al., 2003; Yde et al., 2008). Furthermore, CK2 targets mitotic substrates and is able to generate MPM2 phospho-epitopes (Escargueil et al., 2000). CK2 interacts with and

phosphorylates several sites on Fcp1 (Abbott et al., 2005; Friedl et al., 2003; Palancade et al., 2002). While the effects of these phosphorylations are varied and the subject of debate, one study demonstrates the repression of Fcp1 phosphatase activity upon binding to CK2 (Friedl et al., 2003). Therefore, while purely hypothetical, it is possible that CDK1 and CK2 might phosphorylate and repress Fcp1, suggesting that CK2 might also contribute to the passing of a kinase activity threshold by inhibiting Fcp1. Strikingly, depletion of CK2 also results in a delay in mitotic initiation (Yde et al., 2008), which resembles the phenotype of B α loss, and suggests that they may regulate mitotic initiation through similar or overlapping pathways. Finally, PP2A and CK2 have been shown to interact directly and to colocalize during interphase (Escargueil and Larsen, 2007; Heriche et al., 1997), which suggests that they may engage in a regulatory relationship. Whether PP2A-B α mediates CK2 activation and what role this might play in the stabilization of a kinase activity threshold could be the subject of future investigations.

In summary, control and B δ -depleted G2/M-synchronized HeLa cell populations are able to reach and sustain high levels of G2/M regulator expression and substrate phosphorylation (due to high levels of pre-mitotic kinase activity), even in the absence of CDK1 activity (Fig. 4.10). However, when CDK1 is inhibited, the preservation of high phosphorylation levels is surprisingly dependent on PP2A-B α , possibly through its ability to activate another G2/M kinase, such as CK2. Furthermore, in B α -depleted cells, prolonged RO3306-mediated CDK1 inhibition appears to trigger the reactivation of mitotic substrate-targeting phosphatases (such as PP1 and Fcp1) and the APC/C. Therefore, depletion of B α (single and co-depletion) in G2/M-synchronized HeLa cell populations precipitates the collapse of a pre-mitotic state characterized high expression and phosphorylation of G2/M regulators and substrates (Fig. 4.10). Taken together, these results also suggests that in the absence of CDK1 activity, high activity of other kinases may be essential to maintain the suppression of mitotic antagonists.

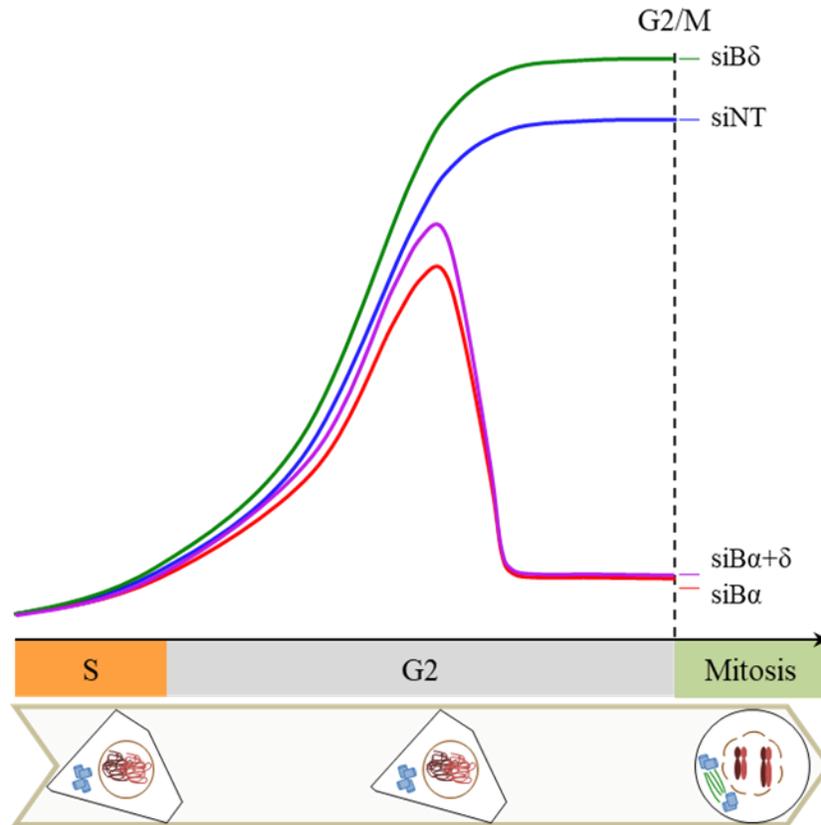


Figure 4.10: Overview of B55-dependent effects on pre-mitotic expression and phosphorylation of G2/M regulators and substrates

The diagram depicts the B55-dependent effects on the stability of a pre-mitotic state characterized by high protein phosphorylation and G2/M regulator expression. Control and B δ -depleted HeLa cell populations synchronized at the G2/M boundary exhibit increasing expression and phosphorylation of G2/M regulators and substrates during G2 phase. Depletion of B δ results in increased phosphorylation of regulators and substrates, suggesting a role for B δ as an antagonist of “mitotic readiness”. In B α -depleted populations (single and co-depleted), pre-mitotic expression and phosphorylation programs are initiated but collapse during G2 phase, suggesting a role for B α as a positive regulator of “mitotic readiness”.

The findings presented in this chapter demonstrate for the first time that PP2A-B α may act as a positive regulator of “mitotic readiness”, by modulating the stability of a pre-mitotic phosphorylation threshold. Furthermore, I provide additional evidence for unique roles of the ubiquitous B α and B δ subunits, where B δ appears to be the prototypical, anti-mitotic B55 subunit,

while B α may actually support mitosis through actions in early G2 phase. Despite the context-specific nature of these findings (CDK1 inhibition), they clearly differentiate these highly related subunits in their cell cycle regulatory roles. The work described here also provides a basis and rationale for more extensive investigations into the roles of the remaining B55 family members (B β and B γ in mammalian systems), as well as for other highly related proteins that currently fall victim to the assumption of redundancy.

Chapter 5: Deregulation of G2 and mitosis due to B α depletion may lead to genome instability

5.1 Rationale and Hypothesis

The fidelity of genome replication in S phase and chromosome segregation during mitosis is essential to ensure accurate propagation of cellular genetic material, and is largely regulated by dynamic protein phosphorylation. Deregulation of the delicate balance of kinase and phosphatase activities that control DNA replication and mitotic progression can undermine these processes and lead to genome instability [reviewed in (Normand and King, 2010; Porter, 2008)]. The regulated activity of CDK1 plays a central role in limiting genome duplication to once per cell cycle, as well as ensuring ordered and timely progression through mitosis. Disruption of CDK1 activity in G2 phase/before mitosis can prompt relicensing and subsequent DNA re-replication (also known as endoreduplication) (Cai et al., 2006; Itzhaki et al., 1997). Accordingly, events that interfere with CDK1 activation, including high levels of CDK inhibitors p21 or p27 (Bates et al., 1998; Niculescu et al., 1998) or reduced levels of mitotic cyclins B and A (Bellanger et al., 2007; L'Italien et al., 2006; Ma et al., 2009; Machida and Dutta, 2007; Zhang et al., 1998; Zhang et al., 1996) trigger endoreduplication and polyploidization. Similarly, reduced CDK1 activity during mitosis causes mitotic defects that precipitate cytokinesis failure and tetraploidization (Cundell et al., 2013; McCloy et al., 2014). Therefore, reaching and maintaining minimal CDK1 threshold activities during G2 and mitosis appear to be essential to prevent processes that lead to polyploidization and destabilize the genome.

As described in Chapter 4, loss of B α leads to unscheduled APC/C-dependent degradation of mitotic cyclins and other G2/M regulators, which induces the collapse of pre-mitotic kinase activity thresholds to culminate in significantly reduced CDK1 activation in G2/M-synchronized HeLa cell populations. These observations strongly suggest that the biochemical environment generated by

depletion of B α may predispose these cells to genome instability and polyploidization. Therefore, I hypothesize that the B α -dependent defects in G2/M kinases and regulators undermine genome stability and enable polyploidization.

5.2 Results

5.2.1 Loss of B α leads to a post-mitotic increase in DNA content and cell size

To determine whether B α -dependent defects in cyclin stability and CDK1 activation precipitate polyploidization, I investigated changes in the post-mitotic DNA content in G2/M-synchronized HeLa cell populations. HeLa cells are known to be hypertriploid (3N+), and therefore the enumeration of polyploid DNA content should utilize multiples of 3N. However, for the sake of simplicity I will designate G1 phase DNA content as 2N and G2/M phase DNA content as 4N through the description and discussion of my results. Therefore, rather than using multiples of 3N, I will use multiples of 2N to describe polyploid genomic states. During the examination of B55-dependent effects on mitotic and G1 progression in RO3306-synchronized HeLa cells (Fig. 3.11), I noticed the appearance of a subpopulation of B α -depleted cells that exhibited as much as two-fold greater DNA content (8N) than that observed in G2 cells (4N) (Fig. 5.1A, black brackets and black arrow head). These ostensibly polyploid cells emerge as B α -deficient cell populations transition through mitosis and into G1 phase between 4 and 10h after release from the G2/M block, and continue to accrue until 14h post-release (Fig. 5.1A and D). Concomitantly, I observed the emergence of a subpopulation of enlarged cells that appeared to be greater in size and granularity than G2 cells (Fig. 5.1B, solid black boxes vs dashed black box). The co-incidence of >4N DNA content and enlarged cells suggests that these cells might comprise the same subpopulation. Visualization of the >4N DNA-containing cells on forward scatter (FSC) versus side scatter (SSC) dot plots confirms this notion and demonstrates that these hyperploid cells are indeed enlarged (Fig.

5.1C, orange boxes, brackets and dots). Quantification of the increase in DNA content demonstrates that polyploid B α -depleted cells accumulate gradually with significant differences relative to the control occurring at 5h and all subsequent timepoints after release (Fig. 5.1D). Similarly, HeLa cells stably expressing eGFP-TUBA and mCherry-H2B and transiently depleted of B α exhibit a significant increase in polyploid (Fig. 5.1E) and enlarged cells (Fig. 5.1F) at 14h following release. Interestingly, ablation of B δ significantly reduces the incidence of enlarged polyploid cells (Fig. 5.1D-F). It has been shown that partial inhibition of CDK1 results in aberrant mitoses that yield polyploid cells, and that partial inhibition of PP2A with okadaic acid rescues these mitotic defects (McCloy et al., 2014). Therefore, it is possible that the basal level of polyploid cells that appears in control populations is due to incomplete wash-out of RO3306 during release and subsequent residual CDK1 inhibition (Fig. 5.1D). In agreement with this, pre- and post-mitotic >4N DNA content is lower in thymidine-synchronized cell populations (~1.5-2% on average; Fig. 5.1G) than it is in RO3306-synchronized control cell populations (3.7-5.5% on average; Fig. 5.1D). Furthermore, the observation that depletion of B δ mitigates the basal level of polyploidization (Fig. 5.1D) implicates the PP2A-B δ complex as the target of the okadaic acid-mediated rescue of mitotic defects described by others (McCloy et al., 2014).

My findings show that depletion of B α results in a post-mitotic increase in DNA content and a concomitant increase in cell size, which strongly suggests that a subset of cells is undergoing polyploidization. Based on previous discoveries that a reduction in mitotic cyclin levels and/or CDK1 activity leads to mitotic defects and polyploidization, it is conceivable that the B α -dependent effects observed here are due to its role in cyclin stabilization and CDK1 activation (described in Chapter 4). These results also provide additional support for distinct roles of the B α and B δ subunits, and reinforce the notion that PP2A-B δ is the predominant CDK1-antagonizing heterotrimer.

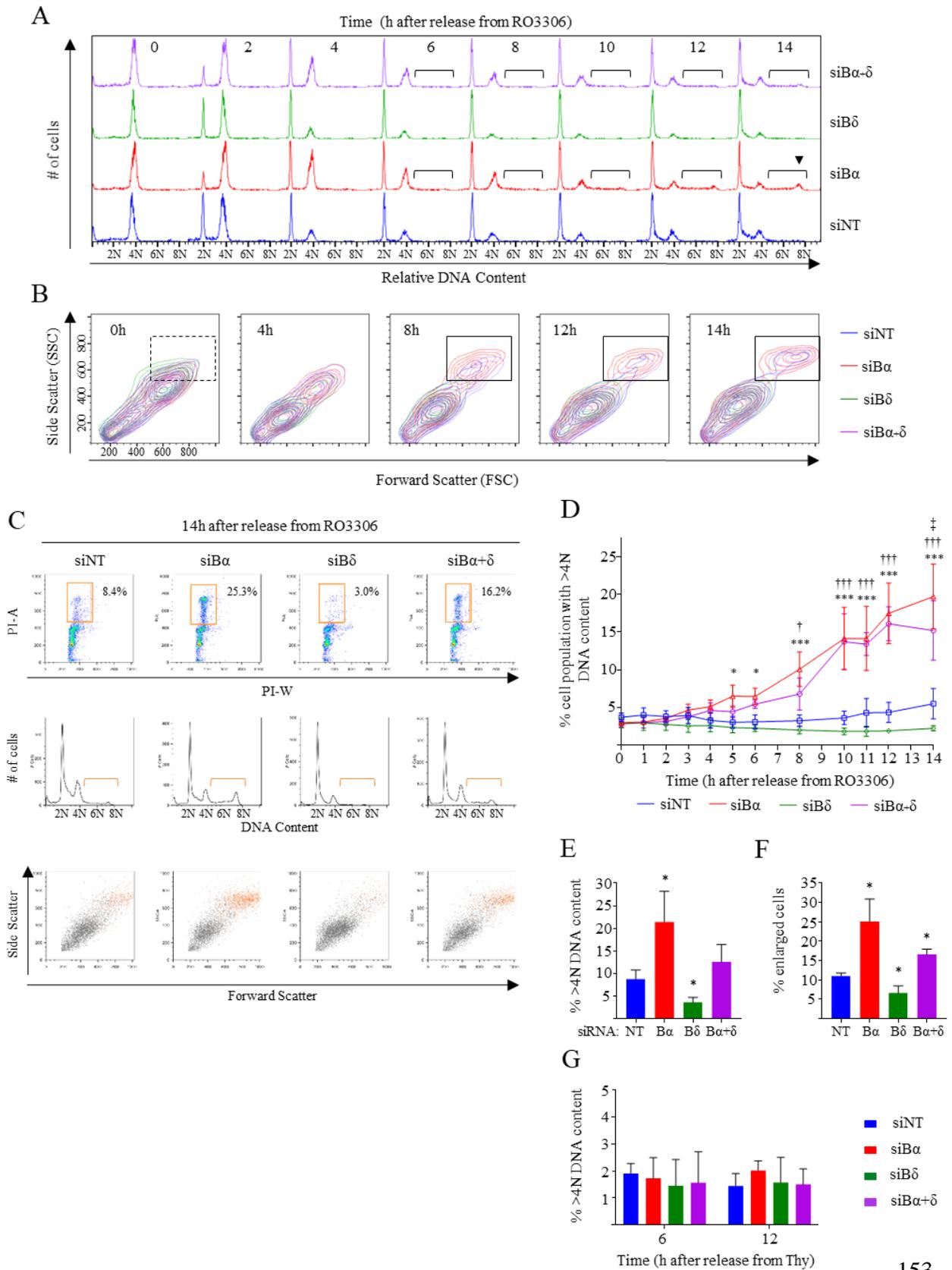


Figure 5.1: Loss of B α leads to a post-mitotic increase in DNA content and cell size

HeLa cell populations were treated with control, B α -, and/or B δ -targeting siRNA duplexes, and synchronized at the G2/M border by consecutive thymidine and RO3306 treatments as previously described. Following release from the RO3306 block, cells were collected at the indicated timepoints and processed for analysis by flow cytometry.

- A. Depletion of B α results in the appearance of cells with >4N DNA content post-mitosis. Samples were analyzed for relative DNA content by flow cytometry. Shown are DNA content histograms at selected timepoints, representative of four independent experiments. Black brackets delineate >4N populations. The black arrow head indicates the 8N DNA content peak at 14h after release from RO3306. Histograms were generated using FlowJo v10.0.1.
- B. Loss of B α produces a subpopulation of enlarged cells post-mitosis. Samples as in (A) were inspected for changes in cell size as determined by forward scatter (FSC) and side scatter (SSC). Forward scatter measures the overall size of individual cells, while side scatter measures surface granularity. Shown are contour plots (FSC versus SSC) of cell populations at selected timepoints, where increased abundance and proximity of lines represent increased cell density. Enlarged cell populations are demarcated by black boxes. Illustrated plots correspond to the same samples shown in (A) and are representative of four independent experiments. Contour plots were generated using FlowJo v10.0.1.
- C. Increased DNA content is associated with enlarged cell size. Upper panels: PI-A (area) versus PI-W (width) plotting was used to identify single cells with >4N DNA content (delineated by the orange boxes). Middle panels: relative DNA content histograms demonstrate the corresponding >4N DNA-containing population (orange brackets). Lower panels: Cells with >4N DNA content (from upper panels) were selected and visualized on a FSC versus SSC dot plot to identify the size distribution of these cells (orange dots). Dot plots, histograms, and visualization of selected populations were generated using FlowJo v 10.0.1. Plots represent samples from a single timepoint (14h following release from RO3306). Observations are representative of four independent experiments.
- D. DNA content gradually increases as B α -depleted cells progress through G2/M and into G1 phase. Cell populations containing >4N DNA content were measured in four independent experiments and quantified at the indicated timepoints following release from RO3306. (n=4, mean \pm s.d., * P <0.05 and *** P <0.001 for NT vs B α , † P <0.05 and ††† P <0.001 for NT vs B α + δ , ‡ P <0.05 for NT vs B δ , ANOVA).
- E. HeLa cells stably expressing eGFP-TUBA and mCherry-H2B were synchronized at the G2/M border as described above, collected at 14h after release from RO3306 treatment, and processed for analysis by flow cytometry. Cells were analyzed for relative DNA content by flow cytometry. The percent cell population with >4N DNA content was determined and graphed as above.
- F. Cell size for HeLa cells as in (E) was measured using FSC vs. SSC plots. Cells occupying the top right corner of FSC vs. SSC plots (as in black boxes in B) were quantified and graphed. Data are representative of three independent experiments (n=3, mean \pm s.d., * P <0.05, ANOVA).
- G. Cells with >4N DNA content were quantified in pre-mitotic (6 h) and post-mitotic (12 h) HeLa cell populations that were synchronized using the double thymidine blocking method.

5.2.2 HEK293 cells also exhibit a B α -dependent post-mitotic increase in DNA content

It is well established that HeLa cells have a highly abnormal genome that is characterized by hypertriploidy (3n+) and an unstable, variable karyotype (Adey et al., 2013; Landry et al., 2013; Macville et al., 1999). Therefore, to investigate whether depletion of B α induces polyploidization in the context of an inherently more stable genome and karyotype, I made use of the HEK293 cell line. Following the same procedure used in HeLa cells, control, B α -, and/or B δ -depleted HEK293 cells were synchronized at the G2/M border by consecutive thymidine and RO3306 treatments. Following release into mitosis, cells were collected at various timepoints and analyzed for relative DNA content by flow cytometry. Depletion of B α (single and co-depletion) from HEK293 cells induced a post-mitotic increase in DNA content at 30 hours after release (Fig. 5.2 A and B). Similar to the observations made in HeLa cells, loss of B α precipitated a two-fold increase in polyploid cells (Fig. 5.2B). Furthermore, B α -deficient HEK293 cells also appear to experience defective S-to-G2 transition, as a large proportion of the cell population remained in G1 and S phase at the end of the synchronization procedure (Fig. 5.2A, 0h timepoint, black arrow head). Interestingly, control and B δ -ablated cells also demonstrate a mild post-mitotic increase in DNA content (Fig. 5.2A and B). However, as suggested above (section 5.2.1) this is likely due to lingering effects of CDK1 inhibition, as parental HEK293 cells synchronized with thymidine alone did not exhibit a marked increase in polyploidization post-mitosis compared to parental cells (not treated with NT siRNA) synchronized with RO3306 (Fig. 5.2C).

Together, these results demonstrate that loss of B α also leads to a post-mitotic increase in polyploidization in the karyotypically more stable HEK293 cell line, indicating the robustness of B α -dependent destabilization of ploidy.

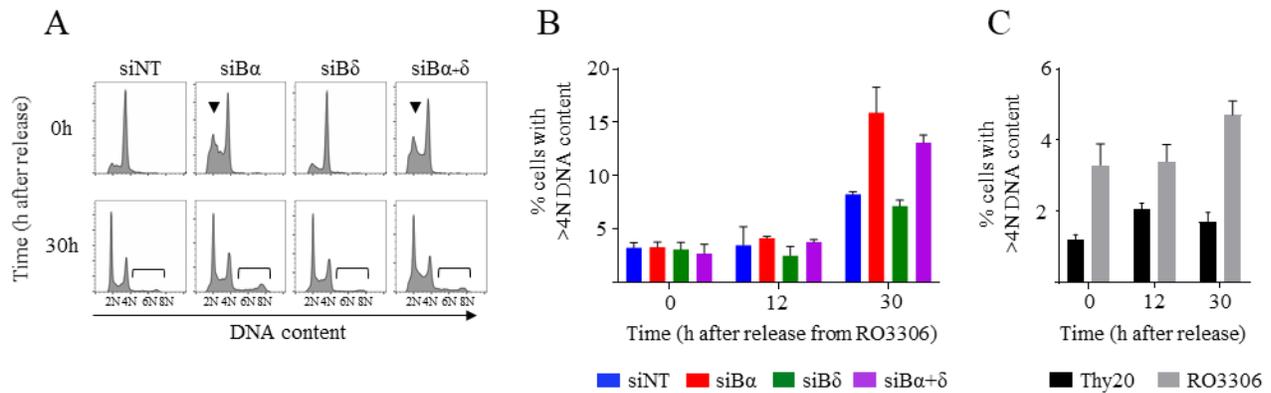


Figure 5.2: HEK293 cells also exhibit a B̑-dependent post-mitotic increase in DNA content

- HEK293 cells were treated with control, B̑-, and/or B̑-targeting siRNA duplexes, and synchronized at the G2/M border by consecutive thymidine and RO3306 treatments. After release from the RO3306 block, cells were collected at the indicated timepoints (0, 12, 30 h) and were processed for relative DNA content analysis by flow cytometry as previously described. Shown are DNA content histograms from samples collected at 0 h and 30 h after release from RO3306. Black brackets indicate cell populations with >4N DNA content.
- The percent cell population with >4N DNA content was quantified in samples from transfected cells in (A). The graph shows the average values calculated from two independent experiments, with the vertical lines indicating the variation between experiments.
- Treatment with RO3306 induces a basal increase in cells with >4N DNA content. Non-transfected HEK293 cells were synchronized to either the G1/S boundary by a single thymidine block (Thy20), or the G2/M border (RO3306) as in (A). Following release from the respective blocks, cells were collected at the indicated timepoints and analyzed for relative DNA content by flow cytometry as previously described. The percent cell population with >4N DNA content was quantified and graphed for each timepoint. The graph shows the average values calculated from two independent experiments, with the vertical lines indicating the variation between experiments.

5.2.3 Post-mitotic polyploidization may result from a B̑-dependent increase in cytokinesis failure

Cytokinesis is the final step of mitosis and proceeds in a highly ordered sequence of events that can be grouped into four stages: cleavage plane specification, cleavage furrow ingression, midbody formation, and abscission (Normand and King, 2010). Proper and timely completion of each stage is crucial to the integrity of the subsequent stage, and defects that cause failure at any of

these steps can disrupt the division process and lead to the fusion of daughter cells. If cells that failed cytokinesis (or mitosis) then undergo another round of DNA replication, they become polyploid. Therefore, cytokinesis failure represents an important mechanism by which cells can acquire polyploid genomic content. To investigate whether B α -dependent polyploidization is linked to cytokinesis failure, I synchronized control and B55-depleted HeLa cells stably expressing eGFP-TUBA and mCherry-H2B at the G2/M border, and monitored their progression through mitosis by live cell time-lapse microscopy (Fig. 5.3A). Cells undergoing cytokinesis failure were quantified as a percentage of the total number of cells that entered viable mitoses, and cells that died during mitosis were excluded from the analysis. Strikingly, the loss of B α results in a marked increase in cytokinesis failure (Fig. 5.3B). Closer inspection of the morphological phenotype of B α -dependent cytokinetic failures shows that these cells are able to specify a cleavage plane, undergo extensive cleavage furrow ingression, and form a midbody (Fig. 5.3A, see 90min). However, they fail to complete abscission, causing daughter cells to fuse (Fig. 5.3A). Interestingly, these cells also frequently display slowed or incomplete breakdown of central spindle microtubules (Fig. 5.3A, white arrow heads), suggesting that microtubule disassembly may be affected by loss of B α and may interfere with abscission.

Timely reactivation of PP2A-B55 complexes during mitotic exit has recently been shown to play an important role in ensuring the ordered/sequential progression of chromosome segregation and cytokinesis (Cundell et al., 2013). Specifically, PP2A-B α was shown to dephosphorylate PRC1 to promote its localization to the anaphase central spindle (Cundell et al., 2013), where it plays an essential role in microtubule bundling and interdigitation, midbody formation, and abscission [reviewed in (Normand and King, 2010)]. In agreement with this report, I observed prolonged phosphorylation of PRC1 at the B α -targeted T481 phosphorylation site in B α -depleted cells that were synchronized in prometaphase using consecutive thymidine and nocodazole treatments (Fig. 5.3C).

These findings suggest that B α -dependent polyploidization may, at least in part, be due to defects that disrupt the late stages of cytokinesis. I also confirm a previous report of impaired dephosphorylation of the central spindle component, PRC1; however, the exact role or relevance this has in B α -dependent cytokinesis failure requires further investigation. B δ depletion in prometaphase-synchronized cells also appeared to delay PRC1 dephosphorylation; however, this is likely due to a B δ -dependent delay in mitotic onset timing (described in section 3.2.7 and 3.2.8).

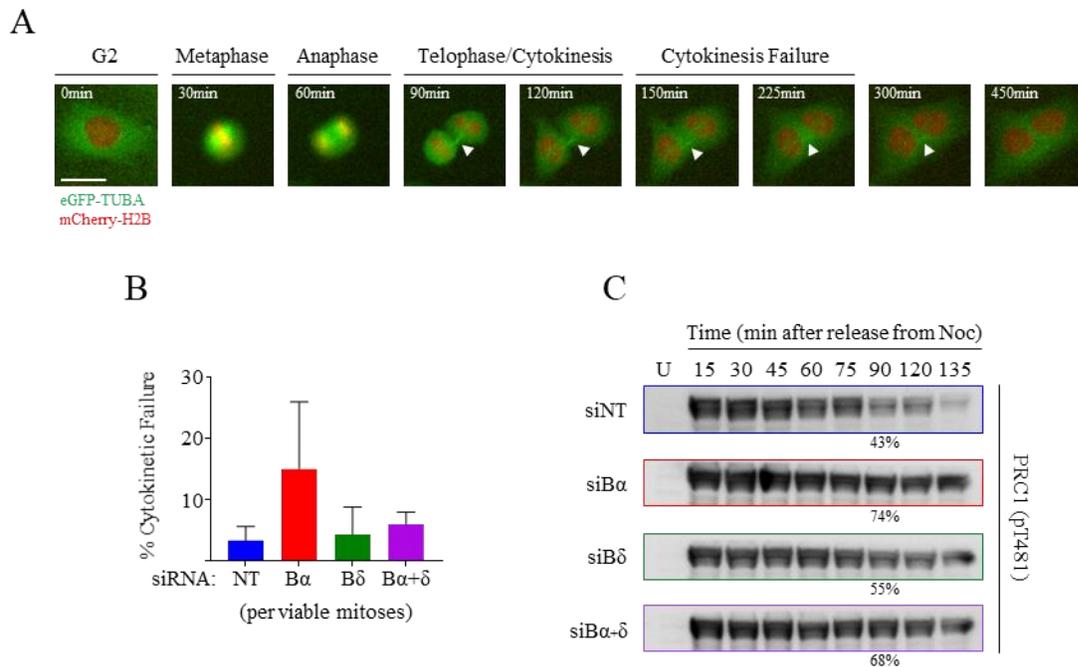


Figure 5.3: Post-mitotic polyploidization may due to a B α -dependent increase in cytokinesis failure

HeLa cells stably expressing eGFP-TUBA and mCherry-H2B were treated with control, B α -, and/or B δ -targeting siRNA duplexes, and synchronized at the G2/M border as described above. Following release, live cells were imaged by time-lapse microscopy as they progressed into and through mitosis. Images were taken every 15 minutes for 20 hours.

- A. Shown is a series of images illustrating a B α -depleted cell undergoing cytokinetic failure. The last frame before initiation of mitosis (defined by rounding up of the cell) was set as timepoint '0 minutes'. White arrow heads indicate the central spindle. Scale bar = 25 μ m. See Fig. 3.9 for images illustrating 'normal mitosis'.

- B. Loss of B α results in an increase in cytokinesis failure. The number of cells that failed cytokinesis throughout the duration of the imaging experiment was measured and quantified as a percentage of the total number of cells that entered viable mitoses (i.e. cells that entered mitosis but died during any of the mitotic phases were excluded from the quantification). Data are representative of three independent experiments, where 100-200 cells were analyzed per treatment in each experiment (n=3, mean \pm s.d.). Despite the lack of statistical significance over three experiments, B α depletion consistently had the highest number of cytokinetic failure in every experiment.
- C. B α -depleted cells exhibit impaired PRC1 pT481 dephosphorylation. HeLa cell populations were synchronized at Prometaphase by consecutive thymidine and nocodazole blocks (as in Fig. 3.7 and 3.8). Mitotic cells were then isolated by the mitotic shake-off method, plated, and collected at the indicated timepoints after plating. Samples were processed for analysis by Western blotting (same samples as in Fig. 3.7 and 3.8), and immunoblotted for PRC1 pT481. Percent values indicate the extent of phosphorylation that remains at 90 min after plating relative to the starting timepoint (15 min). Blots and percent values are representative of three independent experiments.

5.2.4 Depletion of B α leads to an increase in endoreduplication

Genome reduplication (also known as endoreduplication) in absence of nuclear division or mitosis represents another prominent mechanism that generates polyploid cells in both healthy and pathological physiological contexts. In normal cells, genome replication is limited to once per cell cycle by the strict regulation of replication origin licensing. Interestingly, origin licensing occurs in the framework of low CDK activity and high APC/C activity (Sivaprasad et al., 2007), a biochemical setting that appears to be present in B α -depleted, RO3306-synchronized cell populations.

Furthermore, it has been shown that decreased levels of mitotic cyclins (Ma et al., 2009; Machida and Dutta, 2007; Zhang et al., 1996) and disruption of CDK1 activity (Cai et al., 2006; Laronne et al., 2003; Ma et al., 2009; Ullah et al., 2008) can trigger extensive endoreduplication. These findings suggest that the conditions produced by B α depletion and G2/M-synchronization (using RO3306) are highly conducive to genome reduplication. While cytokinetic failure (and a subsequent round of DNA replication) may contribute to B α -dependent polyploidization in cells that entered mitosis, my findings described in Chapter 4 (Fig. 4.1) demonstrated that a significant proportion of G2/M-synchronized, B α -depleted cells do not enter mitosis, indicating that endoreduplication may also

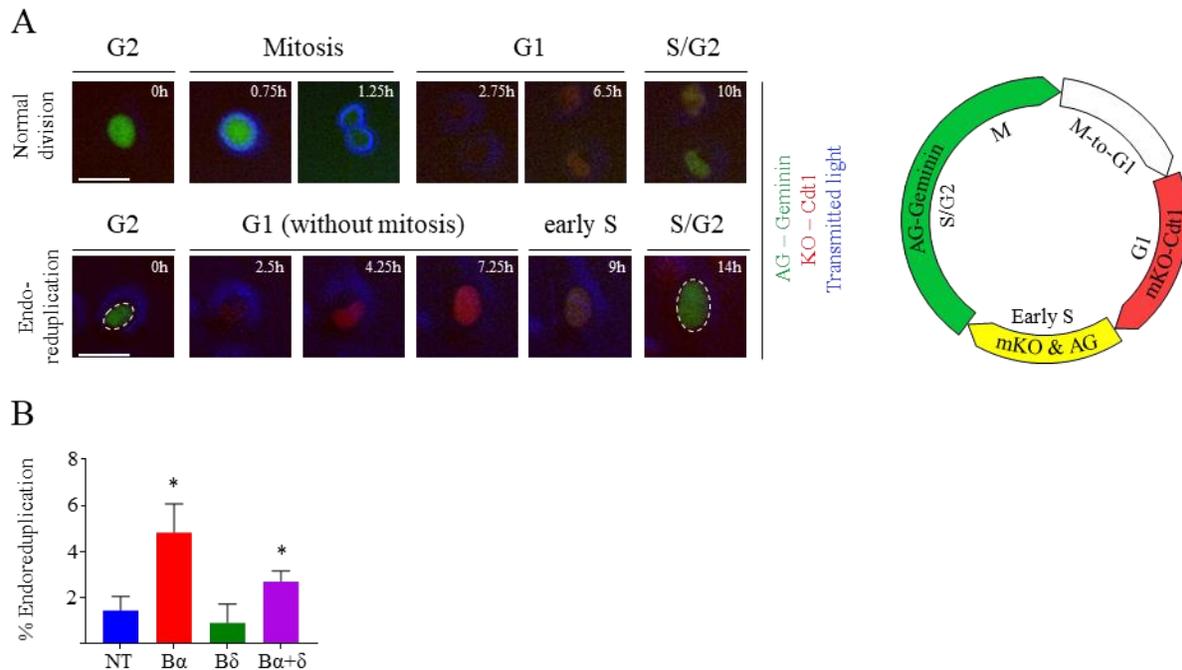


Figure 5.4: Depletion of B α leads to an increase in endoreduplication

HeLa-FUCCI cells stably expressing AzamiGreen-geminin (AG-geminin) and KusabiraOrange-Cdt1 (mKO-Cdt1) were treated with control, B α -, and/or B δ -targeting siRNA duplexes, and synchronized at the G2/M border as described above (Fig. 5.1). Following release, live cells were imaged by time-lapse microscopy as they progressed through at least one round of cell division (starting at the G2/M border). Images were taken every 20 minutes for 24 hours.

- A. The images illustrate cells undergoing normal cell division (from NT control, upper panels) and endoreduplication (from B α -depleted, lower panels). Images were taken using TexasRed (KO-Cdt1), GFP (AG-geminin), and transmitted light channels and were merged at each timepoint. Time indicates hours after release from RO3306. Scale bar = 25 μ m. The schematic illustrates the phase-dependent colour changes associated with the expression of the mKO-Cdt1 and AG-Geminin markers. The white dotted lines delineate the nucleus and serve to indicate the increase in nuclear size associated with reduplication of the genome.
- B. B α depletion results in a significant increase in the proportion of cells undergoing endoreduplication (in single and co-depleted populations). The number of cells undergoing endoreduplication [as illustrated by lower panels in (A)] was quantified as a percentage of the total number of viable cells. Data are representative of three independent experiments, where 150-200 cells were analyzed per treatment in each experiment (n=3, mean \pm s.d., *P<0.05, ANOVA).

contribute to increased DNA content. Therefore, I next investigated whether ablation of B α induces endoreduplication in G2/M-synchronized cells. To this end, control and B55-depleted HeLa-FUCCI

cells were synchronized to the G2/M boundary and imaged for 24 hours following release from RO3306 using live cell time-lapse microscopy. Irrespective of siRNA treatment, cell populations consistently synchronized to G2/M phase with efficiencies of 70-80%, as measured by the proportion of AzamiGreen-Geminin expressing cells (data not shown). Control and B δ -deficient cells mostly underwent normal mitoses, completing division within the first 3 hours after release, and progressed through G1 phase between 3-10 hours (as measured by the re-expression of KusibiraOrange-Cdt1) before initiating the next round of DNA replication (Fig. 5.4A, upper panels). A basal level of endoreduplication (up to 1.5%) was observed in these cell populations. Loss of B α (single and co-depleted) appeared to significantly increase the proportion of cells undergoing endoreduplication (3-5% in single and co-depleted populations) (Fig. 5.4A lower panels; and 5.4B). These cells appeared to “skip” mitosis (whilst still degrading AG-geminin), and progressed from G2 back to G1 phase and underwent another round of DNA replication (Fig. 5.4A, lower panel). Unsurprisingly, these cells also grew in size, with the nuclei enlarging noticeably as the cells entered a second round of DNA replication (Fig. 5.4A, lower panels, white dotted lines, 0 and 14 h timepoint).

These results demonstrate that polyploidization can also occur in B α -deficient cells that do not enter mitosis through the induction of endoreduplication. Based on previous studies, it is likely that B α -dependent endoreduplication is a consequence of the mitotic cyclin destabilization, reduced CDK1 activation, and APC/C reactivation that occurs in B α -depleted, G2/M-synchronized cells (Chapter 4).

5.2.5 B α depletion exacerbates endoreduplication induced by prolonged CDK1 inhibition

The central role of CDK1 inactivation in endoreduplication is generally accepted and has been observed in a wide array of species, ranging from maize (Grafi and Larkins, 1995) to *Drosophila* (Sigrist and Lehner, 1997) and human cells (Cai et al., 2006; Laronne et al., 2003; Ma et

al., 2009; Ullah et al., 2008). A recent study shed additional light on the mechanism by which CDK1 inactivation induces endoreduplication, showing that spontaneous reactivation of the APC/C and the degradation of its target proteins, including geminin, plays an important role in this process (Ma et al., 2009). In this study, the authors showed that prolonged RO3306-mediated inhibition of CDK1 (24-72 h) induces multiple rounds of genome reduplication, yielding large populations of cells with 8N and 16N DNA contents by 72 hours of treatment (Ma et al., 2009). My findings illustrate that cells lacking B α can reduplicate their genomes following release from RO3306-mediated G2/M arrest (section 5.2.4), and therefore in the absence of prolonged (24-72 h) CDK1 inhibition. This suggests that loss of B α may induce endoreduplication through mechanisms beyond its role in regulating CDK1 activation. This prompted me to investigate whether loss of B α may exacerbate RO3306-induced genome reduplication. To examine B55-dependent effects on endoreduplication in the context of CDK1 inhibition, control and B55-depleted, unsynchronized cell populations were treated with RO3306 for 12, 24, and 48 hours. In accordance with previous reports (Ma et al., 2009; Vassilev, 2006), prolonged exposure to RO3306 led to a gradual accumulation of cell populations in G2/M phase, with maximal arrest (65-70%) occurring by 24 hours, irrespective of siRNA treatment (Fig. 5.5A and B). By 48 hours a decrease in the proportion of G2/M cells was observed (Fig. 5.5B), and this was likely due to increased cell death (Fig. 5.5C). Control and B δ -deficient cell populations exhibited only a mild accumulation of 8N DNA-containing cells (2-3%) by 24 and 48 hours (Fig. 5.5D) that may also be attributable to RO3306-mediated toxicity (Fig. 5.5C). In contrast, depletion of B α resulted in a significant increase in 8N polyploid cells at 48 hours (Fig. 5.5D), despite the marked rise of cell death. It is important to note that Ma and colleagues supplemented RO3306 treatment with a pan-caspase inhibitor to prevent apoptosis, which enabled the accumulation and detection of cells with higher DNA content (Ma et al., 2009). In the absence of pan-caspase inhibitors, cells undergoing ectopic/forced endoreduplication likely undergo apoptosis due to cellular surveillance

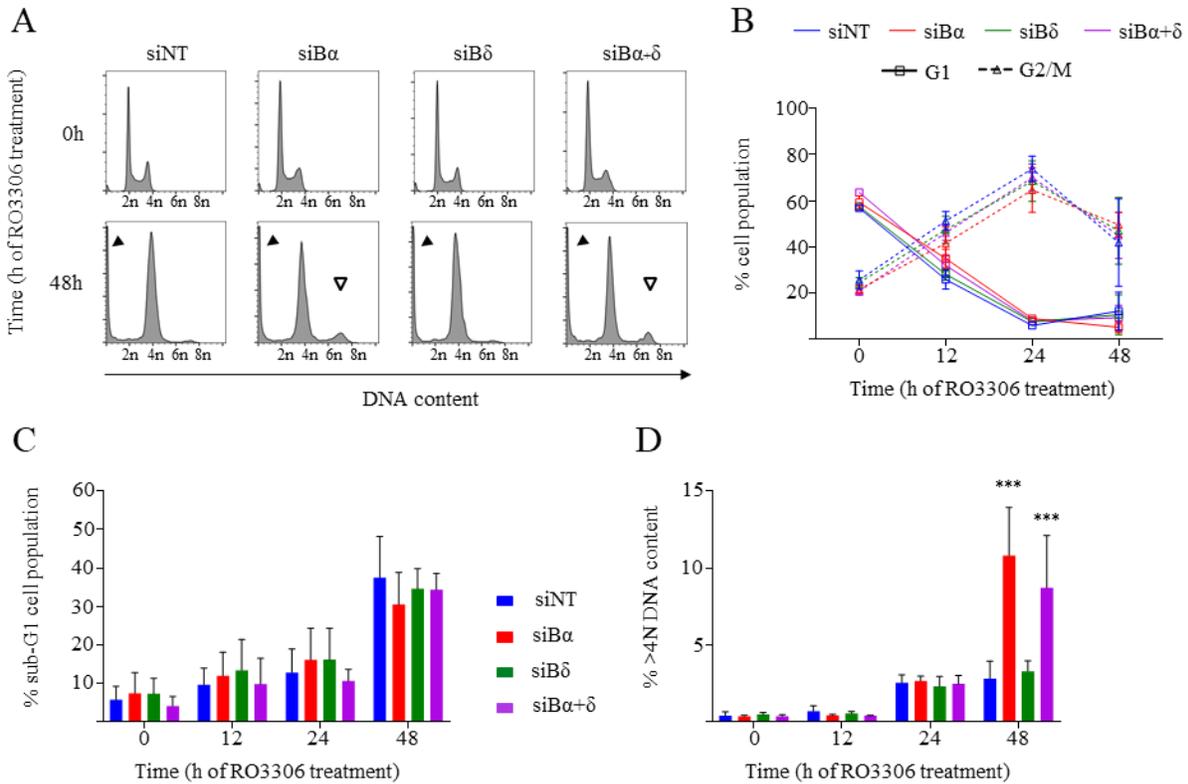


Figure 5.5: *Ba* depletion exacerbates endoreduplication induced by prolonged CDK1 inhibition

Asynchronously cycling control and B55-depleted HeLa cell populations were treated with RO3306 (10 μ M) for 0, 12, 24, and 48 h before being collected and processed for relative DNA content analysis by flow cytometry.

- Loss of *Ba* leads to the accumulation of polyploid cells after 48 h of RO3306 treatment. Relative DNA content histograms from samples collected at 0 and 48 h of RO3306 treatment are shown. White arrow heads indicate polyploid cells with 8N DNA content. Black arrow heads indicate a sub-G1 cell population, which represents dead or dying cells. Histograms are representative of three independent experiments.
- B55 depletion has no effect on RO3306-mediated G2/M arrest or cell cycle distribution at the indicated timepoints. Based on the DNA content histograms as in (A), the percent cell population in G1 and G2/M phase was measured and quantified using the Watson Pragmatic Cell Cycle Module in FlowJo v10.0.1. Data are representative of three independent experiments (n=3, mean \pm s.d.).
- Prolonged treatment with RO3306 induces a substantial amount of cell death, irrespective of siRNA treatment. The percent cell population with <2N DNA content (“sub-G1” population) was determined from DNA content histograms as in (A) by manual gating. Data are representative of three independent experiments (n=3, mean \pm s.d.).

- D. B α -depletion causes a significant increase in polyploid cells after 48 h of RO3306 treatment. The percent cell population with >4N DNA content at each timepoint was determined from DNA content histograms as in (A) by manual gating. Data is representative of three independent experiments (n=3, mean \pm s.d., *** $P\leq 0.001$, ANOVA).

mechanisms that protect cells against excessive genome reduplication. Interestingly, loss of B α does not prevent or reduce apoptosis, yet it allows the generation of cells with higher DNA content. The implications of this are unclear but indicate that endoreduplication is either occurring at a higher rate, or that loss of B α somehow protects polyploid cells from death possibly mimicking the effect of a pan-caspase inhibitor. These findings support the notion that loss of B α can enhance endoreduplication that is induced by CDK1 inhibition.

5.2.6 Gradual depolyploidization occurs following removal of CDK1 inhibition and may be due to increased post-mitotic death

It has previously been shown that cancer cells that undergo polyploidization as a result of forced endoreduplication are capable of depolyploidizing (Erenpreisa et al., 2005a; Erenpreisa et al., 2005b; Puig et al., 2008). Furthermore, others have reported increased apoptosis of cells that become polyploid as a result of aberrant mitoses that take place in the presence of reduced CDK1 activity (McCloy et al., 2014). Therefore, I examined the survival of the polyploid cell populations that emerged as a consequence of B α ablation in G2/M-synchronized cells. To determine whether B α -dependent polyploidization was stable over prolonged periods of time, the DNA content of control and B55-depleted, G2/M-synchronized cell populations was monitored over the course of 24, 48 and 72 hours after release from RO3306 treatment. Consistent with previous studies, I found that the abundance of polyploid cells in both control and B α -deficient contexts gradually decreased between 14 and 72 hours following release (Fig. 5.6A). RO3306 treatment also resulted in a notable level of

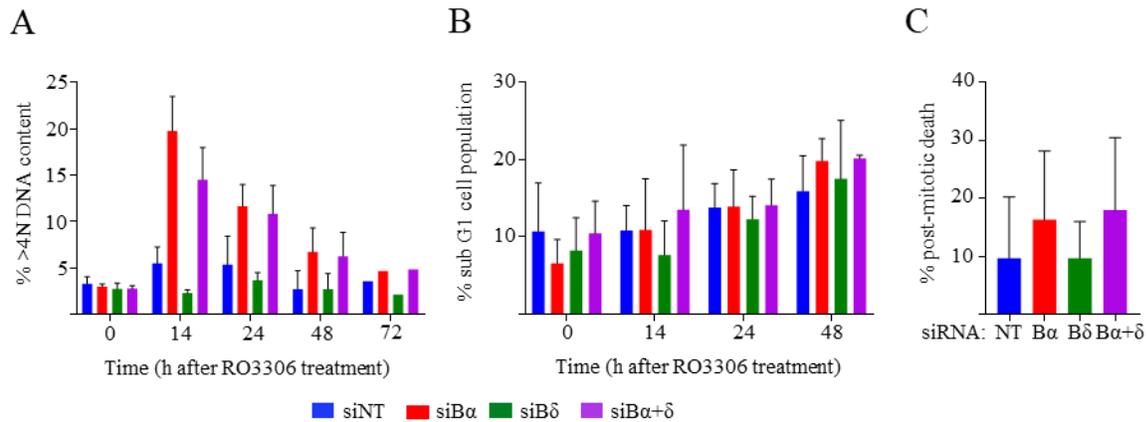


Figure 5.6: Gradual depolyploidization occurs following removal of CDK1 inhibition and may be due to increased post-mitotic death

Control and B55-depleted HeLa cell populations were synchronized at the G2/M border. Following release from RO3306, cells were collected at the indicated timepoints and processed for DNA content analysis by flow cytometry (A and B).

- Polyploid cell populations gradually decrease over prolonged periods of time. The percent cell population with $>4N$ DNA content was determined from DNA content histograms using FlowJo v 10.0.1. Data are representative of six independent experiments for 0 and 14 h timepoints, three independent experiments for 24 and 48 h timepoints, and one experiment for the 72 h timepoint ($n=1-6$, mean \pm s.d.).
- RO3306 induces a moderate, time-dependent increase in toxicity post-release. The percent cell population with $<2N$ DNA content (sub-G1 population) was determined from DNA content histograms. Data are representative of six independent experiments for 0 and 14 h timepoints, and three independent experiments for 24 and 48 h timepoints ($n=3-6$, mean \pm s.d.).
- B α depletion may sensitize cells to post-mitotic cell death. HeLa cells stably expressing eGFP-TUBA and mCherry-H2B were synchronized at the G2/M border, and progression through mitosis was monitored by time-lapse microscopy. The number of cells that died following mitosis was quantified as a percentage of the total number of cells that entered viable mitoses (including cells that exhibited cytokinesis failure). Data are representative of three independent experiments ($n=3$, mean \pm s.d.).

toxicity post-release, which appeared to modestly increase toward 48 hours (Fig. 5.6B). Interestingly, while $B\alpha$ -dependent polyploid populations disappeared at a more pronounced rate than in control cells (Fig. 5.6A), overall time-dependent cell death did not proportionally account for this reduction, as loss of $B\alpha$ induced a negligible increase in cell death as compared to control and $B\delta$ -depleted cells at 48 hours (Fig. 5.6B). An analysis of G2/M-synchronized HeLa cells stably expressing eGFP-TUBA and mCherry-H2B that were monitored by live cell time-lapse microscopy suggested a potential increase in the incidence of post-mitotic cell death in $B\alpha$ -depleted cells (Fig. 5.6C). However, these results were inconclusive and require more a more in-depth investigation. Taken together, it is possible that in the absence of $B\alpha$, cells may be more prone to post-mitotic apoptosis, and that this sensitivity could account for an accelerated rate of loss of polyploid cells.

Overall, my findings suggest that polyploid cell populations that arise in the absence of $B\alpha$ may not be stable over prolonged periods of time. While it is conceivable that these cells disappear as a consequence of eventual cell death, I cannot exclude the possibility that they undergo depolyploidization by other mechanisms that have been observed in cancer cells (Erenpreisa et al., 2005a; Erenpreisa et al., 2005b; Puig et al., 2008).

5.3 Key Findings

1. Loss of the B α (but not B δ) subunit leads to an increase in polyploid cells in G2/M-synchronized HeLa and HEK293 cell populations. Polyploidization of HEK293 cells supports a non-cell type-specific role for PP2A-B α in regulating genome stability.
2. B α depletion may lead to polyploidization by at least two distinct mechanisms:
 - a. Post-mitotic polyploidization may be due to an increase in the incidence of cytokinesis failure.
 - b. Polyploidization in cells that do not undergo mitosis may proceed by endoreduplication.
3. Loss of B α exacerbates endoreduplication caused by prolonged, RO3306-mediated CDK1 inhibition.
4. The B α depletion-mediated polyploid cell population is not stable over prolonged periods of time following release from RO3306 treatment.

5.4 Discussion

The regulation of CDK1 activity is a central theme of studies of the mechanisms that modulate genome integrity. Much of this focus has been directed at understanding the pathways that restrain CDK1 activity in response to DNA damage, and prevent entry into mitosis in the presence of DNA lesions [reviewed in (Donzelli and Draetta, 2003; Zhou and Elledge, 2000)]. A growing body of evidence now supports the notion that disruption of CDK1 activation or sub-threshold levels of CDK1 activity during mitosis can lead to defects that undermine genome integrity and generate polyploid cells (Cai et al., 2006; Itzhaki et al., 1997; Ma et al., 2009; McCloy et al., 2014).

In Chapter 4, I presented evidence that loss of B α results in defective activation of CDK1 and other important mitotic regulators, which led me to hypothesize that these B α -dependent defects

might challenge genome stability. Here, I demonstrate that loss of B α indeed triggers polyploidization by at least two distinct mechanisms, and show that the polyploid state may not be stable over prolonged periods due to depolyploidization or cell death.

In addition to producing cells with >4N DNA content in HeLa cell populations, a role for B α deficiency in polyploidization was further substantiated by experiments in the HEK293 cell line. These experiments showed that loss of B α may prompt genome instability in the context of a more stable genome and karyotype, and is not primarily a by-product of the inherent instability of the HeLa genome. However, the extrapolation of these results is limited by the fact that HEK293 cells are by no means representative of “normal” diploid human cell systems. HEK293 cells were originally immortalized by adenoviral transfection and exhibit a large amount of copy number alterations (CNAs) and other significant chromosomal abnormalities (Lin et al., 2014; Stepanenko and Dmitrenko, 2015). Importantly, constitutive expression of the adenoviral proteins E1A/E1B in these cells (Komorek et al., 2010) deregulates p53-dependent pathways (Stepanenko and Kavsan, 2012) that may be essential to suppress the propagation or survival of polyploid cells that arise due to mitotic defects, including cytokinesis failure (Fujiwara et al., 2005; Livingstone et al., 1992; Margolis et al., 2003; Vogel et al., 2004). Therefore, similar to HeLa cells, HEK293 cells are inherently more susceptible to polyploidization. Despite these abnormalities however, HEK293 cells are widely considered to be non-transformed, are hypo- or pseudotriploid, and exhibit a stable karyotype over many generations (Bylund et al., 2004). Therefore, these findings bolster a role for PP2A-B α in maintaining genomic stability. Nonetheless, further investigation in diploid, karyotypically stable, p53-competent cell lines is warranted to determine the relevance and mechanism of polyploidization in B α -deficient cells. Interestingly, it has been demonstrated that functional p53 does not always prevent tetraploidization resulting from mitotic errors (Uetake and Sluder, 2004; Wong and Stearns, 2005), and it would be interesting to examine whether B α -

dependent polyploidization can occur in a wild-type p53 background. To this end, isogenic p53-competent and p53-null mouse embryonic fibroblast cell lines (MEFs) could be generated and used to determine whether loss of B α may undermine genome integrity by facilitating polyploidization.

It has previously been shown that cells undergoing mitosis in the presence of reduced CDK1 activity can become polyploid as a consequence of cytokinesis failure (followed by a round of DNA replication) (Cundell et al., 2013; McCloy et al., 2014). My results described in Chapter 4 indicate that B α -depleted, G2/M-synchronized cells may be entering and progressing through mitosis in the presence of incompletely activated CDK1, raising the possibility that this effect may translate to cytokinesis failure in these cells. However, the precise mechanism by which cells fail cytokinesis in the B α -deficient context appears to be distinct from that observed in cells with impaired CDK1 activity (Cundell et al., 2013; McCloy et al., 2014). Cells undergoing mitosis in the presence of low doses of RO3306 exhibit premature initiation of cytokinesis despite an active spindle assembly checkpoint. This results in unsegregated chromosomes being trapped in the cleavage plane, leading to cytokinesis failure and yielding daughter cells with polyploid DNA contents (McCloy et al., 2014). Similarly, cells entering mitosis in the presence of the CDK inhibitor flavopiridol initiate cleavage furrow ingression during metaphase and fail to segregate sister chromatids (Cundell et al., 2013). The prolonged presence of chromatin in the cleavage plane then leads to the abortion of cytokinesis (Mullins and Biesele, 1977; Shi and King, 2005). Interestingly, this phenotype resembles that observed in cells depleted of MASTL (Cundell et al., 2013; Diril et al., 2016; Voets and Wolthuis, 2010), suggesting that impaired CDK1 activation decouples mitotic exit events/spindle assembly checkpoint and cytokinesis. However, in the absence of B α no notable chromosome segregation defects occurred, and post-mitotic polyploidization appeared to be due to failed abscission. Therefore, although B α depletion and partial inhibition of CDK1/depletion of MASTL can lead to cytokinesis failure, the mechanisms of collapse differ substantially. This suggests that reduced CDK1

activation in B α -deficient cells is unlikely to be the sole driving force behind the observed cytokinesis failure. While the experimental data provided here are insufficient to elucidate the mechanism by which loss of B α causes cytokinesis failure, a possible mechanism can be proposed. One notable difference that distinguishes the experimental conditions in the work by McCloy *et al.* (2014) and my own experiments is that they specifically inhibited CDK1 activity, whereas depletion of B α results in decreased levels and defective activation of multiple mitotic kinases besides CDK1, including MASTL, PLK1, and AURKA. Importantly, PLK1 is an essential positive regulator of cytokinesis. PLK1 localizes to the central spindle/midzone during anaphase and remains at the midbody during telophase and cytokinesis (Lee *et al.*, 1995). Its precise localization enables it to mediate early and late cytokinetic events ranging from initiation of cleavage furrow ingression (Brennan *et al.*, 2007; Burkard *et al.*, 2007; Petronczki *et al.*, 2007; Santamaria *et al.*, 2007) to priming factors required for abscission (Aumais *et al.*, 2003; Bastos and Barr, 2010; Fabbro *et al.*, 2005; Lowery *et al.*, 2007). The extensive involvement of PLK1 in the regulation of key components that act throughout cytokinesis strongly suggests that it is an important modulator of ordered progression. Therefore, reduced PLK1 activity in B α -depleted, G2/M-synchronized cells may lead to defects or delays in the ordered phosphorylation and regulation of PLK1 substrates during telophase and cytokinesis, which may ultimately undermine the stability of this process. While PLK1 targets many components of the cytokinetic machinery, the microtubule-bundling protein PRC1 is of particular relevance to the failed abscission phenotype because both PP2A-B α and PLK1 converge to regulate its timely localization and activity. PRC1 localizes to the central spindle during anaphase, where it supports microtubule bundling and interdigitation (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). Dephosphorylation of PRC1 at pT481 by PP2A-B α promotes its initial localization and is required for a subsequent activating phosphorylation at T602 by PLK1 (Cundell *et al.*, 2013). Furthermore, PRC1 serves as an important central spindle-associated binding partner for PLK1 and ensures

properly localized action of PLK1 during cytokinesis (Neef et al., 2007). Depletion of B55 subunits has been shown to delay central spindle assembly and contraction (Cundell et al., 2013). Consistent with this report, my findings demonstrate that loss of B α markedly delays pT481 dephosphorylation, suggesting that central spindle assembly may be delayed as well. While it remains to be proven experimentally, it is possible that postponed central spindle assembly and contraction may precipitate defective spindle breakdown, which would interfere with the abscission process. In support of this, my results indicate that microtubule disassembly may be impaired in B α -depleted cells undergoing cytokinesis failure (Fig. 5.3A). Therefore, depletion of B α might have multiple effects that converge on central spindle dynamics and function: (1) it may delay central spindle assembly by reducing the ability of PRC1 to localize properly, (2) this, in turn, would affect PLK1 localization, which combined with (3) reduced PLK1 activity may impair proper ordering of cytokinetic events, ultimately culminating in failed abscission. Further investigation is needed to elucidate the mechanism by which loss of B α may cause abscission failure.

Remarkably, my findings show that disruption of PP2A-B α , CDK1, and MASTL have highly related outcomes (cytokinesis failure), but that the underlying mechanisms are distinct. This may be due to the widespread pre-mitotic effects that depletion of B α exerts on G2/M-synchronized cells. This further suggests that the relationship/interaction between PP2A-B α , CDK1, MASTL, and other mitotic kinases is more complex than currently perceived.

In addition to cytokinesis failure, my results demonstrate that endoreduplication (DNA re-replication in the absence of mitosis) represents another mechanism by which B α -dependent polyploidization can occur. Inactivation of mitotic CDK1 and/or ectopic activation of the APC/C during G2 phase are widely considered to be triggers of endoreduplication. Accordingly, reduced concentrations of the mitotic cyclins B1 and A2 (Ma et al., 2009; Machida and Dutta, 2007; Zhang et al., 1996), and depletion of the APC/C inhibitor Emi1, induce genome reduplication (Di Fiore and

Pines, 2007; Machida and Dutta, 2007). A recent study provided additional insight, demonstrating that prolonged RO3306-mediated inhibition of CDK1 leads to unscheduled activation of the APC/C, and robustly induces multiple rounds of genome reduplication (Ma et al., 2009). The authors showed that activation of the APC/C was dependent on PLK1 and CDK2-cyclin A2 activities. Specifically, it was proposed that in the presence of *persistent* CDK1 inhibition PLK1 triggers SCF ^{β -TrCP1}-dependent degradation of EMI1, relieving inhibition of the APC/C and enabling the degradation of its substrates, including cyclins and geminin. Destruction of geminin then releases the replication licensing factor, CDT1, which, combined with re-expression of cyclin E2, leads to genome reduplication (Ma et al., 2009). Interestingly, endoreduplication in B α -depleted, G2/M-synchronized cells appears to proceed by a related but different mechanism. I observed that while depletion of B α in the presence of CDK1 inhibition also precipitated the reactivation of the APC/C, this resulted in the concomitant degradation of PLK1 and cyclin A2 (in addition to other APC/C substrates), suggesting that activity of PLK1 and CDK2-cyclin A2 is not likely to be involved in or required for APC/C activation. Furthermore, EMI1 levels were unaffected in my experiments, suggesting that the APC/C was reactivated despite the presence of this inhibitory protein. Degradation-independent mechanisms that prevent EMI1 from binding to and inhibiting the APC/C have been described (Lenart et al., 2007; Moshe et al., 2011; Sumara et al., 2004; van Vugt et al., 2004). It is important to point out that depletion of PLK1 or β -TrCP1 in the experiments conducted by Ma *et al.* only partially rescued/reduced endoreduplication, suggesting that this process is not fully dependent on PLK1 (Ma et al., 2009). Therefore, the PLK1- SCF ^{β -TrCP1}-EMI1 axis does not appear to be involved in APC/C reactivation in B α -depleted cells. Strikingly, the APC/C also appeared to be activated more quickly in the absence of B α (at 9h of RO3306 treatment as compared to 16h in the study conducted by Ma and colleagues (Ma et al., 2009). Importantly, the same synchronization timeline prior to addition of RO3306 was used by Ma *et al.* and in my experiments, allowing for this comparison. Furthermore,

endoreduplication in B α -deficient cells did not require persistent chemical inhibition of CDK1 but occurred after RO3306 was washed out. These notable differences reinforce the notion that B α -dependent reduplication proceeds by a different mechanism. In Chapter 4, I proposed that loss of B α leads to the collapse of pre-mitotic phosphorylation due to the inability of these cells to achieve or maintain a minimum kinase activity threshold. I propose that this deficiency may also form the basis for B α -dependent endoreduplication. In general, after initial replication origin licensing and firing during early S phase, CDK activity levels rise from late S phase until mid-mitosis and suppress re-licensing and re-replication of the genome. Endoreduplication is widely perceived to be a consequence of alterations that cause CDK activity to fall below the threshold required to suppress replication licensing, and subsequent re-replication is triggered by a renewed increase in the expression and activities of CDK-cyclin E/A complexes. While the current dogma is focused on the role of CDK activities in this process, my findings suggest that it can be extended more broadly to include the activities of G2 and mitotic kinases. As the activities of these kinases fall due to the pre-mitotic collapse triggered by loss of B α , APC/C is reactivated and results in the degradation of its substrates, including geminin (as observed in B α -dependent endocycles), allowing Cdt1 to reaccumulate and initiate origin re-licensing. Interestingly, in the absence of B α cyclin E1 and E2 levels rise more rapidly than in control and B δ -depleted cells (Fig. 3.12), which would enable the initiation of re-replication. While the mechanism of APC/C reactivation in this model is unclear, it is possible that unscheduled reactivation of Fcp1 (due to a drop in inhibitory kinase activity) is involved, as proposed in Chapter 4. Moreover, in this model, prolonged CDK1 inhibition is not required for endoreduplication, as reactivation of the APC/C results in degradation of mitotic cyclins and other mitotic regulators, thereby substantially impeding entry into mitosis. In this scenario, depletion of B α would partially prevent cells from entering mitosis and at the same time create the conditions necessary for relicensing, thereby enabling re-replication upon accelerated accumulation

of E-type cyclins. Therefore, in the context of B α -depleted and G2/M-synchronized cell populations, destabilization of mitotic cyclins and reduced CDK1 activation may predispose cells to endoreduplication, which is exacerbated by the collapse of other G2 and mitotic kinase activities. The additional role of B α -mediated stabilization of other kinase activities could also explain why loss of B α exacerbates reduplication induced by prolonged CDK1 inhibition. These results also imply that kinase activities besides CDK1 are important in the suppression of genome reduplication.

Endoreduplication has also been proposed to occur as a survival mechanism in response to mitotic catastrophe. Some p53-mutant cancer cells have been shown to engage in endoreduplication instead of undergoing apoptosis following treatment with anti-mitotic compounds such as vinblastine (Erenpreisa et al., 2005a). While the mechanism of action for vinblastine is completely unrelated to that of RO3306, they both belong to a class of “anti-mitotic” compounds. Strikingly, it has also been shown that cancer cells that become polyploid by undergoing endoreduplication can re-enter the mitotic cycle, thereby depolyploidizing (Erenpreisa et al., 2005a; Erenpreisa et al., 2005b; Prieur-Carrillo et al., 2003; Puig et al., 2008). In agreement with this, I observed entry into mitosis of cells that underwent B α -dependent endoreduplication, albeit at a very low frequency. This raises the question as what caused the extensive destabilization/disappearance of the polyploid populations as observed in my experiments. Considering that cytokinesis failure appeared to account for a larger proportion of the polyploid populations, and that I observed a B α -dependent increase in post-mitotic cell death, it is possible that most of these cells fell victim to this fate. This is in agreement with a recent study that demonstrated increased post-mitotic death in cells that became polyploid due to cytokinesis failure (Ohashi et al., 2015).

In summary, I show that loss of B α (but not B δ) can lead to genome instability due to cytokinesis failure and endoreduplication, and propose that this is largely due to a B α -dependent collapse in G2/M kinase activities. The data presented in this chapter underscore the importance of

balanced kinase and phosphatase activities in maintaining cell cycle directionality and genome stability. Historically, studies of the mechanisms that control cell cycle fidelity and genome integrity have favoured kinases as the central players. Based on findings by others and those described by me, the essential contribution of protein phosphatases to these regulatory circuits is rapidly emerging. While it is possible that the effects of B α depletion are indirect and a consequence of B α -dependent loss of kinase activities, my findings nonetheless support a central role for phosphatases in modulating phosphorylation thresholds that drive cellular processes. The importance of kinase and phosphatase activity thresholds are further discussed in Chapter 6.

Chapter 6: Discussion and Conclusions

6.1 Summary

The purpose of this study was to explore the cell cycle regulatory functions of PP2A-B α and -B δ heterotrimers, and to examine whether disruption of these complexes undermines the cell division process and predisposes cells to oncogenic events. The results presented herein demonstrate that these highly related proteins perform distinct functions in cell cycle regulation and provide the basis for in-depth future investigations.

In **Chapter 3**, I used various cell cycle synchronization techniques to demonstrate that the B α and B δ subunits differentially regulate progression through multiple cell cycle phases and phase transitions. My findings illustrate for the first time in a mammalian model system that B α -containing PP2A complexes positively regulate the timely completion of DNA replication. While PP2A-B δ may also play a role in S phase progression, the B α subunit appears to be the predominant effector of B55-dependent activities in S phase (Fig. 6.1A). Isoform-specific roles for the B α and B δ subunits were also identified in the regulation of mitotic kinetics. In the context of G1/S synchronization, PP2A-B δ (but not -B α) regulates mitotic duration, and an analysis of mitotic progression in prometaphase-synchronized cell populations revealed that PP2A-B δ complexes promote the initiation of mitotic exit (Fig. 6.1B). PP2A-B α , on the other hand, appears to drive later stages of mitotic exit and the transition into G1 phase (Fig. 6.1B). While recent studies have significantly improved our understanding of the regulation and functions of PP2A-B55 complexes in mitotic exit, the current dogma still assumes functional redundancy between the B α and B δ isoforms (Cundell et al., 2013; Cundell et al., 2016; Hein et al., 2017; Machado et al., 2010). My findings advance current models by showing that these highly related subunits not only play nonredundant roles but may be activated in a hierarchical temporal order during mitotic exit, where PP2A-B δ activation precedes that of PP2A-B α complexes. An investigation of G1 phase kinetics in G2/M-synchronized cell populations also uncovered previously unknown isoform-specific B55 effects. Specifically, my findings indicate that PP2A-B α restrains progression through G1 phase, possibly by

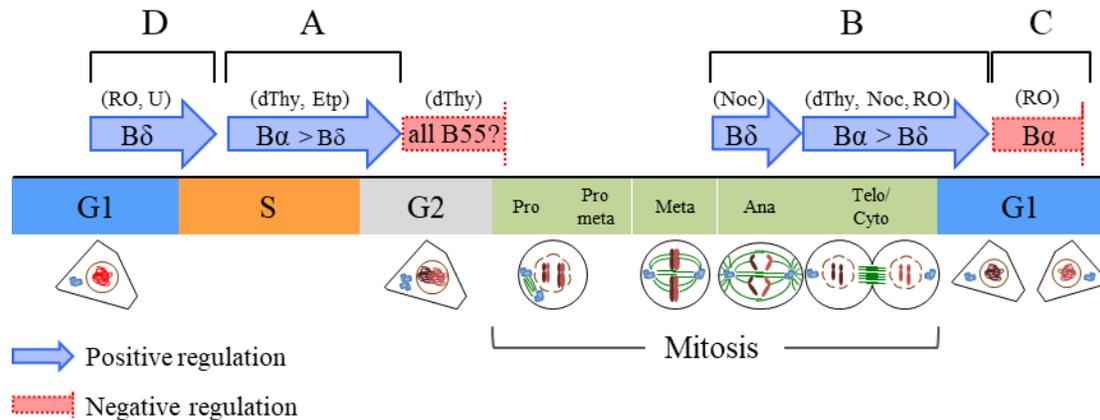


Figure 6.1: Overview of B55-regulated cell cycle progression

Using various cell cycle synchronization techniques, the experiments in **Chapter 3** identified previously undescribed roles and expanded upon established activities of the B α and B δ subunits in cell cycle regulation.

The abbreviations enclosed by parentheses indicate the method of synchronization that was used to reveal the respective regulatory effect. dThy = double thymidine block, synchronization at G1/S boundary; Etp = Etoposide treatment (48 h), G2 arrest; RO = consecutive single thymidine and RO3306 blocks, synchronization at G2/M boundary; Noc = consecutive single thymidine and nocodazole treatments, arrest in Prometaphase; U = unsynchronized. The ‘greater than’ symbol (>) denotes apparent importance of the subunits in a given process.

- E. B α positively regulates timely progression through S and into G2 phase. PP2A-B δ may also play a role, albeit much less prominent.
- F. B δ and B α play nonredundant roles in the regulation of mitotic exit, and may be activated in a sequential order. PP2A-B δ appears to regulate early mitotic exit events, including mitotic exit initiation. PP2A-B α plays a more prominent role during the later stages of mitotic exit and the transition into G1 phase.
- G. B α restrains progression through G1 phase.
- H. B δ may positively regulate G1-to-S phase progression, possibly through regulatory effects on a late G1 checkpoint.

negatively regulating G1/S cyclin expression (Fig. 6.1C). On the contrary, PP2A-B δ appears to positively regulate progression through G1 phase, and may exert its effects on a late G1 phase checkpoint (Fig.

6.1D). Taken together, the data in **Chapter 3** paint a new picture of PP2A-B55 complexes in cell cycle regulation, where B α -containing heterotrimers have emerged as regulators of S phase, late mitosis, and

G1 phase progression, while B δ -containing complexes appear to control early mitotic exit events, and the G1-to-S phase transition.

In **Chapter 4**, I more closely investigated the role of PP2A-B α and -B δ complexes in the regulation of the S-to-G2 and G2-to-M transition. As cell progress through G2 phase and prepare for mitosis, the activity of kinases increases, while that of phosphatases decreases, favouring an overall surge in protein phosphorylation (Domingo-Sananes et al., 2011). Once kinases surpass and phosphatases drop below respective activity thresholds, CDK1-cyclin B1 is activated and drives entry into and progression through mitosis. My findings demonstrate that PP2A-B α plays a crucial role in the stabilization of the ‘high kinase – low phosphatase activity’ environment. Specifically, in cell populations that were arrested at the G2/M border by chemical inhibition of CDK1, depletion of B α triggered the loss of pre-mitotic phosphorylations and the APC/C-dependent degradation of G2 and mitotic regulators, culminating in defective activation of CDK1-cyclin B1 and impaired entry into mitosis. The collapse of the pre-mitotic state was not due to defects in the initiation of G2/M gene expression or substrate phosphorylation programs. Instead, my findings suggested that PP2A-B α may positively regulate the activity of a hitherto unknown kinase that contributes to the attainment of the pre-mitotic kinase activity threshold. Previous studies that investigated the role of PP2A-B55 in the G2-to-M transition concluded that it acts as a negative regulator that counteracts (pre-)mitotic phosphorylations. In mammalian systems it has been largely assumed that all B55 subunits operate in this manner. According to my results, however, this role is largely fulfilled by the B δ isoform. Therefore, the results presented in **Chapter 4** revealed a novel and surprising role for the PP2A-B α complex as a positive regulator of the pre-mitotic state and showed that the widely-accepted anti-mitotic activity of B55 subunits is largely carried out by the B δ isoform.

Proper regulation of CDK1 activity during cell division is essential to the maintenance of genome stability. The experiments in **Chapter 4** illustrated that depletion of the B α subunit leads to defective CDK1-cyclin B1 activation in G2/M-synchronized cell populations and was associated with a reduced mitotic index. Therefore, in **Chapter 5** I investigated whether B α loss, and the concomitant decrease in

G2 and mitotic kinase activities, undermines the stability of the genome in the subsequent cell division. Indeed, I found that B α -depleted cell populations exhibited a significant increase in enlarged polyploid cells over the course of 14 hours following the release from G2/M arrest. This effect was not restricted to HeLa cells and was also observed in the karyotypically more stable HEK293 cell line. Time-lapse imaging of live HeLa cells subsequently showed that loss of B α precipitated an increase in cytokinesis failure in cells that underwent mitosis. Interestingly, the failure always occurred at the abscission stage of cytokinesis. This indicates that the B α -dependent cytokinesis failure was not the result of defective CDK1 activation, as cytokinesis failure due to impaired CDK1 activity is associated with abortive chromosome segregation (McCloy et al., 2014). Within the subpopulation of cells that did not enter mitosis after release from G2/M arrest I also observed a significant increase in cells that underwent endoreduplication. Therefore, loss of B α can lead to genome instability by two distinct mechanisms: cytokinesis failure and endoreduplication. Interestingly, the polyploid population was not stable over prolonged periods of time and gradually disappeared by 72 hours after release from G2/M arrest, suggesting that these cells may have undergone depolyploidization or cell death. Depletion of the B δ subunit did not result in polyploidization. In fact, it exhibited a mild protective effect. Altogether, my findings in **Chapter 5** demonstrate that PP2A-B α (but not-B δ) may support genome stability, possibly through its role in the maintenance of a ‘high kinase – low phosphatase activity’ pre-mitotic state.

The work presented in this study has generated new insights into cell cycle regulatory functions of the most widely expressed B55 family members, B α and B δ , and provides evidence of markedly distinct roles for these subunits. In addition, my findings reveal a novel B α -dependent role in the regulation of mitotic kinase activities, which may ultimately underlie the maintenance of genome stability.

6.2 Proposed working model

On the basis of my experimental data and literature-curated findings, I propose four hypothetical mechanisms (Fig. 6.2A, B, C, and D) by which PP2A-B α and PP2A-B δ heterotrimers might regulate progression through various cell cycle phases and transitions. The objective of these four proposed working models is to stimulate and guide future studies that may lead to a better understanding of not only the functions of PP2A holoenzymes, but cell cycle regulation more broadly.

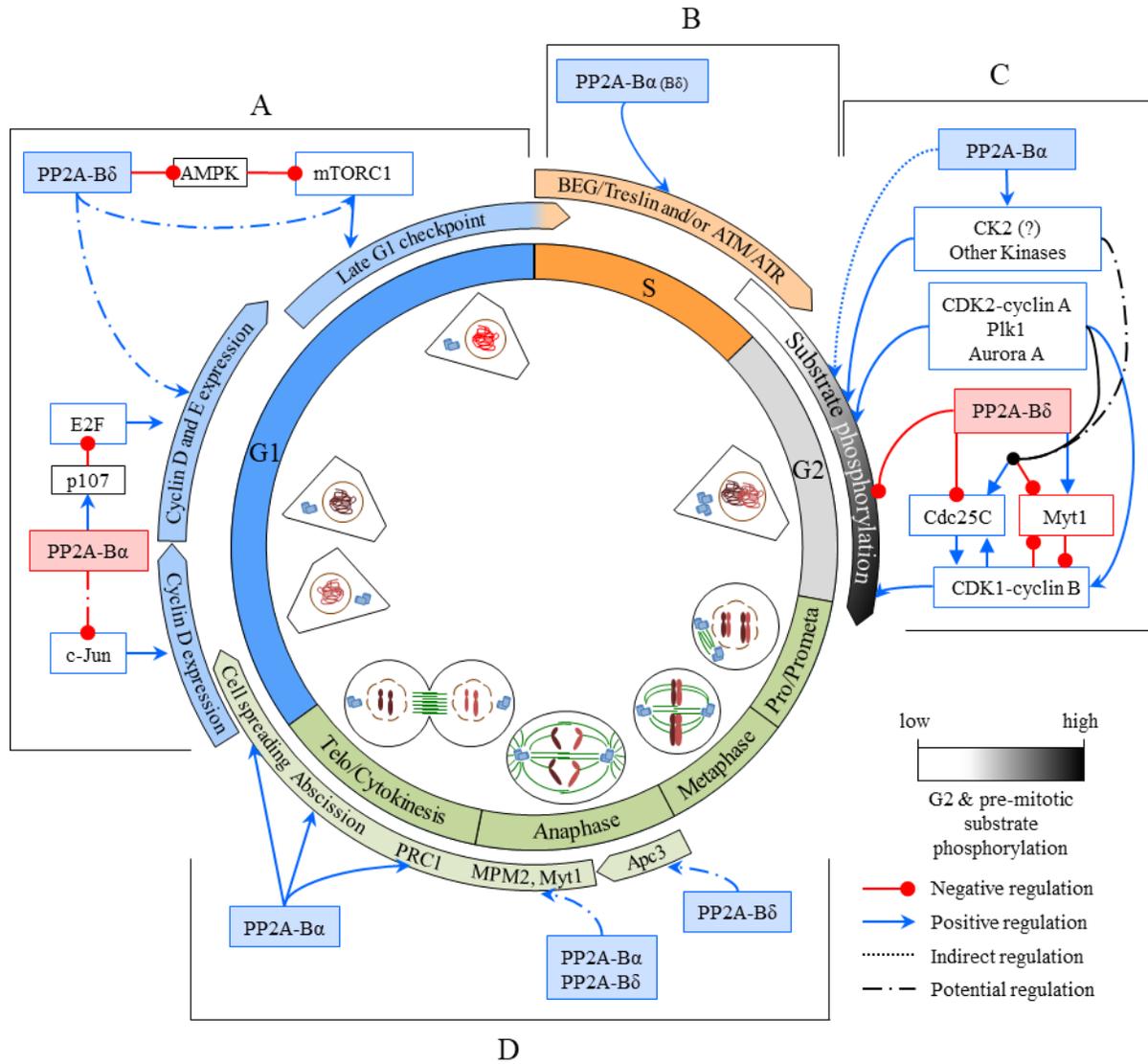


Figure 6.2: Proposed working model

A. ► PP2A-B α may negatively regulate progression through G1 phase by restraining D- and E-type cyclin expression. Hypophosphorylated retinoblastoma-like (RB) proteins, including p107, bind to and inhibit E2F transcription factors. This results in the suppression of E2F target genes, including D- and E-type cyclins (Cobrinik, 2005; Grana et al., 1998). PP2A-B α has been shown to dephosphorylate p107, thereby preventing G1 progression (Jayadeva et al., 2010; Kolupaeva et al., 2013). Furthermore, a hitherto unidentified PP2A heterotrimer dynamically regulates RB protein phosphorylation throughout the cell cycle (Garriga et al., 2004), and my findings implicate the PP2A-B α complex as likely candidate for this activity.

► A currently unspecified PP2A heterotrimer has been shown to dephosphorylate c-Jun, preventing c-Jun-mediated D-type cyclin expression in early G1 phase (Al-Murrani et al., 1999; Tsuchiya et al., 2007). Based on the effects of its depletion on G1 cyclin expression and its role in the M-to-G1

transition, PP2A-B α may represent this elusive phosphatase.

► PP2A-B δ may positively regulate progression through G1 phase. B δ depletion delays D-type and E-type cyclin expression, suggesting that PP2A-B δ may positively modulate their expression. Furthermore, PP2A-B δ has been shown to inhibit AMPK (Joseph et al., 2015), which in turn has been shown to negatively regulate mTORC1-mediated advancement through a late G1 phase checkpoint (Gwinn et al., 2008; Shackelford and Shaw, 2009).

- B. PP2A-B α positively regulates progression through S phase. The B55/Ensa/Gwl (BEG) pathway has been implicated in S phase progression. Nuclear phosphorylated Ensa is required for the stabilization of Treslin, which positively regulates replication fork density and timely completion of DNA replication (Charrasse et al., 2017; Sansam et al., 2015). PP2A-B α may dephosphorylate cytoplasmic Gwl to promote its translocation to the nucleus, where Gwl can phosphorylate Ensa. Alternatively, PP2A-B α may negatively regulate the activity of the ATM/ATR kinases at replication forks, where ATM/ATR has been shown to play a DNA damage-independent role in opposing replication fork progression (Murphy and Michael, 2013; Shechter et al., 2004).
- C. The presence of the B α subunit is essential to the maintenance of pre-mitotic substrate phosphorylation (including phosphorylation of mitotic regulators – Plk1, Aurora A, Cdc25C, and Myt1). This indicates that PP2A-B α may positively regulate the activity of a hitherto underappreciated G2/M kinase, such as CK2. Alternatively, AC core dimers that are freed by the absence of the B α subunit may be recruited by other B-type regulatory subunits (including members of the B56 or PR72 families) resulting in unchecked phosphatase activity and substrate dephosphorylation (not depicted in diagram). PP2A-B δ represents the prototypical anti-mitotic B55 isoform in HeLa cells, and dephosphorylates Cdc25C, Myt1, and pre-mitotic substrates. Therefore, PP2A-B δ may negatively regulate Cdc25C and positively regulate Myt1.
- D. Both PP2A-B α and -B δ heterotrimers positively regulate mitotic exit progression. PP2A-B δ promotes mitotic exit initiation, possibly through dephosphorylation of Apc3. Both complexes appear to regulate the dephosphorylation of MPM2 epitopes and Myt1. PP2A-B α dephosphorylates PRC1 to ensure its timely localization to the central spindle during Anaphase. B α -containing complexes also appear to regulate late mitotic exit events, including abscission and post-cytokinesis cell spreading.

6.3 Exploring the basis of distinct B α - and B δ -dependent functions

6.3.1 Ruminations on sequence variations

The ability of PP2A holoenzymes to regulate a staggering array of cellular processes largely stems from the structural diversity of the regulatory B subunits (Janssens and Goris, 2001; Shi, 2009). Even within B subunit families, structural variations are believed to underlie differences in localization, substrate recruitment/interaction, and ultimately isoform-specific cellular functions (Janssens and Goris, 2001). Members of the well-studied B'/B56 family, for example, contain a conserved central region (80%

sequence identity) that is flanked by highly divergent N- and C-terminal regions, which have been proposed to direct isoform-specific functions through subcellular targeting and substrate selection (Janssens and Goris, 2001). A comparison of the overall sequence conservation within the B'/B56, B''/PR72, and B'''/Striatin regulatory subunit families reveals marked variations between their respective isoforms (63-74%, 21-57%, and 55-65% sequence identity, respectively; see Appendix A.1 for sequence identity matrices). On the contrary, the B55 isoforms exhibit a remarkably high overall sequence conservation (82-89% identical), with the B α and B δ subunits sharing 89% sequence identity (Figure 1.5A). This begs the question as to how these two subunits perform distinct functions throughout the cell cycle, especially in systems where both are abundantly expressed. The crystal structure of the PP2A-B α holoenzyme has been determined (Fig. 6.3) (Xu et al., 2008). This enables an examination of the amino acid substitutions that distinguish the B α and B δ subunits and provides some insight into potential differentiating factors. The B55 subunits contain a highly conserved acidic groove that has been shown to be an essential determinant of substrate binding and selectivity (Fig. 6.4A) (Cundell et al., 2016; Xu et al., 2008). Expectedly, sequence variations are excluded from this important substrate binding interface, strongly suggesting that distinct functions are not due to differences in substrate recognition (Fig. 6.4A). Most of the amino acid substitutions in the B δ isoform are conservative in nature and are situated in surface-exposed loops at the back and sides of the B subunit, away from the region that faces the catalytic subunit (Fig. 6.4B and C; see Appendix A for a complete list of the amino acid substitutions). Such modest alterations are unlikely to cause structural changes, or to significantly affect interactions with potential binding partners. Notably, many of the substitutions occur at the A subunit-binding interface, including the β -hairpin structure that stabilizes the interaction between the B55 and the scaffolding subunit (Fig. 6.4B and C). Interestingly, it has been shown that the B δ subunit exhibits a higher affinity for the A subunit (Batut et al., 2008). While the specific consequences of altered A subunit affinity are unclear, it is possible these isoforms exhibit variable sensitivity to factors that affect association of the B subunit with the AC core dimer, and therefore, PP2A complex formation.

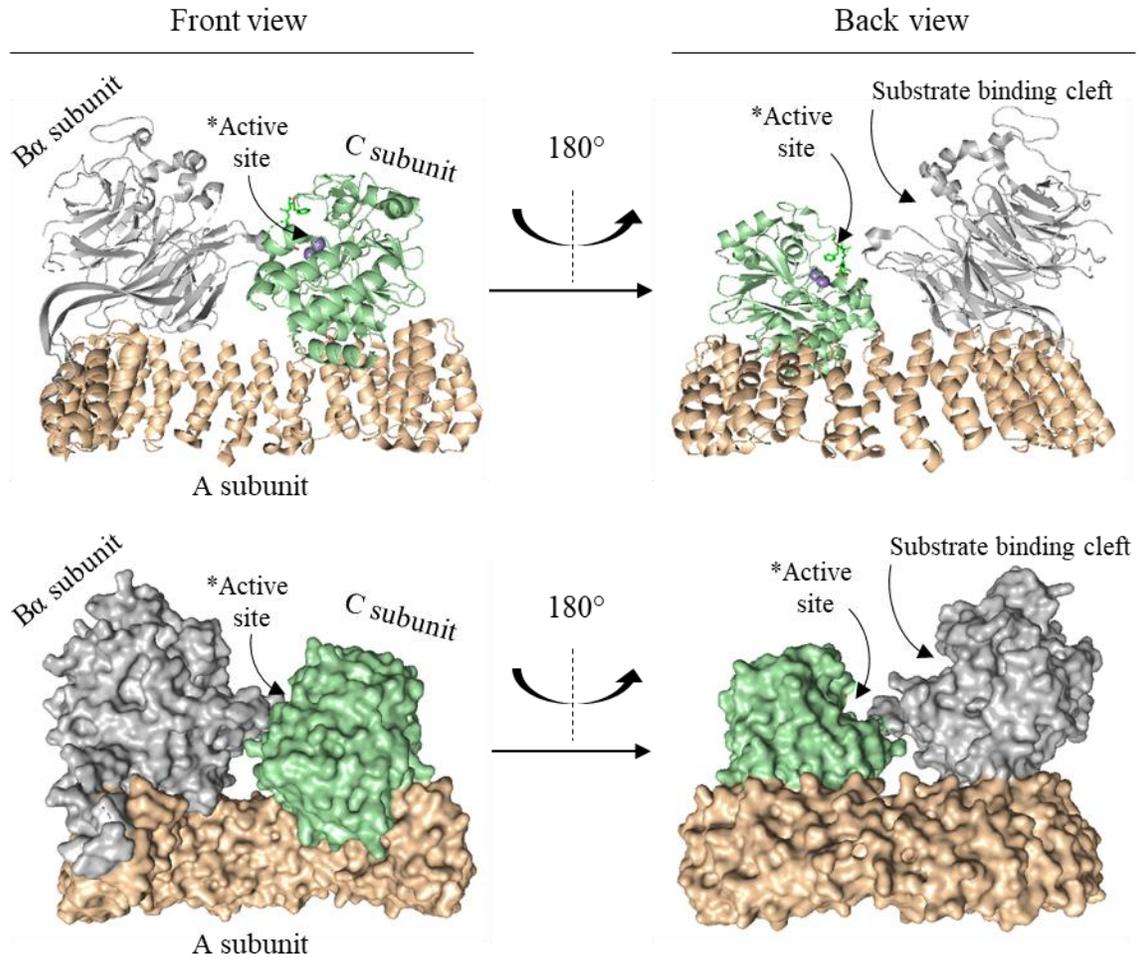


Figure 6.3: Crystal Structure of the PP2A-B α holoenzyme as determined by Xu *et al.* 2008

The crystal structure of the PP2A-B α holoenzyme resolved at 2.46Å (PDB entry: 3DW8) was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/structure/3DW8>) and manipulated using the PyMOL 2.0 software.

The regulatory (B α) subunit is coloured in gray, the scaffolding (A) subunit in 'wheat', and the catalytic (C) subunit in 'palegreen'.

The upper panel illustrates the holoenzyme structure as a cartoon, depicting the β -sheets, α -helices and loops formed by the main chains of each polypeptide.

The lower panel shows the surface topography of each polypeptide in the holoenzyme structure and takes into account the structures of surface-exposed amino acid side chains.

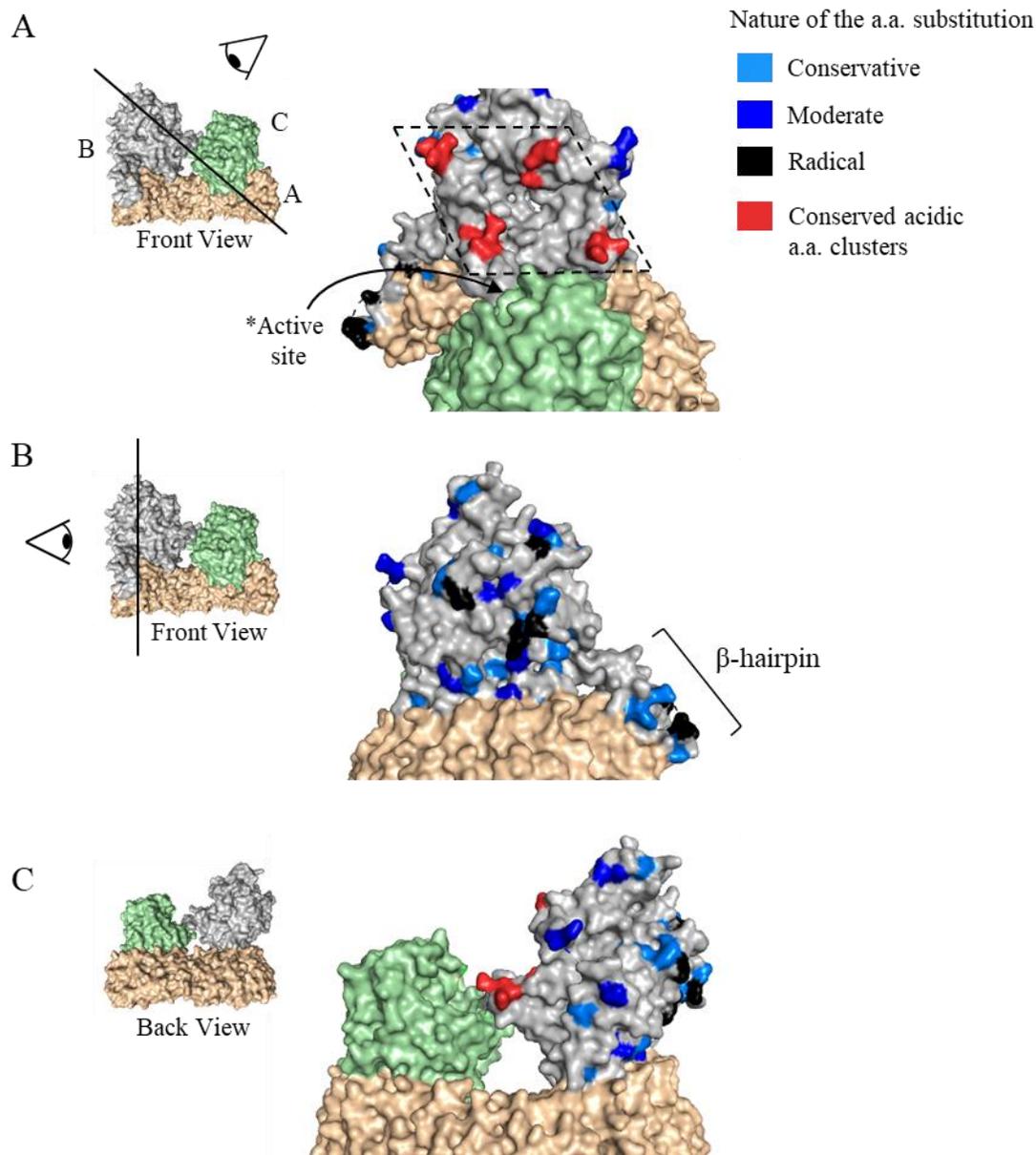


Figure 6.4: Amino acid substitutions in B δ isoform mapped onto crystal structure of PP2A-B α

The crystal structure of the PP2A-B α holoenzyme (as in Fig. 6.3) was used to visualize the locations of the amino acid substitutions present in the B δ isoform. The miniaturized structure to the left of each subfigure illustrates the angle/perspective from which the structure is viewed. Black lines indicate the plane of view. Amino acid (a.a.) substitutions were coloured to reflect the level of a.a. conservation as measured by their physical and chemical properties. Substitutions are treated as conservative when the amino acids pertain to the same class (e.g. non-polar). See legend for colour coding index.

- A. Shown is the conserved acidic substrate binding groove (outlined by dashed box). The most highly conserved acidic residues are highlighted in red. The groove is situated such that it faces the active site (*) in the catalytic cleft of the C subunit.

- B. Amino acid substitutions are mostly conservative to moderate, are located on the protein surface, and concentrate around the back and sides of the B subunit. Shown is the side view. The bracket denotes the β -hairpin structure that stabilizes B55-A subunit association.
- C. See B. Shown is the back view.

Most strikingly, however, eight amino acid substitutions result in the creation of potential phosphorylation sites (Fig. 6.5A and B). All but one (A258S) of these substitutions are positioned in surface-exposed loops that are accessible to kinases when the B δ subunit is in complex with the core AC dimer (Fig. 6.5A and B). Furthermore, several of these potential phosphorylation sites resemble known consensus motifs for various mitotic kinases, including CDK1, Plk1, MPS1, and Aurora A (Fig. 6.5D) (Dou et al., 2011; Nakajima et al., 2003; Santamaria et al., 2011; Suzuki et al., 2015). The V391S and A399T substitutions are particularly intriguing, as the regions flanking these sites are densely populated with basic residues, making them favourable ‘non-S/T-P’ (non-proline-directed) CDK1 target sites (Suzuki et al., 2015). Moreover, the A399T substitution is specifically located in a loop that has been shown to be essential to substrate binding and holoenzyme stability (Fig. 6.5B and C) (Kolupaeva et al., 2013). While speculative, it is conceivable that CDK1-mediated phosphorylation at this (or other) site(s) during mitosis could affect the interaction between the B δ subunit and phosphorylated Ensa, which may underlie the more rapid dephosphorylation of Ensa exhibited by B δ -containing PP2A complexes as compared to B α complexes (Williams et al., 2014). In the context of mitosis at large, phosphorylation at one or more of these sites could drastically alter the function of the B δ subunit (as compared to B α), by modulating its interaction with binding partners, its localization, or affecting substrate selection.

In addition to the surface-exposed sites, the A258S substitution is located at the A subunit-interacting interface and is situated close to a known phosphorylation site present on the B α subunit (S266). Phosphorylation at the S266 site negatively regulates the association of the B α subunit with the AC core dimer (Kolupaeva et al., 2013). This suggests that similar mechanisms of regulation may exist for the B δ subunit.

The crystal structure of the PP2A-B α holoenzyme (as in Fig. 6.3) was used to visualize the locations of the amino acid substitutions that may produce new phosphorylation sites in the B δ isoform. The miniaturized structure to the left of each subfigure illustrates the angle/perspective from which the structure is viewed. Black lines indicate the plane of view. Potential new phosphorylation sites are coloured in magenta, and each amino acid substitution is labelled. Conserved acidic amino acids in the substrate binding groove are coloured in red. Depicted is the cartoon structure and the surface topography (transparency of surface set to 60%).

- A. Shown is the side view of the crystal structure. Potential new phosphorylation sites are located on surface-exposed loops, except for the A258S substitution, which is situated at the B55-A interaction interface.
 - B. See A. Shown is the front view of the crystal structure.
 - C. The loop and short α -helices coloured in blue indicate a region that is critical for substrate binding (Kolupaeva et al., 2013).
 - D. Upper panel: Sequences of potential new phosphorylation sites. Amino acids are coloured according to their biochemical and structural properties (Red=acidic; blue=basic; dark blue=amidic; pink=hydroxylic; dark gray=Proline; light gray=aliphatic; green=aromatic; light orange=sulfur-containing). Phosphorylation site indicated by *.
- Lower panel: minimal consensus motifs for CDK1 (non-S/TP; non proline-directed), Plk1, MPS1, and Aurora A/B kinases.

More broadly speaking, substitutions that generate potential phosphorylation sites may give rise to a plethora of additional regulatory mechanisms throughout the cell cycle and may dictate B δ -specific functions. This additional layer of regulatory complexity is consistent with the emerging picture that cell cycle transitions are driven by finely tuned phosphorylation dynamics (Cundell et al., 2016; Hein et al., 2017; Palmisano et al., 2017; Rogers et al., 2016b).

6.3.2 Subcellular localization and multiprotein complexes

The regulatory B subunits coordinate much of the functional specificity of PP2A holoenzymes by directing the complexes to distinct subcellular compartments and structures (Guergnon et al., 2011; Janssens et al., 2008; Sontag, 2001). Members of the B55 family of regulatory subunits exhibit overlapping and distinct subcellular localization, which is further complicated by cell type-specific differences. The B β subunit, for example, has been shown to associate with the mitotic spindle in HeLa

cells (Torres et al., 2010), while it is cytoplasmic in neuronal cell types (Strack et al., 1998). The B α subunit is not only abundantly expressed across cell types but also localizes to many different cellular structures, including microtubules (Hiraga and Tamura, 2000; Sontag et al., 1995; Sontag et al., 1999), the Golgi apparatus (Sontag et al., 1995), the nucleus (Sontag et al., 1995; Strack et al., 1998; Turowski et al., 1995), and the cytoplasm (Strack et al., 1998; Turowski et al., 1995), amongst others (Sontag, 2001). The localization of the B δ subunit has not been investigated as extensively, but it can localize to the cytoplasm and centrosomes (Schmitz et al., 2010; Strack et al., 1999). Due to the lack of complete information and the overlap between the B α and B δ subunits, their distinct functions cannot be simply explained by differences in subcellular localization.

In addition to the B-type subunits, a vast array of other proteins have been shown to interact with one or more of the PP2A subunits or specific holoenzymes, including many kinases, transmembrane receptors, and transcription factors [reviewed in (Guergnon et al., 2011)]. Consequently, much of the regulation and functional specificity of PP2A is derived from its interaction with other proteins and its engagement in supramolecular multiprotein complexes. The striatin-interacting phosphatase and kinase (STRIPAK) complexes provide a good example of large PP2A-based multiprotein complexes. These complexes are composed of more than a dozen proteins, including PP2A subunits, kinases of the GCK family, adaptor proteins and other proteins that are involved in a wide range of signaling pathways [reviewed in (Goudreault et al., 2009; Hwang and Pallas, 2014; Shi et al., 2016)]. Unsurprisingly, these complexes are involved in a variety of vital cellular pathways, including Hippo, MAP Kinase, and cytoskeletal remodeling, and their disruption is implicated in many diseases, including cancers [reviewed in (Hwang and Pallas, 2014; Shi et al., 2016)]. Importantly, STRIPAK complexes are centered on the B γ /Striatin family of PP2A regulatory subunits, which act as a scaffold for the entire complex. The structure of the B γ /Striatin family members uniquely enables them to form large multiprotein complexes, and other B-type regulatory subunits are not known to anchor such supramolecular signaling nodes. Nonetheless, other B-type subunits specifically engage in various multiprotein complexes. A recent

analysis of the PP2A interactome using chemical cross-linking and mass spectrometry (XL-MS) demonstrated that PP2A heterotrimers engage in a large array of interprotein interactions (Herzog et al., 2012). Interestingly, the study identified protein-protein interactions that were specific to the B α subunit (i.e. not found for the B δ isoform), including an interaction with CDCA4. Strikingly, CDCA4 has been implicated as a negative regulator of E2F-dependent transcriptional activation (Hayashi et al., 2006), and as a midzone factor involved in chromosome segregation or cytokinesis (Wang et al., 2008a). Furthermore, siRNA-mediated depletion of CDCA4 results in a significant increase in multinucleated cells, which was partly attributed to impaired cytokinesis (Wang et al., 2008a). The similarities to B α -depletion phenotypes may be indicative of functional interactions between PP2A-B α and CDCA4. While the study by Herzog *et al.* did not report B55 isoform-specific interactions with components of prominent mitotic protein complexes, such as the spindle assembly checkpoint, the chromosome passenger complex, or central spindle proteins, it may have failed to detect such interactions because these experiments were performed in asynchronously growing cells (HEK293) (Herzog et al., 2012). A similar analysis in mitotic cells may provide further insight into B α - and B δ -specific protein interactions and the basis of their seemingly distinct functions in the regulation of mitosis. Nonetheless, these findings provide concrete examples of differences in interacting partners between B55 isoforms, which may underlie isoform-specific cellular functions (Herzog et al., 2012).

Somewhat analogous to my findings of distinct functions, the B α and B δ subunits have been shown to exert opposite effects on TGF- β /Activin/Nodal signaling pathways. Specifically, B α promotes signaling through the ALK4/5 receptor-like S/T kinases, while B δ antagonizes it (Batut et al., 2008). The underlying mechanism for their contrasting roles was not determined; however, the authors established that the B α and B δ subunits act on distinct substrates.

The findings presented in my study demonstrate that the highly related B α and B δ subunits differentially regulate progression through various cell cycle phases. While the molecular basis for these differences is not entirely clear, it is possible that the presence of additional phosphorylation sites on the

B δ subunit (section 6.3.1) and/or differences in interacting partners underlie distinct functions. Furthermore, additional regulation by phosphorylation may dictate differences in protein-protein interaction and subcellular localization, and therefore, cellular function.

6.4 Perturbations of PP2A subunit composition and tumorigenesis

Proper assembly and composition of PP2A holoenzymes is crucial to the health of the cellular processes that they regulate (Janssens et al., 2008; Sents et al., 2013). Disruption of PP2A complexes results in the deregulation of cellular homeostatic signaling pathways, and has been shown to be involved in tumorigenesis [reviewed in (Arroyo and Hahn, 2005; Kiely and Kiely, 2015; Ruvolo, 2016; Sablina and Hahn, 2008; Westermarck and Hahn, 2008)]. The role of PP2A holoenzyme disruption in tumorigenesis has been appreciated since the discovery that DNA tumour viruses of the *Papovae* family produce small oncoproteins that target the PP2A complex, such as small t antigens and polyomavirus middle T (Campbell et al., 1995; Pallas et al., 1990; Walter et al., 1990). These viral oncoproteins deregulate cellular growth pathways by displacing B subunits from the AC core dimer, effectively abolishing the activity of specific PP2A holoenzymes [reviewed in (Arroyo and Hahn, 2005; Eichhorn et al., 2009; Guernon et al., 2011; Sablina and Hahn, 2008)]. The SV40 small T antigen (ST), for example, is a viral oncoprotein that can competitively displace the B55 α , B56 α/γ , and PR72 subunits, leading to the loss of their respective PP2A holoenzyme activities and the oncogenic activation of the pathways they restrain, including the PI3K/Akt, c-Myc, Wnt and MAP kinase pathways (Chen et al., 2004; Sablina et al., 2010; Sontag et al., 1993).

Holoenzyme composition can also be perturbed by genetic alterations that affect the various PP2A subunits. Indeed, mutations in all PP2A subunits, including deletions, point mutations and gene transcript-altering mutations have been identified in wide variety of cancers (as described in section 1.3.3). Notably, the A subunit isoforms (A α and A β) are mutated most frequently (Sangodkar et al., 2016). Several seminal studies have shown that specific point mutations or decreased expression of the

scaffolding subunit isoforms reduce or abolish binding to specific B subunits (Chen et al., 2005; Ruediger et al., 2001a, b; Ruediger et al., 2011; Sablina et al., 2007). Such mutations or lowered expression produce a state of haploinsufficiency of the scaffolding subunit and result in the loss of specific PP2A holoenzyme activities and the deregulation of the pathways they modulate. The R418W mutation in A α subunit, for example, significantly decreases binding to the B α and B56 ϵ subunits, whereas the E64G and E64D mutations reduce binding to all B56 family subunits (Chen et al., 2005; Ruediger et al., 2001b; Sablina and Hahn, 2008). Chen *et al.* further demonstrated that loss of PP2A-B56 γ activity due to A subunit haploinsufficiency leads to cellular transformation that is specifically associated with increased activation of the AKT pathway (Chen et al., 2005). Cancer-associated genetic alterations in the B subunits are generally less common, but their incidence and relevance to tumorigenesis is becoming increasingly evident. Far more common than holoenzyme-disrupting point mutations are genetic alterations that affect the expression levels and stoichiometry of specific B subunits. Members of the B55 family of regulatory subunits exhibit decreased expression in a variety of cancers through several distinct mechanisms, including methylation-mediated gene silencing (Kurimchak and Grana, 2012a; Muggerud et al., 2010; Tan et al., 2010), miRNA-mediated suppression (Bluemn et al., 2013; Liu et al., 2010; Ruvolo et al., 2011; Ruvolo et al., 2014; Wu et al., 2016; Zhuang et al., 2016), and homo-/heterozygous gene deletions (Cheng et al., 2011; Cristobal et al., 2014; Curtis et al., 2012; Mosca et al., 2013). Strikingly, B α subunit expression is reduced in wider range of cancers (as compared to other B55 subunits) by a multitude of mechanisms, suggesting that disruption of B α -containing complexes (or B α subunit stoichiometry) may be relevant to tumour development and/or progression in a broader context (Cheng et al., 2011; Cristobal et al., 2014; Curtis et al., 2012; Liu et al., 2010; Mosca et al., 2013; Ruvolo et al., 2011). In comparison, reduced expression of the B δ subunit has only been reported in hepatocellular carcinoma cells (Zhuang et al., 2016). These studies highlight the important tumour suppressive functions of PP2A and strongly suggest that disruption of specific PP2A heterotrimers contributes to tumorigenesis in a wide variety of cancers.

PP2A holoenzymes are dynamic complexes that can interchange B subunits rapidly (Janssens et al., 2008). In the absence of binding by a specific B subunit due to point mutations in the scaffolding subunit or loss of expression of the respective B subunit, other B-type subunits may bind to the scaffold instead. Therefore, the AC core dimers that become available by loss of specific B subunit interactions may be recruited and bound by other free subunits (Janssens et al., 2008). Such binding may alter the relative abundance/proportions of the diverse PP2A holoenzymes and their associated activities. Consequently, deregulation of PP2A-dependent signaling pathways may result not only from loss of specific complexes, but also from the increased abundance of other heterotrimers. In my experiments, I depleted the B α and/or B δ subunits, which represent two of the most abundant regulatory subunits in mammalian cells (Kolupaeva et al., 2013). While the consequences of B α and/or B δ depletion described in this study may be largely due to the loss of the activities of these specific holoenzymes, I cannot exclude the possibility these effects may be the consequence of replacement by other B subunits, and therefore, the activity of different holoenzymes. It is therefore conceivable that in the absence of one or both of these subunits, the abundance of heterotrimers containing other B-type subunits, including members of the B56, PR72, and Striatin families, may have increased. Interestingly, B α and B56 α have been shown to directly compete for binding to the AC core dimer in leukemia cells (Ruvolo et al., 2014; Ruvolo et al., 2008). More generally, it has been suggested that subunits of the B55, B56, and PR72 families may compete with each other for binding to the AC core dimer in some cellular contexts (Janssens et al., 2003; Ruvolo, 2016). The question of altered holoenzyme composition in circumstances that severely disturb the equilibrium/stoichiometry of PP2A subunits is further complicated by a multitude of factors: the availability of other B subunits, which in turn depends on their relative expression; the differing affinities that B-type subunit families display for the AC core dimer (i.e. PR72 > B56 > B55) (Janssens et al., 2003); the variable stability of monomeric B subunits (i.e. PR72=stable, B56=unstable, B55=variable) (Chen et al., 2005; Janssens et al., 2003; Janssens et al., 2008; Kolupaeva et al., 2013; Li et al., 2002; Silverstein et al., 2002; Strack et al., 2004; Strack et al., 2002); and the

dependence of B subunit interaction with the AC core dimer on the presence or absence of various post-translational modifications on the B or C subunits [reviewed in (Janssens et al., 2008; Sents et al., 2013)]. Furthermore, experimental manipulation of the expression levels of one B-type subunit has been shown to alter the expression of another. Specifically, the B α and B56 α subunits appear to engage in a reciprocal relationship in leukemia cells. Suppression of B α by shRNA induces B56 α expression, while depletion of B56 α promotes expression of B α (Ruvolo et al., 2014; Ruvolo et al., 2008). While this may be a cell-type or context-specific phenomenon, it further emphasizes the complexity of the mechanisms that regulate PP2A holoenzyme composition. Ultimately, variations in PP2A subunit isoform stoichiometry or alterations in subunit post-translational modifications can result in aberrant cellular signaling events that could promote or drive tumorigenesis.

From the experiments in my study, I cannot determine whether or how cellular PP2A holoenzyme composition is altered, and whether this may play a role in the effects/phenotypes of B55 subunit depletion. Further well-designed studies will be needed to address these questions (see section 6.8).

6.5 Bistability and the kinase-phosphatase balance

Cell cycle transitions must occur in a unidirectional manner to ensure the strict alternation of DNA replication and chromosome segregation, and the genome integrity of daughter cells. The irreversibility (or unidirectionality) of cell cycle transitions is generated by systems-level feedback mechanisms that underlie the bistable, switch-like activation/inactivation of essential cell cycle regulatory kinases/phosphatases, respectively (Kapuy et al., 2009; Novak et al., 2007; Verdugo et al., 2013). The abrupt transition from G2 phase into mitosis, for example, is the result of the switch-like autoactivation of CDK1 through the dephosphorylation of its inhibitory Thr14 and Tyr15 residues (Novak and Tyson, 1993; Solomon et al., 1991; Solomon et al., 1990). As cyclin B1 levels rise during G2 phase, the CDK1 auto-activation loop is triggered by passing a cyclin B1 threshold. Above this threshold, a small pool of CDK1-cyclin B1 complexes is activated, and through a positive feedback loop with Cdc25C

(CDK1→Cdc25C→CDK1) and a negative feedback loop with Wee1/Myt1 (CDK1←Wee1/Myt1←CDK1) triggers the switch-like, auto-amplifying activation of CDK1-cyclin B1. In principle these interlinked loops are sufficient to generate a bistable switch between interphase and mitosis (Novak and Tyson, 1993; Tyson and Novak, 2001), and this was initially proven through experiments in reconstituted *Xenopus* egg extracts (Pomerening et al., 2003; Sha et al., 2003). The recent discovery that CDK1 mediates the inhibition of its counteracting phosphatase, PP2A-B55, through the Gwl/Ensa pathway led to a reconsideration of the basic components of the bistable switch underlying mitotic entry (Castilho et al., 2009; Gharbi-Ayachi et al., 2010; Mochida et al., 2010). Subsequent computer modelling and experimental verification in reconstituted cell-free systems confirmed that the G2/M transition is regulated by two interlinked bistable switches: the CDK1 auto-activation loop and the B55-inhibiting Gwl-Ensa pathway (Domingo-Sananes et al., 2011; Mochida et al., 2016). Similarly, using mathematical modelling and experimental verification in the HeLa cell line, it was shown that the CDK1 autoactivation feedback loop was required for robust and irreversible entry into mitosis in mammalian systems (Potapova et al., 2011; Tuck et al., 2013). Specifically, Potapova *et al.* demonstrated that dual inhibition of Wee1/Myt1 and Cdc25C, while lowering the cyclin B1 threshold required for CDK1 activation and mitotic entry, resulted in insufficient CDK1 activity, mitotic collapse during prophase (associated with mitotic substrate dephosphorylation but not mitotic cyclin proteolysis) and reversion to an interphase state (Potapova et al., 2011). These studies ultimately suggested that feedback-mediated CDK1 activation is important for shifting the kinase-phosphatase balance toward mitotic phosphorylation. Taken together, mathematical modelling and experimental verification in reconstituted cell-free *Xenopus* and mammalian cell systems concluded that the CDK1 autoactivation loop is essential to ensuring proper mitotic entry. Interestingly, while my findings confirm that CDK1 activity itself is required for initiation of mitosis, they also suggest that CDK1 activity is not absolutely required for the phosphorylation-mediated activation of the components of auto-activation loop (Cdc25C and Wee1/Myt1) or the Gwl/Ensa loop. In the presence of prolonged chemical inhibition of CDK1 activity, these hitherto CDK1-dependent

components become phosphorylated and activated/inactivated. Indeed, I found that Cdc25C, Myt1, and MASTL are hyperphosphorylated, and CDK1 T14/Y15 is dephosphorylated in the presence of the CDK1 inhibitor, RO3306 (Chapter 4, section 4.2.5). These observations undermine the assumption of a strict requirement for CDK1 activity for the activation of the interlinked bistable switches that govern mitotic entry. Lindqvist *et al.* have described the regulatory circuitry that controls entry into mitosis as being composed of an outer and an inner feedback loop. In this model, the inner feedback loop consists of the CDK1-Wee1/Myt1-Cdc25C auto-amplification loop, while the outer feedback loop is composed of Plk1, Aurora A and CDK2-cyclin A. The outer feedback loop was proposed to play a largely supportive role that reinforces the amplitude of the inner loop (Lindqvist *et al.*, 2009). While it is possible that this is representative of situations in which CDK1 is not inhibited, my results suggest that other G2/M kinases are able to fully compensate for loss of CDK1 activity to effect the activation of mitotic regulators.

Importantly, the models discussed above were generated using a small number of components that were discovered as central regulators of mitotic entry (CDK1-cyclin B1, Cdc25C, Wee1/Myt1, Gwl, Ensa, PP2A-B55). However, in intact mammalian cells the regulatory loops are likely to be more complex, and other components may play crucial roles in modulating the balance between kinase and phosphatase activities. Strikingly, proteomic screens have revealed that thousands of proteins are phosphorylated during the G2/M transition, including many kinases and phosphatases (Daub *et al.*, 2008; Dephoure *et al.*, 2008; Sharma *et al.*, 2014; Xiang *et al.*, 2008). Therefore, it is conceivable that the central components play a non-exclusive albeit crucial role in a grander mitotic entry network. Indeed, my findings suggest that these models are incomplete and unable to account for additional players, and that bistability relies on the tightly regulated balance of additional kinases and phosphatases.

As discussed in **Chapter 4**, removal of a major negative regulatory component of the mitotic entry feedback loops, such as PP2A-B α , should favour kinase activation and precipitate increased phosphorylation of substrates (including mitotic regulators). Accordingly, the inhibition of Wee1/Myt1 significantly lowers the CDK1 activity threshold required for mitotic entry, and results in a premature and

stable G2-to-M transition (Potapova et al., 2011). Similarly, depletion of the B δ subunit from interphase *Xenopus* egg extracts, or all B55 subunits from G1/S-synchronized HeLa cells, results in accelerated entry into mitosis (Cundell et al., 2013; Mochida et al., 2009). Interestingly, my experiments show that this may be the case for the B δ subunit in HeLa cells, as its ablation consistently resulted in elevated phosphorylation as compared to control cells. Depletion of B α , however, created the opposite effect and led to wide-spread loss of phosphorylation and kinase activity. In **Chapter 4**, I proposed that this implicates PP2A-B α as a positive regulator of some currently unknown kinase activity. Alternatively, however, this may simply indicate that the B α subunit must be present to preserve the regulated balance of kinase and phosphatase activities. As discussed in section 6.4, PP2A heterotrimers are highly dynamic complexes, in which the AC core dimer can readily interchange B subunits and free core dimers can bind available B subunits. Therefore, it is possible that in the absence of the B α subunit another B-type subunit could occupy the freed AC core dimers. Such complexes may not be under the control of the pre-mitotic kinase-phosphatase system and disrupt regulated phosphorylation cascades. This model, however, would also suggest that the B α subunit must be present in much higher quantities than the B δ subunit, because if they were present in similar amounts then B δ loss should also precipitate the generation of such ‘unregulated’ PP2A heterotrimers. Moreover, depletion of both subunits should exhibit a more extreme phenotype than B α loss alone. However, my results indicate that this is not the case.

From the results presented in this study it is unclear whether loss of B α stabilizes an hitherto unknown G2/M kinase activity, or whether it results in an imbalance in kinase-phosphatase activities by enabling the formation of unrestrained PP2A heterotrimers. However, these findings have one clear implication: in addition to their actual activities, the expression levels of mitotic regulators also play an essential role in maintaining the precarious balance between the kinases and phosphatases that regulate these complex processes. The importance of phosphatases in the regulation of cellular processes has long been underappreciated. However, in recent years it has become abundantly clear that phosphorylation-

dependent processes are very carefully regulated by a finely tuned cooperation between kinases and phosphatases (Cordeiro et al., 2017; Gelens et al., 2018).

6.6 A role for PP2A-B α in aneuploidy/polyploidy and tumor aggressiveness

Aneuploidy, which is characterized by abnormalities in the structure and number of chromosomes, is an exceedingly common attribute of tumours and has been implicated in tumour initiation, evolution and drug resistance (Coward and Harding, 2014; Kuznetsova et al., 2015; Rasnick, 2002; Storchova and Kuffer, 2008; Storchova and Pellman, 2004). Approximately 90% of solid tumours and 75% of hematological cancers exhibit some degree of aneuploid chromosome content (Weaver and Cleveland, 2006). Indeed, aneuploidy is one of the oldest recognized features of transformed cells, with its first descriptions dating back over 100 years to David von Hansemann (1890) and Theodor Boveri (1902, 1914) (Boveri, 1902; Boveri, 1914; Hansemann, 1890). To this date, the routes that lead to aneuploidy and tumour initiation remain the subject of much debate and investigation; however, two major genomic mechanisms dominate the discourse. Firstly, oncogenic or tumour-suppressive mutations in mitotic genes can lead to chromosome missegregation and precipitate chromosomal instability (CIN), which through ongoing losses and gains of chromosomes during mitosis propagates an aneuploid genome (Lengauer et al., 1997; Ricke et al., 2011; Shackney et al., 1989; Sotillo et al., 2007; Weaver et al., 2007). Secondly, a growing body of evidence supports a major role for tetraploidization in tumour initiation, and as a precursor of more complex aneuploid karyotypes (Castillo et al., 2007; Davoli and de Lange, 2012; Fujiwara et al., 2005; Lv et al., 2012; Sotillo et al., 2007; Storchova and Kuffer, 2008). Indeed, approximately 40% of tumours undergo tetraploidization at some point during tumorigenesis (Zack et al., 2013), and studies have demonstrated that tetraploid p53-null cells, but not their isogenic diploid counterparts, induce tumor formation *in vivo* (Davoli and de Lange, 2012; Fujiwara et al., 2005; Lv et al., 2012). Moreover, Lundberg *et al.* specifically illustrated in neuroblastoma lines that polyploidization events and clonal cultures with increased genomic content frequently yield aneuploid progeny (Lundberg

et al., 2013). Tetraploidization through whole genome duplication endows cells with an increased tolerance for mitotic errors and CIN, provides a buffer against deleterious mutations, and thereby facilitates aneuploidization and the evolution of a complex tumorigenic karyotype (Castedo et al., 2006; Dewhurst et al., 2014; Kuznetsova et al., 2015; Rasnick, 2002). Aneuploidy and associated CIN ultimately drive tumour heterogeneity and evolution, which represent fundamental obstacles to the development of truly curative cancer therapies (Burrell et al., 2013; Gerlinger and Swanton, 2010; Gillies et al., 2012; Greaves and Maley, 2012; Marusyk et al., 2012; Yap et al., 2012; Yates and Campbell, 2012). Unsurprisingly, increasing chromosome content through polyploidization is a conserved mechanism of adaptation, as it has been shown to underlie the rapid evolution of yeast strains in response to various selective pressures (Chang et al., 2013; Rancati et al., 2008).

Tetraploidization or whole genome duplication can occur by three major pathways: cell fusion, mitotic slippage (including cytokinesis failure), and endoreplication [reviewed in (Storchova and Kuffer, 2008; Storchova and Pellman, 2004)]. Strikingly, my findings demonstrate that loss of the B α subunit can lead to polyploidization/tetraploidization by two of these three mechanisms: cytokinesis failure and endoreplication. Importantly, this was specifically observed in a biochemical setting of low activity of CDK1 and other G2/M kinases. While factors that impair CDK1 activity before or during mitosis may autonomously precipitate endoreplication or cytokinesis failure, respectively, my experiments demonstrated that these polyploidization events occurred at a markedly increased rate in a B α -dependent manner. This suggests that B α loss may not only exacerbate but may also have additional effects that promote polyploidization. The *Drosophila* B55 homologue, *Twins*, has previously been implicated as a regulator of genome integrity in fruit flies; however, this role was associated with PP2A-*twins* activities during DNA damage repair (Merigliano et al., 2017). Interestingly, amongst the B55 subunit, the B α subunit appears to be the most widely silenced in a variety of cancers. This raises the possibility that loss of B α expression by genetic deletion or miRNA-mediated downregulation may be selected for under certain circumstances during tumour evolution. Specifically, in the presence of cellular or environmental

alterations that undermine the activity of CDK1 (and/or other G2/M kinases), concomitant loss of B α may support propagation of tumour cells by facilitating polyploidization. Interestingly, low B α subunit expression has been associated with increased tumour aggressiveness and worse prognosis and patient outcome in a subgroup of luminal breast cancers (Beca et al., 2015; Curtis et al., 2012), and with shorter remission duration in acute myeloid leukemia (AML) (Ruvolo et al., 2011). Furthermore, luminal breast tumours that exhibit loss of B α expression and concomitant overexpression of cyclin D1 are associated with even worse prognosis and patient outcome (Beca et al., 2015). Intriguingly, in my experimental setup where B α depletion induced endoreduplication and cytokinesis failure (i.e. G2/M synchronization using RO3306), I also observed increased cyclin D1 expression (section 4.2.4 and Fig. 4.4). The significance and implications of this are unclear, but the parallels are nonetheless intriguing. Ultimately, it is conceivable that under certain selective conditions, B α loss may promote polyploidization, which in turn may drive tumour evolution and aggressiveness.

Finally, the potential role for B α loss in polyploidization also has therapeutic implications. Specifically, a large number of CDK1 inhibitors have been developed and are undergoing clinical testing for a variety of cancers (Stone et al., 2012). My findings suggest that treatment of tumours that exhibit reduced B α expression with inhibitors of CDK1 might be ill-advised, as it could precipitate the formation of polyploid tumour cells, which may generate more aggressive progeny, and ultimately contribute to treatment resistance (Wang et al., 2013b; Zhang et al., 2014). In addition to CDK1, the B55-inhibitory kinase MASTL has been proposed to be an attractive target for therapeutic development (Alvarez-Fernandez et al., 2017; Wang et al., 2014a). Alvarez-Fernandez *et al.* use mouse models to demonstrate the therapeutic effectiveness of MASTL inhibition against breast tumours, and propose that MASTL inhibition should be specifically considered for treatment of breast cancers. However, my findings suggest that inhibition of MASTL in the presence of low B α expression may generate polyploid cells. Furthermore, B α loss has been described in breast cancers (Curtis et al., 2012; Dupont et al., 2010). Therefore, it may be important to determine the B α expression status of tumours before considering

CDK1 or MASTL (or other mitotic kinase) as therapeutic options for various cancers. My findings suggest that therapeutic use of inhibitors targeting any or multiple G2/M kinases, including Plk1 and Aurora A, may be counterproductive in tumours that exhibit reduced B α expression.

6.7 Caveats to this study

The findings presented in this study were obtained from experiments conducted with the HeLa cell line. HeLa cells are one of the most widely used model cell lines in mammalian cell cycle research, largely due to their ability to tolerate various experimental manipulations, their amenability to cell cycle synchronization and their predictable doubling time. Despite these advantages, HeLa cells exhibit a highly abnormal genome (and transcriptome) that is characterized by extensive chromosomal rearrangements and hypertriploidy (Adey et al., 2013; Landry et al., 2013; Macville et al., 1999). Furthermore, HeLa cells contain a genomic insertion of the human papillomavirus 18 (HPV18) (Landry et al., 2013; Macville et al., 1999), and expression of the HPV E6 and E7 proteins is important to sustain their proliferation and survival (DeFilippis et al., 2003). Importantly, it has been shown that the E7 viral protein may interact with the AC core dimer of PP2A (Pim et al., 2005). Therefore, it is possible that E7-mediated displacement of some B-type subunits inherently disrupts PP2A composition and function in HeLa cells. However, whether E7 truly interacts with PP2A has recently been challenged (White et al., 2015). In addition, HeLa cells are considered to be p53 null (Berglind et al., 2008), which may render these cells inherently more susceptible to polyploidization (Fujiwara et al., 2005; Livingstone et al., 1992). However, as discussed in **Chapter 5**, there is evidence undermining the importance of p53 in preventing tetraploidization resulting from mitotic errors (Uetake and Sluder, 2004; Wong and Stearns, 2005).

Another caveat that affects the findings presented herein is the lack of rescue studies. I did not demonstrate that the B55 subunit-specific effects could be rescued by re-expression of siRNA-resistant clones of the B α and B δ subunits. Interestingly, HeLa cells appear to be particularly sensitive to

overexpression of B α , as cells transfected with varying amounts of B α -encoding DNA plasmids demonstrated markedly reduced viability (data not shown). This suggests that rescue studies may be impeded by this sensitivity. Furthermore, I did not investigate potential off-target effects of RNA interference on the expression levels of the B β and B γ subunits. However, considering that the B α and B δ subunits are the most related B55 isoforms, and that the 3'-UTR sequences targeted by the various siRNA's do not exhibit any notable overlap, it seems unlikely that the expression levels of the other B55 subunits were affected. Furthermore, B γ has not been shown to be expressed in HeLa cells. An investigation of the individual siRNA duplexes comprising the B α - and B δ -targeting siRNA pools demonstrated that the individual siRNAs exerted the same cell cycle effects as the pooled siRNAs used throughout this study (see Appendix A.3).

6.8 Suggested future studies

My findings demonstrate for the first time that the highly related B α and B δ subunits differentially regulate progression through S phase, mitotic exit, and G1 phase. They also extend current models of B55-dependent mitotic exit and demonstrate a novel, context-dependent role for B α in sustaining G2/M kinase activities. Importantly, the results of this study have revealed additional gaps in our understanding of PP2A-mediated cell cycle regulation, and based on the implications of my findings, I suggest a few important follow-up studies below.

My results in **Chapter 3** suggest that the B α and B δ subunits perform temporally distinct functions during mitotic exit, and based on previous findings (Williams et al., 2014) I propose that this may be due to differential kinetics of phosphatase reactivation. Williams *et al.* demonstrate through *in vitro* assays that PP2A-B δ complexes dephosphorylate Gwl-phosphorylated Ensa and myelin basic protein with greater efficiency than PP2A-B α , whereas dephosphorylation of CDK substrates is equivalent (Williams et al., 2014). More detailed *in vitro* and cell-based assays of B55 subunit-specific dephosphorylation kinetics may provide additional insight into whether these subunits indeed exhibit

distinct reactivation kinetics. Recent studies have shed additional light on the regulation of mitotic substrate dephosphorylation during mitotic exit. Using LC/MS Cundell *et al.* specifically identified novel B55 substrates, including TPX2, NUMA, and several nuclear envelope proteins (NUPs) amongst many others (Cundell *et al.*, 2016). However, due to the simultaneous depletion of all B55 family members, this study did not distinguish B α - and B δ -dependent substrates. My findings suggest that these subunits may indeed perform different functions, possibly through regulated targeting to non-overlapping substrates. Cundell *et al.* provide a detailed method to define phosphatase substrates using mass spectrometry, which could be used to more closely investigate potential differences in the substrates and functions between the B α and B δ subunits. The identification of differences in substrate targeting may also provide insight into why cytokinesis failure appears to be specific to B α depletion.

My findings in **Chapter 4** suggest that PP2A-B α may positively regulate pre-mitotic kinase activities, and I further proposed that this may be through a positive regulatory interaction with CK2. This hypothesis could be tested by using the CK-specific inhibitors 4,5,6,7-tetrabromo-2-azabenzimidazole or CX-4945 (silmitasertib) in the same experimental setup. That is, if treatment with the CK2 inhibitor precipitates a B α -like collapse of the pre-mitotic state in control (or B δ -depleted) cell populations, a role for CK2 would be strongly supported.

My analysis of the amino acid substitutions that distinguish the B α and B δ subunits demonstrated that the B δ isoform may contain additional phosphorylation sites, some of which are situated in regions known to be involved in substrate interaction. This suggests that B δ may be subject to additional phosphorylation-dependent regulation. Using mass spectrometric methods (such as IP/MS) it could first be investigated whether these sites are indeed phosphorylated during any of the cell cycle phases. Secondly, site-directed or CRISPR/Cas-mediated mutagenesis could be used to investigate whether these sites contribute to differential functions or activities of these subunits.

Distinct functions of the PP2A-B α and -B δ complexes may also be driven by differential interaction with other proteins, which in turn may be influenced by phosphorylation of the B δ subunit.

This could be further investigated by IP/MS. In section 6.4 and 6.5 I discuss the possibility that altered PP2A holoenzyme composition may underlie some of the effects of B α and/or B δ depletion. Therefore, it would very interesting to investigate the composition of PP2A holoenzymes, especially in cells synchronized at the G2/M transition by RO3306 treatment. This could be accomplished using IP/MS methodologies. Finally, I cannot definitively rule out that the distinct B α and B δ depletion phenotypes are due to differences in the expression levels of these subunits, and an examination of the relative transcript or protein abundance of B α and B δ in the HeLa cell lines may be warranted.

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Appendix A

Table A.1: Percent sequence identity matrix for human B'/B56 (*PPP2R5*) isoforms

	B56 γ	B56 δ	B56 β	B56 α	B56 ϵ
B56 γ	100	77.29	63.56	66.08	67.87
B56 δ	77.29	100	63.1	63.09	66.31
B56 β	63.56	63.1	100	69.48	71.73
B56 α	66.08	63.09	69.48	100	77.52
B56 ϵ	67.87	66.31	71.73	77.52	100

Table A.2 Percent sequence identity matrix for human B''/PR72 (*PPP2R3*) isoforms

	PR72/130	PR48/70	G5PR
PR72/130	100	23.88	21.2
PR48/70	23.88	100	56.74
G5PR	21.2	56.74	100

Table A.3 Percent sequence identity matrix for human B'''/Striatin (*STRN* or *PPP2R6*) isoforms

	Striatin 4	SG2NA	Striatin
Striatin 4	100	56.5	54.65
SG2NA	56.5	100	65.16
Striatin	54.65	65.16	100

Table A.4 List of amino acid substitutions between human B α and B δ isoforms

B α a.a.	a.a position	B δ a.a.	NOTES
I	10	F	
V	21	I	
D	23	E	
H	38	Y	
E	41	D	
Q	57	R	
I	63	S	
Q	64	R	
S	65	P	
K	105	Q	
Q	109	H	
P	131	A	
E	138	D	
Y	143	L	
T	147	F	
T	148	R	
V	149	I	
T	151	A	
V	156	I	
F	157	L	
R	158	K	
I	185	V	
Y	189	H	
N	237	H	
S	238	Q	
T	241	V	
A	258	S	potential new P-site
L	268	F	
N	277	S	potential new P-site

I	310	V	
N	317	S	potential new P-site
Y	323	H	
V	356	A	
V	357	I	
N	372	D	
K	374	R	
I	377	V	
N	385	S	potential new P-site
N	386	S	potential new P-site
T	390	A	
V	391	S	potential new P-site
A	399	T	potential new P-site
S	400	G	
K	404	R	
K	427	V	
E	428	D	
I	430	V	
T	435	A	
V	446	I	