

Cdk1 regulates anaphase onset

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Abstract

Cdk1 is an important cell cycle regulator that, in association with different cyclin regulatory subunits, is responsible for signaling important cell cycle events in all eukaryotic cells. In budding yeast, inhibition of Cdk1 by selective deletion of cyclin subunits has been shown to prevent anaphase onset, suggesting that Cdk1 activity is critically important for triggering anaphase onset. In many eukaryotes, Cdk1 has been shown to phosphorylate subunits of the anaphase promoting complex (APC), an E3 ubiquitin ligase which directly signals anaphase onset by triggering the degradation of the anaphase inhibitor securin. It is currently unclear, however, whether the APC is the sole essential substrate of Cdk1 in anaphase onset or if Cdk1 triggers anaphase onset by phosphorylating additional proteins. Eukaryotic Cdk1 is regulated by the Wee1 family of tyrosine kinases and the Cdc25 family of phosphatases which directly oppose Wee1 activity. Wee1 phosphorylation of Cdk1 on a single tyrosine residue inhibits Cdk1 and has been shown to prevent or delay mitotic entry. In this work we sought to further elucidate the mechanism through which Cdk1 regulates anaphase onset. We showed that, in addition to regulating mitotic entry, the budding yeast Wee1 kinase and Cdc25 phosphatase (Swe1 and Mih1 respectively in *S. cerevisiae*) regulate anaphase onset by modulating Cdk1 activity. Activation of Swe1 delays anaphase onset and cells lacking *SWE1* enter anaphase prematurely, demonstrating that Swe1 regulates anaphase onset in unperturbed cell cycles. Deletion of the *CDC55* regulatory subunit of PP2A has been shown to bypass cell cycle delays due to Swe1 activation. We showed that this is due, in part, to PP2A^{Cdc55} dephosphorylation of Cdk1 sites on the APC. We have also shown that Cdk1 directly phosphorylates separase, the protease that dissolves sister chromatid linkages upon release from inhibitory securin/separase complexes upon APC-mediated securin degradation.

Similar to phosphoregulation of the APC, we showed that Cdk1 phosphorylation of separase is opposed by PP2A^{Cdc55}. Phosphoregulation of separase appears to be important for regulation of the separase substrate Slk19 which cooperates with the conserved kinesin-5 Cin8 and microtubule bundling protein Ase1 to regulate spindle elongation at the spindle midzone.

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Table of Contents

Abstract	ii
Acknowledgements	iv
List of Abbreviations	x
List of Figures	xiii
List of Appendices	xvii
 Chapter 1 – Introduction	 1-44
1.1 The cell cycle	1-2
1.2 The discovery and characterization of Cdk1	2-7
1.2.1 Maturation Promoting Factor (MPF)	2-3
1.2.2 Cell Division Cycle 28 (<i>CDC28</i>)	4-5
1.2.3 Cyclin	5-6
1.2.4 Cyclin Dependent Kinase 1 (<i>CDK1</i>)	6-7
1.3 Cdk1 in the <i>S. cerevisiae</i> cell cycle	7-14
1.3.1 Regulation of Cdk1	8-12
1.3.2 Cdk1 regulation of transcription	12-13
1.3.3 Cdk1 regulation of cell morphogenesis	13
1.3.4 Cdk1 regulation of DNA replication and mitosis	13-14
1.4 Wee1/Cdc25 regulation of mitotic entry	15-21
1.4.1 The discovery and characterization of Wee1/Cdc25	15-17
1.4.2 Wee1/Cdc25 in <i>H. sapiens</i>	18
1.4.3 Wee1/Cdc25 in <i>S. pombe</i>	18-19

1.4.4 Swe1/Mih1 in <i>S. cerevisiae</i>	19-21
1.5 The Anaphase Promoting Complex	21-23
1.6 Anaphase Promoting Complex phosphoregulation	24-25
1.7 Anaphase regulation	25-32
1.7.1 Securin regulation of separase	30-31
1.7.2 Regulation of sister chromatid cohesion	31-32
1.8 Mitotic checkpoints	33-34
1.8.1 The Spindle Assembly Checkpoint (SAC)	33
1.8.2 The DNA Damage Checkpoint	34
1.9 Mitotic exit	34-40
1.9.1 The cdc Fourteen Early Anaphase Release (FEAR) pathway	35
1.9.2 The Mitotic Exit Network (MEN)	35-40
1.10 PP2A regulation of mitosis	40-44
1.10.1 PP2A and PP1 in vertebrate mitotic exit	40-41
1.10.2 <i>S. cerevisiae</i> PP2A	41-44
Chapter 2 – Materials and Methods	45-57
2.1 Strain and plasmid construction	45-51
2.2 Physiology	51
2.3 Immunoblotting	51-53
2.4 Fixed microscopy	54
2.5 Live microscopy	54-55
2.6 FACS analysis	55
2.7 Viability assays	55-56

2.8 In vivo labeling	56
2.9 In vitro phosphorylation and dephosphorylation	56-57
Chapter 3 – Swe1 regulates anaphase onset	58-159
3.1 Purpose	58
3.2 Results	59-140
3.2.1 Swe1 is active during mitosis	59-62
3.2.2 Swe1 regulates anaphase onset in unperturbed cells	62-79
3.2.3 The morphogenesis checkpoint delays anaphase onset	80-98
3.2.4 The morphogenesis checkpoint is not bypassed by <i>PDS1</i> , <i>CLB5</i> or <i>SGO1</i> deletion	98-113
3.2.5 Characterization of the <i>cdc55Δ</i> morphogenesis checkpoint bypass	113-121
3.2.6 PP2A ^{Cdc55} maintains mitotic checkpoints by dephosphorylating the APC	121-137
3.2.7 <i>cdc55Δ</i> rescues mutants with reduced APC activity	137-140
3.3 Discussion	140-159
3.3.1 Swe1 regulates anaphase onset	140-146
3.3.2 Does Wee1 regulate anaphase onset in other organisms?	146-151
3.3.3 How does Cdk1 activity trigger anaphase?	151-152
3.3.4 Kip1 and Cin8 do not regulate spindle formation in budding yeast	152-154
3.3.5 Swe1 and Mhl1 regulate cell size	154-155

3.3.6 Cdc20 is stabilized upon morphogenesis checkpoint	
activation	155
3.3.7 Cells lacking <i>CLB5</i> and <i>CLB6</i> undergo abnormal spindle	
elongation	156
3.3.8 Cells lacking <i>PDS1</i> and <i>SGO1</i> have abnormal mitotic	
spindles	156-157
3.3.9 Deletion of <i>CDC55</i> bypasses the morphogenesis	
checkpoint	157-159
Chapter 4 – Cdk1 phosphorylation of separase regulates spindle elongation	160-252
4.1 Purpose	160
4.2 Results	160-233
4.2.1 Esp1 is activated independently of Pds1 degradation in	
<i>cdc55Δ</i> cells	160-161
4.2.2 Depletion of Pds1 in <i>cdc55Δ</i> cells causes spindle defects	161-171
4.2.3 Deleting <i>SWE1</i> partially rescues <i>cdc55Δ PDS1-AID</i>	
spindle defects	171-177
4.2.4 Esp1 is phosphorylated by Cdk1 ^{Clb2} and dephosphorylated	
by PP2A ^{Cdc55}	177-183
4.2.5 Mimicking phosphorylation on Esp1 recapitulates <i>cdc55Δ</i>	
phenotypes	183-193
4.2.6 Preventing Esp1 phosphorylation does not rescue cell	
cycle defects in <i>cdc55Δ</i> cells	193-199
4.2.7 Mimicking Esp1 phosphorylation causes subtle cell	
cycle defects	199-206

4.2.8 <i>esp1-NLS</i> rescues the lethality of Pds1 depletion in <i>cdc55Δ</i> cells	206--216
4.2.9 Depletion of Pds1 in <i>slk19Δ</i> cells causes spindle defects	216-233
4.3 Discussion	234-252
4.3.1 Depletion of Pds1 causes premature Scc1 cleavage	234
4.3.2 Independent regulation of spindle elongation and sister separation	235-237
4.3.3 <i>CDC55</i> deletion is lethal to Pds1-depleted cells independent of Scc1 phosphorylation	237-238
4.3.4 Cdk1 phosphorylates and PP2A ^{Cdc55} dephosphorylates Esp1	238-239
4.3.5 How does PP2A ^{Cdc55} regulate spindle structure	239-247
4.3.6 Cdk1 regulation of mitotic proteins	247-252
Chapter 5 - Conclusions and Future Directions	253-256
References	257-300
Contributions of Collaborators	301
Appendix 1 – Yeast strains used in this study	302-307

List of Abbreviations

1NM-PP1	C3-1'-naphthylmethyl pyrazolo[3,4- <i>d</i>]pyrimidine
AID	auxin-inducible degron
APC	anaphase promoting complex
C-box	Cdc20-box
CDC	cell division cycle
CHO	chinese hamster
CPC	chromosomal passenger complex
D-box	destruction box
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
FEAR	Cdc14 early anaphase release
G1	gap 1
G2	gap 2
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GVBD	germinal vesicle breakdown
HeLa	Henrietta Lacks
iMT	interpolar microtubule
INCENP	inner centromere protein
IR-tail	isoleucine arginine-tail

KEN box	lysine (K) glutamic acid (E) asparagine (N) box
kMT	kinetochore microtubule
latA	latrunculin A
M-phase, M	mitosis
MBF	Mlu1 cell cycle box binding factor
MCC	mitotic checkpoint complex
MEN	mitotic exit network
MPF	maturation promoting factor
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
ORC	origin recognition complex
PCR	polymerase chain reaction
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
pre-RC	pre-replication complex
RENT	regulator of nucleolar silencing and telophase
RING	really interesting new gene
S-phase	synthesis-phase
SAC	spindle assembly checkpoint
SDS-PAGE	sodium dodecyl sulfate polyacrylamide electrophoresis
SPB	spindle pole body
SBF	Swi4/6 cell cycle box binding factor
SCF	Skp, Cullin, F-box containing complex
SFF	Switching deficient five factor

SPOC	spindle position checkpoint
TEV	tobacco etch virus
TPR	tetratricopeptide repeat

List of Figures

Figure 1-1 – Cdk1 regulation of cell cycle progression

Figure 1-2 – Cdk1 regulation by inhibitory phosphorylation

Figure 1-3 – The mitotic spindle

Figure 1-4 – Regulation of anaphase onset

Figure 1-5 – Regulation of mitotic exit

Figure 1-6 – PP2A^{Cdc55} regulation of mitosis

Figure 3-1 – Swe1 is present and active during mitosis

Figure 3-2 – Deleting *MIH1* stabilizes APC substrates

Figure 3-3 – Monitoring anaphase onset using fluorescence microscopy

Figure 3-4 – Deleting *MIH1* delays mitotic entry and anaphase onset

Figure 3-5 – Deconvolution does not improve three-dimensional spindle measurements

Figure 3-6 – Swe1 and Mih1 regulate anaphase onset in live cells

Figure 3-7 – Deleting *SWE1* or *MIH1* does not affect anaphase spindle elongation

Figure 3-8 - Deleting *SWE1* or *MIH1* affects the size at which cells undergo mitotic transitions

Figure 3-9 – Activation of the morphogenesis checkpoint delays anaphase onset

Figure 3-10 – The morphogenesis checkpoint delays cell cycle progression after bipolar spindle formation

Figure 3-11 – The morphogenesis checkpoint delays anaphase onset through Cdk1 phosphorylation

Figure 3-12 – The morphogenesis checkpoint delays APC substrate degradation

Figure 3-13 – *swe1Δ* does not bypass APC inactivation using the *cdc16-1* allele

Figure 3-14 – *swe1Δ* does not bypass APC inactivation using the *cdc23-1* allele

Figure 3-15 – Morphogenesis checkpoint activation stabilizes Cdc20

Figure 3-16 – Deleting *PDS1* does not bypass the morphogenesis checkpoint

Figure 3-17 – Deleting *PDS1* does not impact Esp1 stability

Figure 3-18 – Deleting *CLB5* and *CLB6* does not bypass the morphogenesis checkpoint

Figure 3-19 – Deleting *CLB5* and *PDS1* does not bypass the morphogenesis checkpoint

Figure 3-20 – Deleting *PDS1* and *SGO1* does not bypass the morphogenesis checkpoint

Figure 3-21 – Depleting Pds1 in *clb5Δ clb6Δ* cells does not bypass the morphogenesis checkpoint

Figure 3-22 – Depleting Clb5 in *pds1Δ sgo1Δ* cells does not bypass the morphogenesis checkpoint

Figure 3-23 – Deleting *CDC55* bypasses the morphogenesis checkpoint

Figure 3-24 – Morphogenesis checkpoint activation partially stabilizes APC substrates in *cdc55Δ* cells

Figure 3-25 – *cdc55Δ* cells re-replicate DNA during morphogenesis checkpoint activation

Figure 3-26 – PP2A^{Cdc55} maintains spindle checkpoint arrest by dephosphorylating the APC

Figure 3-27 – PP2A^{Cdc55} preserves cell viability during spindle checkpoint arrest by dephosphorylating the APC

Figure 3-28 – Preventing APC phosphorylation does not delay anaphase onset in *cdc55Δ* cells treated with latA

Figure 3-29 – PP2A^{Cdc55} maintains morphogenesis checkpoint arrest by dephosphorylating the APC

Figure 3-30 – Preventing APC phosphorylation reduces DNA re-replication in *cdc55Δ* cells treated with latA

Figure 3-31 – Deleting *CDC55* rescues the viability of cells with reduced APC activity

Figure 3-32 – Deleting *CDC55* or *SWE1* does not destabilize APC substrates in cells with reduced APC activity

Figure 3-33 – A new model for the regulation of anaphase onset

Figure 3-34 – A model for spindle formation in yeasts

Figure 4-1 – Esp1 substrates are degraded in *cdc55Δ* cells treated with latA

Figure 4-2 – Pds1 depletion is lethal to *cdc55Δ* cells

Figure 4-3 – Pds1 depletion in *cdc55Δ* cells causes spindle defects

Figure 4-4 – *SCC1-AID* cells display spindle defects in the absence of auxin

Figure 4-5 – Pds1 depletion in *cdc55Δ* cells causes premature sister chromatid separation

Figure 4-6 – Rapid spindle elongation in *cdc55Δ* cells depleted of Pds1 requires Cdk1 inhibitory phosphorylation

Figure 4-7 – The lethality of Pds1 depletion to *cdc55Δ* cells is not due to Sccl phosphorylation

Figure 4-8 – Cdk1^{C1b2} phosphorylates Esp1

Figure 4-9 – PP2A^{Cdc55} dephosphorylates Esp1

Figure 4-10 – Pds1 depletion causes spindle defects in a phosphomimetic Esp1 mutant

Figure 4-11 – *esp1-3A* and *esp1-3D* are dominant

Figure 4-12 – Pds1 depletion does not cause premature sister chromatid separation in a phosphomimetic Esp1 mutant

Figure 4-13 – Preventing Esp1 phosphorylation does not rescue phenotypes associated with deleting *CDC55*

Figure 4-14 – Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to Pds1 depletion

Figure 4-15 – Preventing Esp1 phosphorylation does not perturb mitotic progression

Figure 4-16 – Spindle defects in *cdc55Δ* and *esp1-3D* cells depleted of Pds1 are not due to spindle checkpoint activation

Figure 4-17 – Mimicking Esp1 phosphorylation does not perturb mitotic progression

Figure 4-18 – Mimicking Esp1 phosphorylation partially rescues sickness due to Mps1 overexpression

Figure 4-19 – Mimicking Esp1 phosphorylation is lethal to cells deficient in Esp1 nuclear import

Figure 4-20 – *esp1-NLS* rescues the lethality of *cdc55Δ* cells depleted of Pds1

Figure 4-21 – The *esp1-NLS* allele is dominant

Figure 4-22 – *esp1-NLS* rescues the spindle defects displayed by *cdc55Δ* cells depleted of Pds1

Figure 4-23 – The lethality of Pds1 depletion in *cdc55Δ* cells is not due to FEAR pathway activation

Figure 4-24 – *slk19Δ* exhibits different genetic interactions than *spo12Δ*

Figure 4-25 – Pds1 depletion causes spindle defects in cells lacking *SLK19*

Figure 4-26 – *cdc55Δ* and *slk19Δ* cells depleted of Pds1 missegregate sister chromatids

Figure 4-27 – Pds1 depletion is lethal to cells lacking spindle midzone proteins

Figure 4-28 – Cdc14 is not released from the nucleolus prematurely in *swe1Δ cdc55Δ* cells depleted of Pds1

Figure 4-29 – Cdk1 sites in eukaryotic separases

Figure 4-30 – A model for Esp1/Slk19 regulation by spindle midzone proteins

Figure 4-31 – A model for cohesin cleavage and spindle stabilization

List of Appendices

Appendix 1 – Yeast strains used in this study

Chapter 1

Introduction

1.1 The cell cycle

Cell cycle progression in all cells is the ordered series of events required for the formation of a pair of daughter cells from a single mother cell. At its most basic level, the cell cycle requires the duplication of the mother cell's genetic material and the subsequent partitioning of the duplicated genome into two daughter cells. In eukaryotic cells, the genetic material is composed of multiple discrete chromosomes which each must be copied separately during synthesis (S-phase). At the entry to mitosis (M-phase), these replicated chromosomes condense, resulting in structures small enough to be successfully separated by the mitotic spindle. After spindle elongation and chromosome segregation into daughter cells, chromosomes decondense in preparation for the subsequent round of replication. Thus the eukaryotic cell cycle consists of ordered rounds of replication and segregation in which the entire genome is replicated before being segregated, and segregated before being subsequently replicated. This process can therefore be regulated by molecules that are activated and inactivated in a temporally cyclic fashion.

In prokaryotic cells, however, cell cycle progression occurs in a fundamentally different manner (reviewed in (1)). Generally, prokaryotic cells have a single circular genome that replicates from a single origin of replication. If prokaryotic cells had distinct DNA synthesis and segregation phases, the speed at which they were capable of dividing could not exceed the time it took to replicate the entire chromosome. However, it has been shown that in optimal conditions *Escherichia coli* cells can divide in half the time it takes for

a single round of DNA replication (1). They do this by initiating multiple rounds of DNA replication within a single as-yet undivided cell. Multiple concurrent rounds of replication in a single cell thus requires DNA segregation to happen concurrently to DNA replication in a single cytoplasm. This limits the ability of cyclically activated/inactivated cell cycle regulators to signal cell cycle transitions as is the case in eukaryotic cells. Therefore the biochemical pathways discussed here, though broadly applicable to eukaryotic cells, are in general not conserved in prokaryotes.

1.2 The discovery and characterization of Cdk1

Our current understanding of the biochemical control of the cell cycle is fundamentally informed by research beginning in the 1970s and 1980s into cell cycle regulation in a variety of model organisms, most notably the frog *Xenopus laevis*, clam *Spisula solidissima*, starfish *Asterina pectinifera*, sea urchin *Arbacia punctulata*, fission yeast *Schizosaccharomyces pombe* and budding yeast *Saccharomyces cerevisiae*. Individually, each of these model organisms provided distinct advantages in terms of the biochemical and genetic techniques available to researchers. More importantly, concurrent research on all of these organisms allowed for the identification and characterization of the most fundamental biochemical processes regulating cell cycle progression.

1.2.1 Maturation Promoting Factor (MPF)

The first hints regarding how eukaryotic cells regulate cell cycle progression came from studies of meiotic cell divisions in frog and starfish oocytes. During meiosis, immature oocytes arrest at prophase I until hormone stimulation causes them to progress through meiosis I and arrest at metaphase of meiosis II in a process called maturation. Fertilization by

a sperm cell stimulates mature oocytes arrested at metaphase II to complete meiosis, resulting in the formation of a zygote. Stimulation of immature prophase I-arrested oocytes in vitro with progesterone causes oocyte maturation, thereby allowing researchers to easily study this process (2). It had previously been shown that nuclei injected from interphase somatic cells into sufficiently matured oocytes immediately underwent germinal vesicle breakdown (GVBD; nuclear breakdown), a process characteristic of maturation (3). This experiment suggested that matured oocytes contained a cytoplasmic factor that induced cell cycle progression into meiosis. To characterize this factor, frog oocytes were induced to mature using the hormone progesterone and cytoplasm was periodically withdrawn from these maturing oocytes to determine if these extracts could in turn induce hallmarks of maturation in immature oocytes (4, 5). These experiments demonstrated that shortly after the beginning of maturation, a factor accumulated in oocytes that could in turn induce maturation in immature oocytes. Cytoplasm could induce maturation even after serial transfer through 10 donors (6). This demonstrated that the meiosis-inducing factor produced in maturing oocytes was amplified during maturation. This factor was termed maturation promoting factor (MPF) (4).

MPF activity was soon identified in many different eukaryotic organisms and cell types including salamanders (*Ambystoma mexicanum*), humans (HeLa cells), chinese hamsters (CHO cells) and budding yeast (6-9). It was also determined that cytoplasm from maturing oocytes was able to induce GVBD in oocytes from both vertebrates and invertebrates (10). MPF was further shown to cycle during mitotic cell cycles in frog embryos (11, 12), and could induce not only meiotic but mitotic events (13, 14). Together, these data demonstrated that MPF was a fundamental and highly conserved eukaryotic cell cycle regulator.

1.2.2 Cell Division Cycle 28 (CDC28)

At the same time that MPF was first being characterized, yeast researchers were using genetic techniques to identify key cell cycle regulators. In budding and fission yeasts it had been shown that specific gene products could be generated that functioned at low ‘permissive’ temperatures but failed to function at high ‘restrictive’ temperatures (15). This allowed essential gene products to be conditionally knocked-out using temperature-shift experiments. In a landmark study, Leland Hartwell mutagenized *S. cerevisiae* using a chemical mutagen to induce temperature-sensitive mutations (16-20). Mutagenized cells were grown at the permissive temperature, shifted to the restrictive temperature and mutations were identified that arrested cells at a uniform stage in the cell cycle. This experiment identified 32 cell division cycle (*CDC*) regulators that are important for signaling specific cell cycle transitions. Among these 32 regulators Hartwell identified one, *CDC28*, which was necessary for both bud emergence and DNA replication.¹

Similar analysis was subsequently undertaken in fission yeast to identify *cdc* genes (21). This analysis identified mutations in the gene *cdc2+* that, similar to budding yeast *CDC28*, resulted in G1 arrest (22). *cdc2* temperature sensitive alleles also caused defects in mitotic entry, a phenotype that was soon also demonstrated for *cdc28* temperature sensitive mutants (23). The functional homology of *S. cerevisiae* Cdc28 and *S. pombe* Cdc2 was subsequently demonstrated by the ability of *CDC28* to complement *cdc2-* in fission yeast (24). Once cloned, it became clear the conservation of Cdc2 extended beyond budding and fission yeasts. Cdc2 homologs were soon found in humans and the fruit fly *Drosophila melanogaster* (25, 26).

¹ Accepted *S. cerevisiae* nomenclature: wildtype allele (*CDC28*); recessive allele *cdc28-1*; deletion (*cdc28Δ*); protein (Cdc28). Accepted *S. pombe* nomenclature: wildtype allele (*cdc2+*); recessive allele (*cdc2-1*); deletion (*cdc2-*); protein (Cdc2).

It was quickly determined that Cdc2 was a key component of MPF (27, 28). Injection of purified *S. pombe* Suc1, a protein that had been shown to bind fission yeast Cdc2, blocked the ability of MPF to induce maturation in *X. laevis* oocytes (29). Antibodies raised against fission yeast Cdc2 recognized a Cdc2 protein in *X. laevis*, and *S. pombe* Suc1 bound both *X. laevis* Cdc2 and depleted MPF activity from crude *X. laevis* MPF preparations. Though Cdc2 was clearly a component of MPF, researchers had not yet identified the mechanism that up-regulated MPF activity during maturation.

1.2.3 Cyclin

Concurrent work in marine invertebrates such as the sea urchin *Arbacia punctulata* and the clam *Spisula solidissima* had identified proteins that were expressed in a cyclic manner over the course of the cell cycle (30). It had previously been observed that fertilized sea urchin oocytes accumulated different protein species than unfertilized controls (31, 32). Crucially, Evans *et al.* observed that upon fertilization of sea urchin oocytes, the amount of some protein species increased linearly, while others appeared to increase and decrease in a cyclic manner (30). They termed these cyclically expressed proteins ‘cyclins’. The timing of peak cyclin expression preceded oocyte cleavage during each cleavage cycle. The presence of these proteins in both sea urchin and clam embryos suggested that cyclin proteins were conserved through evolution.

Indeed, in the years following the discovery of cyclin in sea urchins and clams, they were found in starfish (*A. pectinifera*), *X. laevis*, *D. melanogaster* and *S. pombe* (33-37). These cyclin proteins were divided into two classes, A-type and B-type, based on sequence homology (33), kinetics of appearance and molecular weight (30).

Purification of MPF consistently co-purified Cdc2 and a single additional protein in *X. laevis*, *S. cerevisiae*, *H. sapiens* and *S. pombe* (38-41). The first demonstration that this component was likely cyclin came when cyclin A from the clam *S. solidissima* was cloned (42). Injecting clam cyclin A mRNA into immature *X. laevis* oocytes induced maturation, suggesting that cyclin was the regulated component of MPF. Degradation of cyclin mRNA was shown to prevent mitotic entry in *X. laevis* cell free extracts, demonstrating that, like Cdc2, cyclin was conserved between vertebrates and invertebrates and was necessary for mitosis (33, 43). Cyclin mRNA was also shown to be sufficient to trigger mitotic entry (43). Cyclin was shown to interact directly with Cdc2, first in *S. solidissima* (44) and later *A. punctulata*, *A. pectinifera* and *X. laevis* (45-47). This data together demonstrated that MPF was composed of a cyclin subunit and a Cdc2 subunit. This data also suggested that MPF activity was regulated by the periodic expression of the cyclin component.

1.2.4 Cyclin Dependent Kinase 1 (CDK1)

Early on it was clear that MPF activity was closely associated with protein phosphorylation. A burst of phosphorylation preceded GVBD in progesterone-activated oocytes and followed MPF addition to unactivated oocytes (48). The first evidence that MPF itself was a kinase came from the observation that partially purified MPF activity could be greatly induced with the addition of ATP (49). It was then determined that, in maturing starfish oocytes, levels of MPF activity closely match levels of cellular phosphorylation (50). Sequencing of *CDC28* and *cdc2+* showed that they shared sequence homology with several vertebrate oncogenes that function as protein kinases (51, 52), and purified Cdc28 and later Cdc2 were shown to display kinase activity (53, 54). Direct evidence for a Cdc2 kinase in human cells was demonstrated in 1987 when Draetta et al. used antibodies to *S. pombe* Cdc2

to immunoprecipitate a protein from HeLa cells dubbed p34, which displayed kinase activity and interacted with p13, the human homolog of *S. pombe* Suc1 (55). Further, peptide mapping of p34 suggested that Cdc28 and p34 displayed similarities in amino acid sequence. Lee and Nurse were able to show the functional homology of human p34 and *S. pombe* Cdc2 by demonstrating that *S. pombe* cells lacking Cdc2 could survive if they expressed human p34 (25). Thus it became clear that the Cdc2/cyclin complex functioned in all eukaryotic organisms as an important cell cycle regulator. Cdc2 was subsequently renamed Cdk1 in all organisms in recognition of its conserved role.

Though in budding yeast Cdk1 is responsible for the majority of Cdk-dependent cell cycle regulation, continued work demonstrated that vertebrates had multiple essential Cdks. *Xenopus* was shown to express two different Cdc2 homologs (56). One associates with cyclin A and is important for regulating DNA replication. Another binds cyclins B1 and B2 and is important for signaling mitotic entry. Human cells were also shown to express multiple Cdks, suggesting a higher degree of complexity in cell cycle control (57).

1.3 Cdk1 in the *S. cerevisiae* cell cycle

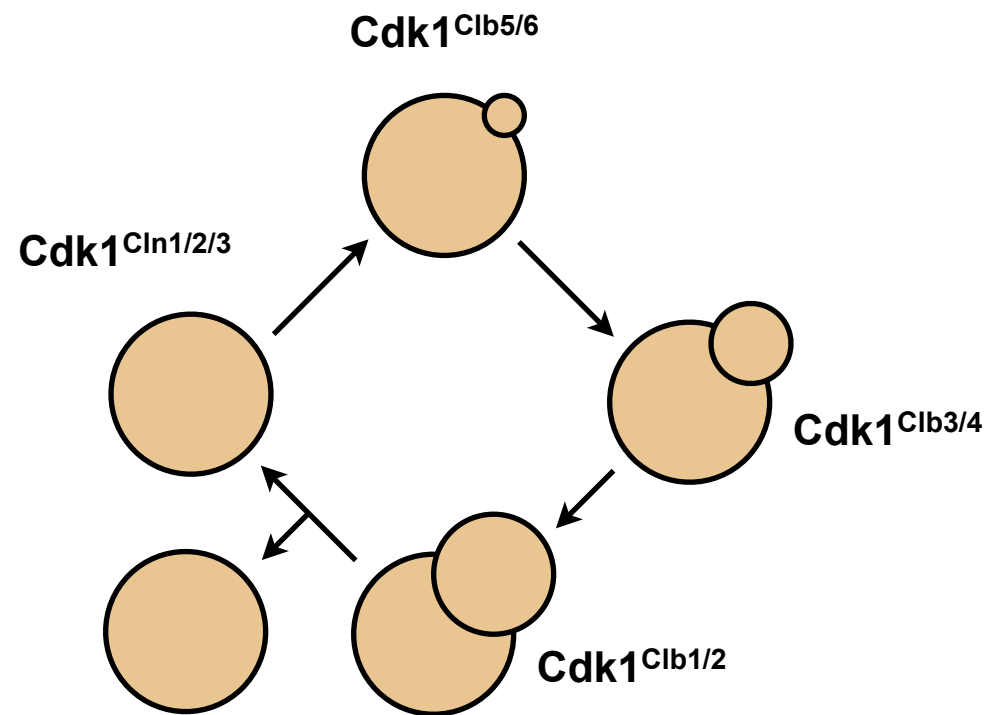
S. cerevisiae has six Cdks; Cdk1, Pho85, Kin28, Ssn3, Ctk1 and Bur1 (51, 58-62). Ssn3, Ctk1 and Bur1 are non-essential and appear to function mainly in transcriptional regulation (reviewed in (63))(60, 61, 64). Pho85 has a role in G1 regulating cell morphology (reviewed in (65)), though it is also non-essential and overexpressing it cannot rescue the *cdc28-1* allele (58, 66). Kin28 is essential but overexpression cannot rescue mutants expressing the *cdc28-6* allele, suggesting that Cdk1 and Kin28 have different cellular functions (59). Similar to Ssn3, Ctk1 and Bur1, Kin28 has been implicated mainly in transcriptional regulation (reviewed in (63)). In contrast to the other *S. cerevisiae* Cdks,

Cdk1 is essential and has been implicated in a wide variety of cellular processes (67). There are at least 75 well-documented Cdk1 substrates (reviewed in (68)), though one recent large-scale screen has identified 308 Cdk1 substrates, suggesting that many more have yet to be characterized (69).

1.3.1 Regulation of Cdk1

Cdk1 activity through the cell cycle is regulated by cyclin-association, inhibitor binding and phosphorylation. Cdk1 interacts with nine different cyclins in budding yeast (Figure 1-1A) (70-75). The G1 cyclins Cln1, Cln2 and Cln3 are involved in the passage from G1 to S phase and are highly redundant, as only a triple knockout is lethal (76). Analysis of the specific roles of the six mitotic cyclins Clb1-6 can be difficult as there is also significant redundancy between them (77, 78). Of the six mitotic cyclins, Clb5 and Clb6 are expressed the earliest in the cell cycle and are thought to signal the transition to S-phase, as deletion of *CLB5* delays DNA replication and this phenotype is exacerbated by deleting *CLB6* (74, 75, 79). Clb3 and Clb4 expression peaks after that of Clb5 and Clb6. Clb3 and Clb4 are thought to act redundantly to Clb5/6 to promote DNA replication and Clb1/2 to promote mitosis (80). Clb3 and Clb4 also appear to have an important role in the formation of the mitotic spindle (77). Clb1 and Clb2 expression peaks following Clb3/4, and Cdk1^{Clb2} and Cdk1^{Clb1} primarily regulate mitotic events such as spindle elongation (80). Total Cdk1 activity gradually increases from G1 to mitosis and then abruptly decreases as cells exit mitosis and enter the subsequent G1 (Figure 1-1B) (81). Cell cycle transitions are therefore likely signaled both by targeted substrate phosphorylation by specific Cdk1^{cyclin} complexes and by the gradual increase in Cdk1 activity.

A



B

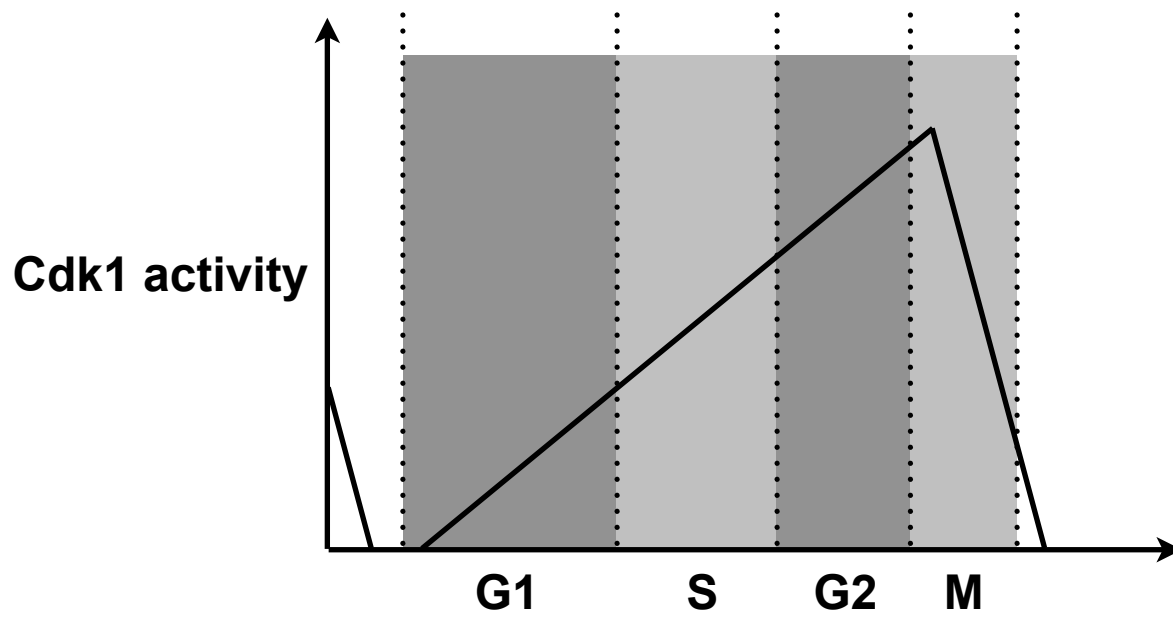


Figure 1-1

Figure 1-1. Cdk1 regulation of cell cycle progression.

(A) Cell cycle progression is characterized by an ordered series of Cdk1^{cyclin} interactions.

The G1 cyclins Cln1, Cln2 and Cln3 are expressed during G1. Peak Clb5 and Clb6 expression occurs during S-phase. Peak Cln3 and Cln4 expression follows that of Clb5 and Clb6. Clb1 and Clb2 expression levels are highest during mitosis.

(B) Cell cycle progression is characterized by gradually increasing total Cdk1 activity from G1 until mitosis.

Cdk1 binds to, and is activated by, the small protein Cks1 (71). Deletion of *CKS1* is lethal and mutants arrest cells in either G1 or mitosis, similar to mutations that inactivate Cdc28 (67, 71). This data suggests Cks1 activity is crucial for proper Cdk1 function, however the mechanism through which Cks1 functions is unclear. Recently it has been shown that Cks1 may be involved in recognition of phosphorylated proteins by Cdk1^{cyclin/Cks1} complexes (82, 83).

Cdk1 is also bound by inhibitory molecules during G1 to prevent progression through START. During vegetative growth and in response to mating pheromone, Far1 binds and inhibits Cdk1^{Clⁿ1} and Cdk1^{Clⁿ2} directly (84-86). During G1, Cdk1^{Cl^b} complexes are bound and inhibited by Sic1 (39, 79). This inhibition is relieved at START when Cdk1^{Clⁿ} complexes promote the phosphorylation of Far1 and Sic1, thereby signaling their degradation by the SCF (Skp, Cullin, E-box containing complex) associated with the F-box protein Cdc4 (87-90). Notably, the only essential substrate of Cdk1^{Clⁿ} is Sic1 as the lethality of a strain lacking *CLN1*, *CLN2* and *CLN3* can be rescued by deleting *SIC1* (91).

Cdk1 is regulated both positively and negatively by phosphorylation. Cdk1 is activated by phosphorylation of its T-loop, a large flexible loop that blocks the active site when unphosphorylated. T-loop phosphorylation on threonine 169 is catalyzed by Cak1 (Cdk1 activating kinase 1) (92, 93). This results in a conformational change that exposes the substrate binding site and improves Cdk1 affinity for cyclins (94). Phosphorylation of the T-loop does not appear to be a highly regulated process as threonine 169 phosphorylation does not vary significantly over the cell cycle (94).

In addition to activating phosphorylation, Cdk1 undergoes inhibitory phosphorylation. This inhibitory phosphorylation on tyrosine 19 is catalyzed by the kinase Swe1 (95). Swe1 activity is opposed by the phosphatase Mih1 (96). Swe1 phosphorylates

Cdk1 in response to reduced cell size or perturbed bud morphogenesis and delays cell cycle progression prior to mitotic entry (97, 98). Though *SWE1* and *MIH1* are non-essential, *swe1* Δ cells enter mitosis prematurely at reduced cell size and *mih1* Δ cells delay mitotic entry, demonstrating that Y19 phosphorylation of Cdk1 regulates unperturbed cell cycles (96, 99-104). Inhibitory phosphorylation of Cdk1 will be discussed in greater detail in later sections.

Cdk1 has also been shown to be regulated by acetylation on lysine 40 within its kinase domain (105). Mutation of the acetylated residue lysine 40 to the acetyl-mimic glutamine is lethal, as is mutation to the non-acetylatable residue arginine. This suggests that regulation of Cdk1 acetylation status is important for cell cycle progression, though the downstream processes that Cdk1 acetylation regulates have yet to be determined.

1.3.2 Cdk1 regulation of transcription

Cdk1 is important for regulating many diverse cellular processes. Cdk1 regulation of transcription and cell morphogenesis takes place throughout the cell cycle, whereas regulation of DNA replication and mitotic processes is confined to specific cell cycle stages. Approximately 200 genes, known as the G1 cluster, are specifically expressed in the G1 stage of the cell cycle (106). Transcription of these genes is triggered by the transcription factors SBF (Swi4/6 cell cycle box binding factor) and MBF (Mlu1 cell cycle box binding factor) (107, 108). Cdk1^{Cln3} promotes transcription of the G1 cluster by phosphorylating the SBF inhibitory component Whi5 and promoting the dissociation of Whi5 from SBF (109-113). In G2/M, another set of approximately 35 genes is expressed termed the *CLB2* cluster (106). This set of genes is activated by the SFF (Switching deficient five factor) transcription factor, which is composed of Fkh1/Fkh2, and its coactivator Ndd1 (114-116). Cdk1^{Clib5}

phosphorylates Fkh2 and Cdk1^{Clb2} phosphorylates Ndd1 (117-120). These phosphorylations promote Ndd1/Fkh2 association and transcription of the *CLB2* cluster.

1.3.3 Cdk1 regulation of cell morphogenesis

Cdk1 also regulates cell morphology throughout the cell cycle. At START Cdk1^{Clb3} phosphorylates Far1 and signals Far1 degradation by the SCF^{Cdc4} (87, 90). Degradation of Far1 releases the sole budding yeast guanine nucleotide exchange factor (GEF) Cdc24 from nuclear Far1/Cdc24 complexes (121). This allows for activation by Cdc24 of the Rho-type GTPase Cdc42 at the site of bud formation (122). Cdc24-activated Cdc42 then promotes cell polarization and bud formation (123, 124). Upon bud formation, bud growth also requires Cdk1 activity. Cdk1^{Clb2} phosphorylates the Cdc24-associated protein Boi1 and this phosphorylation is essential for bud growth (125). When buds have reached approximately two-thirds the size of the mother they undergo a switch from apical to isotropic growth (126). This switch is regulated by both Cdk1^{Clb} and Cdk1^{Clb}, as inhibiting Cdk1^{Clb} activity or overexpression of Clb2 results in uninhibited apical growth (127).

1.3.4 Cdk1 regulation of DNA replication and mitosis

Cdk1 regulation is also important for regulating chromosome replication in S-phase and segregation during mitosis. Cdk1^{Clb} ensures DNA replication occurs only once per cell cycle and alternates with mitosis by preventing pre-replication complex (pre-RC) formation during G2 and mitosis (reviewed in (128)). Cdk1^{Clb} inhibition in G1 allows pre-RC formation and subsequent replication in S-phase. The pre-RC is composed of the six subunit origin recognition complex (ORC), Cdc6, Cdt1 and Mcm2-7. Cdk1^{Clb} inhibits the formation of this complex in three redundant ways and each must be bypassed in order for replication

to proceed (129). Cdk1^{C_{1b}} phosphorylates Cdc6 and targets it for degradation by the SCF^{C_{dc4}} (130, 131). Cdk1^{C_{1b}} promotes nuclear export of Mcm2-7 proteins, which spatially segregates them from replication origins (132, 133). Finally, Cdk1^{C_{1b}} prevents pre-RC formation by phosphorylating Orc2 and Orc6 directly (129).

Though the involvement of Cdk1 in regulating mitotic progression will be discussed in greater detail in later sections, Cdk1 also regulates mitotic structures through numerous substrates. The mitotic spindle is stabilized by Fin1, a self-associating coiled-coil protein that, when activated, forms filaments between spindle pole bodies (SPBs) (134, 135). Fin1 is phosphorylated by Cdk1^{C_{1b5}} from S-phase to metaphase (135, 136). Phosphorylation inhibits Fin1 association with the spindle until Fin1 is dephosphorylated by Cdc14 at anaphase onset. Fin1 dephosphorylation targets it to the poles and microtubules of the elongating spindle where it contributes to spindle integrity and efficient chromosome segregation.

Cdk1 also contributes to spindle stabilization by phosphorylating components of the chromosomal passenger complex (CPC), which consists of Ipl1, Bir1, Sli15 and Nbl1. The CPC localizes to kinetochores to facilitate biorientation, but relocates to the spindle during anaphase to control spindle stabilization and elongation. Cdk1 phosphorylates Bir1 (137) recruits Ndc10, an inner kinetochore protein to the spindle midzone (126, 137, 138). Cdk1 also phosphorylates Sli15 (inner centromere protein; INCENP) preventing its localization to the spindle (139). Finally, Cdk1 phosphorylates Ase1, a microtubule bundling factor and spindle midzone component (140-142). Cdk1 phosphorylation of different substrates can either stabilize or destabilize the mitotic spindle and further work is needed to elucidate these pathways.

1.4 Wee1/Cdc25 regulation of mitotic entry

1.4.1 The discovery and characterization of Wee1/Cdc25

Perhaps the best-characterized Cdk1-regulated event is mitotic entry (Figure 1-2). In 1975, Paul Nurse used fission yeast to screen for mutations that caused cells to undergo cell division at a reduced cell size ('*wee*' mutants) (143). He isolated mutants in two genes termed *wee1*⁺ and *wee2*⁺ (*wee2*⁺ was soon determined to be *cdc2*⁺) that underwent cytokinesis at approximately half the size of wildtype controls (21, 143). An additional gene, *cdc25*⁺ was implicated in this signaling pathway by the observations that a) deletion of *cdc25*⁺ caused inviability due to the cell's inability to enter mitosis; b) defective *cdc25*⁺ could be rescued by deleting *wee1*⁺; and c) expressing multiple copies of *cdc25*⁺ caused a *wee* phenotype (143-145). This suggested the model that Wee1 prevented mitosis by inhibiting Cdc2 and Cdc25 promoted mitosis by directly antagonizing Wee1 activity towards Cdc2 (146). Cloning of *wee1*⁺ demonstrated that the carboxy-terminal region of Wee1 contains protein kinase consensus sequences (147). Work in other eukaryotic organisms suggested that Wee1 might be a conserved Cdk1 regulator. Cdc2 dephosphorylation during hormone induced maturation correlated with activation in *A. pectinifera* and *X. laevis* (148, 149). Also, Cdc2 tyrosine phosphorylation in HeLa cells was shown to be cell cycle regulated (150). Using phospho-tyrosine antibodies it was shown in *X. laevis* and mouse 3T3 cells that tyrosine phosphorylation was inversely correlated with Cdc2 activity (151, 152). The mechanism by which Wee1 inhibits Cdc2 was determined in *S. pombe*. Gould and Nurse showed that Cdc2 was phosphorylated on a conserved tyrosine residue (Y15) within the nucleotide binding domain (153). Preventing phosphorylation of this residue caused a *wee* phenotype and rescued the conditional lethality of a *cdc25-22* strain.

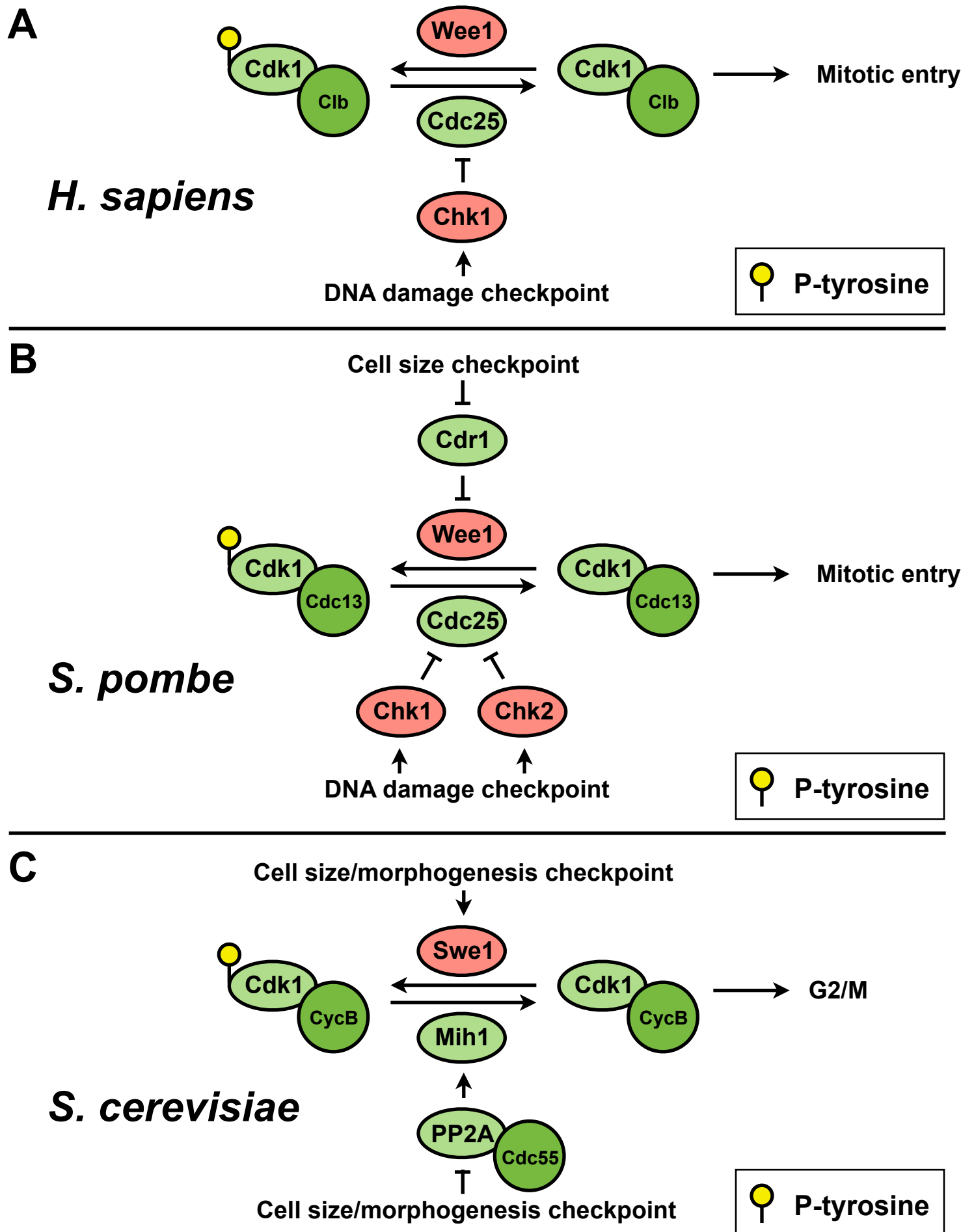


Figure 1-2

Figure 1-2. Cdk1 regulation by inhibitory phosphorylation.

- (A) In human cells, activation of the DNA damage checkpoint induces Cdk1 inhibitory phosphorylation.
- (B) In fission yeast, activation of the DNA damage or cell size checkpoints induces Cdk1 inhibitory phosphorylation.
- (C) In budding yeast, activation of the cell size/morphogenesis checkpoint induces Cdk1 inhibitory phosphorylation.

1.4.2 Wee1/Cdc25 in H. sapiens

In most eukaryotic cells, Cdk1-inhibitory phosphorylation prevents mitotic entry in response to DNA damage or stalled replication forks (Figure 1-2A) (reviewed in (154)). In human cells, DNA damage checkpoint activation results in the phosphorylation of three Cdc25 isoforms: Cdc25A, Cdc25B and Cdc25C, by the essential checkpoint kinase Chk1 (155, 156). This a) downregulates Cdc25 activity directly (157, 158); b) promotes Cdc25 binding to 14-3-3 proteins which prevents Cdc25 nuclear translocation (155, 159, 160); and c) initiates Cdc25 proteolytic degradation (161, 162). Cdc25A, B and C repression through these mechanisms prevents Cdk1 dephosphorylation and mitotic entry to allow for DNA damage repair.

1.4.3 Wee1/Cdc25 in S. pombe

In fission yeast, the DNA damage checkpoint also targets Cdk1 inhibitory phosphorylation and acts through a very similar mechanism to mammalian cells (Figure 1-2B) (163). Activation of this checkpoint due to DNA damage or stalled replication forks causes the checkpoint kinases Chk1 and Chk2 to phosphorylate Cdc25 (164, 165). This inactivates Cdc25 and prevents mitotic entry. There is some evidence that phosphorylation of Cdc25 causes Cdc25 to bind 14-3-3 proteins, thereby preventing Cdc25 from dephosphorylating nuclear Cdk1 (166). However, cells expressing a Cdc25 allele that targets Cdc25 to the nucleus (Cdc25-NLS; nuclear localization signal) are competent for checkpoint signaling, suggesting that Chk1/Chk2 phosphorylation may regulate Cdc25 in other ways (167). Indeed, Cdc25 phosphorylation has been shown to inhibit Cdc25 activity directly in vitro (159).

In fission yeast, Cdk1 is also targeted by a ‘cell size’ checkpoint (Figure 1-2B). As mentioned previously, cells lacking *wee1*⁺ enter mitosis prematurely at a smaller size, demonstrating that Cdk1 inhibitory phosphorylation may delay mitotic entry until a critical cell size is reached (143, 168). In fission yeast, Wee1 localizes to interphase medial nodes along with its inhibitor Cdr1 in a Cdr2 dependent manner (169-171). The cell polarity factor Pom1 is concentrated at cell tips and its concentration decreases towards the centre of the cell (172, 173). Therefore, as cells grow and cell length increases, medial cortical nodes are exposed to decreasing concentrations of Pom1 (170, 171). Decreasing Pom1 concentration relieves Cdr2 inhibition of Cdr1 at medial cortical nodes thereby promoting Cdr1 inhibition of Wee1 (170, 171). The medial cortical nodes are located in close proximity to the nucleus where Cdk1 is localized to the spindle pole bodies (174, 175). Therefore it is thought that Wee1 inactivation at medial cortical nodes results in Cdk1 activation at spindle pole bodies and the initiation of mitotic entry (174).

1.4.4 Swe1/Mih1 in S. cerevisiae

In *S. cerevisiae*, phosphorylation of Cdk1 has also been shown to regulate cell cycle progression (Figure 1-2C). In budding yeast, Cdk1 phosphorylation is not the target of the DNA damage checkpoint (176, 177). Instead a checkpoint delays the cell cycle during G2/M in response to perturbations in bud formation and bud growth (97). There is some debate as to whether this checkpoint detects morphogenesis defects directly through the actin cytoskeleton (a ‘morphogenesis’ checkpoint) or if it simply senses daughter cell size in G2 (in a ‘cell size’ checkpoint analogous to the fission yeast cell size checkpoint) and delays mitosis until daughter cells are large enough to be viable at cell division (102, 178).

In unperturbed cell cycles, Swe1 is localized to the bud neck along with polo kinase (Cdc5) by Hsl1 and Hsl7 (179-183). The current model of the morphogenesis checkpoint proposes that at the bud neck Cdc5 and Cdk1 cooperatively phosphorylate Swe1 and this phosphorylation triggers Swe1 degradation and mitotic onset (179). According to this model bud morphogenesis defects prevent the activation of Hsl1, thereby preventing Swe1 localization to the bud neck and subsequent degradation (183). However, Swe1 must be regulated by additional mechanisms, as forced localization of Swe1 away from the bud neck does not prevent Swe1 regulation and degradation (184). Also, the mechanism and importance of Swe1 degradation *in vivo* has not been shown. It occurs as cells exit mitosis and may simply be a downstream effect of cell cycle progression (102).

Swe1 and Mih1 both undergo significant cell cycle regulated phosphorylation. Swe1 is largely absent in G1 cells and as the cell cycle progresses Swe1 becomes progressively more phosphorylated (185). This phosphorylation is largely due to Cdk1 activity, though Cla4 and Cdc5 have also been shown to phosphorylate Swe1 (101, 182). Initial phosphorylation of Swe1 by Cdk1 promotes Swe1 association with and phosphorylation of Cdk1 (82, 101). This phosphorylation is opposed by protein phosphatase 2A in association with its regulatory subunit Cdc55 (PP2A^{Cdc55}) (186). Cdk1 is maintained in this phosphorylated and inhibited form until some, yet to be identified, signal causes a small pool of Cdk1 to be activated (Harvey Kellogg 2011). Activation of this small pool of Cdk1 causes hyperphosphorylation and release of Swe1 from Cdk1/Swe1 complexes (101). This reduces Swe1 inhibition of Cdk1 and causes further hyperphosphorylation of Swe1 in a positive feedback loop resulting in full Cdk1 activation. Mih1 is also hyperphosphorylated throughout the cell cycle, with most Mih1 becoming dephosphorylated during mitosis (103). Mih1 is phosphorylated by casein kinase 1 and dephosphorylated by PP2A^{Cdc55}, and

dephosphorylation of Mih1 by PP2A^{Cdc55} requires Cdk1 activity. This data is consistent with a model in which a small amount of Cdk1 activation leads to hyperphosphorylation of Swe1 and dephosphorylation of Mih1. Hyperphosphorylated Swe1 and dephosphorylated Mih1 exhibit reduced ability to inhibit Cdk1, thereby resulting in a positive feedback loop leading to full Cdk1 activation and mitotic entry. Fundamental aspects of this model appear to be conserved in other eukaryotic organisms, as increased Cdk1 activity ultimately inhibits Wee1 kinases and activates Cdc25 phosphatases in positive and double negative feedback loops (187-191).

Recent data has greatly improved our understanding of how Swe1 and Mih1 are regulated in response to signals regarding cell size/morphogenesis. Anastasia *et al.* showed that blocking the fusion of secretory vesicles to the site of bud growth caused a *SWE1*-dependent G2/M delay (192). In these cells, actin organization appeared normal, demonstrating that the morphogenesis/cell size checkpoint monitored secretory vesicle delivery or bud growth rather than actin organization. Anastasia *et al.* outlined a model in which cells monitored bud growth through a signaling pathway composed of Rho1, Pkc1, Zds1/2, PP2A^{Cdc55}, Swe1 and Mih1. Further work will be required to determine how proteins implicated in regulating Swe1 at the bud neck interact with this signaling pathway.

1.5 The Anaphase Promoting Complex

In order to pass from mitosis into the following G1, Cdk1^{Cib} complexes must be inactivated. The cyclical pattern of cyclin expression originally observed by Evans *et al.* suggested that Cdk1^{Cib} inactivation was caused by cyclin degradation (30). The first direct evidence of the mechanism regulating cyclin destruction came in 1991 when Glotzer *et al.* showed that cyclin was ubiquitinated and Hershko *et al.* showed that preventing

polyubiquitination of proteins in cell-free clam oocytes prevented cyclin A and B degradation (193, 194). Since polyubiquitination was known to cause protein degradation through the 26S proteasome, this suggested that cyclins were ubiquitinated and targeted for degradation by the 26S proteasome. Sudakin *et al.* identified a large protein complex in clam oocytes that exhibited cell cycle dependent ubiquitin ligase activity towards cyclins A and B and dubbed it the cyclosome (195).

Concurrent studies in budding yeast elucidated the importance of a group of tetratricopeptide repeat (TPR) containing proteins Cdc16, Cdc23 and Cdc27. These three proteins were found to form a complex (196). Cdc23 and Cdc16 were shown to be necessary for both anaphase onset and the instability of Clb2 in G1 cells; and inactivation of Cdc16, Cdc23 and Cdc27 prevented Clb2 and Clb3 ubiquitination (197, 198). In HeLa cells, Cdc16 and Cdc27 were found to colocalize and inhibition of Cdc27 prevented anaphase onset, suggesting that the function of these proteins was conserved (199).

Fractionation of mitotic *X. laevis* extracts identified a 20S cell cycle regulated ubiquitin ligase containing homologs of yeast Cdc27 and Cdc16 (200). They named this complex the anaphase promoting complex (APC; the APC/cyclosome will be heretofore referred to simply as the APC). The APC was later shown to contain a cullin-like protein, Apc2, which shared homology to the Cdc53 subunit of the SCF ubiquitin ligase (88, 201-203).

Ubiquitin ligases covalently attach ubiquitin proteins to their substrates to signal their degradation through the 26S proteasome (reviewed in (204-206)). The process of ubiquitination begins with ubiquitin, a small 8kDa protein, bound to a ubiquitin-activating enzyme (E1). The ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) and then an E3 ubiquitin ligase transfers the ubiquitin to a specific lysine residue on a target

substrate. Ubiquitin can be added to any of seven lysine residues found within ubiquitin or the N-terminal methionine, though lysine (K) 48 linkages are the most common in yeast (207). Recently, it has been shown that the APC can catalyze the formation of branched ubiquitin chains and these branched chains promote protein degradation (208).

The APC is a multi-subunit cullin-RING (really interesting new gene) ubiquitin ligase composed of 13 subunits in yeast and human cells. The catalytic core is composed of a cullin-like protein Apc2, which serves as a scaffold for the complex, and a RING-finger protein Apc11 that recruits an E2 enzyme (209-211). The observation that ubiquitination activity can be achieved *in vitro* with only the E2 Ubc5 and the RING-finger containing component Apc11 suggests that the rest of the complex is primarily involved in generating substrate specificity. APC regulation is achieved by the binding of adaptor subunits, APC inhibitors and phosphorylation of APC subunits. The mitotic APC can be activated by binding either Cdc20 (fizzy in *D. melanogaster*) or Cdh1 (fizzy-related in *D. melanogaster*) (212-217). In both *S. cerevisiae* and *D. melanogaster* it was shown that fizzy/Cdc20 and fizzy-related/Cdh1 confer substrate specificity on the APC. These proteins are characterized by the presence of a Cdc20-box (C-box) and IR-tail (isoleucine arginine – tail), which are important for binding to the APC (218-220). APC^{Cdc20} and APC^{Cdh1} recognize substrates containing specific motifs called destruction boxes (D-boxes). In addition APC^{Cdh1} recognizes substrates containing KEN boxes (lysine (K) glutamic acid (E) asparagine (N) – boxes) (193, 221-223). This facilitates the destruction of substrates with different temporal patterns. Initial degradation of mitotic cyclins by APC^{Cdc20} causes a reduction in Cdk1 activity (224, 225). This, along with Cdc14 activation by the mitotic exit network (discussed in more detail later), activates APC^{Cdh1}, which is responsible for degrading Cdc20 and maintaining low Cdk1 activity during G1 (217, 226-230).

1.6 Anaphase Promoting Complex phosphoregulation

Early research suggested that the APC was activated by phosphorylation. Partially purified APC from clam oocytes displayed the ability to be activated by purified Cdc2 (231). Lambda phosphatase treatment of *X. laevis* APC decreased its activity and changed the mobility of APC subunits on SDS-PAGE gels (232). The fission yeast homolog of Cdc16 is hyperphosphorylated at metaphase (233). In clam embryos Cdc20 activation of interphase APC requires prior Cdk1^{cyclin B} incubation (234). Joint phosphorylation of human APC by Cdk1^{cyclin B} and polo kinase was required for full APC^{Cdc20} activation in vitro (235).

To determine whether Cdk1^{Clb} phosphorylation of the APC was required for its Cdc20-dependent activity Rudner and Murray mutated 12 consensus Cdk1 sites (S/TP) in the TPR subunits Cdc16, Cdc23 and Cdc27 to unphosphorylatable alanine residues (236). These mutations nearly abolished APC phosphorylation and inhibited APC^{Cdc20} activity *in vivo*. Consistent with this Cdk1 phosphorylation of human APC was later shown to increase Cdc20 binding and promote APC activity (237). In budding yeast, inhibition of Clb1 and Clb2, or Clb3 and Clb2 results in cell cycle arrest with short spindles and stabilized APC substrates, suggesting that Cdk1 phosphorylation of the APC may be required for anaphase onset (104). Consistent with this, in *D. Melanogaster*, a Cdc27 allele with two potential Cdk1 sites mutated to alanine cannot complement a nonfunctional Cdc27 allele, suggesting that phosphorylation of these sites may be required for APC activity (238). Preventing phosphorylation of budding yeast APC, however, results in only a 15 minute delay in metaphase (236). A single Cdk1 phosphorylation site in the fission yeast homolog of Cdc26 (Hcn1) has been identified. Preventing phosphorylation of this site is not lethal (239). It is presently unclear whether the viability of these mutants is due to the phosphorylation of

different residues on APC subunits or if Cdk1 phosphorylation of the APC is not required for anaphase onset in some organisms.

In contrast to the TPR subunits, Cdk1 phosphorylation of the APC activator Cdh1 is inhibitory and is thought to allow for declining Cdk1 activity after anaphase to promote APC activity and facilitate G1 entry (240-243).

1.7 Anaphase regulation

During metaphase, cohesin protein complexes physically connect sister chromatids and prevent their segregation (Figure 1-3). Sister chromatids are connected to the mitotic spindle at the centromere, where a large proteinaceous structure called the kinetochore assembles. Kinetochores on sister chromatids bind microtubules (kinetochore microtubules; kMTs) emanating from opposite spindle pole bodies and, even before anaphase, are pulled toward the poles so that chromosomal DNA forms a cruciform structure. These pulling forces are generated at the spindle midzone, where proteins that crosslink microtubules (interpolar microtubules; iMTs) slide iMTs past each other thereby pushing spindle pole bodies apart.

The APC triggers anaphase by targeting the mitotic cyclins and the anaphase inhibitor securin (Pds1 in budding yeast) for degradation (Figure 1-4). In fact, the APC can be rendered non-essential by the deletion of *PDS1* and *CLB5*, one of the six mitotic cyclins, (225, 244). Mitotic cyclin degradation inhibits Cdk1 activity and allows cells to re-enter a new cell cycle at G1 (195, 197, 200, 245, 246). Pds1 degradation liberates the protease separase (Esp1 in budding yeast) from inhibitory Pds1/Esp1 complexes (247). Esp1 then cleaves the Scc1 subunit of cohesin protein complexes (248). Scc1 cleavage allows the mitotic spindle to pull sister chromatids apart at anaphase onset.

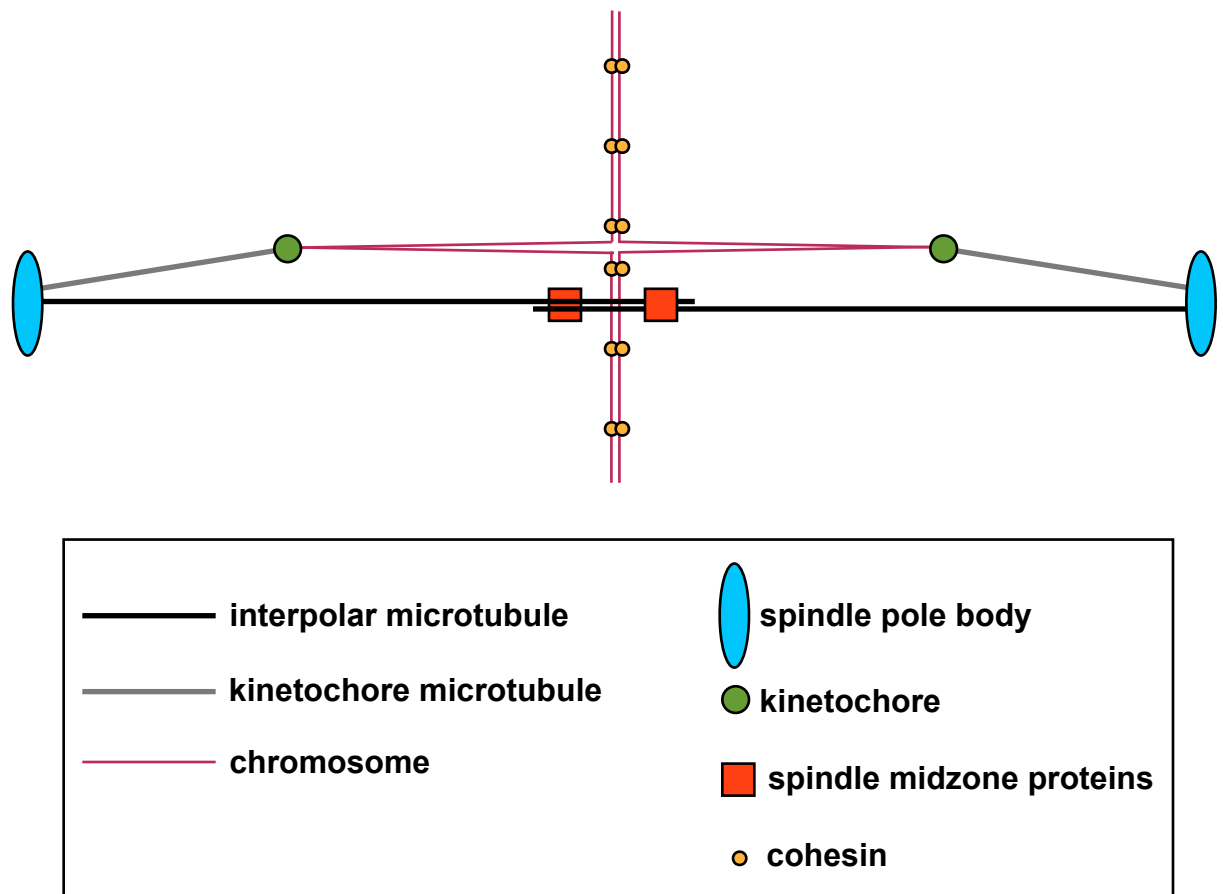


Figure 1-3

Figure 1-3. The mitotic spindle.

The mitotic spindle mediates chromosome segregation at anaphase onset. Proteins found at the spindle midzone act to push spindle pole bodies apart by sliding interpolar microtubules past each other. This outward force causes kinetochores, which assemble on centromeric regions on each sister chromatid to be pulled away from each other by kinetochore microtubules. This force is opposed by cohesin protein complexes which bind the arms of sister chromatids together. These balancing forces cause the region on each chromosome surrounding the centromere to be pulled into a cruciform structure. Each pair of sister chromatids adopts a similar structure, though for the sake of simplicity a single pair is depicted. At anaphase onset, cleavage of the Scc1 component of cohesin allows the poleward directed force mediated by the mitotic spindle to segregate sister chromatids into daughter cells.

A

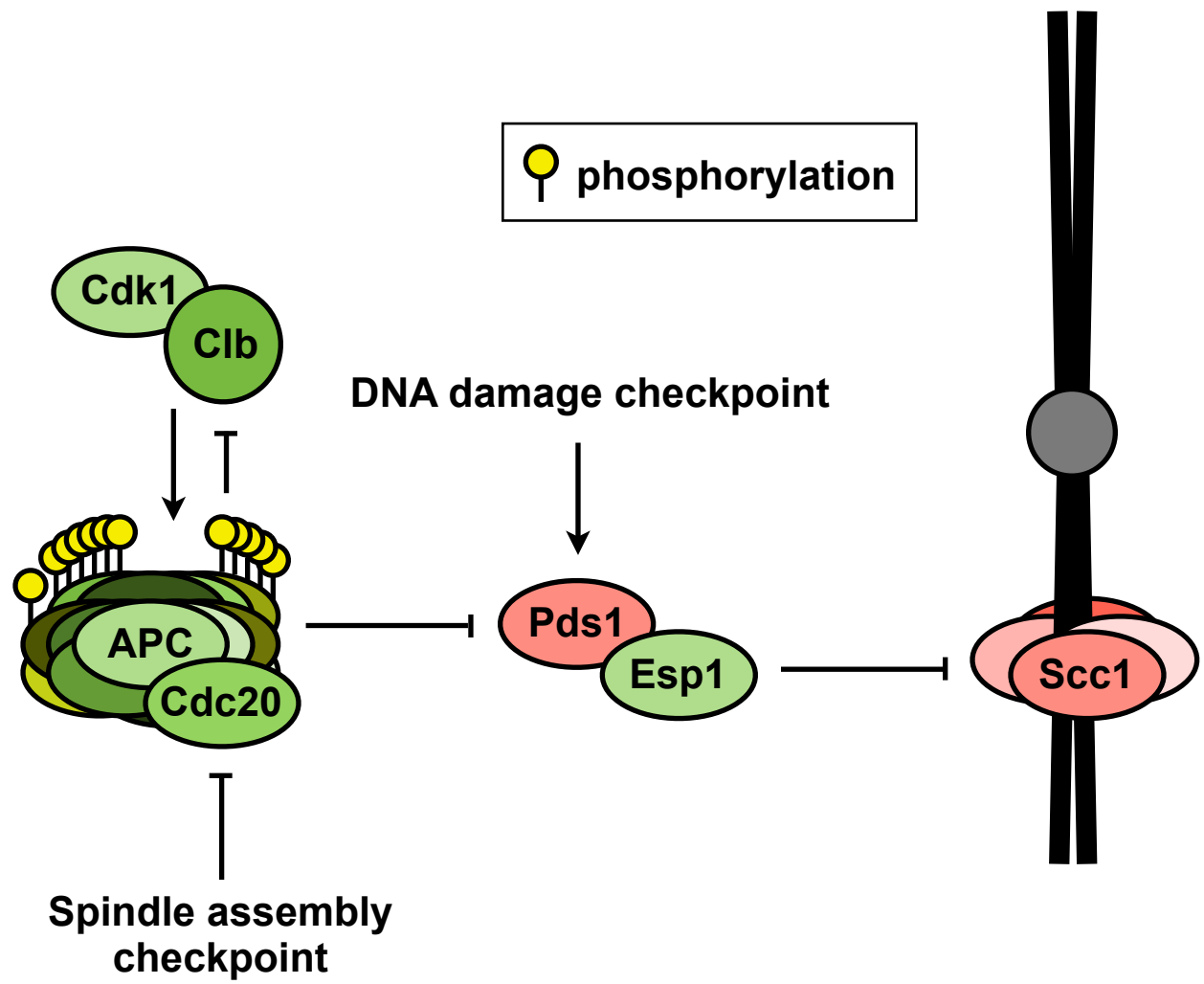


Figure 1-4

Figure 1-4. Regulation of anaphase onset.

Anaphase onset is triggered by the APC. The APC^{Cdc20} is activated by Cdk1^{Clb} phosphorylation. Active APC^{Cdc20} targets mitotic cyclins and Pds1 for degradation. Cyclin degradation reduces Cdk1 activity and allows G1 re-entry. Pds1 degradation liberates Esp1 from inhibitory Pds1/Esp1 complexes. Esp1 cleaves the Scc1 subunit of cohesin. Scc1 cleavage allows sister chromatids to be pulled into daughter cells by the mitotic spindle.

1.7.1 Securin regulation of separase

Though degradation of mitotic cyclins was the first activity characterized for the APC, it was quickly observed that spindle elongation occurred independently of cyclin degradation (249, 250). A key inhibitor of anaphase onset was identified as securin, a protein that was required to prevent spindle elongation in cells with an activated spindle checkpoint, DNA damage checkpoint or inhibited APC (251). Pds1 and its functional homologue in fission yeast Cut2 were shown to undergo APC-dependent degradation at anaphase onset, and preventing their degradation prevented anaphase onset (252, 253). Securin was later shown to inhibit anaphase initiation by binding the anaphase promoter separase in inhibitory complexes until securin degradation at anaphase onset liberated active separase (247).

In addition to this inhibitory role, securin has been shown to activate separase. Indeed, securin and separase were first identified as Cut2 and Cut1 in a screen for fission yeast mutants that undergo cytokinesis before signaling nuclear division, suggesting that both proteins function as anaphase promoters (254, 255). In fission yeast it was determined that Cut2 was required for Cut1 loading onto spindle pole bodies and the mitotic spindle during mitotic entry (256). Deletion of *PDS1* in budding yeast results in temperature sensitivity and incubation at the restrictive temperature results in delayed anaphase onset, suggesting it is also an anaphase activator (257). Pds1 activation of Esp1 appears to involve promoting nuclear localization. Separase nuclear targeting and spindle localization requires *PDS1* (258), and Cdk1^{Cib} phosphorylation of three sites in the Pds1 carboxy-terminal domain regulates Pds1/Esp1 interaction and Esp1 import into the nucleus (259). Securin also appears to activate separase in *D. melanogaster* and human cells (260-262). In budding yeast Cdk1 phosphorylation of Pds1 on different sites near its D-box slows its APC-mediated

ubiquitination, and dephosphorylation of these sites by Cdc14 is thought to increase the rapidity of anaphase progression once it has been triggered (263).

1.7.2 Regulation of sister chromatid cohesion

After APC-dependent degradation of Pds1, liberated Esp1 triggers anaphase onset by destroying the proteinaceous linkages that bind sister chromatids together during metaphase (reviewed in (264)). In all eukaryotic cells, cohesin complexes connect replicated chromosomes over their entire length (265-269). The cohesin complex consists of the four core subunits Scc1, Scc3, Smc1 and Smc3 (270-272). Smc1 and Smc3 fold into rod-shaped molecules composed of 'hinge' and 'head' regions separated by a 45nm long coiled-coil (reviewed in (273)). Smc1 and Smc3 interact at their hinge regions creating a V-shaped heterodimer (274). The 'head' regions of Smc1 and Smc3 are bound by Scc1 to form a tripartite ring structure. Scc3 binds to Scc1 outside the ring and is not required for the maintenance of cohesion (274, 275). This ring-like structure led to the hypothesis that cohesin could maintain sister chromatid cohesion by topologically entrapping replicated chromatids (274). The best evidence for the 'ring theory' of cohesion is that specific chemical cross-linking of the three protein interfaces in the cohesin tripartite ring and subsequent protein denaturation results in cohesion loss from linear minichromosomes but not circular minichromosomes (276, 277). Despite this data, there is no consensus model on how cohesin proteins mediate sister chromatid cohesion on chromosomes, and whether cohesin rings might promote cohesion in more than one way (278, 279).

Cohesion is established in S-phase by the acetyltransferase Eco1. *ECO1* was originally identified as *CTF7* in a screen for mutations that resulted in chromosome loss (280). Interestingly, its function in promoting sister chromatid cohesion is required in S-

phase, not mitosis, suggesting that it is involved in the establishment, not the maintenance of cohesion (270, 281). Eco1 in multiple eukaryotes was subsequently demonstrated to display acetyltransferase activity (282-285). Smc3 is the key target of Eco1 in cohesion establishment, as preventing acetylation of two lysine residues, K112 and K113, causes cohesion defects and cell inviability, and mutation of K113 to asparagine renders *ECO1* non-essential (286, 287). The importance of Eco1 acetylation of Smc3 appears to be conserved in human cells (288).

In vertebrates, cohesin dissociates from sister chromatids in two phases. At mitotic entry most cohesin dissociates from chromosome arms in a process termed the ‘prophase pathway’ (289-292). This process is not dependent on APC activation and is not accompanied by anaphase spindle elongation. In both vertebrates and invertebrates, loss of cohesion and anaphase spindle elongation is ultimately mediated by cleavage of the cohesin subunit Scc1 (292, 293). Scc1 is cleaved by the CD clan cysteine protease separase (248, 292). This appears to be the major function of separase in sister chromatid segregation, as artificially cleaving an engineered Scc1 containing a TEV (tobacco etch virus) protease cleavage site with a TEV protease is sufficient to trigger chromosome segregation (248). In sum, APC^{Cdc20} activation during metaphase causes Pds1 degradation and the liberation of Esp1 from Pds1/Esp1 complexes. Liberated Esp1 then cleaves Scc1, thereby destroying the linkages between sister chromatids and allowing sister chromatid segregation by the mitotic spindle.

1.8 Mitotic checkpoints

1.8.1 The Spindle Assembly Checkpoint (SAC)

Due to the irreversible nature of sister chromatid segregation, anaphase onset is delayed in response to checkpoints that monitor a) correct sister chromatid attachment to the mitotic spindle and b) DNA damage (Figure 1-4). Attachment of one member of each pair of replicated sister chromatids to opposite poles of the mitotic spindle is essential in ensuring that each daughter cell inherits a single copy of each chromosome. Early research showed that inhibiting spindle assembly using microtubule depolymerizing drugs delayed cell cycle progression, presumably due to a ‘checkpoint’ that ensured proper spindle formation before allowing anaphase onset (294). To identify the proteins involved in this spindle assembly checkpoint (SAC) Li *et al.* and Hoyt *et al.* screened for yeast mutants that were unable to delay cell cycle progression in the presence of the spindle depolymerizing drug benomyl (295, 296). They termed these mutants the mitotic arrest deficient (*MAD*; *mad1*, *mad2* and *mad3*) and budding uninhibited by benzimidazole (*BUB*; *bub1*, *bub2* and *bub3*) mutants. Bub2 was later shown to participate in the mitotic exit network, not the SAC per se and will be discussed in greater detail in subsequent sections. The SAC was later shown to require the essential kinase Mps1 (297, 298). Mps1 phosphorylation of the kinetochore protein Spc105 is required for Bub1/Bub3 recruitment to the kinetochore (299, 300). Subsequent phosphorylation of Bub1 recruits Mad1 to unattached kinetochores (301, 302). Mad1 binding to kinetochores recruits Mad2, and promotes a conformational change in Mad2 from an ‘open’ to a ‘closed’ form (303, 304). Mad2, Mad3 and Bub3 then bind Cdc20 in a complex termed the mitotic checkpoint complex (MCC), thereby preventing Cdc20-mediated activation of the APC (305-308).

1.8.2 The DNA Damage Checkpoint

The DNA damage checkpoint also inhibits anaphase onset during mitosis, however this checkpoint functions to inhibit the degradation of securin, rather than inhibiting the APC directly. This is in contrast to humans and fission yeast, which inhibit Cdk1 dephosphorylation to delay cell cycle progression in response to DNA damage checkpoint activation (as discussed previously). The first clues to the biochemical regulation of the DNA damage checkpoint in budding yeast emerged when Weinert and Hartwell screened previously identified genes important for maintaining viability upon exposure to radiation for the inability to delay cell cycle progression in response to X-irradiation (309, 310). They identified the *RAD9* gene as a mediator of the DNA damage checkpoint. Subsequent work identified the additional components *MEC1*, *DDC2*, *CHK1* and *PDS1* (311-314). Mec1 is recruited to damaged sites by Ddc2 (312, 315). Mec1 recruitment activates a signaling pathway that ultimately activates Chk1 (Reviewed in (316)). Activated Chk1 phosphorylates Pds1 (251, 313, 314, 317). Chk1 phosphorylation of Pds1 prevents its degradation by the APC (318). Satisfaction of the DNA damage checkpoint allows for the APC^{Cdc20} dependent degradation of Pds1 and anaphase onset.

1.9 Mitotic exit

In order for cells to exit mitosis and re-enter G1, Cdk1 substrates must be dephosphorylated. As will be discussed in greater detail later, the primary Cdk1-opposing phosphatase in budding yeast mitotic exit is Cdc14. Cdc14 activation involves release from nucleolar RENT (regulator of nucleolar silencing and telophase) complexes containing Cdc14, Net1 and Sir2 (319, 320). Cdc14 is released in two stages. During early anaphase it is transiently released into the nucleus through a signaling network termed the Cdc fourteen

early anaphase release (FEAR) pathway (Figure 1-5A) (321-323). Later in anaphase, full Cdc14 release is signaled by the mitotic exit network (MEN)(Figure 1-5B) (324).

1.9.1 The cdc Fourteen Early Anaphase Release (FEAR) pathway

During early anaphase Cdc14 is transiently released by activation of the FEAR pathway (Figure 1-5A). Components of the FEAR pathway include Esp1, Cdc5, Slk19, Spo12, Bns1, Zds1, Zds2, and Fob1 (321-323, 325-327). The FEAR pathway appears to contain two branches. In one branch Esp1 promotes Cdc14 release through Slk19 and Zds1/2 in a protease-independent manner (325, 327-329). This pathway inhibits PP2A^{Cdc55}, which opposes Cdk1 and likely Cdc5 phosphorylation of Net1 (322, 330-333). Net1/Cdc14 interaction is stabilized during metaphase by PP2A^{Cdc55} dephosphorylation of Net1. At anaphase onset FEAR pathway activation results in PP2A^{Cdc55} inhibition and a resultant increase in Net1 phosphorylation. This destabilizes Cdc14/Net1 complexes and allows Cdc14 release. Spo12 and Bns1 appear to act in a parallel branch of the FEAR pathway by inhibiting the Net1 stabilizer Fob1 (325, 326). Cdc5 likely has additional roles in FEAR pathway regulation as Cdc5 overexpression causes Cdc14 release in all identified FEAR mutants (325).

1.9.2 The Mitotic Exit Network (MEN)

After FEAR activation has led to transient Cdc14 release, the mitotic exit network is activated in response to spindle elongation (Figure 1-5B). The upstream components of the MEN compose the *BUB2*-dependent spindle position checkpoint (SPOC) (334)(reviewed in (335)). Along with Cdc5, which appears to have an independent function, SPOC components serve to promote mitotic exit by localizing Cdc15 to the spindle pole body. Identification of

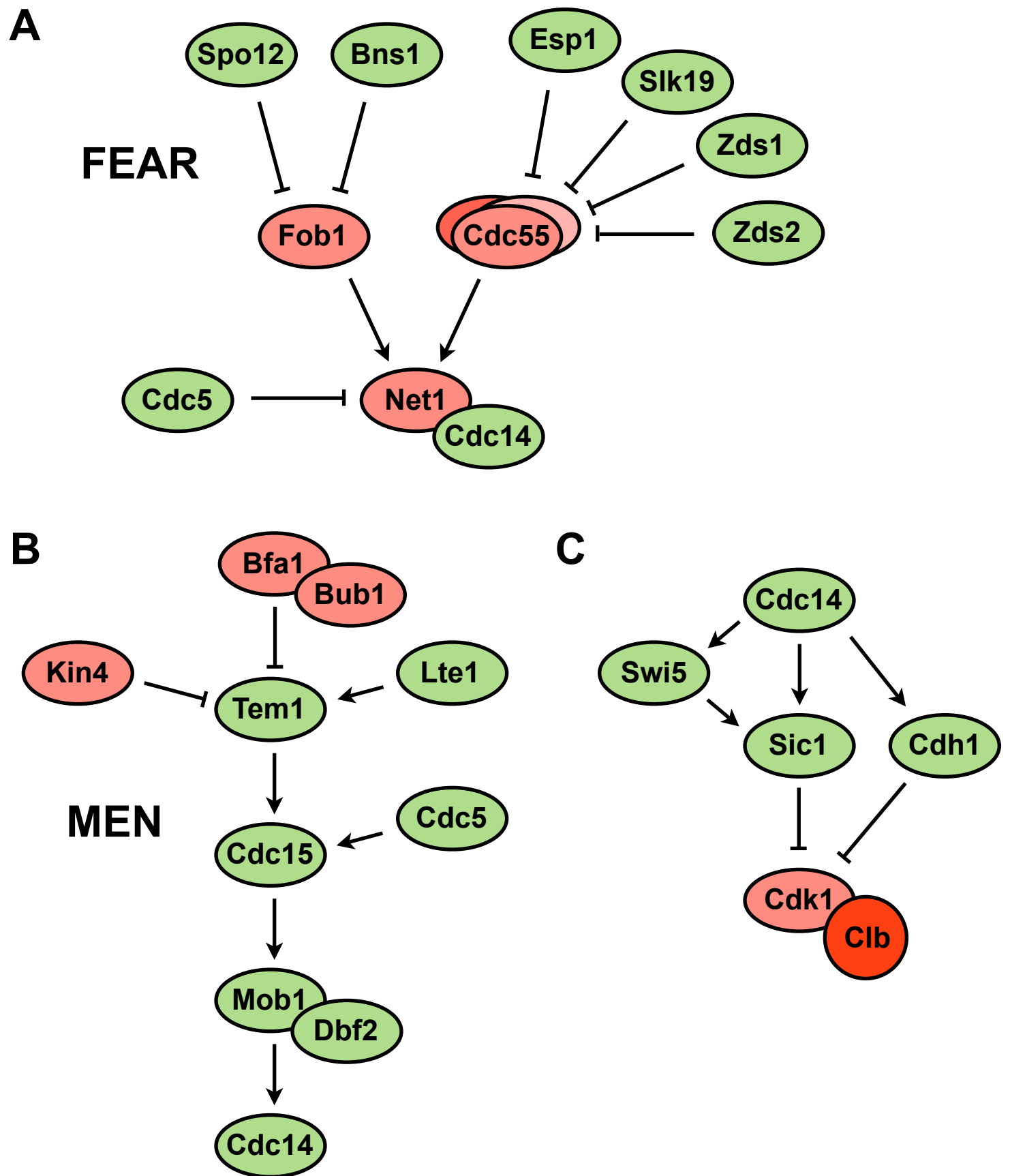


Figure 1-5

Figure 1-5. Regulation of mitotic exit.

(A) The FEAR network causes transient release of Cdc14 from the nucleolus during early anaphase. The FEAR network components Esp1, Slk19, Zds1 and Zds2 promote Cdc14 release from Net1/Cdc14 complexes by inhibiting PP2A^{Cdc55} and promoting Net1 phosphorylation. The FEAR network components Bns1 and Spo12 promote Cdc14 release by inhibiting Fob1, a protein that stabilizes Net1/Cdc14 interaction. Cdc5 promotes Cdc14 release by phosphorylating Net1 and destabilizing Net1/Cdc14 interaction.

(B) MEN causes full release of Cdc14 from the nucleolus during late anaphase. Tem1 is localized to the daughter spindle pole body (dSPB). Before anaphase, the daughter spindle pole body is found in the mother cell, a region of relatively high Kin4 activity, which inhibits Tem1. Passage of the dSPB into the daughter cell causes Tem1 activation due to reduced Kin4 inhibition and increased activation by the bud localized protein Lte1. Tem1 activation, as well as Cdc5 activity promote dSPB localization of Cdc15, which activates Mob1/Dbf1. Mob1/Dbf1 subsequently promotes Cdc14 nucleolar release.

(C) Cdc14 inhibits Cdk1 activity. Cdc14 dephosphorylates Swi5, a transcription factor that promotes Sic1 expression. Cdc14 also directly dephosphorylates Sic1 and promotes Sic1 association with and inhibition of Cdk1^{Cln}. Cdc14 also dephosphorylates the APC co-activator Cdh1. APC^{Cdh1} signals cyclin degradation during G1 and thereby maintains low Cdk1 activity.

SPOC was catalyzed by the observation that despite being identified in the same screen as Bub1 and Bub3, it was clear that cells lacking Bub2 responded to spindle checkpoint activation differently than *mad1*, *mad2*, *mad3*, *bub1* and *bub3* mutants (336-339). An additional mutation, in *BFA1*, displayed a similar phenotype to *bub2* and was found to be required for cell cycle delay upon anaphase spindle misalignment (334, 340, 341). SPOC functions through the spindle pole body associated small GTPase Tem1 (342). Tem1 is localized to the bud spindle pole body during S-phase and mitosis along with Bfa1 and Bub2 which together act as a GAP (GTPase-activating protein) and inhibit Tem1 (343-345). Lte1, which may act as a GEF (guanine-nucleotide exchange factor) for Tem1 is localized to the bud, and Kin4, a kinase that inhibits mitotic exit is localized to the mother cell (342, 345-349). The current model posits that translocation of the daughter spindle pole body into the bud results in Tem1 activation by Lte1 and the recruitment of the kinase Cdc15 to the spindle pole body (345, 350). Recent data has suggested that Lte1 activation of Tem1 may not require Lte1 GEF activity and instead Lte1 may promote Bfa1 preferential localization to the daughter spindle pole body, which has been shown to coincide with mitotic exit signaling (351-357). In either case, correct spindle positioning results in Tem1 activation, which promotes Cdc15 localization to the spindle pole body.

Cdc15 spindle pole body localization is also signaled independently by the polo kinase Cdc5. Polo kinase is a highly conserved kinase which has a single isoform, Cdc5, in budding yeast (67, 358-363). Cdc5 is expressed during mitosis, is required for mitotic exit, and is degraded by the APC^{Cdh1} in G1 (226, 361, 364, 365). Cdc5 has multiple roles regulating mitotic progression and exit. Cdc5 promotes mitotic entry by phosphorylating the Cdk1-inhibitor Swe1 and promoting its degradation (182). Cdc5 phosphorylates the cohesin subunit Scc1 on at least two conserved sites (366). This phosphorylation promotes Scc1

cleavage by Esp1. During mitotic exit Cdc5 phosphorylates Bfa1, thereby inhibiting Bfa1/Bub2 GAP activity towards Tem1, and promoting Bfa1 asymmetric localization, thereby promoting mitotic exit (357, 367-369). It has also been shown that localization of Cdc15 to the SPB is the only essential function of Cdc5 in MEN (370). Though it appears not to phosphorylate Cdc15 directly, Cdc5 does phosphorylate the spindle pole body component Nud1, which acts to localize MEN components to the SPB (348, 371, 372). In this way, Cdc15 activation integrates temporal signals regarding cell cycle progression from Cdc5 along with spatial signals from Tem1 regarding signal elongation to trigger mitotic exit (371). Cdc5 has also been shown to be important later in the cell cycle during cytokinesis (373-375).

Tem1 and Cdc5-dependent localization of Cdc15 to the spindle pole body triggers the downstream components of MEN. SPB localized Cdc15 promotes Dbf2 activity by phosphorylating the SPB protein Nud1, which promotes Mob1-Dbf2 associating with the SPB and allows Cdc15 to subsequently phosphorylate Dbf2 directly (371, 376-378). Dbf2-Mob1, in turn, phosphorylates Cdc14 on residues adjacent to its nuclear localization signal (NLS), thereby promoting Cdc14 nuclear export (379). Dbf2-Mob1 must regulate Cdc14 in other ways, though, as preventing Cdc14 phosphorylation does not recapitulate the phenotype of deleting *DBF2*.

In contrast to MEN, the FEAR network is not essential, as deleting either *SLK19* or *SPO12* and *BNS1* is not lethal (380, 381). However, FEAR network activation is thought to prime MEN activation in multiple ways. PP2A^{Cdc55} inactivation results in increased phosphorylation of Bfa1 and Mob1, both of which promote MEN-dependent Cdc14 release (382, 383). FEAR-released Cdc14 itself is thought to promote its own sustained release by dephosphorylating and activating Cdc15 (321, 384).

The result of MEN activation is sustained release of Cdc14 from the nucleolus. Cdc14 is a protein phosphatase that is essential for mitotic exit in budding yeast (67, 385). Cdc14 signals the transition from mitosis to G1 by dephosphorylating multiple Cdk1 substrates (Figure 1-5C) (228, 240, 242). Cdc14 dephosphorylates and stabilizes Sic1, thereby inhibiting Cdk1^{Cib} (228). Cdc14 also dephosphorylates the transcription factor Swi5 thereby promoting Sic1 transcription (228). Cdc14 inactivates mitotic cyclins by dephosphorylating and activating the APC subunit Cdh1 (228, 240, 242). Thus release of Cdc14 results in Cdk1^{Cib} inhibition through Sic1 and APC^{Cdh1}, thereby allowing progression from mitosis to G1.

1.10 PP2A regulation of mitosis

The most important, and best characterized, Cdk1-opposing phosphatase in budding yeast mitotic exit is Cdc14. Though three Cdc14 homologs have been identified in vertebrate cells (CDC14A, CDC14B and CDC14C), vertebrate Cdc14 homologs do not appear to play a similar role to budding yeast Cdc14 (reviewed in (386)). Instead, Cdc14 homologs have been implicated in processes such as centrosome duplication (387, 388), the DNA damage checkpoint (389), DNA repair (390) and regulation of mitotic entry (391). PP2A-B55 and PP1 (protein phosphatase 1) appear to be primarily responsible for dephosphorylating Cdk1 substrates during vertebrate mitotic exit (reviewed in (392)).

1.10.1 PP2A and PP1 in vertebrate mitotic exit

PP2A is a trimeric protein complex composed of a scaffold (A), regulatory (B) and catalytic (C) subunit (reviewed in (393)). In vertebrates, at least 15 different regulatory subunits have been identified in 4 families. PP2A in complex with B55 (PP2A-B55) appears

to play an important role in dephosphorylating Cdk1 substrates during mitotic exit. PP2A-B55 α has been shown to be required for timely nuclear envelope reassembly, postmitotic Golgi assembly, spindle breakdown and chromatin decondensation in HeLa cells, though the exact substrates responsible for these phenotypes are unknown (394). In *X. laevis* egg extracts, PP2A-B55 δ is the primary phosphatase that dephosphorylates CDK substrates in interphase (395).

PP1 is a dimeric phosphatase composed of catalytic and regulatory subunits (reviewed in (396)). In *X. laevis* egg extracts, PP1 inhibition leads to delays in Cdk1 substrate dephosphorylation (397). PP1 has been shown to be required for timely nuclear envelope reassembly in human HL60 cells (398, 399). PP1 in complex with its regulatory subunits Repo-Man and PNUTS have been implicated in histone dephosphorylation and chromatin decondensation in a variety of vertebrate cell types (400-402). PP1 may not dephosphorylate some CDK1 substrates directly, though, as phosphatase activity towards the CDK1 substrates high mobility group protein-I(Y), caldesmon and histone H1 copurify with PP2A but not PP1 from rat liver, human fibroblasts and *X. laevis* eggs (403). Though much work remains to be done in identifying specific PP2A and PP1 substrates in vertebrate mitotic exit, it is clear that Cdc14 does not play a similar role in vertebrates and budding yeast.

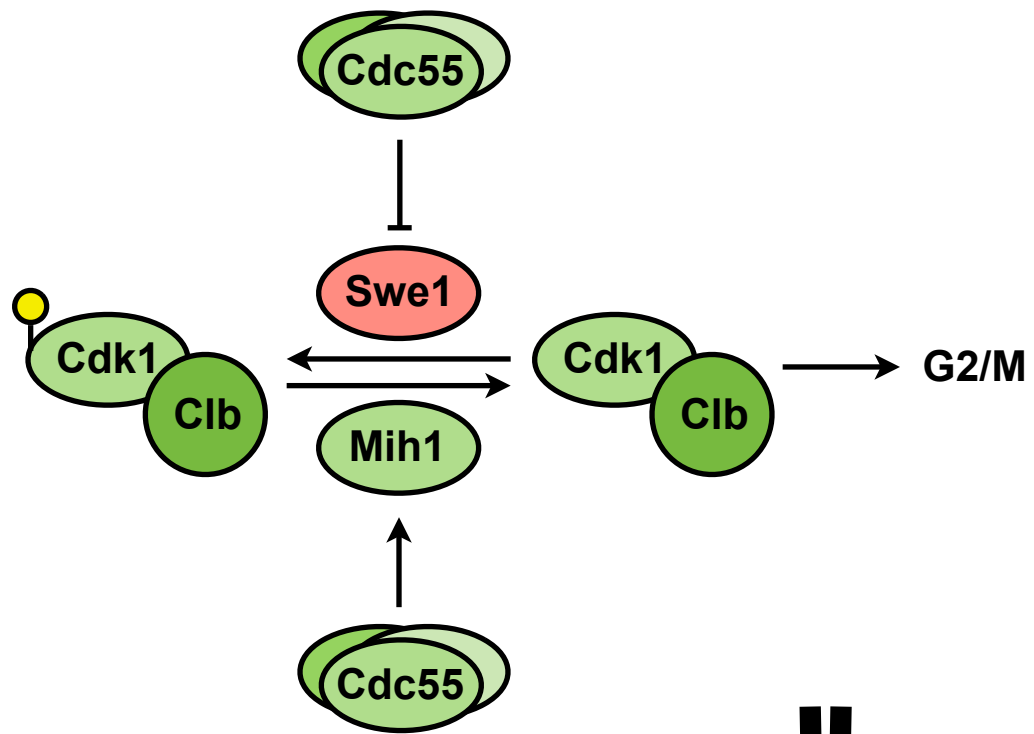
1.10.2 S. cerevisiae PP2A

S. cerevisiae cells express two redundant PP2A catalytic subunits Pph21 and Pph22, a single scaffold subunit Tpd3 and two regulatory subunits Cdc55 and Rts1 which confer substrate specificity on the complex (reviewed in (404)). Rts1 is best characterized as a protector of centromeric cohesion during meiosis (405, 406). However, PP2A^{Rts1} is also

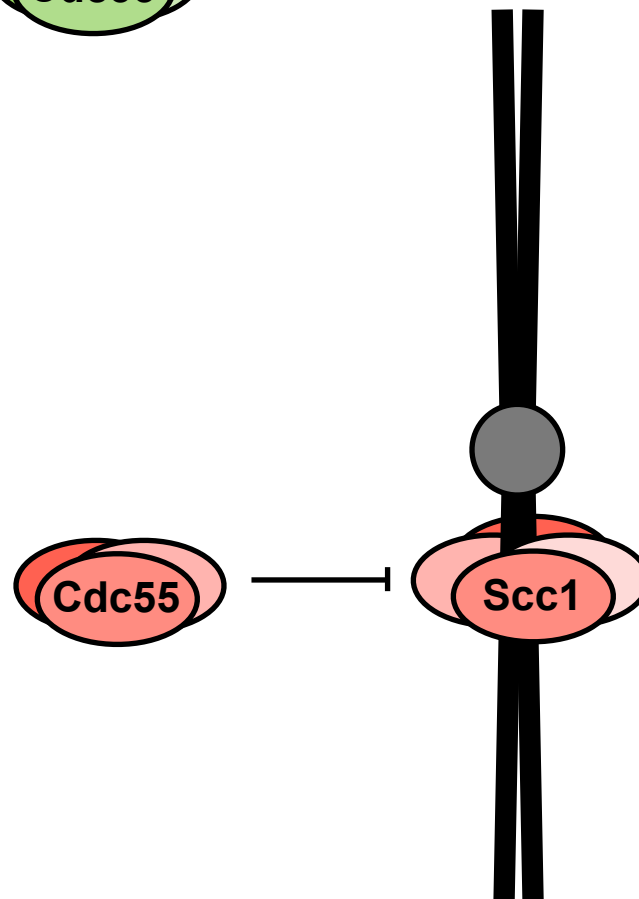
involved in pathways regulating cell size and pericentric chromatin composition (407-409).

During mitosis, PP2A^{Cdc55} plays an important role dephosphorylating multiple mitotic substrates (Figure 1-6). PP2A^{Cdc55} dephosphorylates Swe1 and Mih1 thereby promoting mitotic entry (Figure 1-6A) (103, 186, 192, 410). PP2A^{Cdc55} appears to dephosphorylate Scc1 and inhibit Scc1 cleavage before anaphase onset (Figure 1-6B) (411). PP2A^{Cdc55} also dephosphorylates Net1 and prevents Cdc14 release from Net1/Cdc14 complexes during metaphase (Figure 1-6C) (320, 331, 333).

A



B



C

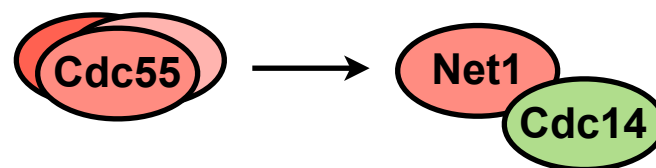


Figure 1-6

Figure 1-6. PP2A^{Cdc55} regulation of mitosis.

(A) PP2A^{Cdc55} promotes mitotic entry. PP2A^{Cdc55} activation of Mhl1 and inhibition of Swe1 promotes mitotic entry.

(B) PP2A^{Cdc55} inhibits sister chromatid separation. PP2A^{Cdc55} dephosphorylation of Scc1 inhibits Scc1 cleavage and anaphase onset.

(C) PP2A^{Cdc55} inhibits FEAR-dependent Cdc14 release. Before anaphase onset PP2A^{Cdc55} stabilizes Net1/Cdc14 interaction by dephosphorylating Net1.

Chapter 2

Materials and Methods

2.1 Strain and plasmid construction

All yeast strains were constructed in the W303 strain background (W303-1a).

Relevant genotypes for all strains can be found in Appendix 1. All alleles were confirmed by immunoblotting, PCR and/or phenotypically. The sequences of all primers used in this study are available upon request. The bacterial strains TG1 and DH5 α were used for DNA amplification, and Rosetta (Novagen) was used for protein purifications.

The *PDS1-myc18-leu2::HIS3* allele was originally obtained as *PDS1-myc18-LEU2* in K6445, a gift from Kim Nasmyth. *LEU2* was then converted to *HIS3* with pLH7 (412). *cdc16-1* and *cdc23-1* are derived from the A364A strains H16C1B1 and H23C1A1 (19) and have been backcrossed at least five times to W303-1a. *cdc16-6A-TRP1*, *cdc23-A-HA* and *cdc27-5A-KAN^R* alleles were made as described previously (236). The *trp1::pSCC1-SCC1-HA3-TRP1*, *trp1::pSCC1-scc1-10A-HA3-TRP1* and *leu2::pSCC1-scc1-10A-HA3-LEU2* alleles were from strains Y1287, Y1288 and Y1296 respectively provided as a gift by Frank Uhlmann (413). Strains Y1287, Y1288 and Y1296 all contained the *scc1::pGAL-SCC1-myc18-URA3* allele. The *ESP1-myc18-TRP1* allele was originally from K7024, a gift from Frank Uhlmann. The *ura3::pGAL-MPS1-myc-URA3* allele is from pAFS120 (414). The *cdc20 Δ ::LEU2* and *trp1::pGAL-CDC20-TRP1* alleles are from RTK237, a gift from Rachel Tinker-Kulberg. The *pds1-38-URA3* allele is from the strain RA2815 and was given to us by Orna Cohen-Fix (259). *esp1-NLS-URA3*, *esp1-3A-NLS-URA3-NAT^R* and *esp1-3D-NLS-URA3-HYG^R* were constructed by cutting pFD026 (415) with BstAPI to integrate it at *ESP1*.

his3::pCUP1-GFP12-lacI12::HIS3 was made by integrating pSB116 (416). *PDS-AID-KAN^R* and *SCC1-AID-KAN^R* were constructed by amplifying *AID* and *KAN^R* off pAID1, a gift from Leon Chan, Thomas Eng and Vinny Guacci (417) and integrating the resulting PCR product at *PDS1* or *SCC1* respectively. *PDS-AID-NAT^R* was made by switching *KAN^R* to *NAT^R* using pAG25. *leu2::pGPD1-OsTIR1-LEU2* was constructed by cutting pTIR4, a gift from Leon Chan, Thomas Eng and Vinny Guacci (417) with PmeI to integrate it at *LEU2*.

SPC42-eGFP-KAN^R, *SPC42-eGFP-sphis5⁺*, *NDC80-eGFP-sphis5⁺*, *CDC14-eGFP-Sphis5⁺*, *ESP1-eGFP-Sphis5⁺*, *esp1-3A-eGFP-sphis5⁺-NAT^R* and *esp1-3D-eGFP-sphis5⁺-NAT^R* were made by using PCR-targeted recombination with pKT127 or pKT128 (418) and gene specific primers. *SPC42-eGFP-HYG^R* and *SPC42-eGFP-NAT^R* were made by switching the marker in *SPC42-eGFP-KAN^R* with *HYG^R* or *NAT^R* cassettes by homologous recombination using pAG32 or pAG25 (419).

KIP1-myc13-NAT^R, *CIN8-GFP-HYG^R*, *SGO1-myc13-Sphis5⁺*, *SLK19-myc13-KAN^R*, *SCC1-GFP-Sphis5⁺* and *ESP1-myc13-KAN^R* were made using PCR-targeted recombination using pFA6a-GFP(S65T)-kanMX6, pFA6a-13Myc-kanMX6, pFA6a-13Myc-HIS3MX or pFA6a-GFP(S65T)-HIS3MX6 (420) and gene specific primers. Markers were switched by homologous recombination using a *HYG^R* or *NAT^R* cassette amplified off pAG32 or pAG25 (419). The *ESP1-GFP-TRP1* allele was made using PCR-targeted recombination with pAR1179, a gift from Yoshida (415), and *ESP1* specific primers. The *CDC55-TAP-KITRPI* allele was made using PCR-targeting recombination with pBS1479 and *CDC55* specific primers.

The *clb5Δ::hisG-URA3-hisG* and *clb6Δ::LEU2* alleles were originally from YSC664, a gift from Kim Nasmyth (University of Oxford, Oxford, UK). *BARI* was deleted using pJGsst1 (a gift of Jeremy Thorner, University of California, Berkeley, CA). *MIH1* was

deleted using pIP33 (a gift of Peter Sorger, Massachusetts Institute of Technology, Cambridge, MA). *swe1Δ::TRP1* strains were made by crossing JM449 (a gift of Jeremy Minshull, DNA2.0, Menlo Park, CA) to the appropriate strains. *mad2Δ::URA3* was made using pRC10.1 (421). *cin8Δ::TRP1* was made using pMA1186, a gift from Andrew Hoyt. *PDS1*, *SGO1*, *CDC55*, *ESP1*, *SLK19*, *SPO12*, *RTS1* and *ASE1* were deleted using cassettes amplified from pAG32, pAG25 or pFA6a-kanMX6. *cdc55Δ::HIS3* was created using pJM6 (422) and *cdc55Δ::his3::LEU2* converted to *LEU2* using pHL3 (412). The *mad3Δ::LEU2* allele was from KH125 (414).

KAN^R-CDK1-Y19F was created from two overlapping PCR fragments: *KAN^R* (amplified from pFA6a-kanMX6) and the promoter (from -334 to -1) and 5' region of the *CDK1-Y19F* allele, including the mutation (amplified from pSF38 (a gift of Peter Sorger, Harvard Medical School, Boston, MA)). These two fragments were used to create a single long cassette that was integrated by homologous recombination. Positive transformations were determined by amplifying the 5' region of *CDK1* and digesting this fragment with NdeI, which was inserted near the Y19F substitution in pSF38. Additionally, positive *KAN^R-CDK1-Y19F* strains have no detectable Cdk1-Y19 phosphorylation as determined by immunoblotting using an α -Cdk1-P-Tyr antibody.

KAN^R-pMET3-CLB5 and *KAN^R-pMET3-PDS1* were created by amplifying the *KAN^R-pMET3* cassette from pAR704 with *CLB5* and *PDS1* specific primers respectively. pAR704 was made by inserting a PCR-amplified *pMET3* (from -626 to -1) into the BglII and SalI sites of pFA6a-kanMX (423).

The CEN-pESP1-ESP1-URA3, CEN-pESP1-ESP1-LEU2 and CEN-pESP1-ESP1-HIS3 plasmids were constructed as follows. A 5kb region containing the *ESP1* ORF was amplified and cloned into pRS316 between XhoI and NotI resulting in pAR745 (CEN-

pESP1-ESP1-URA3). The XhoI/NotI fragment from pAR745 was cloned into pRS315 and pRS313 resulting in pAR797 (CEN-pESP1-ESP1-LEU2) and pAR795 (CEN-pESP1-ESP1-HIS3) respectively.

The *esp1-2A-NAT^R*, *esp1-3A-NAT^R*, *esp1-1A-NAT^R*, *esp1-2A+3A-NAT^R*, *esp1-2A+1A-NAT^R*, *esp1-2A+3A+1A-NAT^R*, *esp1-2D-NAT^R*, *esp1-3D-NAT^R*, *esp1-1D-NAT^R*, *esp1-2D+3D-NAT^R*, *esp1-2D+1D-NAT^R* and *esp1-2D+3D+1D-NAT^R* alleles were constructed as follows. We had six DNA sequences synthesized (DNA 2.0) corresponding to each of the N-terminal (containing S13 and T16), central (containing T1014, S1027 and T1034) and C-terminal (containing S1280) sites and surrounding residues with each site mutated to either alanine or tandem aspartic acid residues. Restriction sites were engineered into the codons for the mutated residues for later identification. Each mutated region was amplified and cut with restriction sites found in the *ESP1* gene (AvrII/MscI for *esp1-2A* and *esp1-2D*; SpeI/NheI for *esp1-3A* and *esp1-3D*; and SalI/AatII for *esp1-1A* and *esp1-1D*). These fragments were then cloned into pAR797 to make pAR873 (CEN-pESP1-*esp1-2A*-LEU2), pAR875 (CEN-pESP1-*esp1-3A*-LEU2), pAR871 (CEN-pESP1-*esp1-1A*-LEU2), pAR872 (CEN-pESP1-*esp1-2D*-LEU2), pAR874 (CEN-pESP1-*esp1-3D*-LEU2) and pAR870 (CEN-pESP1-*esp1-1D*-LEU2). pAR875 was subsequently used to make pAR902 (CEN-pESP1-*esp1-2A* + 3A-LEU2); pAR873 to make pAR924 (CEN-pESP1-*esp1-2A* + 1A-LEU2); pAR871 to make pAR886 (CEN-pESP1-*esp1-3A* + 1A-LEU2); pAR872 to make pAR903 (CEN-pESP1-*esp1-2D* + 3D-LEU2); pAR870 to make pAR915 (CEN-pESP1-*esp1-2D* + 1D-LEU2); pAR874 to make pAR901 (CEN-pESP1-*esp1-3D* + 1D-LEU2); pAR886 to make pAR1005 (CEN-pESP1-*esp1-2A* + 3A + 1A-LEU2); and pAR915 to make pAR927 (CEN-pESP1-*esp1-2D* + 3D + 1D-LEU2). To add a marker to pAR797, pAR873, pAR875, pAR871, pAR872, pAR874, pAR870, pAR902, pAR924, pAR886, pAR903, pAR915,

pAR901, pAR1005 and pAR927 each was cut with SnaBI and cotransformed into yeast with a PCR product containing the *NAT^R* cassette off pAG25 and ends overlapping the cut backbone plasmid. Plasmids were rescued and checked by restriction digest. The resulting plasmids were pAR906 (CEN-pESP1-ESP1-*NAT^R*-LEU2), pAR936 (CEN-pESP1-*esp1*-2A-*NAT^R*-LEU2), pAR905 (CEN-pESP1-*esp1*-3A-*NAT^R*-LEU2), pAR904 (CEN-pESP1-*esp1*-1A-*NAT^R*-LEU2), pAR921 (CEN-pESP1-*esp1*-2D-*NAT^R*-LEU2), pAR922 (CEN-pESP1-*esp1*-3D-*NAT^R*-LEU2), pAR920 (CEN-pESP1-*esp1*-1D-*NAT^R*-LEU2), pAR990 (CEN-pESP1-*esp1*-2A + 3A-*NAT^R*-LEU2), pAR992 (CEN-pESP1-*esp1*-2A + 1A-*NAT^R*-LEU2), pAR930 (CEN-pESP1-*esp1*-3A + 1A-*NAT^R*-LEU2), pAR991 (CEN-pESP1-*esp1*-2D + 3D-*NAT^R*-LEU2), pAR932 (CEN-pESP1-*esp1*-2D + 1D-*NAT^R*-LEU2), pAR931 (CEN-pESP1-*esp1*-3D + 1D-*NAT^R*-LEU2), pAR1006 (CEN-pESP1-*esp1*-2A + 3A + 1A-*NAT^R*-LEU2), and pAR994 (CEN-pESP1-*esp1*-2A + 3A + 1A-*NAT^R*-LEU2). *ESP1* mutants along with the *NAT^R* cassette were amplified off these plasmids and transformed into wildtype yeast. The presence of mutated phosphorylation sites was verified by amplifying the mutated region and digesting the amplified product with the appropriate restriction enzyme. *esp1*-3D-*HYG^R* and *esp1*-3A-*HYG^R* were constructed by switching *NAT^R* for *HYG^R* using pAG32. *esp1*-3A-*ADE2* and *esp1*-3D-*ADE2* were constructed by amplifying *ADE2* off pRS412 and using it to replace *NAT^R* and *HYG^R* respectively.

FLAG tagged *ESP1* mutants were constructed in the following manner. The *ESP1* ORF was amplified off pAR745 and cloned into pBSKS between SalI and NotI to make pAR868. A BglII site was inserted downstream of the *ESP1* stop codon in pAR868 using site-directed mutagenesis to make pAR877. *ESP1*-*BglII* was amplified off pAR877 and cotransformed into yeast along with pAR797 cut with SnaBI and NcoI and rescued to get pAR888. pAR888 was then cut with BglII and cotransformed into yeast with *FLAG3x-KAN^R* amplified off

pDAM278 and rescued to get pAR911. *3FLAG-KAN^R* was then cut out of pAR911 with BsgI and NotI and cloned into pAR797 (CEN-pESP1-ESP1-LEU2), pAR873 (CEN-pESP1-esp1-2A-LEU2), pAR875 (CEN-pESP1-esp1-3A-LEU2), pAR871 (CEN-pESP1-esp1-1A-LEU2), pAR902 (CEN-pESP1-esp1-2A + 3A-LEU2), pAR924 (CEN-pESP1-esp1-2A + 1A-LEU2), pAR886 (CEN-pESP1-esp1-3A + 1A-LEU2) and pAR1005 (CEN-pESP1-esp1-2A + 3A + 1A-LEU2) to get pAR911 (CEN-pESP1-ESP1-3FLAG-KAN^R-LEU2), pAR965 (CEN-pESP1-esp1-2A-3FLAG-KAN^R-LEU2), pAR968 (CEN-pESP1-esp1-3A-3FLAG-KAN^R-LEU2), pAR973 (CEN-pESP1-esp1-1A-3FLAG-KAN^R-LEU2), pAR971 (CEN-pESP1-esp1-2A + 3A-3FLAG-KAN^R-LEU2), pAR964 (CEN-pESP1-esp1-2A + 1A-3FLAG-KAN^R-LEU2), pAR966 (CEN-pESP1-esp1-3A + 1A-3FLAG-KAN^R-LEU2) and pAR975 (CEN-pESP1-esp1-2A + 3A + 1A-3FLAG-KAN^R-LEU2). These plasmids were then transformed into the appropriate yeast strain.

To create the *ura3::lacO-URA3* allele the lacO array was cut out of pLAU43 from XbaI to BamHI and cloned into pRS406 to make pAR615. Concurrently a 416bp region ~1000 bp from *CEN5* was amplified by PCR and cloned into pRS416 between EcoRI and BamHI to make pAR634. The *CEN5* region from pAR634 was cloned into pAR615 between EcoRI and BamHI to make pAR660. pAR660 was cut with StuI and transformed into yeast to integrate the lacO array at URA3.

The 2 μ -pGAL-CLB2-TAP-URA3 plasmid (pAR546) was created as follows. The *CLB2* ORF was amplified and the resultant PCR, designed to have overlapping homology was cotransformed into yeast along with pRS-AB1234 (a gift from Christopher Carroll and David O. Morgan, University of California San Francisco, San Francisco, CA) cut with BamHI and HindIII.

SPC42-mCherry-NAT^R was constructed in the following manner. The *mCherry* coding sequence (BBa_K165004) was obtained in the vector BBa_J63009 (iGEM). *mCherry* was amplified by PCR as a PacI/AscI fragment and cloned into pKT127 resulting in pAR733. *mCherry* along with the *KAN^R* marker was amplified by PCR off pAR733 with *SPC42* specific primers and integrated. *SPC42-mCherry-KAN^R* was switched to *SPC42-NAT^R* using pAG25.

2.2 Physiology

Unless otherwise noted in the figure legend, cells were grown in yeast extract peptone media + 2% dextrose (YPD) at 30°C. Nocodazole was used at 10µg/mL (Sigma-Aldrich). 100ng/mL α -factor (Biosynthesis) was used to induce G1 cell cycle arrest. Cells were arrested in nocodazole or α -factor for 3 hours. Cells were re-arrested with 800ng/mL α -factor. The morphogenesis checkpoint was activated using 2.5-5µM latA (Sigma-Aldrich or Tocris Biosciences). latA efficacy varied between batches and suppliers so the amount needed to induce a fully Swe1-dependent checkpoint arrest was determined empirically. 5mM L-methionine was added to deplete proteins expressed from the *MET3* promoter.

2.3 Immunoblotting

Cell extracts were prepared by bead beating frozen cell pellets containing 3.0×10^7 cells in a Mini-Beadbeater (BioSpec Products) into 1X SDS sample buffer (2% SDS, 80mM Tris-Cl pH 6.8, 10% glycerol, 10mM EDTA, 0.02% bromophenol blue, 1mM Na₃VO₄, 1mM PMSF and 5mM NaF) and an equal volume of acid washed glass beads (BioSpec Products) for 60s. Samples were run on 12.5% protein gels and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S to ensure equal protein loading,

blocked for at least 30 minutes in tris-buffered saline (TBS) + 0.1% Tween-20 and 4% fat-free milk powder (Carnation) at room temperature and incubated overnight at 4°C in a primary antibody solution. Blots were washed with TBS + 0.1% Tween-20 (TBS-T) for at least 30 minutes at room temperature and incubated in a secondary antibody solution for at least 30 minutes at room temperature. Blots were washed with TBS-T for at least 30 minutes at room temperature and incubated in Western Lightning Plus-ECL reagents (Cedarlane) using the manufacturer's instructions. Blots were then exposed to HyBlot CL autoradiographic film (Denville Scientific).

The following antibodies were used for immunoblots. 9E10 ascites (BabCO), α Clb2 and α Clb3 were used as described previously, except that primary antibody solutions for these antibodies was typically prepared by diluting the antibody into TBS-T with 4% Fat Free Milk Powder (Carnation), 5% glycerol and 0.02% NaN_3 (236, 424). α Clb5, α Pds1, α Sgo1, α Cdk1, α Swe1 and α Cdc20 rabbit polyclonal antibodies were used at 1:1000 and α GFP at 1:4000 in TBS-T with 4% Fat Free Milk Powder (Carnation), 5% glycerol and 0.02% NaN_3 . α Phospho-cdc2(Tyr15) (Antibody #9111, Cell Signaling) was used to detect Y19-phosphorylated Cdk1 and α G-6-PDH (A9521, Sigma-Aldrich) was used to detect Zwfl. α Phospho-cdc2 (α Cdk1-P-tyr) and α G-6-PDH (α Zwfl) were diluted 1:1000 into TBS-T with 4% Fat Free Milk Powder (Carnation), 5% glycerol and 0.02% NaN_3 . α -mouse and α -rabbit secondary antibody solutions were prepared by diluting IgG from mouse and rabbit serum (Sigma-Aldrich) respectively 1:5000 into TBS-T with 4% Fat Free Milk Powder (Carnation).

The generation of the α Clb5, α Pds1, α Sgo1, α Cdk1, α Swe1, α Cdc20 and α GFP antibodies were described previously (425). Plasmids used to generate α Swe1 and α Cdk1 were a gift from Vu Thai and Doug Kellogg (University of California Santa Cruz, Santa

Cruz, CA) (185). α GFP serum was a gift from Aaron Straight and Andrew Murray (Stanford University, Stanford, CA and Harvard University, Cambridge, MA respectively).

α Esp1 was generated as follows. Coding sequences for the truncated proteins Esp1₂₁₁ and Esp1₂₃₀₋₄₁₄ were amplified using PCR and cloned into pHIS-parallel2 (426) as BamHI/SalI fragments to create pAR880 and pAR882 respectively. His₆-tagged Esp1 protein fragments were induced, purified on Ni-NTA columns and ~0.5mg of each fusion protein was injected into rabbits every 4 weeks for 8 to 16 weeks (UOttawa animal facility). Rabbit serum was harvested, clarified by centrifugation and loaded on Affigel-15 (Bio-rad) columns coupled to purified Esp1₂₃₀₋₄₁₄-His₆. α Esp1 was eluted from Affigel columns with either 100mM triethylamine pH 11.5 or 100mM glycine pH2.3. These elutions were neutralized, dialyzed in PBS + 50% glycerol and stored at -80°C.

α Sccl was generated as follows. Coding sequence for the truncated protein Sccl₂₀₁₋₃₀₁ was amplified by PCR and cloned into pGEX6P-1 (Promega) as a BamHI/EcoRI fragment to create pAR742. GST-tagged Sccl₂₀₁₋₃₀₁ was induced, purified on a glutathione-coupled column and 1mg of the fusion protein was injected into rabbits every 4 weeks for 8 to 16 weeks (UOttawa animal facility). Rabbit serum was harvested, clarified by centrifugation and loaded on Affigel-10 (Bio-rad) columns coupled to purified malE-Sccl. malE-Sccl was expressed from the plasmid pAR1117 which contains Sccl₂₀₁₋₃₀₁ cloned as a BamHI/SalI fragment into pMAL-c2 (NEB). α Sccl was eluted from the Affigel column with either 100mM triethylamine pH 11.5 or 100mM glycine pH 2.3. The triethylamine and glycine elutions were neutralized, dialyzed in PBS + 50% glycerol and stored at -80°C.

2.4 Fixed microscopy

~ 2.0×10^6 cells were harvested and fixed with 4% paraformaldehyde in PBS pH 7.5 for 15 minutes. Cells were washed with 100mM KPO₄/1.2M sorbitol pH 7.5, sonicated to break cell adhesions and resuspended in KPO₄/1.2M sorbitol. Samples were imaged using a Nikon TI microscope (Nikon) with a Nikon Plan Apo 60X 1.4 NA objective and FITC and/or TRITC filter sets (FITC (41001); TRITC (41002c), Chroma) at room temperature. Images were obtained using a Photometrics CoolsnapHQ2 camera (Photometrics) and NIS-Elements software (Nikon). Unless otherwise noted a minimum of 200 cells were visually scored per data point. Unless otherwise noted budding morphology and spindle type was scored qualitatively. Unless otherwise noted, in experiments where spindle lengths were measured, spindles were measured in three dimensions using a stack of 17 fluorescence images spaced every 0.5μm, covering the entire height of the cell. All measuring was done using NIS-Elements software (Nikon).

2.5 Live microscopy

Live imaging pads were made by dissolving 25% gelatin (w/v) in either SC or YEPD media at 56°C, pipetting 50μL of this media between two microscope slides and allowing it to cool. ~2μL of yeast culture was pipetted onto these pads and sealed with a coverslip and VALAP (1:1:1 vaseline:lanolin:paraffin). Strains were imaged at room temperature for two hours using brightfield and a FITC (for imaging eGFP) filter set ((41001) Chroma). Where applicable mCherry was also imaged using a TRITC filter set ((41002c) Chroma). Imaging was done on either a Leica DMI600B fluorescence microscope (Leica Microsystems) with a Leica Plan Apo 63X 1.40 NA objective and a Hamamatsu Orca AG Camera (Hamamatsu Photonics) or on a Nikon TI microscope (Nikon) with a Nikon Plan Apo 60X 1.4 NA

objective and a Photometrics CoolsnapHQ2 camera (Photometrics). 17 fluorescence images were acquired spaced every 0.5µm, covering the entire height of the cell. Spindle lengths and inter-kinetochore distances (where applicable) were measured in three dimensions using these image stacks. Mother and daughter cell size was determined as follows. Cell length and width were measured from a single brightfield image taken at the middle plane of the cell (along the Z-axis). Cell volume was then calculated using the formula for calculating the volume of an ellipsoid; $\text{volume} = 4/3\pi ab^2$ where a is the cell length (longest distance in XY plane) and b is the cell width (distance in direction perpendicular to 'a' measurement). All measuring was done using either Volocity (PerkinElmer) or NIS-Elements software (Nikon). Where applicable example images were prepared using ImageJ software, and look up tables were adjusted linearly. Mitotic spindle morphology in live cell timecourses was characterized qualitatively.

2.6 FACS analysis

For each timepoint $\sim 1.0 \times 10^7$ cells were harvested and resuspended in 70% ethanol. Samples were rotated overnight at 4°C, pelleted and treated with 0.25mg/mL RNase (Fisher) for 1 hour at 50°C. Cells were then treated with 0.25mg/mL proteinase K (Fisher) for 1 hour at 60°C and Sytox Green (Molecular Probes) for 30 minutes at room temperature. Samples were sonicated to disrupt cell adhesions and analyzed using a 488nm laser on a CyAn ADP (Beckman Coulter) flow cytometer.

2.7 Viability assays

For plate-based viability assays cell dilutions were plated using a multi-pronged serial dilution fork (DAN-KAR) on the following media as indicated in the appropriate figure.

YPD (yeast extract peptone media + 2% dextrose), YPD + auxin (YPD + 500uM indole-3-acetic acid (Sigma-Aldrich)), YPD + benomyl (YPD + 7.5μg benomyl (Sigma-Aldrich)). YPG (yeast extract peptone media + 2% galactose).

For viability assays in liquid culture, samples were diluted 1000X and/or 10000X into YPD and sonicated to disrupt cell adhesion. 200μL of the sonicated cultures were plated. Viability was determined relative to viability at $t = 0$ by counting the number of colony forming units (cfu) on the plate containing $100 < \text{cfu} < 1000$ and multiplying by the appropriate dilution factor.

2.8 In vivo labeling

10×10^7 cells from log-phase cultures were harvested and labeled in 2mL phosphate-free medium containing 0.5-1mCi $^{32}\text{PO}_4$ (GE or PerkinElmer). Scintillation counting (TriCarb 2910TR; PerkinElmer) was used to monitor uptake of the label and exceeds 98%. Esp1-myc13 or Esp1 was immunoprecipitated using the 9E10 or αEsp1 antibody respectively as described below.

2.9 In vitro phosphorylation and dephosphorylation

Cdk1^{C1b2} was purified from ADR7432 cells induced to overexpress Clb2 by addition of 2% galactose. PP2A^{Cdc55-TAP} was purified from asynchronous ADR5465 cells. Endogenous Esp1 protein was purified from asynchronous ADR4006 cells. Esp1-FLAG and Esp1-mutant-FLAG proteins were purified from the indicated strains.

Yeast extracts were made by bead beating frozen cell pellets in lysis buffer (50mM Hepes-KOH pH 7.8, 700mM NaCl, 150mM NaF, 150 mM Na-β-glycerophosphate pH 8.3,

1mM EDTA, 1mM EGTA, 5% glycerol, 0.25% NP-40, 1mM DTT, 1mM PMSF, 1mM Na₃VO₄, 1mM benzamidine, 1mM leupeptin, 1mM bestatin 1mM pepstatin A and 1mM chymostatin) and an excess of acid washed glass beads for two 90 second pulses separated by 5 minutes during which samples were maintained on ice. The resulting lysate was centrifuged in a microcentrifuge at 14000rpm for 5 minutes to remove insoluble material. Protein concentration was measured using Bradford reagent (Bio-rad) and normalized. A portion of this lysate was mixed with 2X sample buffer and run on a protein gel. The remaining lysate was incubated with 0.5 to 3.0μg of either αClb2 (for Cdk1^{Clb2} purification), αEsp1 (for Esp1 purifications) or αFLAG-M2 (F1804, Sigma-Aldrich), incubated on ice for 30 minutes and centrifuged in a microcentrifuge at 14000 rpm for 5 minutes at 4°C. The supernatants were transferred to 10 to 15μL of equilibrated proteinA magnetic beads (Invitrogen) in the case of Cdk1^{Clb2}, Esp1 and Esp1-FLAG purifications and IgG magnetic beads in the case of PP2A^{Cdc55-TAP}. The beads were rotated at 4°C for 2 to 4 hours. The beads were then washed with lysis buffer and residual buffer removed. Purified FLAG-tagged Esp1 proteins were resuspended in 1.5X SDS sample buffer, heated to 65°C for 15 minutes and run on a protein gel.

Phosphorylation of Esp1 was assayed by incubating bead-bound Esp1 with purified Cdk1^{Clb2} complexes. Kinase reactions were performed with cold ATP (1mM for cold reactions and 0.01mM for hot reactions), and 1μCi γ-[³²P]ATP. Dephosphorylation of Esp1 was measured by incubating in vitro phosphorylated Esp1 still bound to beads with purified PP2A^{Cdc55-TAP}. Okadaic acid (LC laboratories) was used at 2nM.

Chapter 3

Swe1 regulates anaphase onset

3.1 Purpose

Due to the importance of Wee1 kinases in regulating mitotic entry in other eukaryotic cells, Swe1 has been thought to regulate mitotic entry in *S. cerevisiae* as well (Figure 1-2C) (99). Indeed, deletion of *SWE1* has been shown to result in mitotic entry prematurely at a reduced cell size and deletion of *MIH1* has been shown to result in delayed mitotic entry at an increased cell size (101-103, 186).

However, activation of the *SWE1*-dependent morphogenesis checkpoint causes a cell cycle arrest in which most cells have formed a short mitotic spindle (97, 185, 427-429). This data conflicts with claims that Cdk1 dephosphorylation is essential for spindle formation in budding yeast (99, 430). We hypothesized that, unlike in other organisms, in budding yeast Swe1 and Mih1 regulated anaphase onset. We show that, in addition to regulating mitotic entry, inhibitory phosphorylation of Cdk1 plays an important role in the metaphase to anaphase transition in both unperturbed cells and during morphogenesis checkpoint activation. We show that this is due, in part, to the prevention of Cdk1 activation of the APC^{Cdc20}. We also show that PP2A^{Cdc55} inhibits anaphase onset, in part, by dephosphorylating Cdk1 sites on the APC. PP2A^{Cdc55} dephosphorylation of the APC is important in maintaining a mitotic arrest during spindle checkpoint and morphogenesis checkpoint activation.

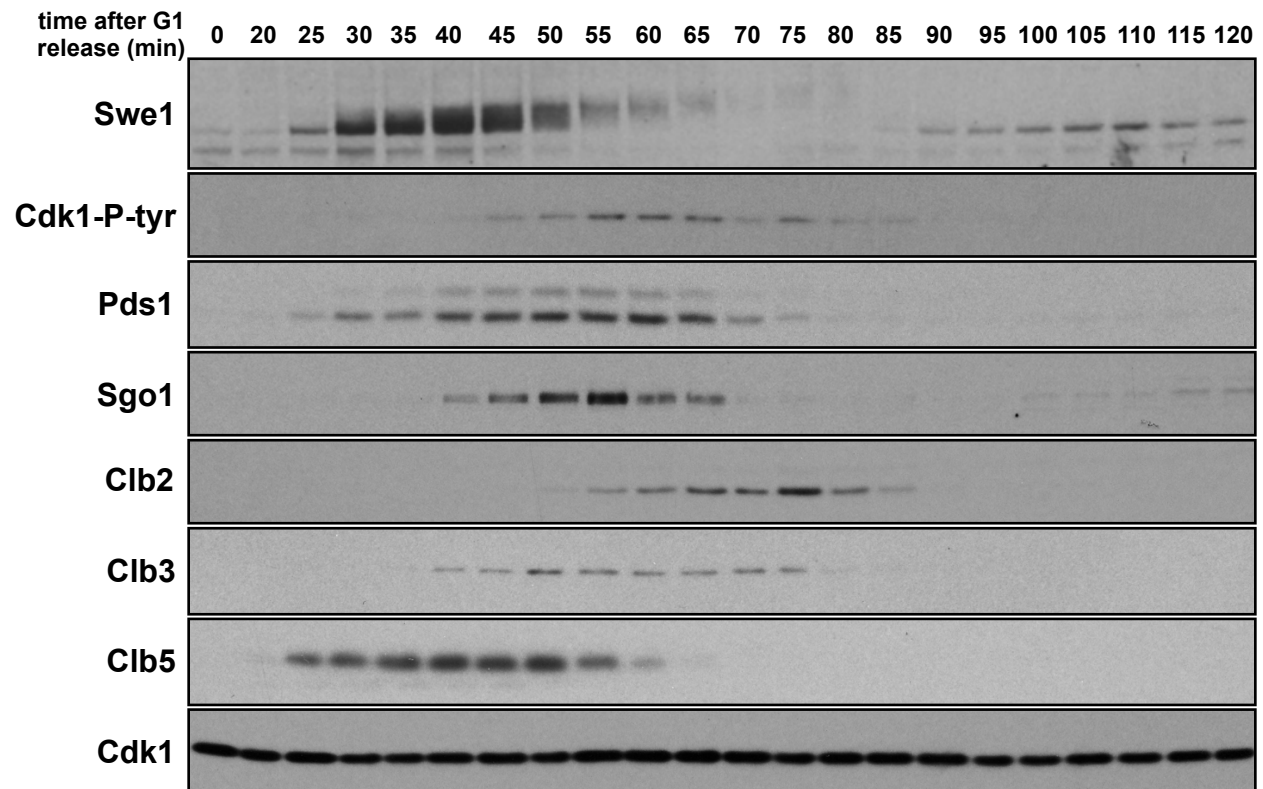
3.2 Results

3.2.1 *Swe1 is active during mitosis*

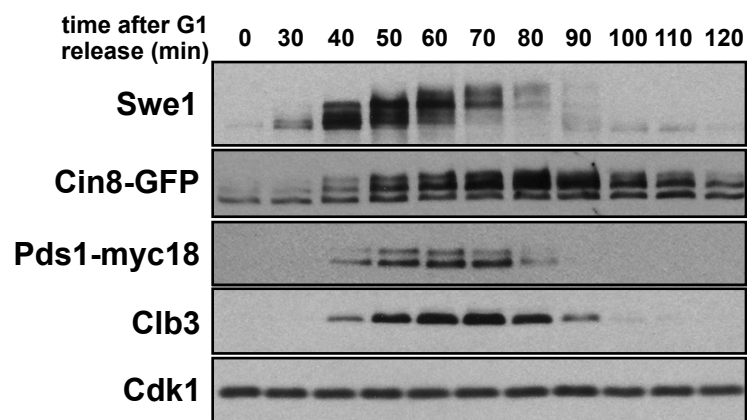
To better understand how Swe1 regulates unperturbed cell cycles, we sought to determine the timing of Swe1 activation. We did this by monitoring Swe1 protein and Cdk1 tyrosine 19 phosphorylation levels in cells synchronously undergoing a single cell cycle (Figure 3-1A). During G1 ($t = 0$) both Swe1 and Cdk1 tyrosine 19 phosphorylation are absent. Swe1 expression begins 30 minutes after G1 release, and over the ensuing timepoints Swe1 becomes highly phosphorylated (185). It is unclear when Swe1 degradation is initiated as Swe1 phosphorylation causes Swe1 to migrate as a smear between $t = 60$ and $t = 80$ minutes. Cdk1 tyrosine 19 phosphorylation peaks somewhat later than Swe1 protein, at $t = 55$ to $t = 65$ minutes. Between $t = 75$ and $t = 85$ minutes, tyrosine 19 phosphorylation disappears. Interestingly, tyrosine 19 phosphorylated Cdk1 disappears with similar timing to the APC substrate Clb2 and only shortly after Pds1. Clb5 and Clb3, which are also APC substrates, are degraded earlier than Clb2. Sgo1, which is likely to be an APC substrate as well (425), is degraded at approximately the same time as Pds1. This data demonstrates that Swe1 phosphorylation regulates Cdk1 activity during mitosis. It further suggests, along with the observations that Swe1 activation arrests cells with short mitotic spindles (97, 185, 427-429), that Cdk1 dephosphorylation may be necessary for anaphase onset.

This data contradicts a previous observation that Cdk1 dephosphorylation was required for spindle formation at mitotic onset (430). Crasta *et al.* showed that Cdk1 tyrosine 19 phosphorylation peaks before mitosis and disappears precisely when mitotic spindles form and Clb2 and the microtubule motors Cin8 and Kip1 begin to accumulate. Our data clearly shows that Cdk1 tyrosine 19 phosphorylation does not disappear before Clb2 accumulation. We therefore looked at Cin8 and Kip1 levels by immunoblot during a single

A



B



C

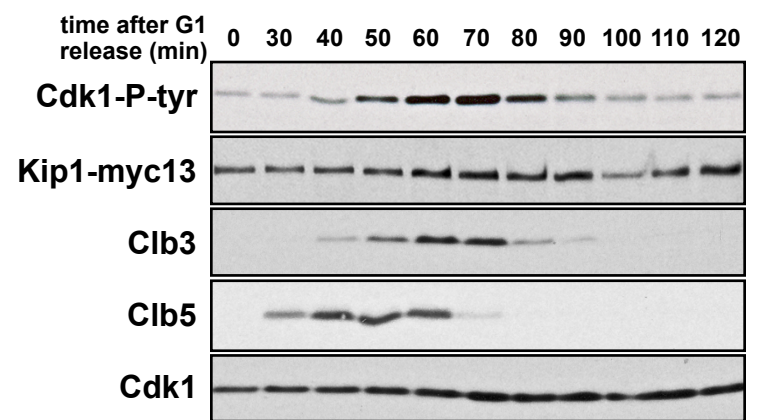


Figure 3-1

Figure 3-1. Swe1 is present and active during mitosis.

(A) Swe1 is present and active during mitosis. Wildtype (ADR4006) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

(B) Cin8 stabilization does not coincide with Swe1 degradation. *CIN8-GFP PDS1-myc18* (ADR4193) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

(C) Kip1 stabilization does not coincide with Cdk1 dephosphorylation. *KIP1-myc13* (ADR4191) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

synchronous cell cycle (Figure 3-1B and C). Though Cin8 accumulates during mitosis, this occurs while Swe1 is still present, suggesting that Cdk1 tyrosine 19 phosphorylation may not regulate Cin8 expression (Figure 3-1B). We were unable to see any significant cell cycle dependent regulation of Kip1 protein levels (Figure 3-1C).

3.2.2 Swe1 regulates anaphase onset in unperturbed cells

Swe1 has previously been shown to inhibit mitotic entry in unperturbed cells (102). Our observation that Cdk1 tyrosine 19 phosphorylation disappearance coincides with the degradation of APC substrates suggested that Cdk1 phosphorylation regulates unperturbed cells during mitosis. To observe this directly, we monitored cyclin and Pds1 degradation in cells lacking Cdk1 tyrosine 19 phosphorylation (*swe1Δ*) and cells with increased tyrosine 19 phosphorylation (*mih1Δ*) (Figure 3-2). Deleting *MIH1* causes increased Cdk1 tyrosine 19 phosphorylation and delays APC activation, as shown by the persistence of the APC substrates Pds1, Clb2, Clb3 and Clb5. By $t = 95$, Pds1, Clb2, Clb3 and Clb5 are all degraded in wildtype cells, but only Clb5 is partially degraded in *mih1Δ* cells. Deletion of *SWE1*, however, does not appear to cause earlier degradation of APC substrates. We independently monitored mitotic progression in these cells by visualizing mitotic spindles using fluorescence microscopy of Spc42-eGFP (Figure 3-3). Spc42 is a component of the spindle pole body (SPB, a homologous structure to the mammalian centrosome). Early in the cell cycle, SPBs duplicate but cannot be resolved by conventional widefield fluorescence microscopy, as they lie close together in the nuclear envelope (Figure 3-3A and B). At mitotic entry, SPBs migrate to opposite sides of the nucleus and a short spindle ($\sim 1\text{-}2\text{ }\mu\text{m}$ in length). After the successful biorientation of each pair of sister chromatids, sister chromatid cohesion is dissolved and spindles rapidly elongate. By measuring the distance between

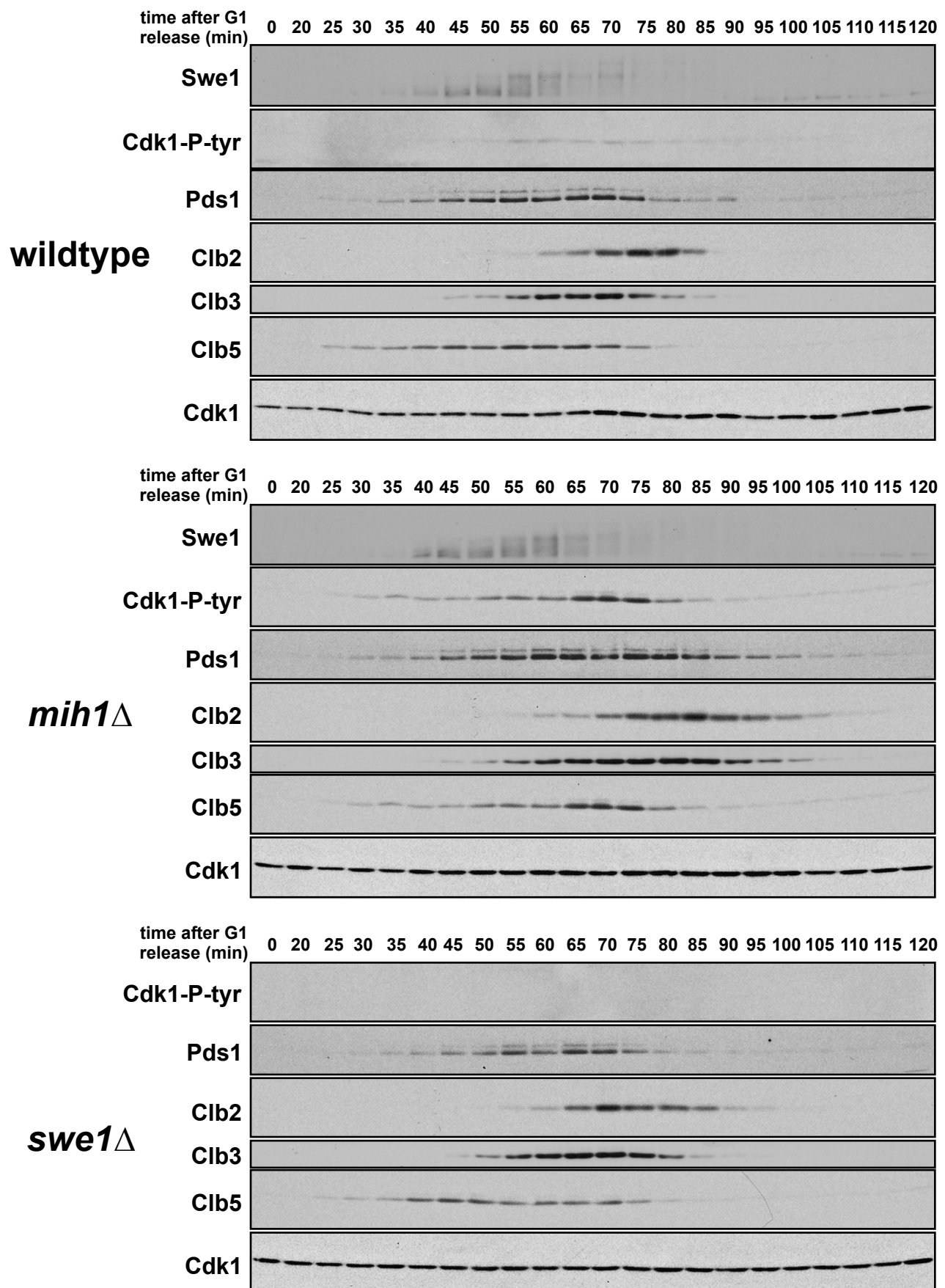


Figure 3-2

Figure 3-2. Deleting *MIH1* stabilizes APC substrates.

SPC42-eGFP (ADR4009), *mih1* Δ *SPC42-eGFP* (ADR4012) and *swel* Δ *SPC42-eGFP* (ADR4015) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting (this Figure) and samples were fixed for microscopy (Figure 3-3) at the indicated timepoints.

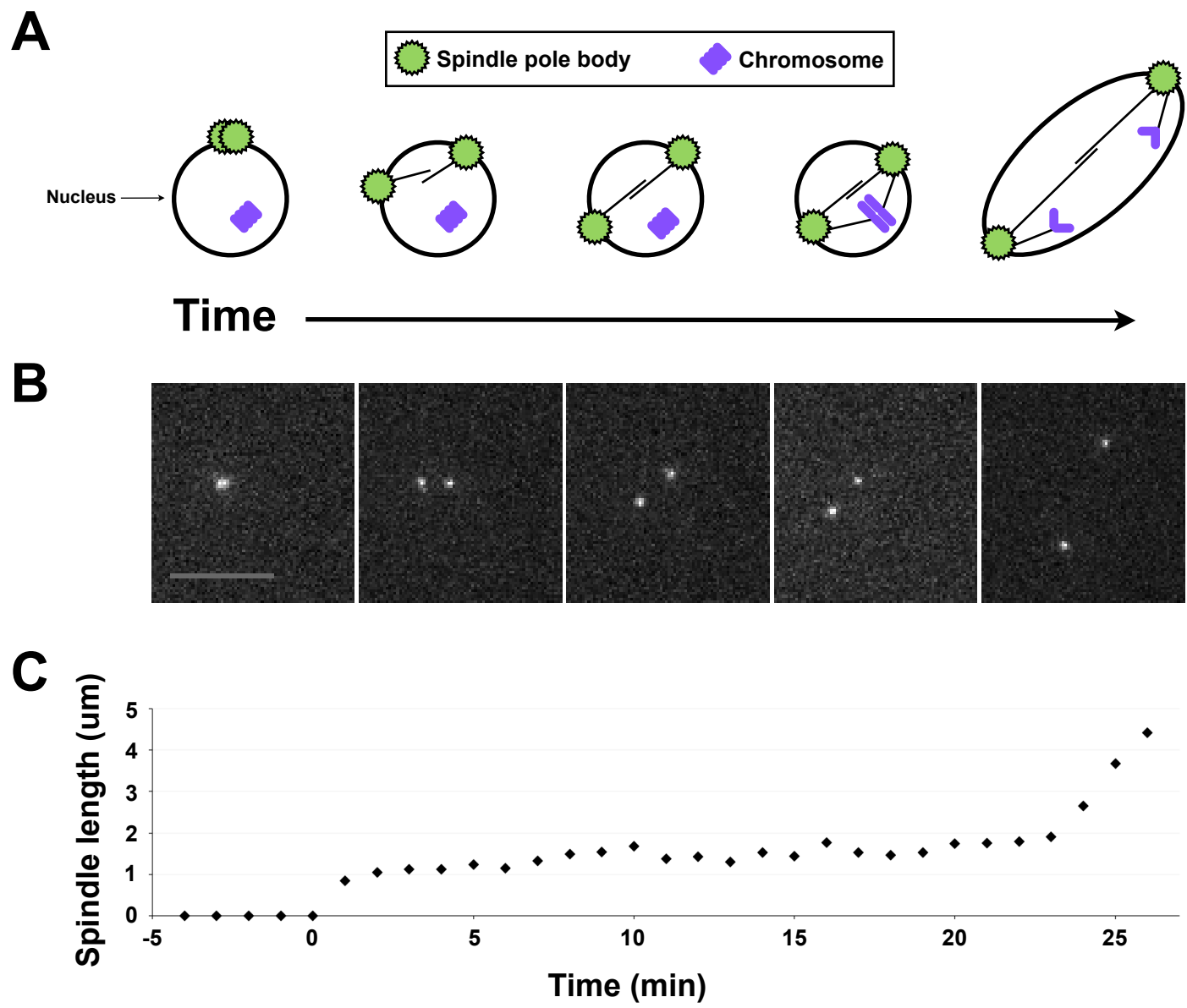


Figure 3-3

Figure 3-3. Monitoring anaphase onset using fluorescence microscopy.

Anaphase onset can be monitored directly in live or fixed cells by expressing a fusion protein between a component of the spindle pole body (Spc42) and a fluorescent reporter (eGFP).

(A-B) Before spindle formation, duplicated spindle pole bodies lie close together and cannot be differentiated using standard widefield fluorescence microscopy techniques. Upon spindle formation spindle pole bodies migrate to opposite sides of the nucleus and remain separated by ~1 to 2 μm until anaphase onset. At anaphase, when cohesion between sister chromatids is dissolved, spindle pole bodies rapidly separate. The scale bar is 4 μm .

(C) By measuring the distance between Spc42-eGFP foci over time, mitotic entry and anaphase onset can be monitored.

SPC42-eGFP foci as cells traverse the cell cycle, the timing of mitotic entry and anaphase onset can be determined in single live cells (Figure 3-3C). Alternatively, visualizing SPC42-eGFP foci in fixed cells can be used to monitor cell cycle progression in a population of cells (Figure 3-4). Wildtype, *swe1Δ* and *mih1Δ* cells undergo budding with similar timing after release from G1 arrest (Figure 3-4A). Consistent with the observation that increasing Cdk1 phosphorylation delays mitotic entry (102), *mih1Δ* cells form short mitotic spindles later than wildtype cells (Figure 3-4B). *mih1Δ* cells also elongate their spindles at anaphase later than wildtype cells, suggesting that perhaps Cdk1 tyrosine 19 phosphorylation regulates anaphase onset (Figure 3-4C). The timing of spindle formation and anaphase onset in *swe1Δ* cells appears to be similar to wildtype cells. Due to the design of these experiments we were unable to obtain high temporal resolution, and cell asynchrony during mitosis could mask small changes in cell cycle progression in *swe1Δ* mutants. Therefore, we decided to look at spindle formation and anaphase onset directly in live *swe1Δ* and *mih1Δ* cells. We first determined how to best obtain accurate spindle length measurements in these experiments (Figure 3-5). We measured spindles of a variety of lengths in a) a three-dimensional image stack, b) the same image stack deconvolved to improve image quality and c) a two-dimensional maximum projection of the three-dimensional image stack. As expected, spindles measured in two dimensions were often found to be shorter than when the same spindles were measured in three dimensions (Figure 3-5A and B). Deconvolved image stacks, however, did not improve the accuracy of spindle length measurements as compared to non-deconvolved image stacks (Figure 3-5C). Therefore in live imaging experiments, we measured spindle lengths in three dimensions using undeconvolved image stacks.

To determine if *SWE1* deletion causes premature anaphase, we monitored spindle formation and anaphase onset in live wildtype, *mih1Δ* and *swe1Δ* cells (Figure 3-6A). In

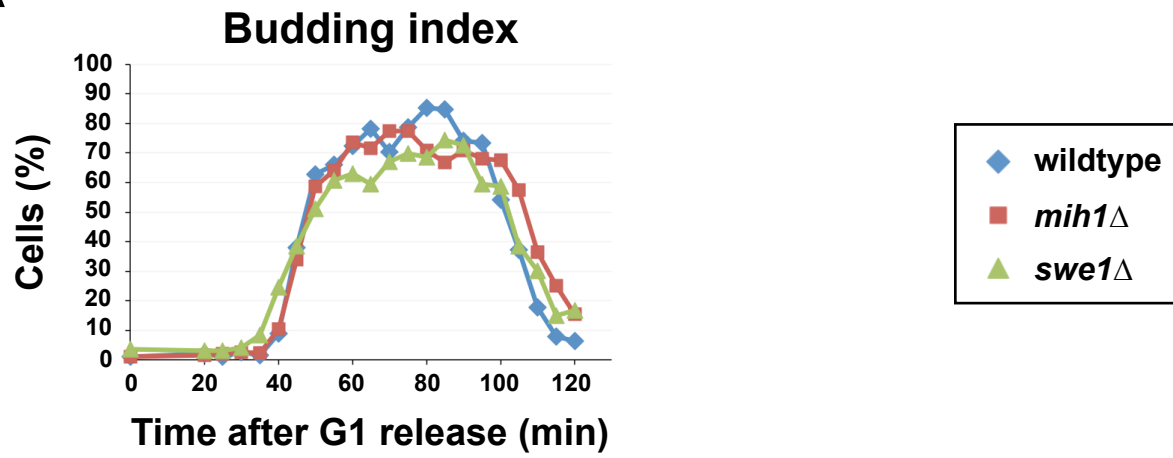
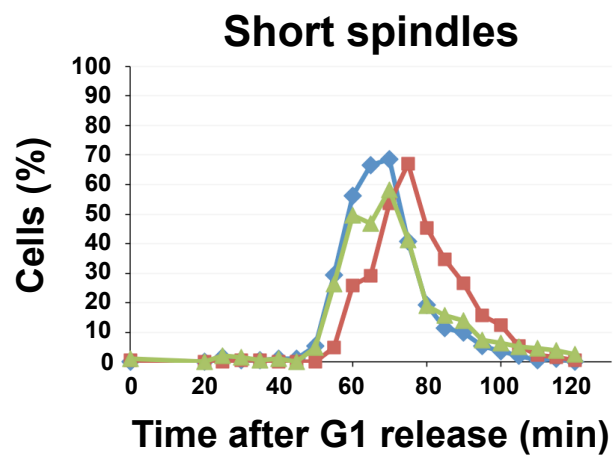
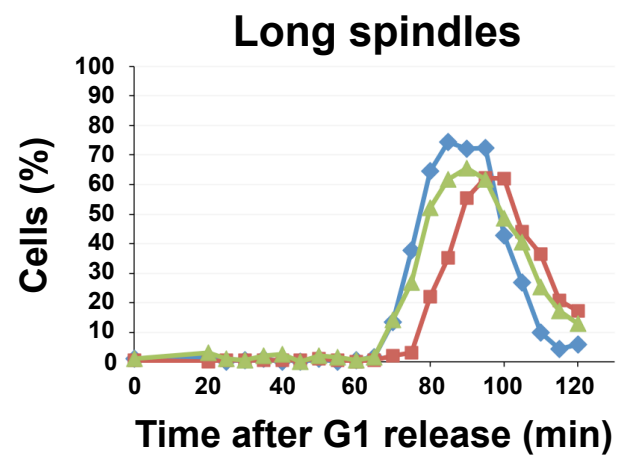
A**B****C****Figure 3-4**

Figure 3-4. Deleting *MIH1* delays mitotic entry and anaphase onset.

(A-C) As described in Figure 3-2, *SPC42-eGFP* (ADR4009), *mih1Δ SPC42-eGFP* (ADR4012) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

(A) Deleting *MIH1* or *SWE1* does not affect bud emergence.

(B) Deleting *MIH1* delays mitotic entry.

(C) Deleting *MIH1* delays anaphase onset.

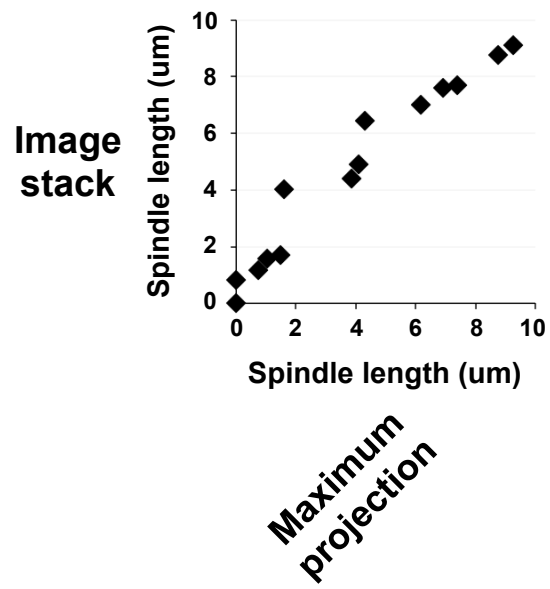
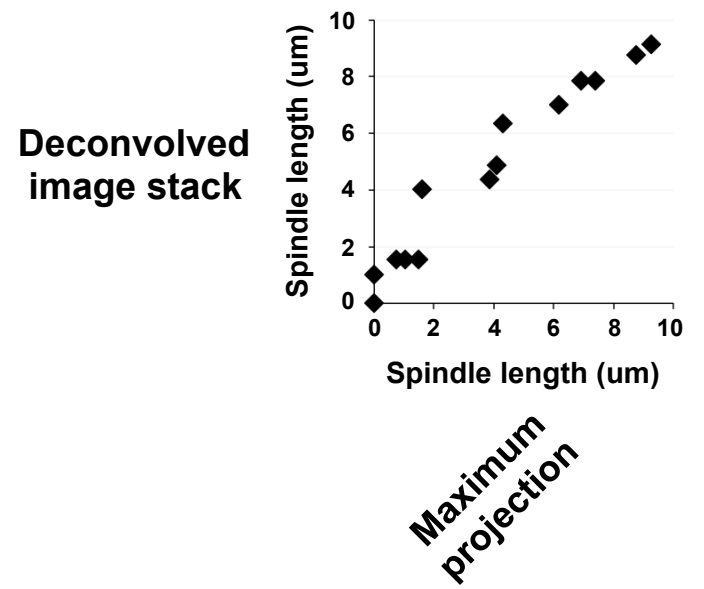
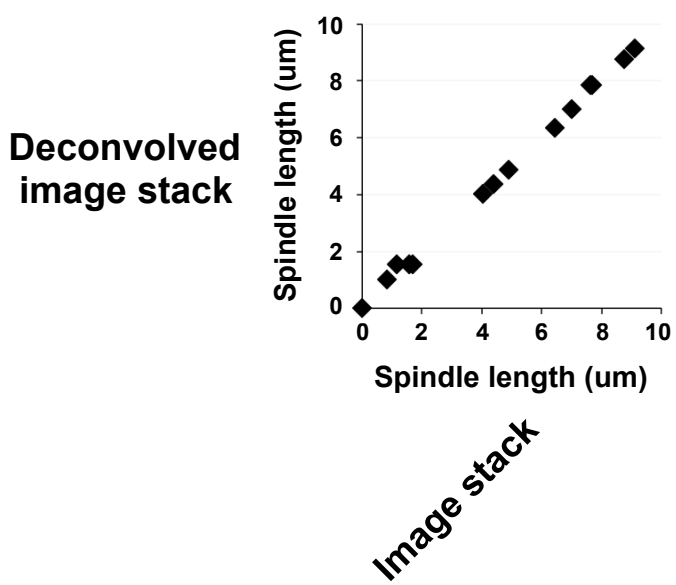
A**B****C****Figure 3-5**

Figure 3-5. Deconvolution does not improve three-dimensional spindle measurements.

(A-D) *SPC42-eGFP* (ADR4009) cells were grown to log phase. A single sample of asynchronous cells was fixed for microscopy. A single fluorescence image stack was obtained using a FITC filter set. Spindle lengths ($n = 14$) were measured in the raw 3-dimensional *raw image stack*, a 2-dimensional *maximum projection* of the image stack and a 3-dimensional *deconvolved image stack*.

(A) 2-dimensional measurement underestimates spindle length. For each measured spindle, the length measured using the 3D deconvolved image stack was plotted versus the length measured using the maximum projection.

(B) Raw 3-dimensional image stacks provide better estimates of spindle length than 2-dimensional projections. For each measured spindle, the length measured using the 3D image stack was plotted versus the length measured using the maximum projection.

(C) Deconvolution does not improve 3-dimensional spindle measurements. For each measured spindle, the length measured using the 3D deconvolved image stack was plotted versus the length measured using the 3D image stack.

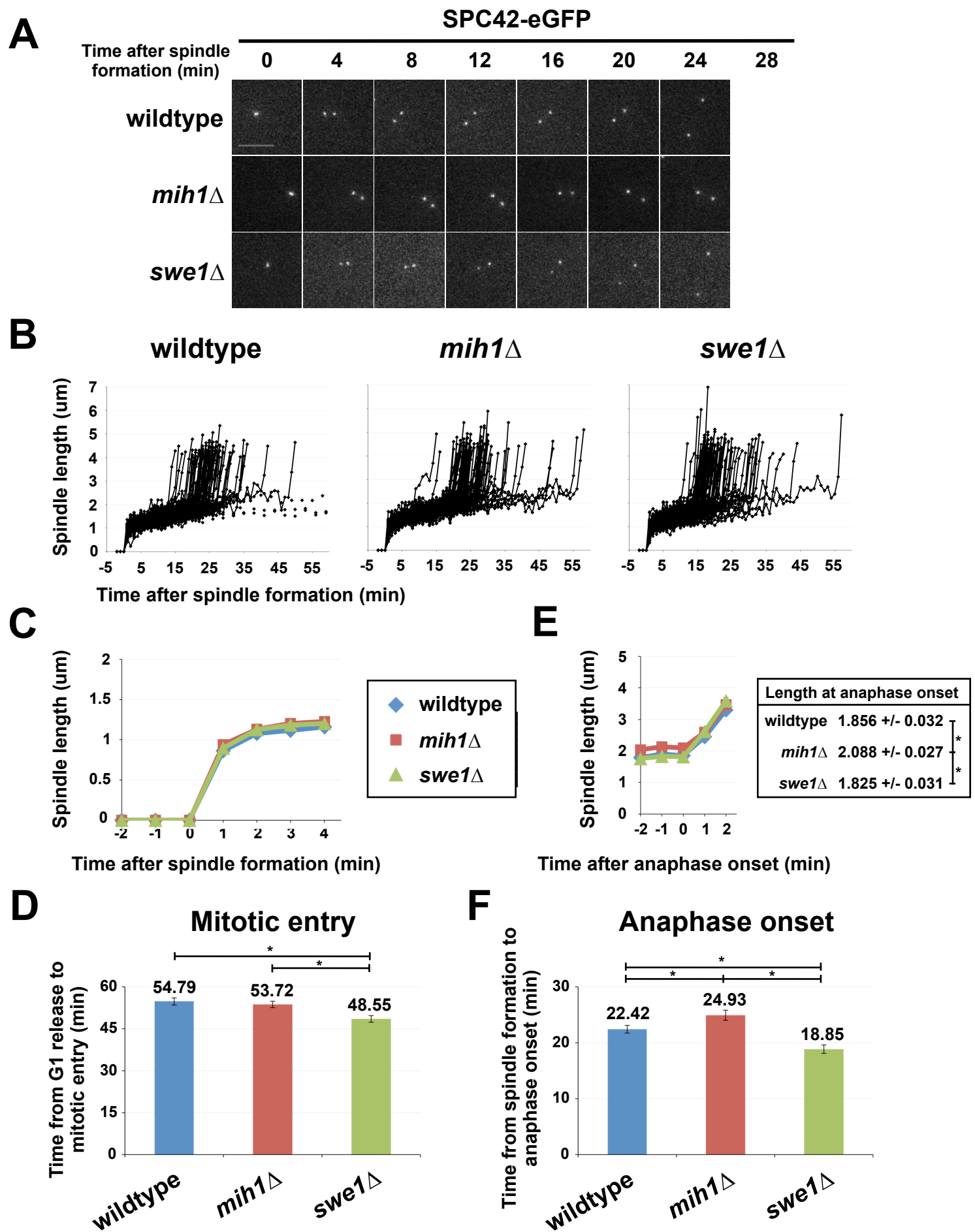


Figure 3-6

Figure 3-6. Swe1 and Mih1 regulate anaphase onset in live cells.

(A-F) *SPC42-eGFP* (ADR4009), *mih1Δ SPC42-eGFP* (ADR4012) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$). At $t = 25$ minutes cells were plated onto minimal media live microscopy pads and imaged. Strains were imaged three times and the data combined (wildtype ($n = 75$), *mih1Δ* ($n = 73$), *swe1Δ* ($n = 89$)). The scale bar is 4 μm .

(A) 2-dimensional maximum projections of spindles in example live wildtype, *mih1Δ* and *swe1Δ* cells.

(B) Traces of spindle length during mitotic progression for all imaged wildtype, *mih1Δ* and *swe1Δ* cells. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μm .

(C) Deleting *MIH1* or *SWE1* does not affect spindle pole body separation. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean $\pm$ SEM).

(D) Deleting *SWE1* promotes mitotic entry. The time spent between G1 release and spindle formation was calculated for each cell. Displayed values are (mean $\pm$ SEM). Statistically significant differences were determined using one-way ANOVA ($p < 0.001$) and a Tukey HSD post-hoc test ($\text{HSD}_{.05} = 3.34$).

(E) *mih1Δ* cells begin anaphase at an increased spindle length. The timepoint before anaphase spindle elongation began was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after anaphase spindle elongation began were calculated. Displayed values are (mean $\pm$ SEM). Statistically significant differences were determined using one-way ANOVA ($p < 0.001$) and a Tukey HSD post-hoc test ($\text{HSD}_{.05} = 0.102$).

(F) Deleting *SWE1* promotes anaphase onset and deleting *MIH1* delays anaphase onset. The time spent between spindle formation and anaphase onset was calculated for each cell. Displayed values are (mean $\pm$ SEM). Statistically significant differences were determined using one-way ANOVA ($p < 0.001$) and a Tukey HSD post-hoc test ($\text{HSD}_{.05} = 2.19$).

wildtype cells, spindles initially elongate to a metaphase length of between 1 and 2 μ m (Figure 3-6A, t = 4 to 20). At anaphase onset, spindles undergo a rapid elongation (t = 24). The majority of wildtype cells undergo anaphase onset between 15 and 25 minutes after spindle formation (Figure 3-6B). Deletion of *MIH1* or *SWE1* does not grossly perturb spindle dynamics, as *mih1* Δ and *swe1* Δ cells exhibit a period with relatively short metaphase spindles and then rapid spindle elongation at anaphase (Figure 3-6B). Contrary to what would be expected if Cdk1 phosphorylation regulated spindle formation through motor proteins (430), spindle lengths in the timepoints immediately after spindle formation were similar in wildtype, *mih1* Δ and *swe1* Δ cells (Figure 3-6C). This live imaging data provided more sensitive measurements of the timing of mitotic entry and anaphase onset and revealed that, consistent with previously published data (102), deletion of *SWE1* significantly reduces the amount of time spent before spindle formation, though deletion of *MIH1* did not appear to affect the timing of mitotic entry (Figure 3-6D). Increased time in metaphase resulted in significantly longer spindles at anaphase onset in *mih1* Δ cells (Figure 3-6E). Deleting *MIH1* significantly increased the length of time cells spent between spindle formation and anaphase onset, and deleting *SWE1* significantly decreased this time (Figure 3-6F), even after controlling for the timing of spindle formation. This demonstrates that Cdk1 tyrosine 19 phosphorylation regulates anaphase onset in unperturbed cells.

Since Cdk1 tyrosine 19 phosphorylation regulated anaphase onset, we considered whether Cdk1 phosphorylation status might impact spindle elongation following anaphase onset. Anaphase in budding yeast is characterized by a relatively rapid early phase and then a relatively slow later phase (431). Deletion of *MIH1* or *SWE1* does not grossly impact anaphase elongation, as both *mih1* Δ and *swe1* Δ cells undergo a rapid early phase and then a relatively less rapid stage of spindle elongation (Figure 3-7A and B). Elongation rates during

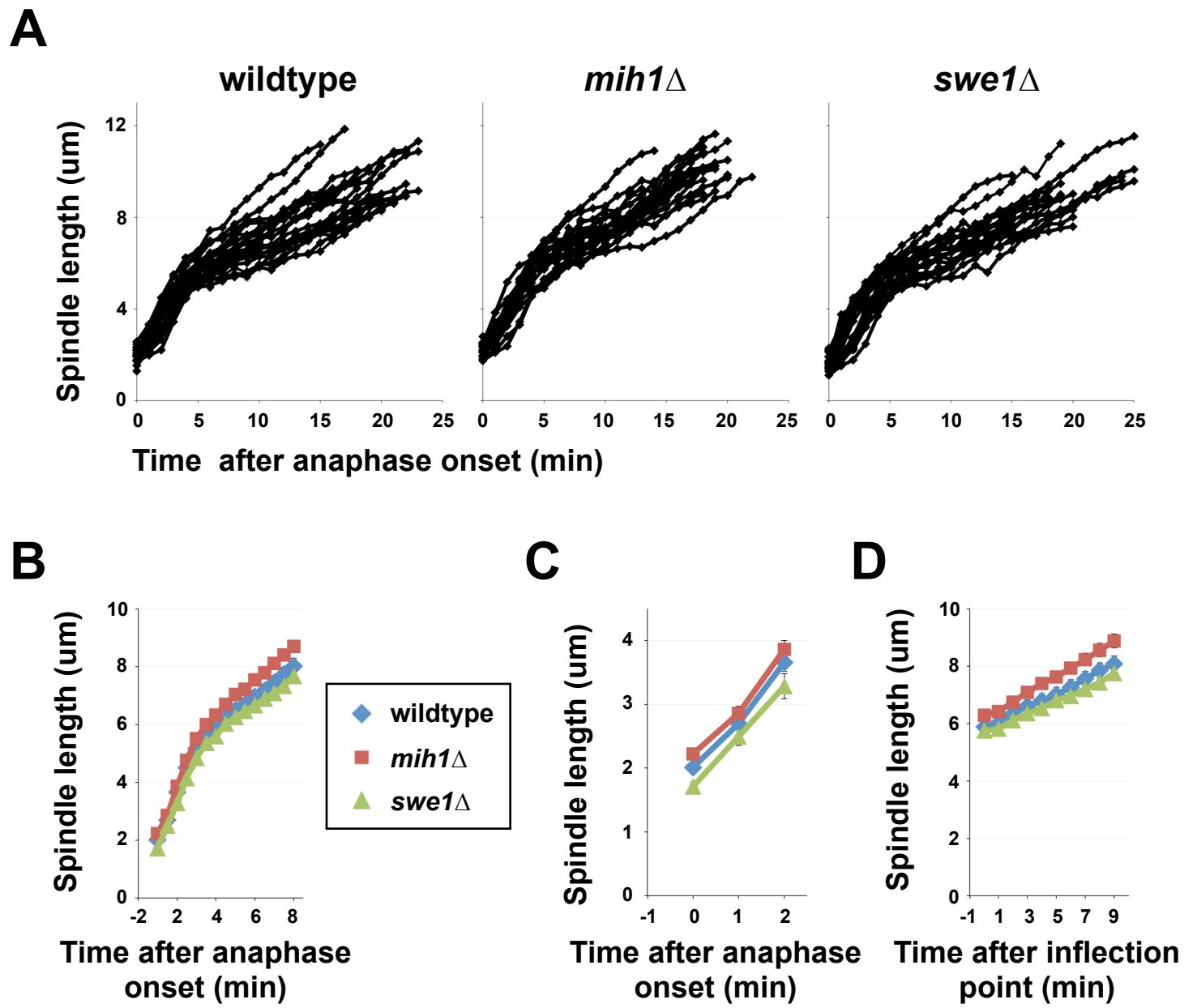


Figure 3-7

Figure 3-7. Deleting *SWE1* or *MIH1* does not affect anaphase spindle elongation.

(A-D) Ten cells from each of two timecourses of *SPC42-eGFP* (ADR4009), *mih1Δ SPC42-eGFP* (ADR4012) and *swe1Δ SPC42-eGFP* (ADR4015) cells obtained for Figure 3-5 were analyzed further. Spindle lengths were measured at each timepoint in each cell between anaphase onset and spindle disassembly.

(A) Traces of spindle length during anaphase for all imaged wildtype, *mih1Δ* and *swe1Δ* cells. The timepoint before anaphase spindle elongation began was defined as $x = 0$ for each cell.

(B) Deleting *MIH1* or *SWE1* does not affect anaphase spindle elongation. The timepoint before anaphase spindle elongation began was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints after anaphase spindle elongation began were calculated. Displayed values are (mean $\pm$ SEM).

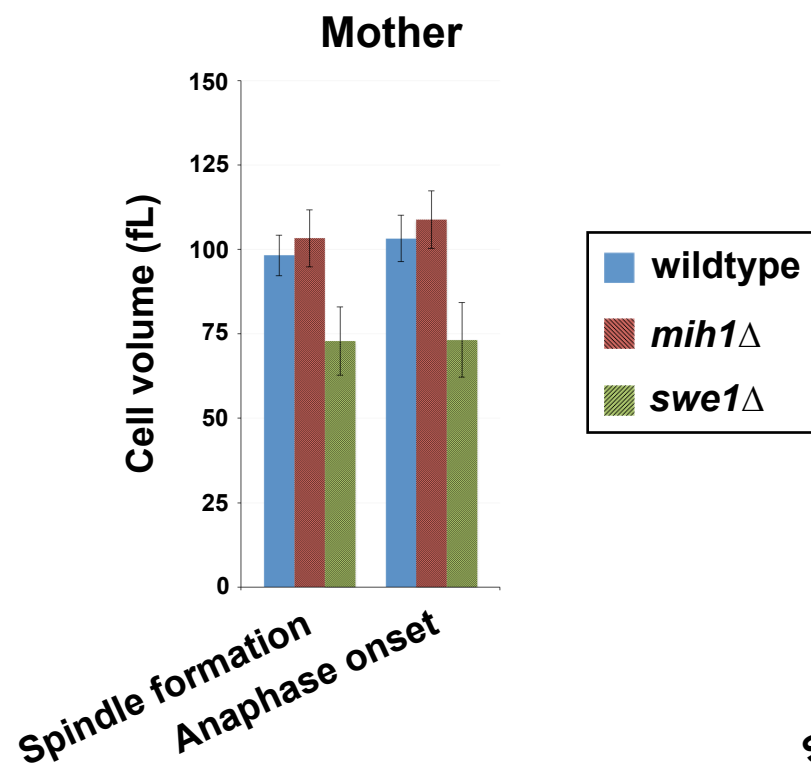
(C) Deleting *MIH1* or *SWE1* does not affect fast anaphase spindle elongation. The timepoint before anaphase spindle elongation began was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints after anaphase spindle elongation began were calculated. Displayed values are (mean $\pm$ SEM).

(D) Deleting *MIH1* or *SWE1* does not affect slow anaphase spindle elongation. The timepoint at the inflection point between ‘fast’ and ‘slow’ phases of anaphase was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints after the inflection point were calculated. Displayed values are (mean $\pm$ SEM).

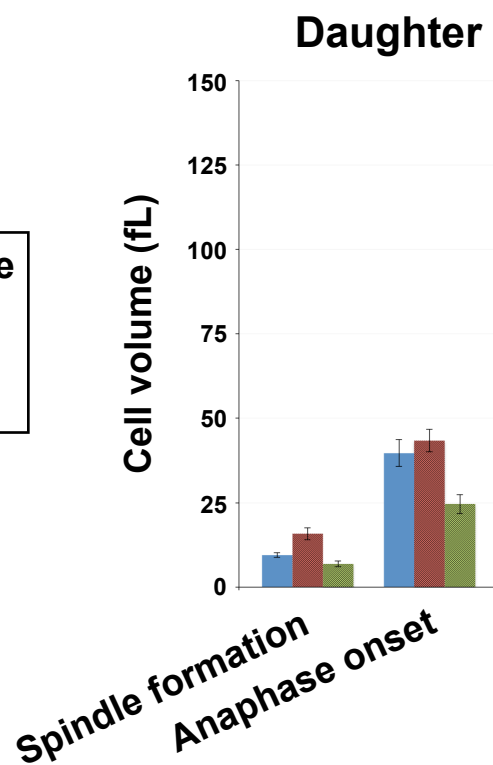
the rapid phase were similar for wildtype, *mih1* Δ and *swe1* Δ cells (Figure 3-7C). Elongation rates during the slow phase were also relatively similar for wildtype, *mih1* Δ and *swe1* Δ cells (Figure 3-7D).

There has been controversy over whether budding yeast Swe1 responds to a cell size checkpoint as it does in fission yeast cells (102) or a budding yeast-specific morphogenesis checkpoint (98). If Swe1 is involved in a cell size checkpoint, we might expect that deletion of *SWE1* would result in cells undergoing mitotic entry and/or anaphase onset at a smaller size than wildtype, and deletion of *MIH1* would result in cells undergoing mitotic entry and/or anaphase onset at a larger size than wildtype. To determine if this was the case, we measured cell size at spindle formation and anaphase onset in live, asynchronous, unperturbed wildtype, *mih1* Δ and *swe1* Δ cells (Figure 3-8). As expected, cell growth between spindle formation and anaphase onset occurs primarily in the bud in wildtype, *mih1* Δ and *swe1* Δ cells (Figure 3-8A, B and C). *SWE1* deletion causes significantly reduced cell size in both the mother and bud at both mitotic entry and anaphase onset (Figure 3-8A, B, C and D). Deletion of *MIH1* appears to cause increased cell size in both the mother and bud at both mitotic entry and anaphase onset (Figure 3-8A, B and C), however some differences between wildtype and *mih1* Δ cells were not statistically significant (Figure 3-8D). These experiments, though clearly showing that Swe1 and Mih1 regulate cell size, do not elucidate whether Swe1 responds to a cell size checkpoint per se or to morphological changes due to small cell size. Recent work on this topic will be addressed in detail in the discussion.

A



B



C

		Volume (fL)		
		wildtype	<i>mih1</i> Δ	<i>swe1</i> Δ
Mother	Spindle formation	98.23 +/- 6.04	103.30 +/- 8.44	72.86 +/- 10.15
	Anaphase onset	103.23 +/- 6.84	108.85 +/- 8.50	73.24 +/- 11.04
Daughter	Spindle formation	9.53 +/- 0.71	15.80 +/- 1.78	6.88 +/- 0.83
	Anaphase onset	39.68 +/- 3.96	43.39 +/- 3.33	24.58 +/- 2.78

D

		wildtype vs <i>mih1</i> Δ	wildtype vs <i>swe1</i> Δ	<i>mih1</i> Δ vs <i>swe1</i> Δ	ANOVA p-value	Tukey HSD _{.05}
Mother	Spindle formation	n.s.	*	*	0.043	9.52
	Anaphase onset	n.s.	*	*	0.024	10.10
Daughter	Spindle formation	*	*	*	< 0.001	1.50
	Anaphase onset	n.s.	*	*	0.003	3.97

Figure 3-8

Figure 3-8. Deleting *SWE1* or *MIH1* affects the size at which cells undergo mitotic transitions.

(A-D) *SPC42-eGFP* (ADR4009), *mih1Δ SPC42-eGFP* (ADR4012) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown to log phase, resuspended in minimal media, plated onto minimal media live microscopy pads and imaged. Strains were imaged two times and the data combined (wildtype (n = 8), *mih1Δ* (n = 9), *swe1Δ* (n = 7)). The timepoints at which spindles formed and anaphase began were identified in each cell. Mother and bud volumes were calculated at these timepoints.

(A) Deleting *SWE1* allows mitotic progression in smaller mother cells. The average mother cell volume at spindle formation and anaphase onset are shown (mean +/- SEM).

(B) Deleting *SWE1* allows mitotic progression in smaller daughter cells. The average daughter cell volume at spindle formation and anaphase onset are shown (mean +/- SEM).

(C) Cell size at spindle formation and anaphase onset summary data.

(D) Statistical comparisons between cell size at spindle formation and anaphase onset for wildtype, *mih1Δ* and *swe1Δ* cells.

3.2.3 *The morphogenesis checkpoint delays anaphase onset*

Since we have shown that Cdk1 tyrosine 19 phosphorylation regulates anaphase onset in unperturbed cell cycles, we decided to examine whether Swe1 activation due to the cell size/morphogenesis checkpoint (hereafter referred to as the morphogenesis checkpoint) inhibited anaphase onset. To do this, we treated wildtype and *swe1Δ* cells with the actin depolymerizing drug latrunculin A (latA) at G1 release ($t = 0$), and monitored spindle formation and anaphase onset using Spc42-eGFP. latA has previously been shown to cause a *SWE1*-dependent delay in mitotic entry (432). Checkpoint activation causes wildtype, but not *swe1Δ* cells, to delay cell cycle progression before anaphase onset (Figure 3-9A, B and C). While doing these experiments it became clear that latA treatment causes delays in both spindle formation and anaphase onset. To quantify these two phenotypes in an unbiased manner, cells were characterized as exhibiting *delayed mitotic entry* (> 90 minutes between G1 release and spindle formation), *delayed anaphase* (> 60 minutes between spindle formation and anaphase onset) or *normal progression of mitosis* (Figure 3-9F and G). latA treatment causes an increase in the number of cells that delay mitotic entry from 2.6% in untreated controls to 31.5% in latA treated cells. The number of cells that delayed anaphase onset, however, increased from 3.9% to 55.6%. Due to experimental constraints, cells that delayed mitotic entry could not be scored for delayed anaphase. Of the cells that could be characterized, 81.1% exhibited a delay before anaphase onset. This data suggests that, though Swe1 clearly does regulate mitotic entry, Swe1 may primarily inhibit cell cycle progression by preventing anaphase onset. To facilitate further study of how Swe1 activation delays anaphase onset, we delayed latA treatment until 25 minutes after G1 release (Figure 3-9D, E, F and G). This causes a *SWE1*-dependent delay to mitotic entry in only 14.8% of cells but delays anaphase onset in 83.3%. Regardless of the timing of latA addition $> 90\%$ of

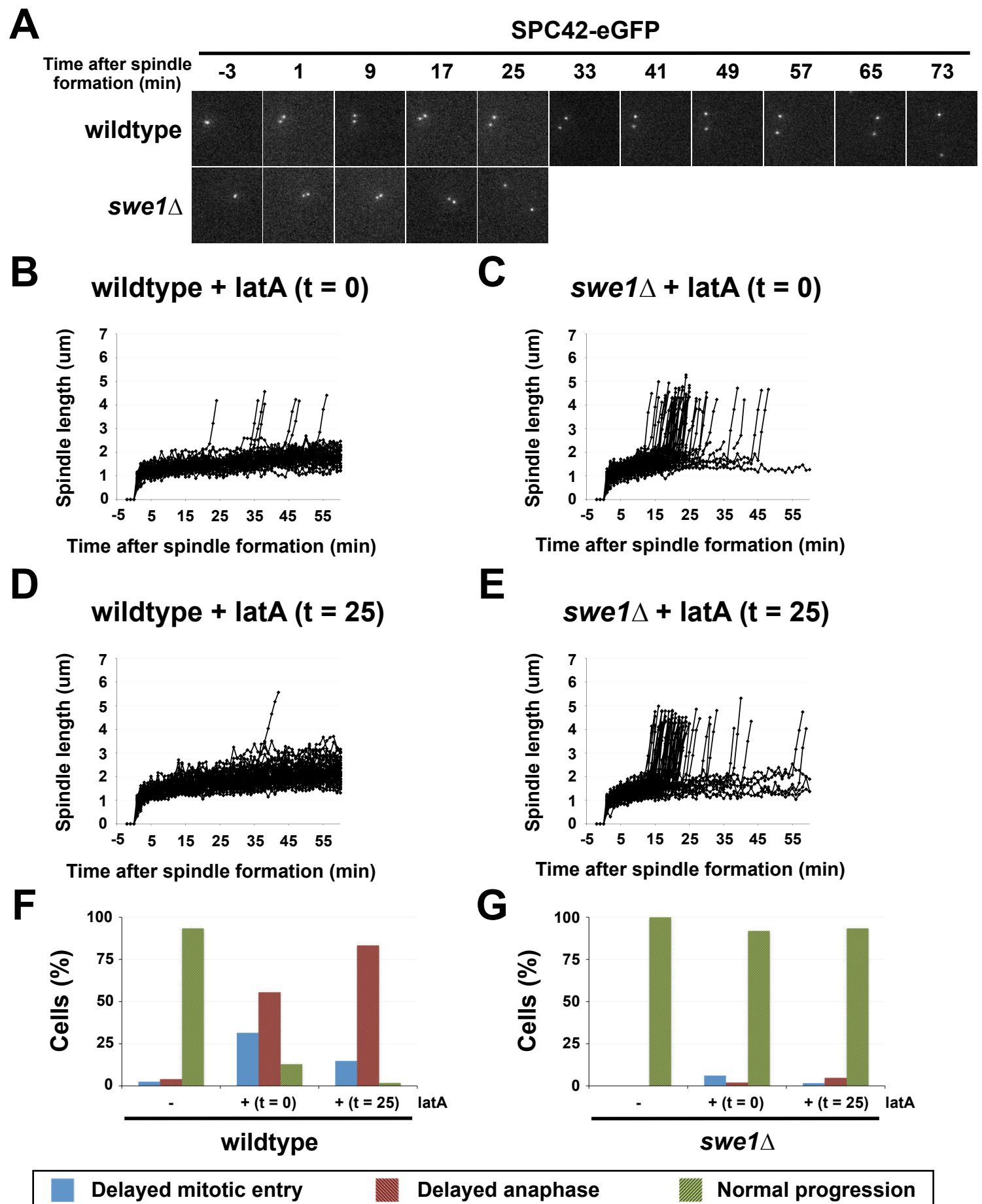


Figure 3-9

Figure 3-9. Activation of the morphogenesis checkpoint delays anaphase onset.

(A-C) *SPC42-eGFP* (ADR4009) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$) containing latA. At $t = 25$ minutes cells were plated onto minimal media live microscopy pads containing latA and imaged. Strains were imaged two times and the data combined (wildtype ($n = 37$), *swe1Δ* ($n = 46$)).

(A) 2-dimensional maximum projections of spindles in example live wildtype and *swe1Δ* cells treated with latA.

(B) Activation of the morphogenesis checkpoint delays anaphase onset. Traces of spindle length during mitotic progression for all imaged wildtype cells treated with latA at $t = 0$. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $4\ \mu\text{m}$.

(C) latA-dependent delay of anaphase onset requires *SWE1*. Traces of spindle length during mitotic progression for all imaged *swe1Δ* cells treated with latA at $t = 0$. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $4\ \mu\text{m}$.

(D-E) *SPC42-eGFP* (ADR4009) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$). At $t = 25$ minutes cells were plated onto minimal media live microscopy pads containing latA and imaged. Strains were imaged two times and the data combined (wildtype ($n = 46$), *swe1Δ* ($n = 61$)).

(D) Late addition of latA delays anaphase onset. Traces of spindle length during mitotic progression for all imaged wildtype cells treated with latA at $t = 25$ minutes. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $4\ \mu\text{m}$.

(E) Late addition of latA does not delay anaphase onset in *swe1Δ* cells. Traces of spindle length during mitotic progression for all imaged *swe1Δ* cells treated with latA at $t = 25$ minutes. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $4\ \mu\text{m}$.

(F) Late addition of latA causes a uniform mitotic arrest. The cells from (B) and (D) as well as wildtype cells from Figure 10B as a control were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

(G) *swe1Δ* fully bypasses the latA-dependent mitotic delay. The cells from (C) and (E) as well as *swe1Δ* cells from Figure 10B as a control were each characterized as either *delayed mitotic entry*, *delayed anaphase* or *normal progression* as in (F).

swe1Δ cells enter mitosis and anaphase in a timely manner, confirming that the observed cell cycle defects depend on *SWE1* (Figure 3-9F and G).

We have shown that repression of the microtubule motor proteins Cin8 and Kip1 is unlikely to be responsible for delaying spindle formation in response to Cdk1 tyrosine 19 phosphorylation (Figure 3-1B and C and Figure 3-6C). To determine if spindle pole body separation is perturbed in latA treated cells, we measured average spindle lengths in the timepoints immediately after spindle formation in latA treated wildtype and *swe1Δ* cells (Figure 3-10A). latA treatment does not appear to perturb spindle pole body separation. We next tested whether latA treatment impacted anaphase indirectly by inhibiting spindle assembly. In unperturbed cells, after spindle pole bodies have separated, kinetochores must be captured by kinetochore microtubules (kMTs) before anaphase onset. Kinetochore capture results in the separation of the kinetochores and centromeres of sister chromatids by $\sim 1\mu\text{m}$ in the timepoints preceding anaphase onset (433). To determine if latA treatment delayed mitotic progression before or after kinetochore capture, we imaged mitotic progression in cells in which the kinetochore component Ndc80 had been tagged with eGFP (Figure 3-10B). In untreated control cells, 76.7% of Ndc80-GFP signals undergo a characteristic transition from a $\sim 1\mu\text{m}$ separation at metaphase to $> 3\mu\text{m}$ after anaphase onset (Figure 3-10B and C). latA treatment largely prevents anaphase separation and instead 87.1% of cells delay cell cycle progression with kinetochores separated by $\sim 1\mu\text{m}$. The average distance between separated kinetochores at anaphase onset in untreated cells ($1.32\mu\text{m} \pm 0.09$) is similar to the distance between separated kinetochores 150 minutes after G1 release in latA treated cells ($1.28\mu\text{m} \pm 0.08$) (Figure 3-10C). This data strongly suggests that Swe1 delays anaphase onset, not through spindle regulation per se, but by inhibiting the signal that triggers anaphase onset.

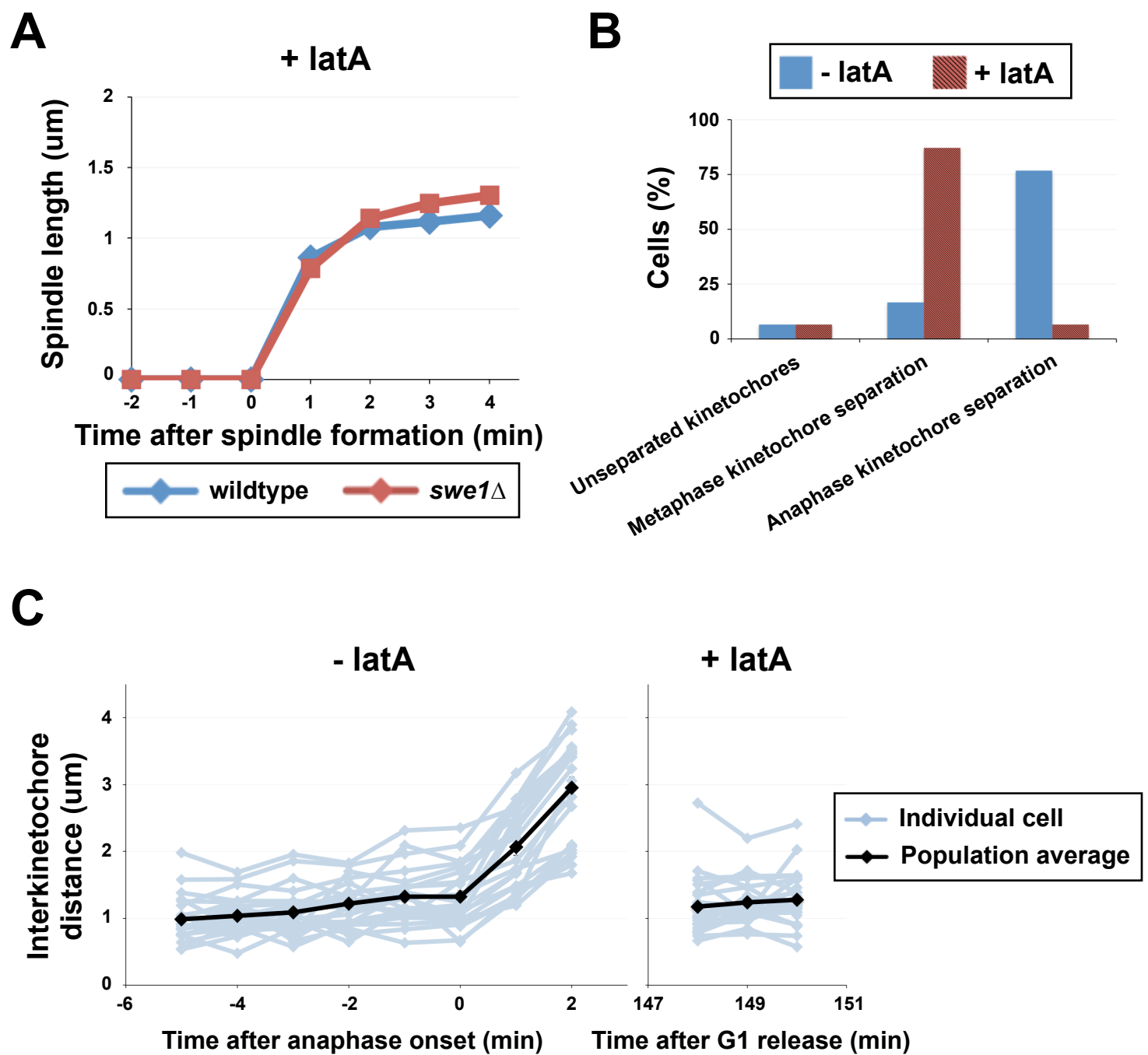


Figure 3-10

Figure 3-10. The morphogenesis checkpoint delays cell cycle progression after bipolar spindle formation.

(A) Morphogenesis checkpoint activation does not perturb spindle pole body separation. The average lengths of spindles in the timepoints immediately before and after spindle formation were determined in cells from Figure 3-8D and E. Displayed values are (mean $\pm$ SEM).

(B-C) *NDC80-eGFP* (ADR5026) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$). At $t = 25$ minutes cells were plated onto minimal media live microscopy pads $\pm$ latA and imaged. The *NDC80-eGFP* strain was imaged two times in each condition and the data combined ($-$ latA ($n = 30$), $+$ latA ($n = 31$)).

(B) The morphogenesis checkpoint arrests cells with separated kinetochores. Cells were characterized as either *unseparated kinetochores* (> 150 minutes from G1 release to kinetochore separation), metaphase kinetochore separation (< 150 minutes from G1 release to kinetochore separation and > 150 minutes from G1 release to anaphase kinetochore separation) or *anaphase kinetochore separation* (< 150 minutes from G1 release to anaphase kinetochore separation).

(C) latA treatment does not prevent metaphase kinetochore separation. The timepoint before anaphase kinetochore separation was defined as $x = 0$ for untreated cells. Average kinetochore separation in the timepoints before and after kinetochore separation were calculated (left). Kinetochore separation in latA treated cells was determined in the timepoints 148, 149 and 150 minutes after G1 release (right). Average kinetochore separation was calculated from these values.

Swe1 inhibition of cell cycle progression is thought to be due to Cdk1 phosphorylation (97). However, it has been suggested that Swe1 also has a tyrosine 19-independent role in regulating cell cycle progression (183). To determine if the anaphase delay we observed upon latA treatment is caused solely by Cdk1 phosphorylation, we tested whether preventing Cdk1 inhibitory phosphorylation using the *cdk1-Y19F* allele would prevent latA-dependent anaphase inhibition (Figure 3-11). 86.0% of *cdk1-19F* cells treated with latA undergo timely anaphase onset (Figure 3-11A and B) as compared to 95.3% in untreated controls, suggesting that Cdk1 inhibitory phosphorylation is the primary mechanism through which Swe1 inhibits anaphase onset.

Anaphase onset is signaled by the APC. Cdk1 has been shown to promote anaphase onset by phosphorylating the APC subunits Cdc16, Cdc27, and Cdc23 (236). This suggests that Cdk1 tyrosine 19 phosphorylation could delay onset by preventing Cdk1 activation of the APC. If this were the case, we would expect that APC substrates would be stabilized during latA treatment. To test this, we looked at the stability of the APC substrates Pds1, Clb5 and Sgo1 by immunoblot during latA treatment (Figure 3-12A). latA treatment stabilizes all three of these APC substrates. latA-dependent stabilization of APC substrates depends on *SWE1*, as latA-treated *swe1Δ* cells degrade Pds1, Clb2 and Clb3, similar to untreated controls (Figure 3-12B). To ensure that APC substrate stabilization upon latA treatment is due to Swe1 phosphorylation of Cdk1, we tested whether preventing Cdk1 phosphorylation in latA-treated cells could prevent APC substrate stabilization. Similar to *swe1Δ* cells, *cdk1-Y19F* cells do not stabilize APC substrates in response to latA treatment (Figure 3-12C).

If the morphogenesis checkpoint prevents anaphase onset by inhibiting the APC, we would expect that *swe1Δ* would not be able to bypass morphogenesis checkpoint activation

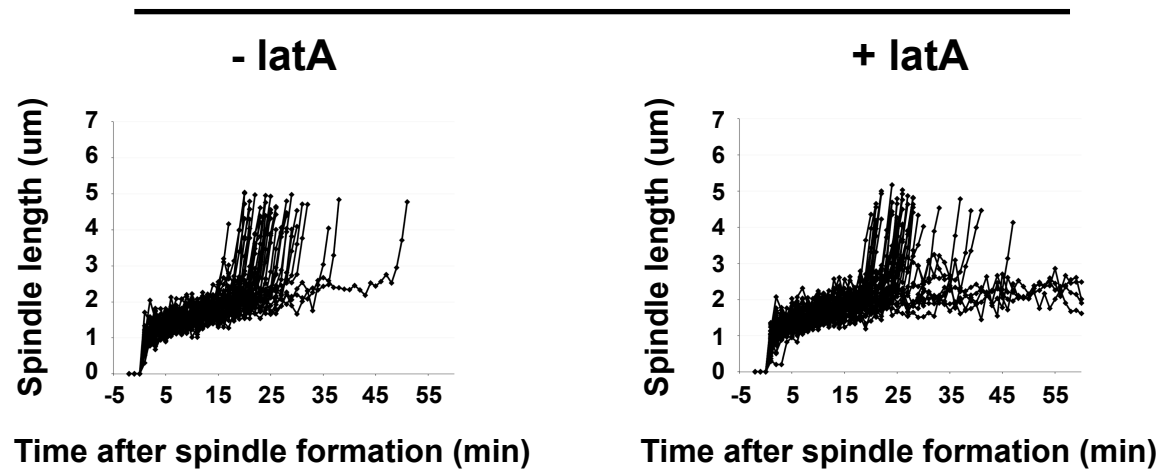
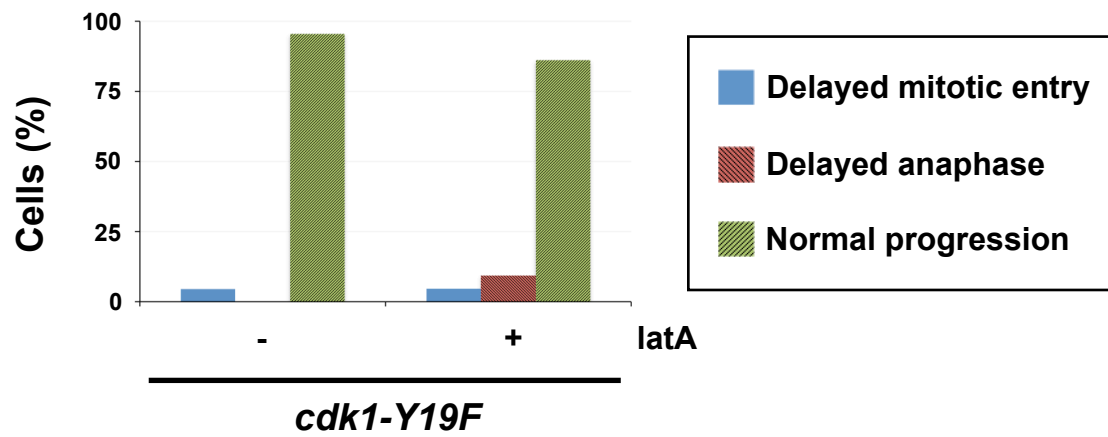
A***cdk1-Y19F*****B****Figure 3-11**

Figure 3-11. The morphogenesis checkpoint delays anaphase onset through Cdk1 phosphorylation.

(A-B) *cdk1-Y19F SPC42-eGFP* (ADR4313) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$). At $t = 25$ minutes cells were plated onto minimal media live microscopy pads +/- latA and imaged. Each condition was imaged two times and the data combined (- latA ($n = 41$), + latA ($n = 41$)).

(A) The morphogenesis checkpoint delays anaphase onset through Cdk1 phosphorylation. Traces of spindle length during mitotic progression for all imaged *cdk1-Y19F* cells. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μm .

(B) *cdk1-Y19F* fully bypasses the latA-dependent mitotic delay. Cells were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

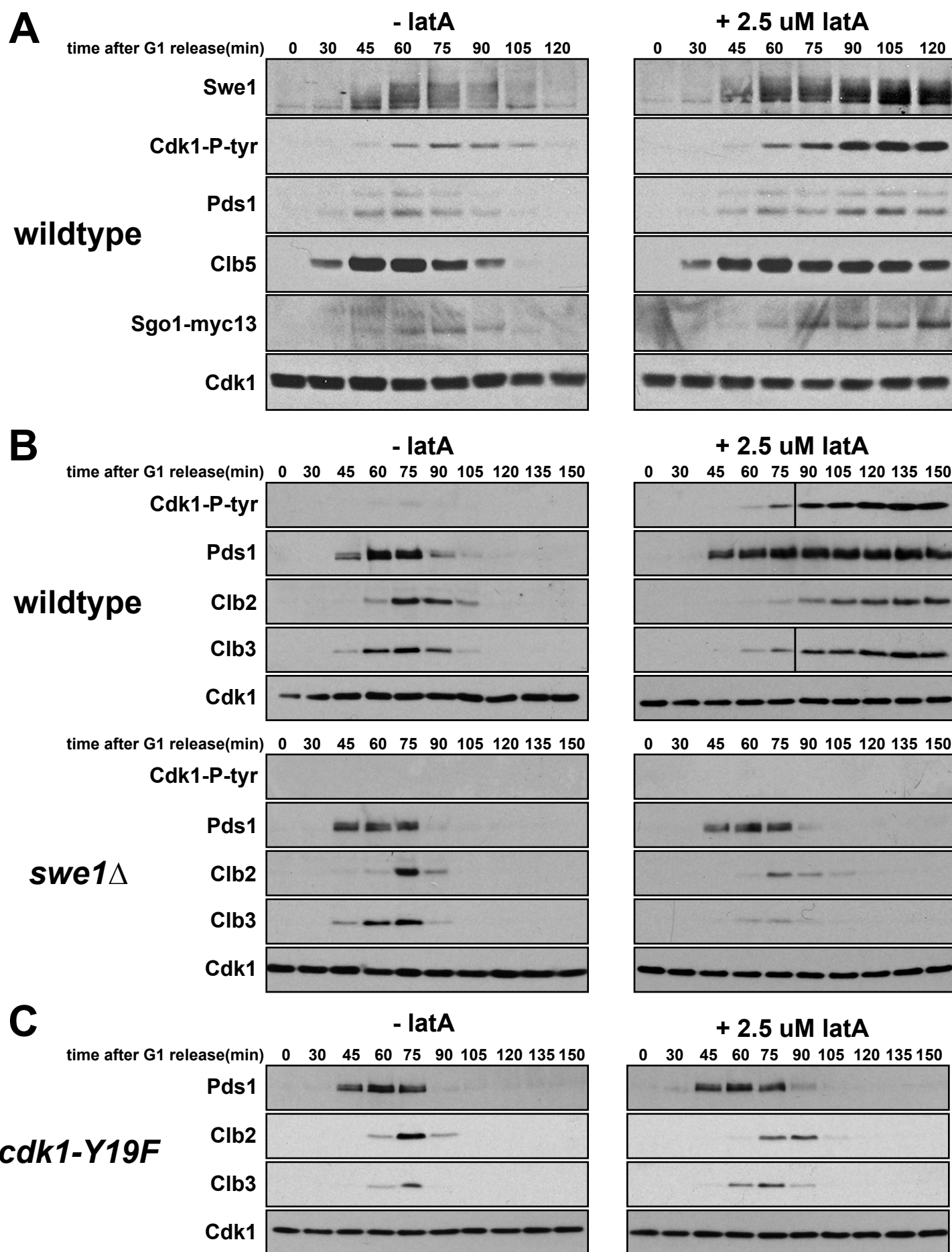


Figure 3-12

Figure 3-12. The morphogenesis checkpoint delays APC substrate degradation.

(A) The morphogenesis checkpoint delays APC substrate degradation. *SGO1-myc13* (ADR4517) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

(B) APC substrates are degraded in *swe1 Δ* cells treated with latA. Wildtype (ADR4006) and *swe1 Δ* (ADR4100) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

(C) APC substrates are degraded in *cdk1-Y19F* cells treated with latA. *cdk1-Y19F* (ADR4313) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

in cells with an inactive APC. To test this, we inactivated the APC using the *cdc16-1* allele and monitored mitotic progression in fixed cells (Figure 3-13). latA treatment delays bud formation in wildtype, *swe1Δ*, *cdc16-1* and *swe1Δ cdc16-1* cells (Figure 3-13A to D). In wildtype cells, latA causes a delay in spindle formation and short spindles persist (Figure 3-13A). *SWE1* deletion abrogates the delay in spindle formation and the persistence of short spindles (Figure 3-13B). Inactivation of the APC prevents anaphase onset and thus cells arrest with short spindles (Figure 3-13C) and *SWE1* deletion does not prevent the persistence of short spindles in *cdc16-1* cells (Figure 3-13D). This data shows that active APC is required for morphogenesis checkpoint bypass. To corroborate this data, we did a similar experiment using the *cdc23-1* allele to inactivate the APC (Figure 3-14). In this experiment, latA treatment delayed spindle formation and caused the persistence of short spindles in wildtype cells (Figure 3-14A). Deletion of *SWE1* prevents the persistence of short spindles (Figure 3-14B). APC inactivation using *cdc23-1* prevents anaphase onset, and thus cells arrest with short spindles (Figure 3-14C) and deletion of *SWE1* cannot prevent the persistence of short spindles in *cdc23-1* cells (Figure 3-14D).

While this work was being completed, it was shown that rapid Cdk1 inhibition using the *cdk1-as1* allele can lead to the destabilization of the APC regulatory subunit Cdc20 (434). This could provide a simple mechanism for how latA treatment prevents APC substrate degradation and anaphase onset. To test this, we looked at Cdc20 levels directly by immunoblot during latA treatment (Figure 3-15). Contrary to the published report, we observed Cdc20 stabilization upon Cdk1 inhibition. This led us to conclude that Cdc20 destabilization could not account for APC substrate stability upon latA treatment.

Taken together, this data shows that Swe1 delays anaphase onset by preventing activation of the APC without destabilizing Cdc20. Work from our lab has shown that

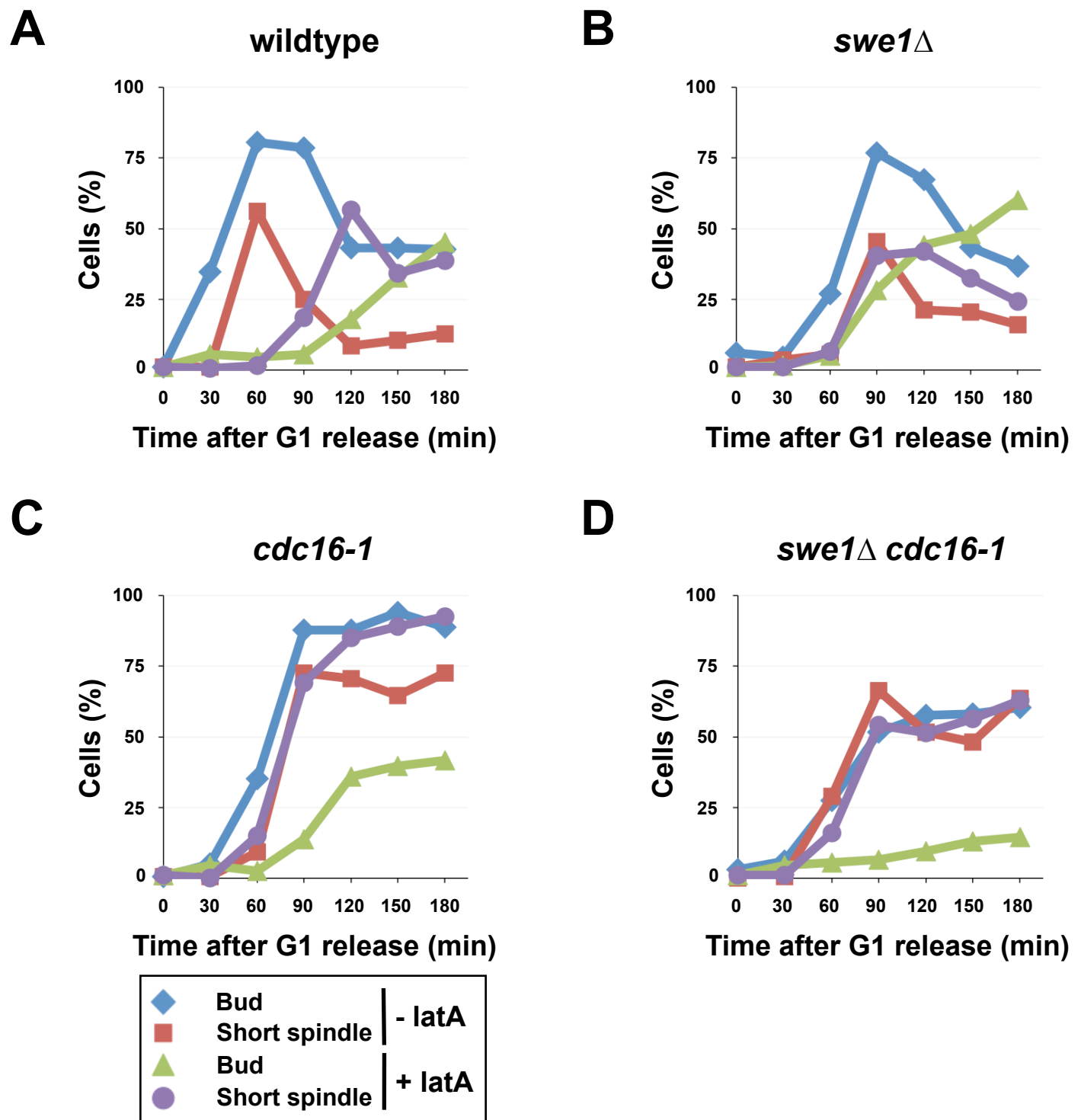


Figure 3-13

Figure 3-13. ***swe1Δ* does not bypass APC inactivation using the *cdc16-1* allele.**

(A-D) *SPC42-eGFP* (ADR4009), *swe1Δ SPC42-eGFP* (ADR4015), *cdc16-1 SPC42-eGFP* (ADR4979) and *swe1Δ cdc16-1 SPC42-eGFP* (ADR4984) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release cells were shifted to 37°C. Cells were released from G1 arrest and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

- (A) latA treatment delays budding and mitotic progression.
- (B) latA delays budding but not mitotic progression in *swe1Δ* cells.
- (C) Inactivating the APC prevents spindle elongation independently of latA treatment.
- (D) *swe1Δ* cannot bypass mitotic arrest due to APC inactivation.

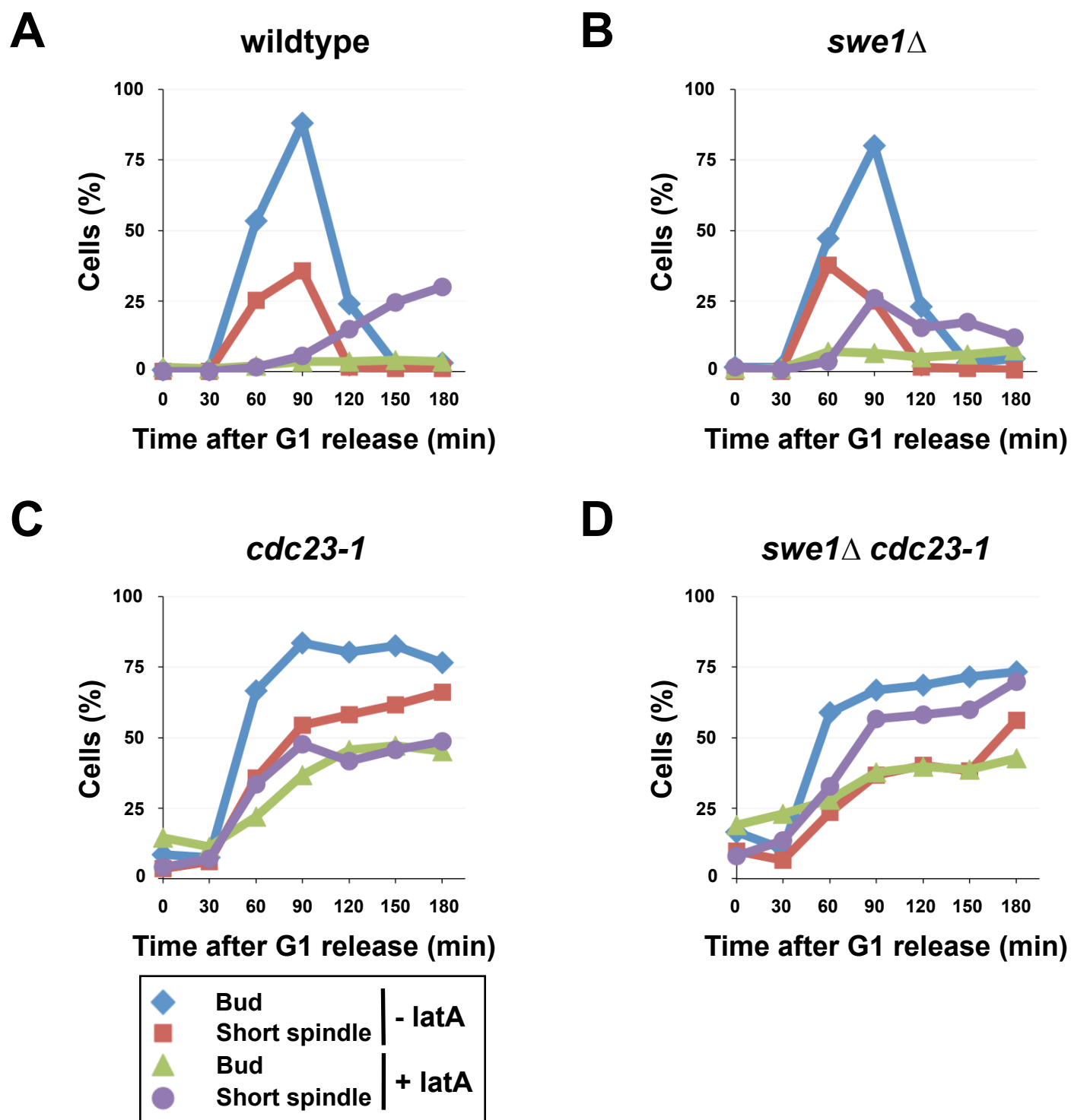


Figure 3-14

Figure 3-14. ***swe1*Δ does not bypass APC inactivation using the *cdc23-1* allele.**

(A-D) *SPC42-eGFP* (ADR4009), *swe1*Δ *SPC42-eGFP* (ADR4015), *cdc23-1 SPC42-eGFP* (ADR5599) and *swe1*Δ *cdc23-1 SPC42-eGFP* (ADR5604) cells were grown at 25°C to log phase and arrested in G1 with α-factor. 30 minutes before α-factor release cells were shifted to 37°C. Cells were released into the cell cycle (t = 0) at 37°C. Cells were treated with +/- latA at t = 25 minutes and α-factor was re-added at t = 55 minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

(A) latA treatment delays budding and mitotic progression.

(B) latA delays budding but not mitotic progression in *swe1*Δ cells.

(C) Inactivating the APC prevents spindle elongation independently of latA treatment.

(D) *swe1*Δ cannot bypass mitotic arrest due to APC inactivation.

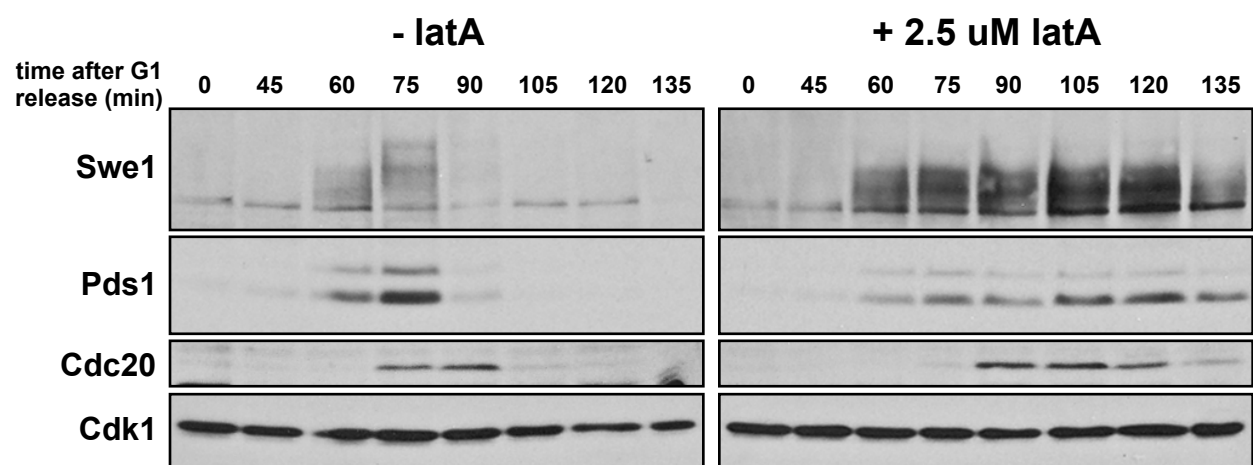


Figure 3-15

Figure 3-15. Morphogenesis checkpoint activation stabilizes Cdc20.

Wildtype (ADR4006) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

overexpression of Swe1 during mitosis reduces APC phosphorylation in vivo and reduces APC^{Cdc20} activity in vitro (425). Further, APC purified from cells overexpressing Swe1 can be reactivated by phosphorylating it with purified Cdk1^{Clb2}. Preventing APC phosphorylation using the *cdc16-6A* and *cdc27-5A* alleles reduces APC reactivation by purified Cdk1^{Clb2}. This data demonstrates that Swe1 delays anaphase onset by preventing Cdk1 phosphorylation of the APC.

3.2.4 The morphogenesis checkpoint is not bypassed by PDS1, CLB5 or SGO1 deletion

The APC signals anaphase onset by targeting the anaphase inhibitor Pds1 for degradation, thereby liberating the protease Esp1 and allowing it to cleave the Scc1 subunit of cohesin protein complexes which hold metaphase sister chromatids together. If APC inhibition were preventing anaphase onset in latA treated cells, *PDS1* deletion should bypass the morphogenesis checkpoint. Therefore, we imaged live *pds1Δ* cells undergoing mitosis in the presence of latA (Figure 3-16). Untreated *pds1Δ* cells undergo relatively normal, though slightly delayed, anaphase onset (Figure 3-16A). Treatment of *pds1Δ* cells with latA completely prevents anaphase onset (Figure 3-16B and D). This data is consistent with experiments done in fixed cells (429). Under similar experimental conditions, 78.5% of *swe1Δ* cells are able to successfully initiate anaphase onset (Figure 3-16C and D). latA treatment of *pds1Δ* cells does not perturb spindle pole body separation (Figure 3-16E). In addition to its role as an Esp1 inhibitor, Pds1 has been shown to be required for full Esp1 activity (257-259). We therefore wondered whether *pds1Δ* cells were unable to bypass the morphogenesis checkpoint due to reduced Esp1 activity. As a first step in determining this we have shown that Esp1 protein levels are not reduced in *pds1Δ* cells during unperturbed

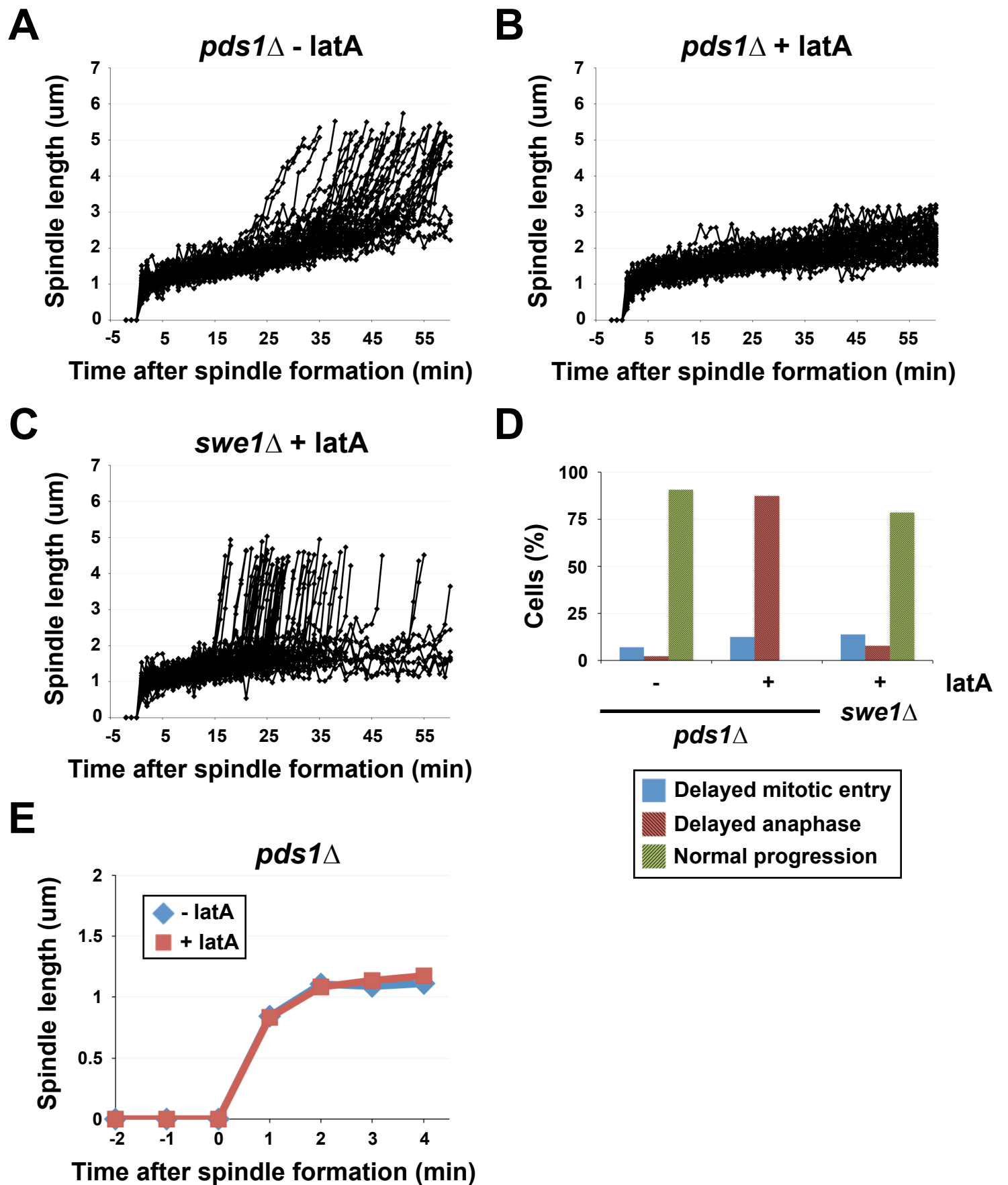


Figure 3-16

Figure 3-16. Deleting *PDS1* does not bypass the morphogenesis checkpoint.

(A-E) *pds1Δ SPC42-eGFP* (ADR4491) and *swe1Δ SPC42-eGFP* (ADR4015) cells were grown at 25°C to log phase, arrested in G1 with α -factor, and released into minimal media (t = 0). At t = 25 minutes *swe1Δ SPC42-eGFP* cells were plated onto minimal media live microscopy pads + latA and *pds1Δ SPC42-eGFP* cells were plated onto minimal media live microscopy pads +/- latA and imaged. Each condition was imaged two times and the data combined (*pds1Δ* - latA (n = 40), *pds1Δ* + latA (n = 42), *swe1Δ* + latA (n = 56)).

(A) Anaphase onset is delayed in *pds1Δ* cells. Traces of spindle length during mitotic progression for all untreated *pds1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 5 μ m.

(B) The morphogenesis checkpoint delays anaphase onset in *pds1Δ* cells. Traces of spindle length during mitotic progression for all latA treated *pds1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μ m.

(C) *swe1Δ* bypasses the morphogenesis checkpoint at 25°C. Traces of spindle length during mitotic progression for all latA treated *swe1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μ m.

(D) *pds1Δ* cells treated with latA delay anaphase onset. Cells were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

(E) latA does not perturb spindle pole body separation in *pds1Δ* cells. The average lengths of spindles in the timepoints before and after spindle formation were determined for untreated and latA treated *pds1Δ* cells. Displayed values are (mean +/- SEM).

cell cycles or during morphogenesis checkpoint activation (Figure 3-17). It is possible that in the absence of Pds1, Esp1 is stable but restricted from the nucleus. This does not explain, however, how latA treatment prevents activation of the population of Esp1 that is able to signal anaphase onset in *pds1Δ* cells. This suggests that either 1) stabilization of a different APC substrate must prevent anaphase onset in *pds1Δ* cells, or 2) Cdk1 plays an additional role downstream of the APC in regulating anaphase onset.

Clb5 is an APC substrate that has been shown to prevent sister chromatid separation during multiple mitotic checkpoints (435). Cells lacking *CLB5* are able to successfully undergo anaphase onset, though it is delayed in comparison to wildtype cells (Figure 3-18A). Treatment of *clb5Δ* cells with latA resulted in an anaphase delay, demonstrating that Clb5 is not responsible for delaying anaphase onset in latA-treated cells (Figure 3-18B).

Interpretation of this result is complicated by the fact that, in the absence of Clb5, Cdk1^{Clb6} complexes may phosphorylate Cdk1^{Clb5} targets. Therefore we determined if deletion of both *CLB5* and *CLB6* bypassed the morphogenesis checkpoint (Figure 3-18C, D, E and F).

Interestingly, *clb5Δ clb6Δ* cells undergo rapid spindle elongation immediately upon spindle formation (Figure 3-18C and D). The elongated spindles in *clb5Δ clb6Δ* cells shrink to a more characteristic metaphase length before eventually elongating during what appears to be a stereotypical anaphase. Treatment of *clb5Δ clb6Δ* cells with latA does not prevent early spindle elongation (Figure 3-18E and F) but it does largely prevent anaphase onset (Figure 3-18E and G). This demonstrates that, though Clb5 and Clb6 are clearly involved in the regulation of spindle morphology through an as yet unidentified mechanism, Clb5 and Clb6 are not specifically involved in delaying anaphase in response to latA treatment.

To see if Clb5 and Pds1 act redundantly to prevent anaphase onset in response to latA treatment, we looked at anaphase onset in live *clb5Δ pds1Δ* cells (Figure 3-19). Anaphase

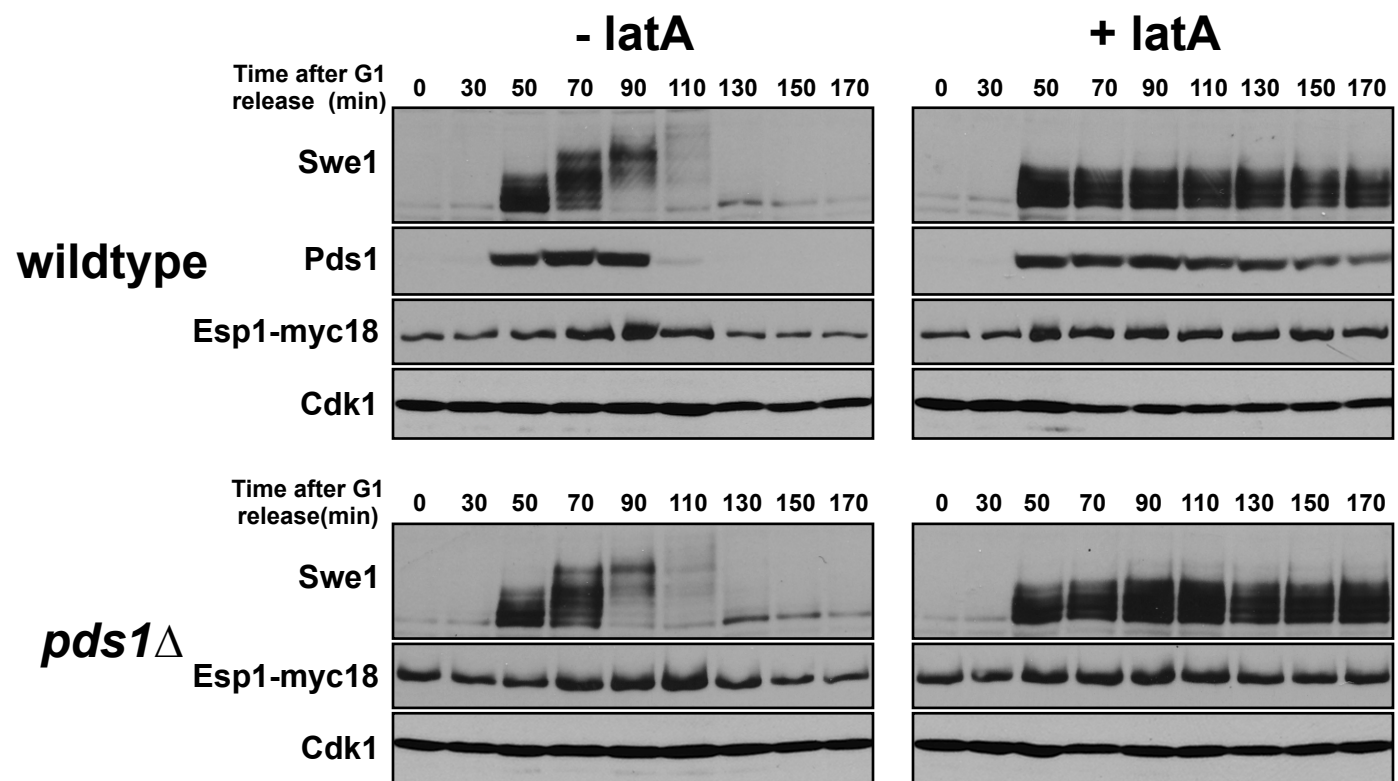


Figure 3-17

Figure 3-17. Deleting *PDS1* does not impact Esp1 stability.

ESP1-myc18 (ADR5274) and *pds1Δ ESP1-myc18* (ADR5300) cells were grown at 25°C to log phase, arrested in G1 with 10µg/mL α -factor, and released into the cell cycle (t = 0). Cells were treated with +/- latA at t = 25 minutes and 100µg/mL α -factor was re-added at t = 55 minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

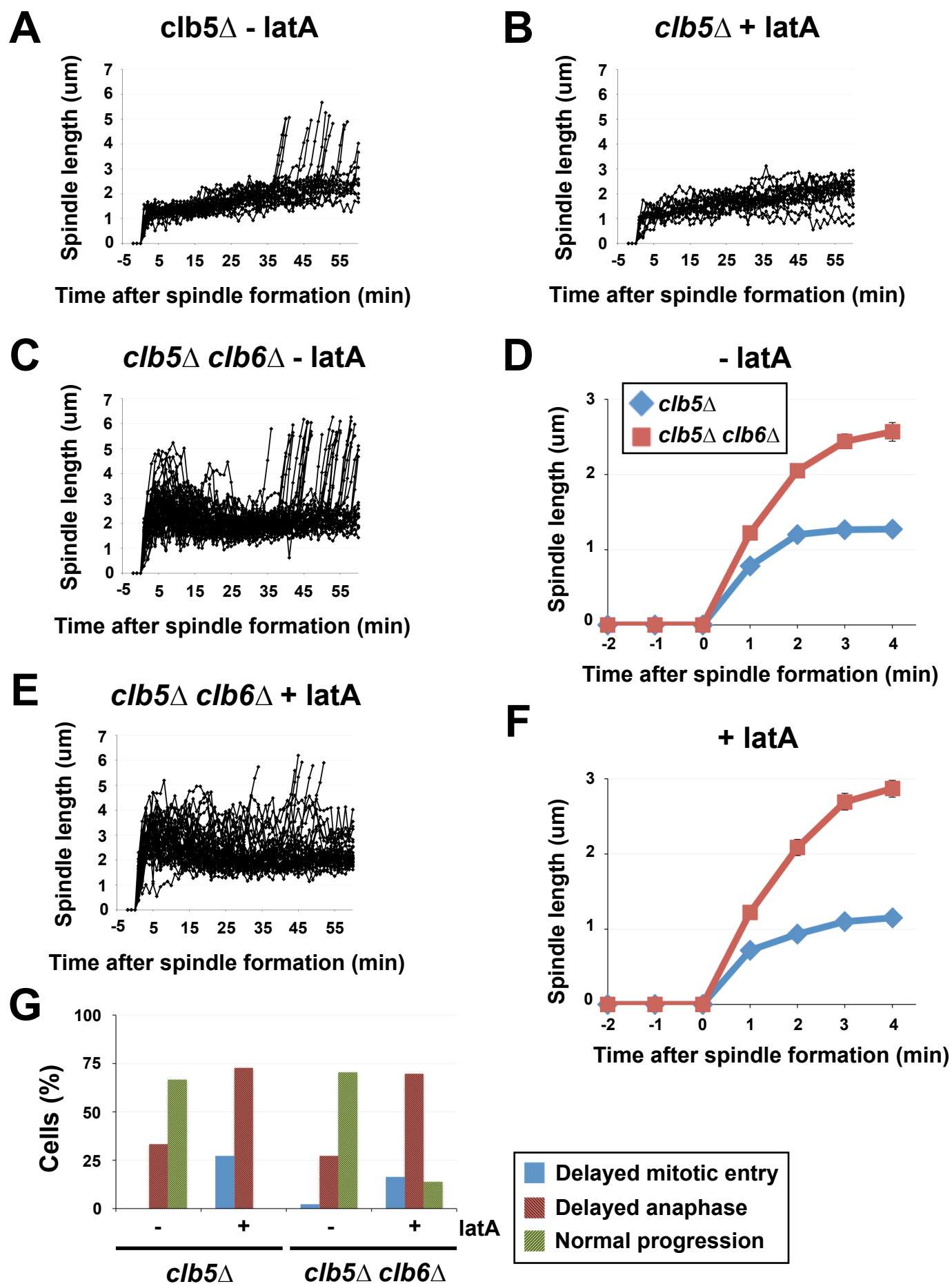


Figure 3-18

Figure 3-18. Deleting *CLB5* and *CLB6* does not bypass the morphogenesis checkpoint.

(A-G) *clb5Δ SPC42-eGFP* (ADR4381) and *clb5Δ clb6Δ SPC42-eGFP* (ADR4403) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media (t = 0). At t = 25 minutes cells were plated onto minimal media live microscopy pads +/- latA and imaged. The *clb5Δ SPC42-eGFP* strain was imaged once in each condition. The *clb5Δ clb6Δ SPC42-eGFP* strain was imaged twice in each condition (*clb5Δ* - latA (n = 21), *clb5Δ* + latA (n = 16), *clb5Δ clb6Δ* - latA (n = 43), *clb5Δ clb6Δ* + latA (n = 36)).

(A) Anaphase onset is delayed in *clb5Δ* cells. Traces of spindle length during mitotic progression for all untreated *clb5Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of ~5 μ m.

(B) The morphogenesis checkpoint delays anaphase in *clb5Δ* cells. Traces of spindle length during mitotic progression for all latA treated *clb5Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μ m.

(C) *clb5Δ clb6Δ* cells undergo abnormal spindle elongation upon spindle pole body separation. Traces of spindle length during mitotic progression for all untreated *clb5Δ clb6Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Anaphase spindle elongation is not depicted after spindles reach a length of ~5 μ m.

(D) Spindle pole body separation is perturbed in *clb5Δ clb6Δ* cells. The average lengths of spindles in the timepoints before and after spindle formation were determined for untreated *clb5Δ* and *clb5Δ clb6Δ* cells. Displayed values are (mean +/- SEM).

(E) The morphogenesis checkpoint delays anaphase in *clb5Δ clb6Δ* cells. Traces of spindle length during mitotic progression for all latA treated *clb5Δ clb6Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Anaphase spindle elongation is not depicted after spindles reach a length of ~5 μ m.

(F) Spindle pole body separation is perturbed in *clb5Δ clb6Δ* cells treated with latA. The average lengths of spindles in the timepoints before and after spindle formation were determined for latA treated *clb5Δ* and *clb5Δ clb6Δ* cells. Displayed values are (mean +/- SEM).

(G) *clb5Δ* and *clb5Δ clb6Δ* cells treated with latA delay anaphase onset. Cells were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

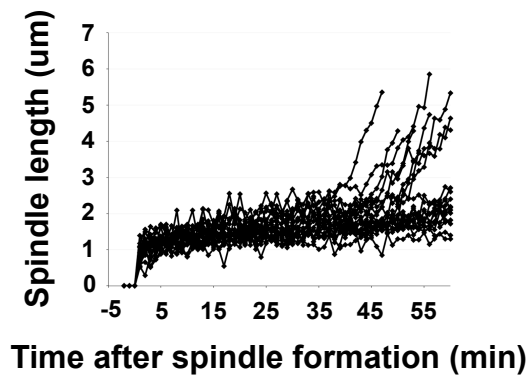
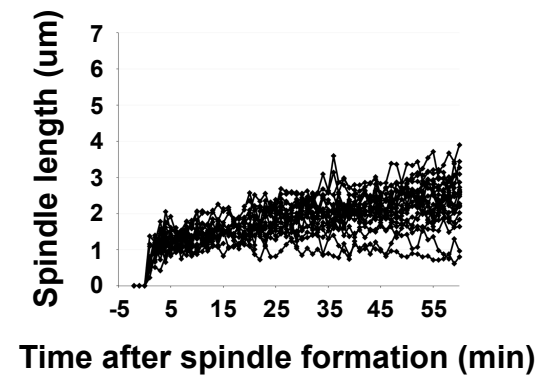
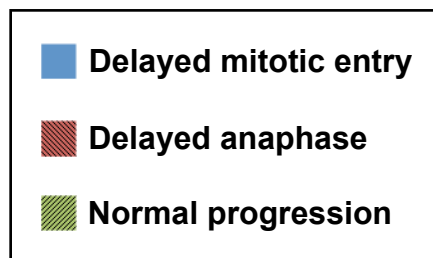
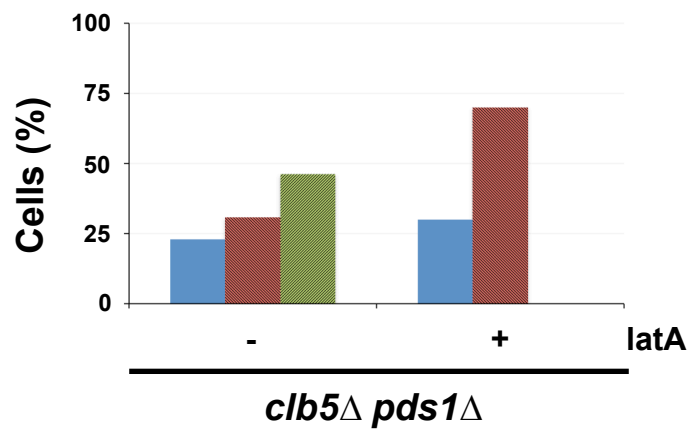
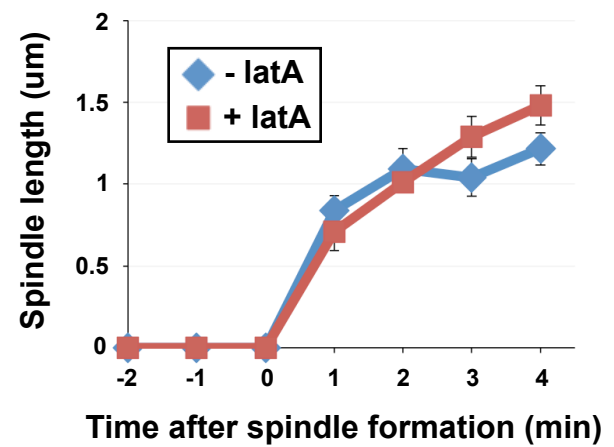
A*clb5* Δ *pds1* Δ - latA**B***clb5* Δ *pds1* Δ + latA**C****D***clb5* Δ *pds1* Δ **Figure 3-19**

Figure 3-19. Deleting *CLB5* and *PDS1* does not bypass the morphogenesis checkpoint.

(A-D) *clb5Δ pds1Δ SPC42-eGFP* (ADR4486) cells were grown at 25°C to log phase, arrested in G1 with α -factor, and released into minimal media ($t = 0$). At $t = 25$ minutes cells were plated onto minimal media live microscopy pads +/- latA and imaged. The *clb5Δ pds1Δ SPC42-eGFP* strain was imaged once in each condition (- latA ($n = 9$), + latA ($n = 8$)).

(A) Anaphase onset is delayed in *clb5Δ pds1Δ* cells. Traces of spindle length during mitotic progression for all untreated *clb5Δ pds1Δ* cells. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $\sim 5 \mu\text{m}$.

(B) The morphogenesis checkpoint delays anaphase onset in *clb5Δ pds1Δ* cells. Traces of spindle length during mitotic progression for all latA treated *clb5Δ pds1Δ* cells. The timepoint before spindle formation was defined as $x = 0$ for each cell. Spindle elongation is not depicted after spindles reach a length of $4 \mu\text{m}$.

(C) latA treatment delays anaphase onset in *clb5Δ pds1Δ* cells. Cells were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

(D) Spindle pole body separation is not perturbed in *clb5Δ pds1Δ* cells treated with latA. The average lengths of spindles in the timepoints before and after spindle formation were determined for untreated and latA treated *clb5Δ pds1Δ* cells. Displayed values are (mean +/- SEM).

onset is significantly delayed in untreated *clb5Δ pds1Δ* cells, though 46.2% of cells still undergo timely anaphase onset (Figure 3-19A and C). Treatment of these cells with latA completely prevents anaphase onset (Figure 3-19B and C). Spindles in either latA-treated or untreated *clb5Δ pds1Δ* cells elongate abnormally immediately upon formation, though not to the same extent as in *clb5Δ clb6Δ* cells (Figure 3-19D).

The shugoshin (Sgo1) protein has been shown to be important for protecting sister chromatid cohesion in the absence of securin (436). Human shugoshin has been shown to be an APC substrate (437), and we have shown that Sgo1 degradation depends on the APC (425). We therefore looked at whether Sgo1 was responsible for maintaining sister chromatid cohesion during morphogenesis checkpoint activation. To do this, we tested whether *SGO1* deletion allowed anaphase onset during latA treatment (Figure 3-20). *SGO1* deletion causes a delay in anaphase onset (Figure 3-20A). Deletion of *SGO1*, however, does not allow anaphase onset upon latA treatment (Figure 3-20B and E). Sgo1 and Pds1 do not act redundantly to maintain sister chromatid cohesion during latA treatment, as *pds1Δ sgo1Δ* cells maintain a mitotic arrest during latA treatment (Figure 3-20C, D and E). Deletion of both *PDS1* and *SGO1* does cause spindle abnormalities, with many cells exhibiting an increased metaphase spindle length (Figure 3-20C and F). As a result, the cells enter anaphase with an increased spindle length (Figure 3-20G).

Cells lacking *PDS1*, *CLB5* and *CLB6* are inviable (438). To determine if depleting these three proteins would allow anaphase during latA treatment, we constructed a strain lacking *CLB5* and *CLB6* and expressing Pds1 from the *MET3* promoter. We grew these cells in media lacking methionine, added methionine to repress Pds1 expression, and then treated them with latA to activate the morphogenesis checkpoint (Figure 3-21). Cells lacking *CLB5* and *CLB6* form buds more readily in the presence of latA (Figure 3-21A). Therefore in this

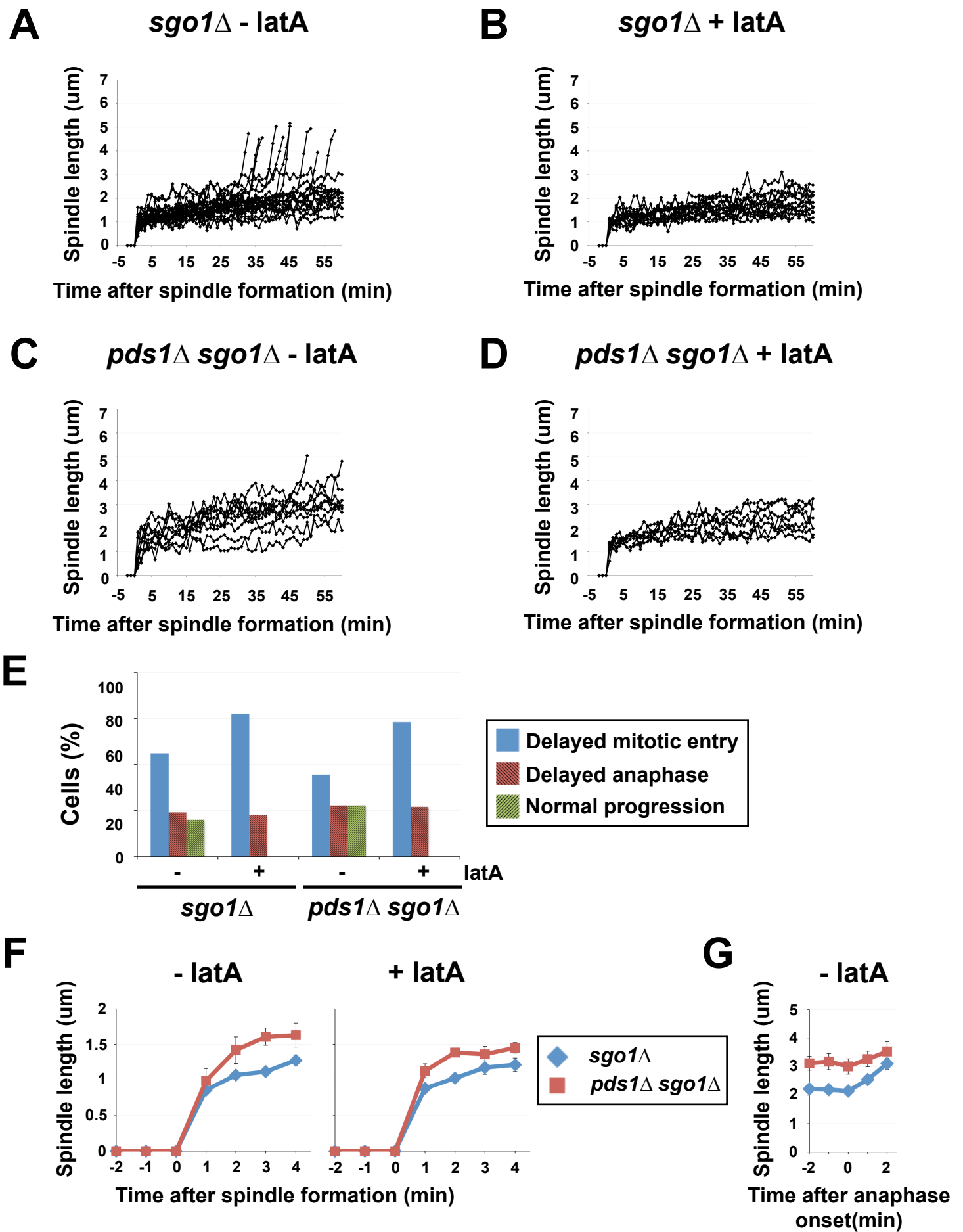


Figure 3-20

Figure 3-20. Deleting *PDS1* and *SGO1* does not bypass the morphogenesis checkpoint.

(A-G) *sgo1Δ SPC42-eGFP* (ADR4502) and *pds1Δ sgo1Δ SPC42-eGFP* (ADR4533) cells were grown to log phase, arrested in G1 with α -factor, and released into minimal media (t = 0). At t = 40 minutes cells were plated onto minimal media live microscopy pads +/- latA and imaged. The *sgo1Δ SPC42-eGFP* strain was imaged twice in each condition and the *pds1Δ sgo1Δ SPC42-eGFP* strain was imaged once in each condition (*sgo1Δ* - latA (n = 22), *sgo1Δ* + latA (n = 13), *pds1Δ sgo1Δ* - latA (n = 9), *pds1Δ sgo1Δ* + latA (n = 7)).

(A) Anaphase onset is delayed in *sgo1Δ* cells. Traces of spindle length during mitotic progression for all untreated *sgo1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of ~4 μ m.

(B) The morphogenesis checkpoint delays anaphase onset in *sgo1Δ* cells. Traces of spindle length during mitotic progression for all latA treated *sgo1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μ m.

(C) Anaphase onset is delayed in *pds1Δ sgo1Δ* cells. Traces of spindle length during mitotic progression for all untreated *pds1Δ sgo1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of ~4 μ m.

(D) The morphogenesis checkpoint delays anaphase onset in *pds1Δ sgo1Δ* cells. Traces of spindle length during mitotic progression for all latA treated *pds1Δ sgo1Δ* cells. The timepoint before spindle formation was defined as x = 0 for each cell. Spindle elongation is not depicted after spindles reach a length of 4 μ m.

(E) *sgo1Δ* and *pds1Δ sgo1Δ* cells treated with latA delay anaphase onset. Cells were each characterized as either *delayed mitotic entry* (> 90 minutes from G1 release to spindle formation), *delayed anaphase* (> 60 minutes from spindle formation to anaphase onset) or *normal progression* (< 90 minutes from G1 release to spindle formation and < 60 minutes from spindle formation to anaphase onset).

(F) Spindle pole body separation is perturbed in *pds1Δ sgo1Δ* cells. The average lengths of spindles in the timepoints before and after spindle formation were determined for untreated (left) and latA treated (right) *sgo1Δ* and *pds1Δ sgo1Δ* cells. Displayed values are (mean +/- SEM).

(G) Anaphase onset occurs at an increased spindle length in *pds1Δ sgo1Δ* cells. The timepoint before anaphase spindle elongation began was defined as x = 0 for each cell. Average spindle lengths in the timepoints before and after anaphase spindle elongation began were calculated. Displayed values are (mean +/- SEM).

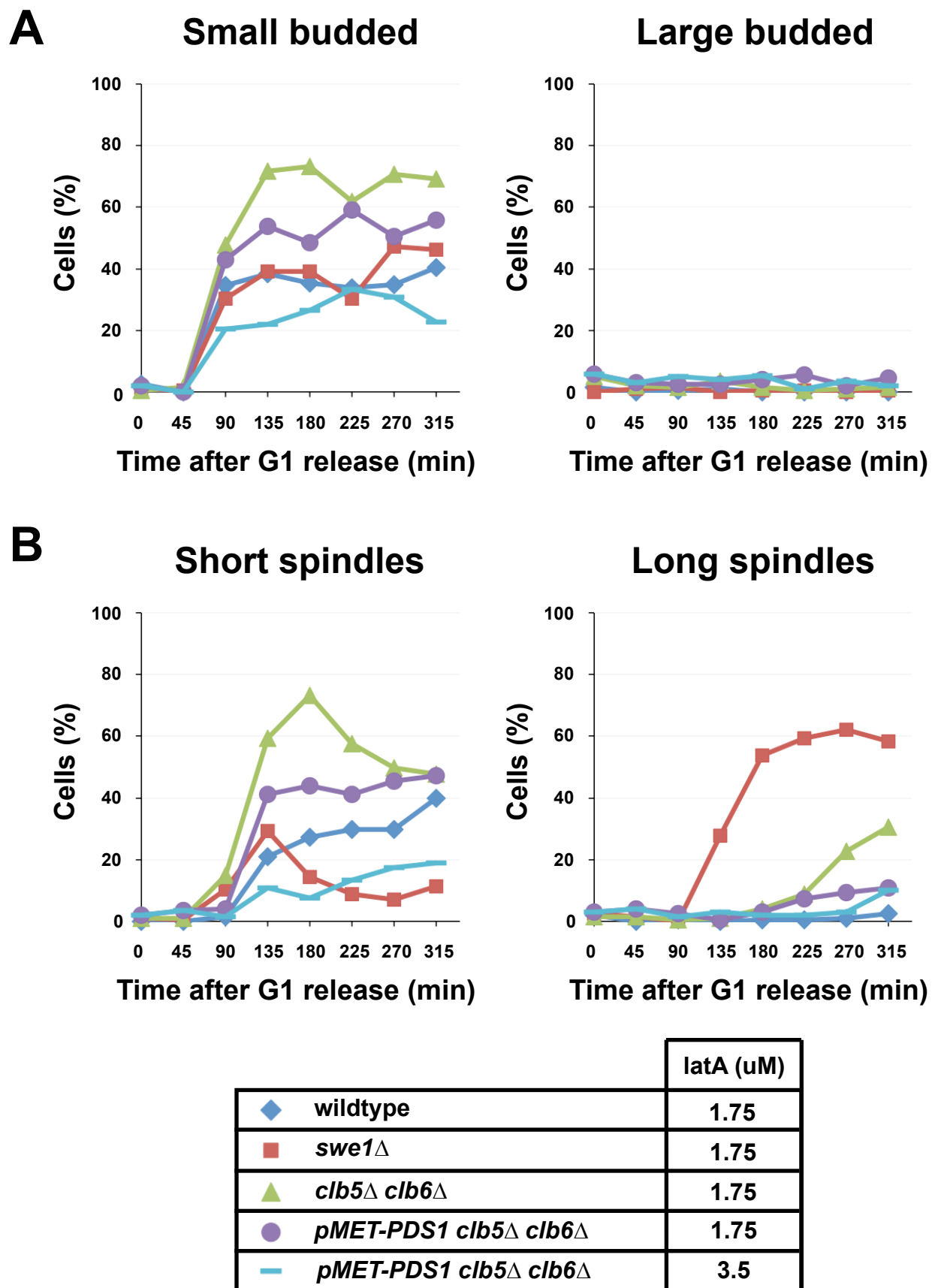


Figure 3-21

Figure 3-21. Depleting Pds1 in *clb5* Δ *clb6* Δ cells does not bypass the morphogenesis checkpoint.

(A-B) *SPC42-eGFP* (ADR4009), *swe1* Δ *SPC42-eGFP* (ADR4015), *clb5* Δ *clb6* Δ *SPC42-eGFP* (ADR5410) and *pMET-PDS1 clb5* Δ *clb6* Δ *SPC42-eGFP* (ADR5504) cells were grown at 25°C to log phase in minimal media lacking methionine and arrested in G1 with α -factor. 30 minutes before α -factor release L-methionine was added. Cells were released from G1 arrest at t = 0, cells were treated with latA at t = 25 minutes and α -factor was re-added at t = 55 minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

(A) *clb5* Δ *clb6* Δ cells are somewhat refractory to latA treatment.

(B) *clb5* Δ *clb6* Δ cells depleted of Pds1 do not bypass the morphogenesis checkpoint.

experiment latA was added to *pMET-PDS1 clb5Δ clb6Δ* cells at two different concentrations. Addition of twice the amount of latA resulted in similar bud growth inhibition in *pMET-PDS1 clb5Δ clb6Δ* cells as wildtype and *swe1Δ* controls (Figure 3-21A). Under these conditions the majority of *swe1Δ* cells successfully elongate their spindles (Figure 3-21B). Wildtype and *pMET-PDS1 clb5Δ clb6Δ* cells were completely unable to elongate their spindles, suggesting that they were unable to bypass the morphogenesis checkpoint.

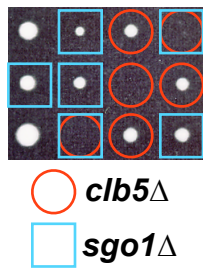
Cells lacking *CLB5* and *SGO1* are inviable (Figure 3-22A) (439). To determine if Clb5, Pds1 and Sgo1 all act redundantly to prevent anaphase onset in response to morphogenesis checkpoint activation, we constructed a strain lacking *PDS1* and *SGO1* that expressed Clb5 from the *MET3* promoter. We grew these cells in media lacking methionine, added methionine to repress Clb5 expression, and then treated them with latA to activate the morphogenesis checkpoint (Figure 3-22B and C). Methionine addition effectively depleted Clb5 in this experiment (Figure 3-22B). Cells lacking Clb5, Pds1 and Sgo1 arrested with short spindles upon latA treatment (Figure 3-22C). This tells us that Clb5, Pds1 and Sgo1 are not responsible for collectively preventing anaphase onset in response to latA treatment.

Though we were unable to identify any APC substrates that either singly or in combination bypassed the morphogenesis checkpoint, our data clearly suggests that Swe1 delays cell cycle progression in unperturbed and morphogenesis checkpoint-activated cells by preventing Cdk1 phosphorylation and activation of the APC^{Cdc20}.

3.2.5 Characterization of the *cdc55Δ* morphogenesis checkpoint bypass

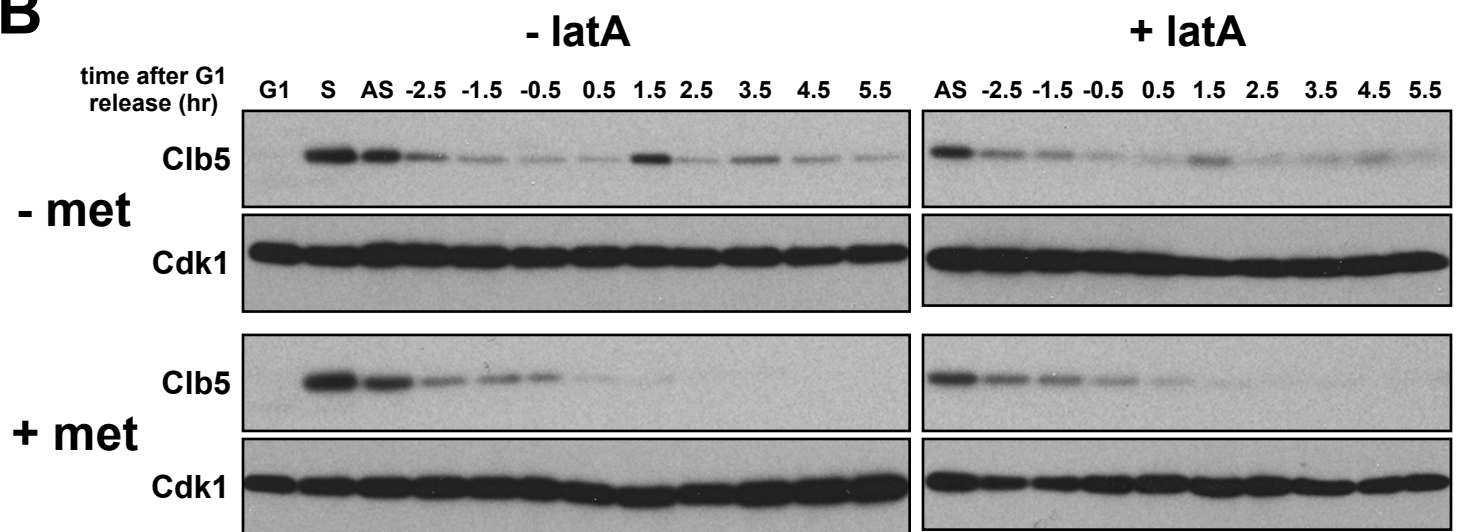
While we were characterizing how Swe1 activation by the morphogenesis checkpoint delayed anaphase onset, it was shown that deletion of *CDC55*, which codes for a regulatory subunit of PP2A, caused bypass of the morphogenesis checkpoint (429). In order to better

A



	Viability
wildtype	100% (17/17)
<i>clb5</i> Δ	67% (14/21)
<i>sgo1</i> Δ	67% (14/21)
<i>clb5</i> Δ <i>sgo1</i> Δ	0% (0/17)

B



C

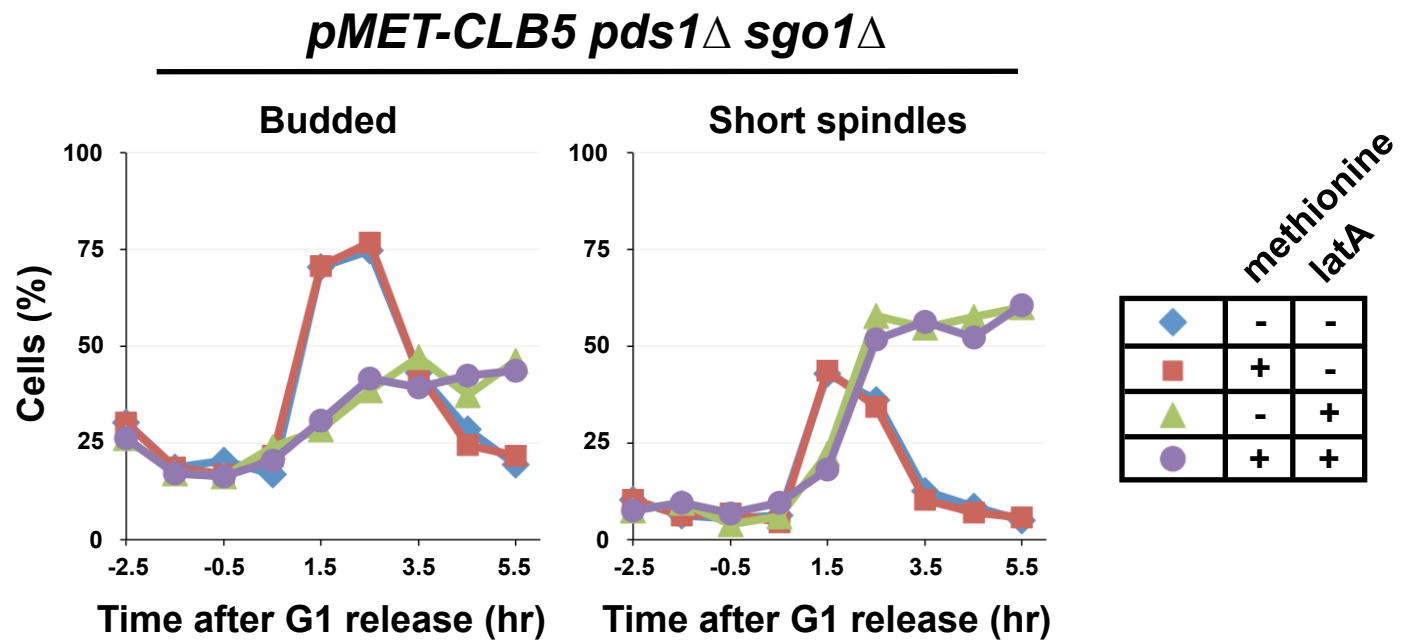


Figure 3-22

Figure 3-22. Depleting Clb5 in *pds1Δ sgo1Δ* cells does not bypass the morphogenesis checkpoint.

(A) *clb5Δ* and *sgo1Δ* are synthetically lethal. *MATα clb5Δ* (ADR1555) and *MATα sgo1Δ* (ADR4502) cells were mated, sporulated and the resultant tetrads dissected. A portion of the dissection is shown (left) with the genotype of each spore. Tetrads in which the genotype of each spore could be determined unequivocally were used to determine the viability of each genotype (right). A total of 76 spores were scored.

(B-C) Depleting Clb5 in *pds1Δ sgo1Δ* cells does not bypass the morphogenesis checkpoint. *pMET-CLB5 pds1Δ sgo1Δ SPC42-eGFP* (ADR4647) cells were grown at 25°C to log phase in minimal media lacking methionine and arrested in G1 with α -factor. 30 minutes before α -factor release +/- L-methionine was added. Cells were released from G1 arrest at t = 0, and treated with +/- latA at t = 25 minutes. Samples were taken for immunoblotting (B) and samples were fixed for microscopy (C) at the indicated timepoints.

(B) Methionine addition depletes Clb5 in *pMET-CLB5 pds1Δ sgo1Δ* cells.

(C) *pds1Δ sgo1Δ* cells depleted of Clb5 do not bypass the morphogenesis checkpoint.

understand morphogenesis checkpoint signaling, we decided to characterize this bypass. We first monitoring spindle morphology in a synchronous culture of latA-treated wildtype, *pds1Δ*, *cdc55Δ* and *swe1Δ* cells transiting mitosis (Figure 3-23). Cell cycle progression proceeds relatively similarly in unperturbed wildtype, *swe1Δ*, *cdc55Δ* and *pds1Δ* cells, and within 210 minutes of G1 release, the majority of cells have completed one complete cell cycle (Figure 3-23A). Treatment with latA inhibits bud growth in these cells. In wildtype and *pds1Δ*, as we had seen in live cells, latA treatment delayed mitotic entry and anaphase onset (Figure 3-23B and C). In *swe1Δ*, similar to what we had observed in live cells, mitotic entry and anaphase onset were not delayed in response to latA treatment (Figure 3-23D). *cdc55Δ* cells exhibited slight delays in mitotic entry and anaphase onset as compared to untreated controls (Figure 3-23E).

In order to determine how this bypass occurred, we examined APC substrate stability in wildtype, *swe1Δ* and *cdc55Δ* cells treated with latA (Figure 3-24). As we had previously seen in wildtype cells, morphogenesis checkpoint activation causes the stabilization of the APC substrates Pds1, Clb2, Clb3, Clb5 and Sgo1. Deletion of *SWE1* allows the degradation of APC substrates with timing similar to untreated controls. Deletion of *CDC55*, in the absence of latA, causes an increase in Swe1 phosphorylation, and a concomitant increase in Cdk1 tyrosine 19 phosphorylation. This increase in Y19 phosphorylation is because *CDC55* deletion on its own has been shown to activate Swe1 (103, 422, 440). Upon latA treatment, *cdc55Δ* cells exhibit partial activation of the APC as compared to wildtype and *swe1Δ* cells. APC substrates are stabilized compared to *swe1Δ* but degrade more quickly than in wildtype cells. The observations that *cdc55Δ* cells have increased levels of Cdk1 tyrosine 19 phosphorylation and partially stabilized APC substrates during morphogenesis checkpoint

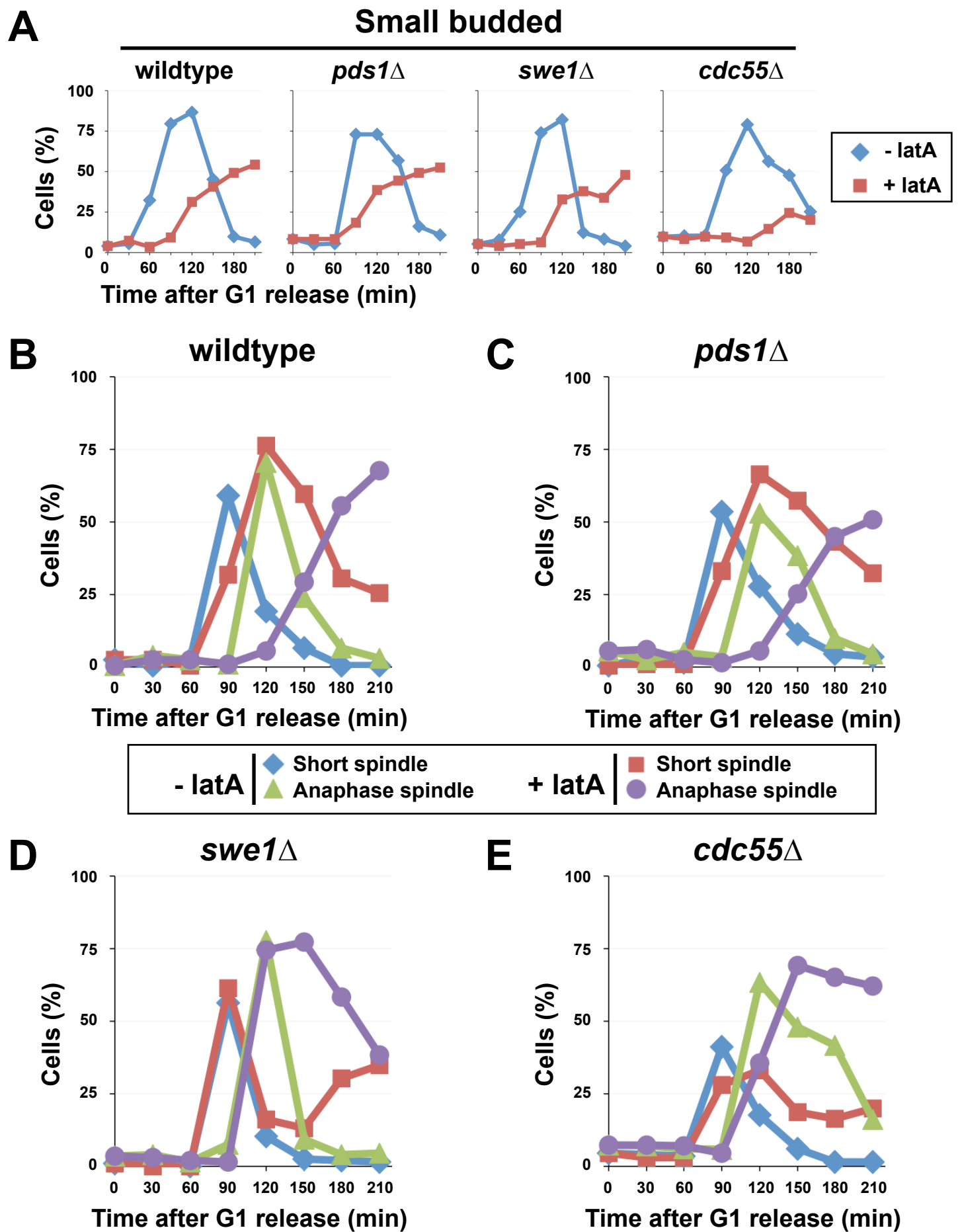


Figure 3-23

Figure 3-23. Deleting *CDC55* bypasses the morphogenesis checkpoint.

(A-E) Wildtype (ADR4009), *pds1* Δ (ADR4491), *swe1* Δ (ADR4015) and *cdc55* Δ (ADR4738) cells were grown at 25°C to log phase and arrested in G1 with α -factor. Cells were released from G1 arrest at t = 0, treated with +/- latA at t = 25 minutes and α -factor was re-added at t = 55 minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

- (A) latA inhibits bud growth in *cdc55* Δ cells.
- (B) latA delays mitotic progression.
- (C) latA delays mitotic progression in *pds1* Δ cells.
- (D) latA does not delay mitotic progression in *swe1* Δ cells.
- (E) latA does not delay mitotic progression in *cdc55* Δ cells.

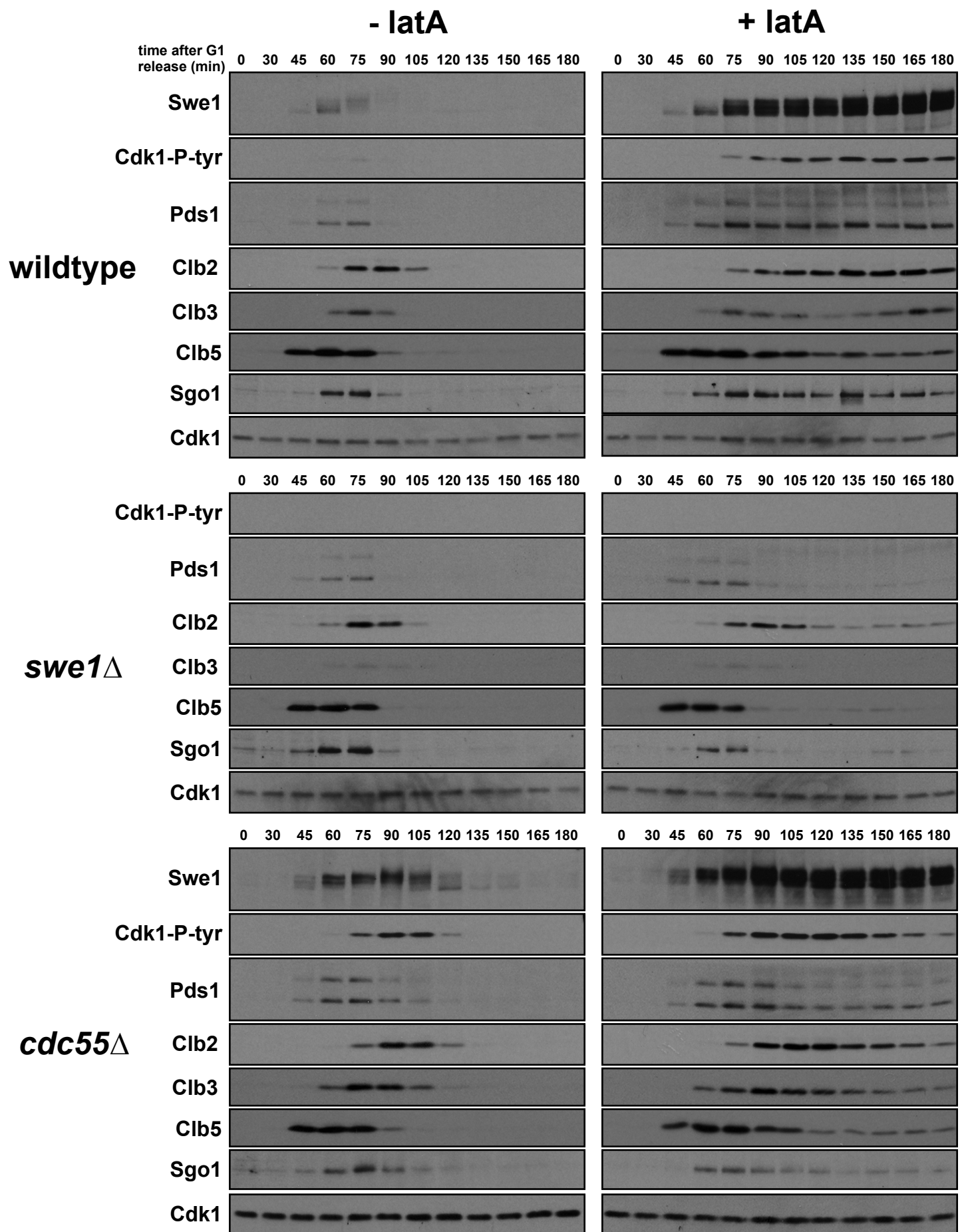


Figure 3-24

Figure 3-24. Morphogenesis checkpoint activation partially stabilizes APC substrates in *cdc55*Δ cells.

Wildtype (ADR4009), *swe1*Δ (ADR4015) and *cdc55*Δ (ADR4738) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

activation demonstrates that *cdc55Δ* bypasses the morphogenesis checkpoint through a different mechanism than *swe1Δ*.

Since *cdc55Δ* bypass of the morphogenesis checkpoint, as observed by spindle morphology or APC substrate levels, was not as striking as *swe1Δ* bypass, we assayed checkpoint bypass using flow cytometry. Activation of the morphogenesis checkpoint arrests wildtype cells after S-phase with 2C DNA content (441). *swe1Δ* cells, on the other hand, undergo a subsequent round of chromosome replication within a single unbudded cell and accumulate 4C DNA content. To see if *cdc55Δ* cells also undergo an additional round of DNA synthesis during morphogenesis checkpoint activation, we looked at the DNA content of synchronous populations of latA-treated wildtype, *swe1Δ* and *cdc55Δ* cells by flow cytometry (Figure 3-25). As expected, treatment of wildtype cells with latA causes cell cycle arrest with 2C DNA content and deletion of *SWE1* allows cells to undergo an additional round of DNA replication in the absence of budding. Similar to *swe1Δ*, *cdc55Δ* cells undergo an additional round of DNA replication during latA treatment. This demonstrates that *cdc55Δ* cells are indeed able to bypass the morphogenesis checkpoint.

3.2.6 *PP2A^{Cdc55}* maintains mitotic checkpoints by dephosphorylating the APC

Our observation that APC substrates are partially destabilized in *cdc55Δ* cells treated with latA suggested that *cdc55Δ* cells may hyper-activate the APC. This hypothesis would predict that *PP2A^{Cdc55}* slows the activation of *APC^{Cdc20}* by dephosphorylating Cdk1 sites on Cdc16, Cdc23 and Cdc27. If this were true, preventing APC phosphorylation should prevent morphogenesis checkpoint bypass in *cdc55Δ* cells. To test this hypothesis, we used a strain expressing the *cdc16-6A*, *cdc23-A* and *cdc27-5A* alleles (*apc-12A* collectively), which have been shown to abolish phosphorylation of the APC in vivo and in vitro (236). Due to the

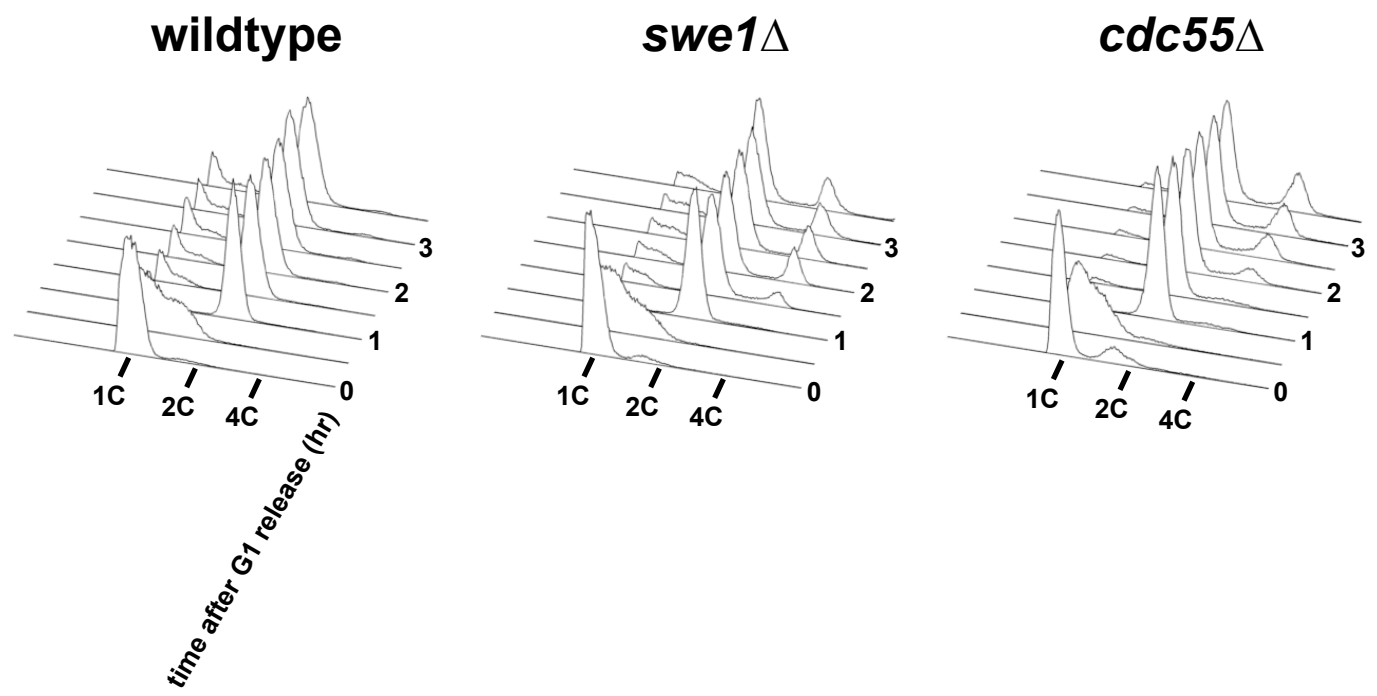


Figure 3-25

Figure 3-25. ***cdc55*Δ cells re-replicate DNA during morphogenesis checkpoint activation.** Wildtype (ADR4009), *swe1*Δ (ADR4015) and *cdc55*Δ (ADR4738) cells were grown to log phase, arrested in G1 with α -factor, released into the cell cycle ($t = 0$) and treated with latA at $t = 25$ minutes. Samples for flow cytometry were taken at the indicated timepoints.

difficulty and expense of working with latA, we decided to first explore whether increased APC phosphorylation was responsible for *cdc55Δ* bypass of the spindle checkpoint (422). The spindle checkpoint arrests cell cycle progression during mitosis in response to incompletely or improperly attached kinetochore microtubules (294). Wildtype cells treated with the microtubule poison nocodazole activate the spindle checkpoint and block APC activation (442). To examine spindle checkpoint bypass in *cdc55Δ* and *cdc55Δ apc-12A* cells, we monitored APC substrate degradation in synchronized cells treated with nocodazole (Figure 3-26). Similar to morphogenesis checkpoint activation, spindle checkpoint activation causes the stabilization of APC substrates in wildtype cells. In contrast to the morphogenesis checkpoint, which prevents APC activation by activating Swe1 and inhibiting Cdk1, Swe1 is degraded during spindle checkpoint activation. APC substrates are also stabilized in *apc-12A* cells. Mad2 is required for spindle checkpoint signaling and *mad2Δ* cells degrade APC substrates normally during nocodazole treatment (296). As previously observed (422), *cdc55Δ* cells also degrade APC substrates during nocodazole treatment, though more slowly than *mad2Δ* cells. Strikingly, preventing APC phosphorylation using the *apc-12A* allele stabilizes APC substrates in *cdc55Δ* cells to levels similar to *apc12-A* and wildtype controls. This demonstrates that PP2A^{Cdc55} dephosphorylation of the APC maintains cell cycle arrest during spindle checkpoint activation.

We next considered whether cell viability during spindle checkpoint signaling is maintained by PP2A^{Cdc55} dephosphorylation of the APC. To test this, we measured cell viability during nocodazole exposure of *cdc55Δ* and *cdc55Δ apc-12A* cells (Figure 3-27). Wildtype cells have a functional spindle checkpoint and maintain viability during short exposure to nocodazole (Figure 3-27A). *mad2Δ* and *cdc55Δ* cells, which bypass the spindle checkpoint, quickly lose viability when treated with nocodazole. Preventing APC

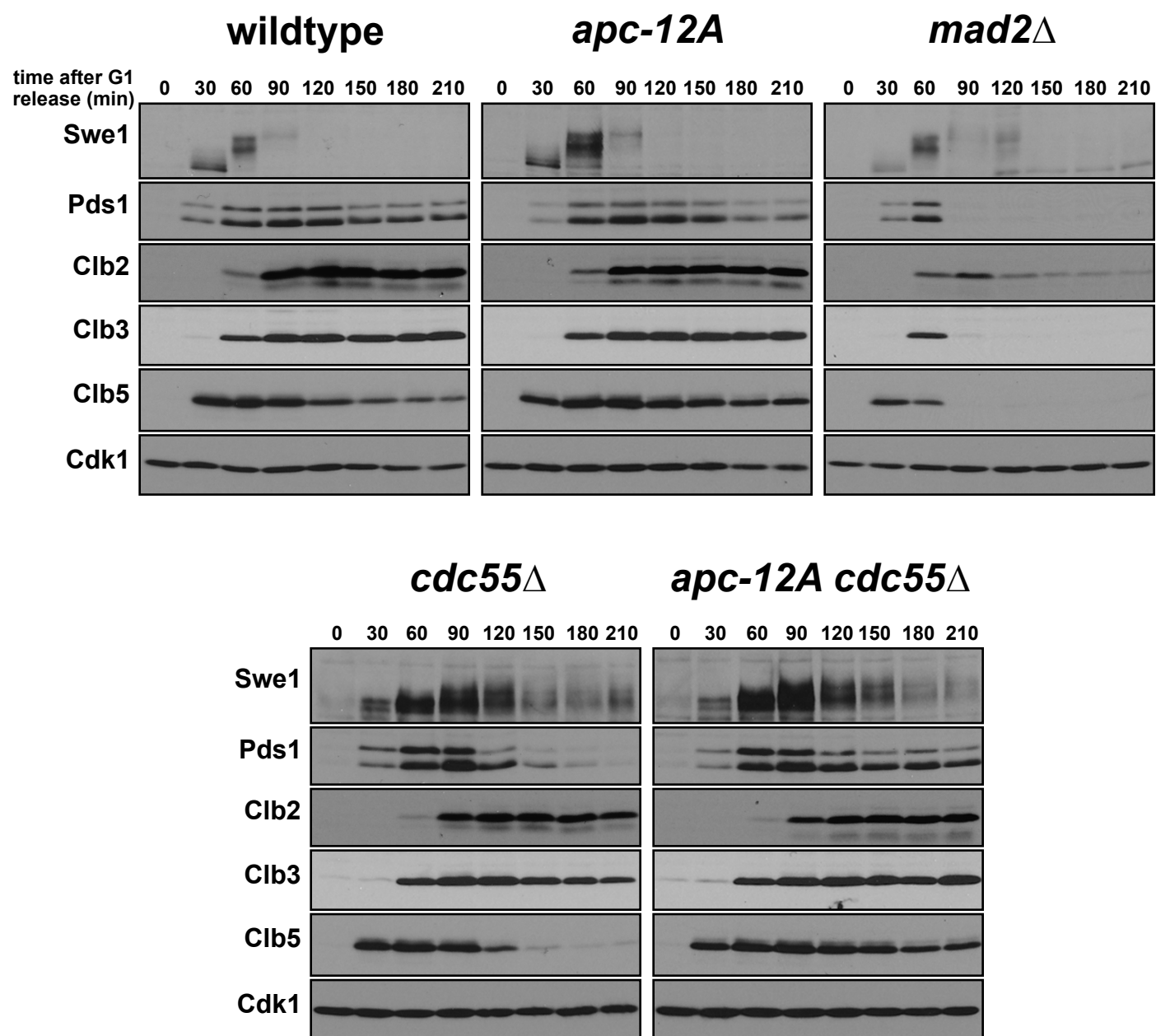


Figure 3-26

Figure 3-26. PP2A^{Cdc55} maintains spindle checkpoint arrest by dephosphorylating the APC.

Wildtype (ADR4009), *apc-12A* (ADR4973), *mad2Δ* (ADR4099), *cdc55Δ* (ADR4738) and *apc-12A cdc55Δ* (ADR4902) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with nocodazole at $t = 35$ minutes and α -factor was re-added at $t = 80$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

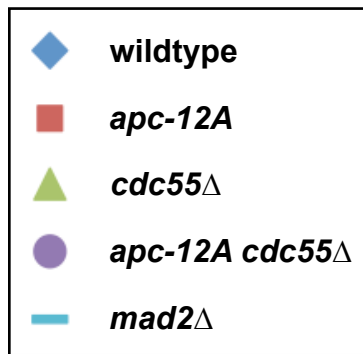
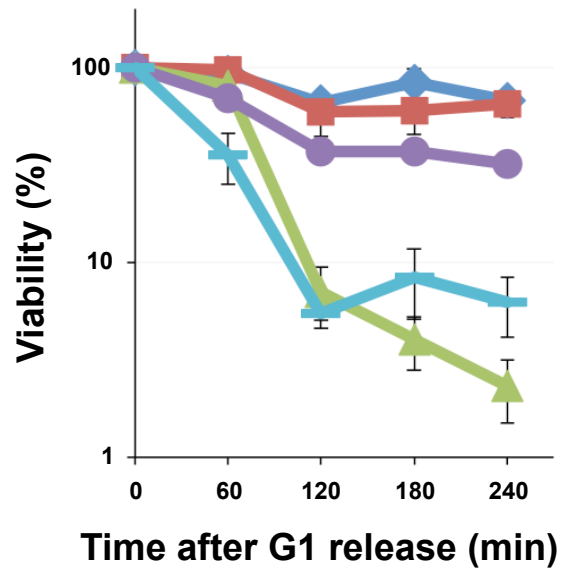
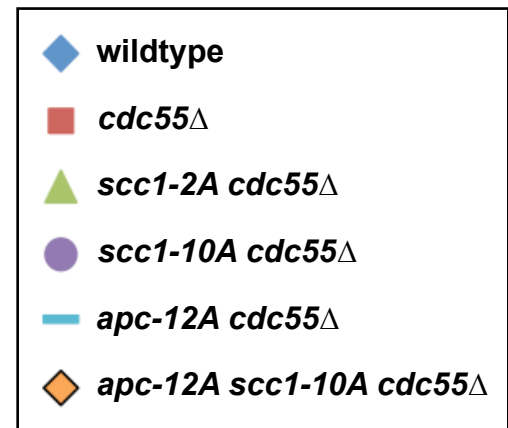
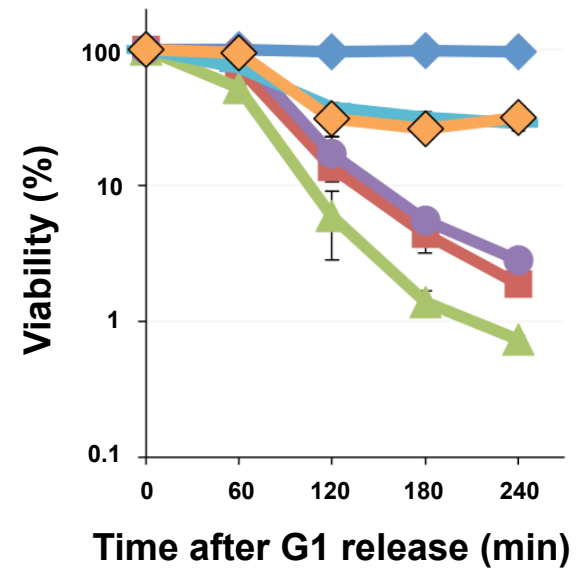
A**B****Figure 3-27**

Figure 3-27. PP2A^{Cdc55} preserves cell viability during spindle checkpoint arrest by dephosphorylating the APC.

(A) Preventing APC phosphorylation rescues the sensitivity of *cdc55Δ* cells to nocodazole. Wildtype (ADR4009), *apc-12A* (ADR4973), *cdc55Δ* (ADR4738), *apc-12A cdc55Δ* (ADR4902) and *mad2Δ* (ADR4099) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with nocodazole at $t = 35$ minutes and samples were taken for viability assays at the indicated timepoints. Viability assays were repeated four times. Displayed values are (mean $\pm$ SEM).

(B) Preventing Scc1 phosphorylation does not rescue the sensitivity of *cdc55Δ* cells to nocodazole. Wildtype (ADR4009), *cdc55Δ pGAL-SCC1* (ADR5044), *scc1-2A cdc55Δ pGAL-SCC1* (ADR5056), *scc1-10A cdc55Δ pGAL-SCC1* (ADR5049), *apc-12A cdc55Δ* (ADR4902) and *apc-12A scc1-10A cdc55Δ pGAL-SCC1* (ADR5417) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with nocodazole at $t = 35$ minutes and samples were taken for viability assays at the indicated timepoints. Viability assays were repeated two times. Displayed values are (mean $\pm$ SEM).

phosphorylation greatly improves the viability of *cdc55Δ* cells treated with nocodazole, demonstrating that PP2A^{Cdc55} dephosphorylation of the APC is crucially important for maintaining cell viability during spindle checkpoint activation.

While we were completing this work it was shown that PP2A^{Cdc55} may dephosphorylate ten Cdc5 phosphorylation sites on Scc1 (411, 413). Cdc5 phosphorylation of two of these ten sites has been shown to be critical to maintaining cell viability in the absence of Pds1 (366). Since Scc1 phosphorylation promotes Scc1 cleavage, increased Scc1 cleavage and premature sister chromatid separation could also potentially explain why *cdc55Δ* cells are sensitive to nocodazole. To test this hypothesis, we measured the viability of *cdc55Δ*, *cdc55Δ scc1-2A*, *cdc55Δ scc1-10A*, *cdc55Δ apc-12A* cells and *cdc55Δ apc-12A scc1-10A* cells after short exposure to nocodazole (Figure 3-27B). In contrast to *apc-12A*, neither *scc1-2A* nor *scc1-10A* improve the viability of *cdc55Δ* cells treated with nocodazole. The *scc1-10A* allele also doesn't further improve the viability of *cdc55Δ apc-12A* cells treated with nocodazole. Thus it seems that PP2A^{Cdc55} regulation of the APC, but not Scc1, is important for maintaining spindle checkpoint arrest.

Since APC dephosphorylation maintains spindle checkpoint arrest during spindle checkpoint activation, we next determined whether morphogenesis checkpoint arrest is maintained by PP2A^{Cdc55} dephosphorylation of the APC. We first determined whether preventing APC phosphorylation prevents spindle elongation during morphogenesis checkpoint activation in *cdc55Δ* cells (Figure 3-28). The majority of untreated wildtype, *apc-12A*, *cdc55Δ* and *cdc55Δ apc-12A* cells undergo a complete cell cycle during the course of this experiment and latA treatment effectively inhibits bud formation in these strains (Figure 3-28A). Wildtype and *apc-12A* cells exhibit a delay in anaphase onset in response to latA treatment (Figure 3-28B and C). *cdc55Δ* cells largely bypass the latA induced delay in

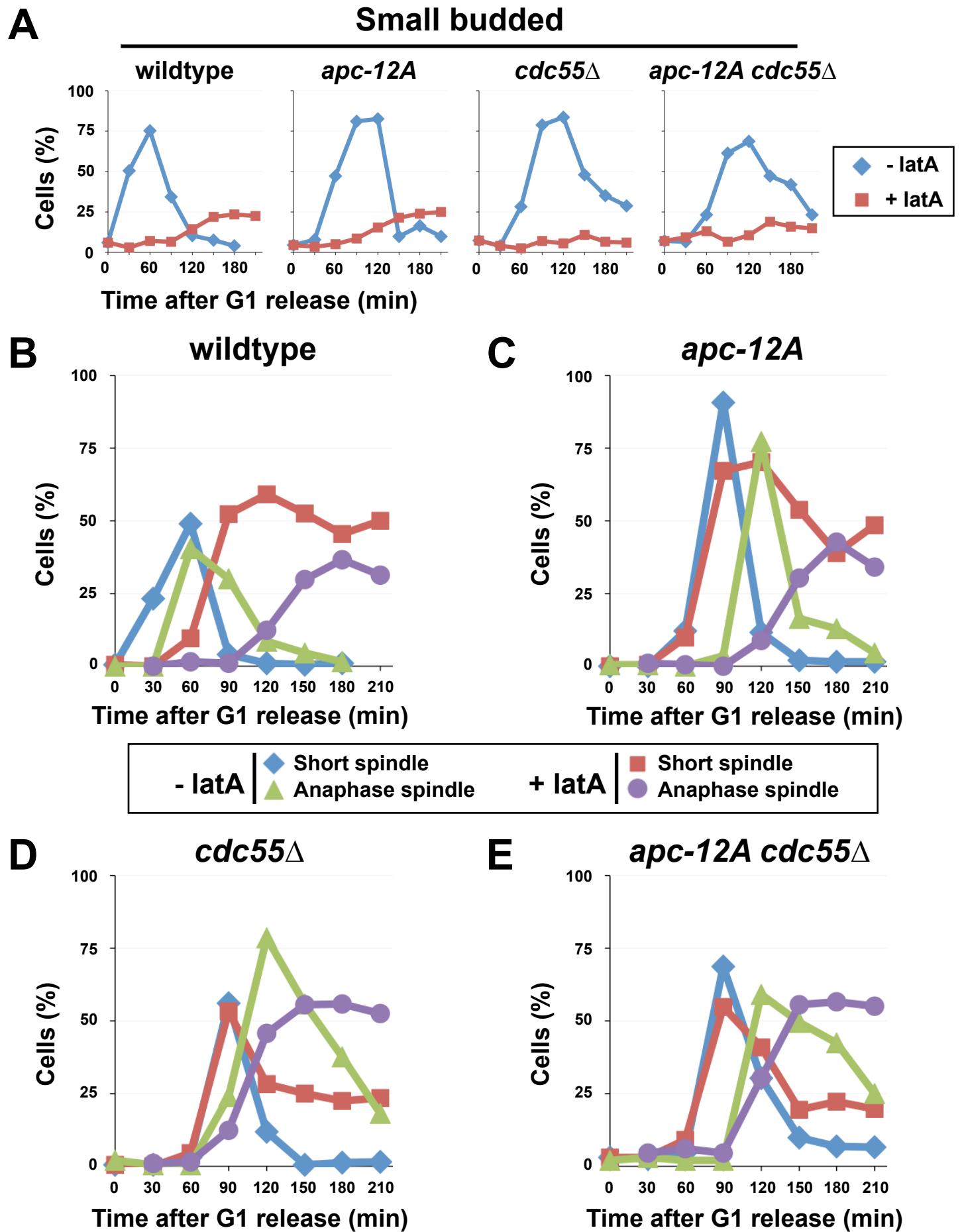


Figure 3-28

Figure 3-28. Preventing APC phosphorylation does not delay anaphase onset in *cdc55*Δ cells treated with latA.

(A-E) Wildtype (ADR4009), *apc-12A* (ADR4973), *cdc55*Δ (ADR4738) and *apc-12A cdc55*Δ (ADR4902) cells were grown to log phase and arrested in G1 with α -factor. Cells were released from G1 arrest at t = 0, treated with +/- latA at t = 25 minutes and α -factor was re-added at t = 55 minutes to arrest cells in the following G1. Samples were fixed for microscopy at the indicated timepoints.

(A) latA inhibits bud growth.

(B) latA delays mitotic progression.

(C) latA delays mitotic progression in *apc-12A* cells.

(D) latA does not delay mitotic progression in *cdc55*Δ cells.

(E) Preventing APC phosphorylation does not delay anaphase onset in *cdc55*Δ cells treated with latA.

anaphase onset (Figure 3-28D). Preventing APC phosphorylation does not delay anaphase onset in *cdc55Δ* cells (Figure 3-28E).

We next sought to determine if APC substrate destabilization in latA-treated *cdc55Δ* cells was due to APC phosphorylation. We monitored APC substrate degradation in synchronous *apc-12A*, *cdc55Δ* and *cdc55Δ apc-12A* cells treated with latA (Figure 3-29). Untreated *cdc55Δ* and *cdc55Δ apc-12A* cells transit mitosis relatively normally, with increased Cdk1 phosphorylation causing a slight stabilization of APC substrates. APC substrates are degraded more quickly in *cdc55Δ* than *apc-12A* cells. Preventing APC phosphorylation in *cdc55Δ* cells restabilizes APC substrates to levels similar to *apc-12A* cells. This suggests that PP2A^{Cdc55} dephosphorylation of the APC is important for the maintenance of mitotic arrest during morphogenesis checkpoint activation.

Due to the subtle effect of preventing APC phosphorylation on *cdc55Δ* mutants, we used flow cytometry as an independent assay of cell cycle progression in *cdc55Δ* and *cdc55Δ apc-12A* cells treated with latA. *apc-12A* cells treated with latA are unable to undergo an additional round of DNA synthesis and arrest with 2C DNA content (Figure 3-30). Deletion of *CDC55* allows cells to progress into another S phase and a 4C DNA peak emerges. Preventing APC phosphorylation partially prevents *cdc55Δ* cells from entering another S phase. We attempted to develop a latA sensitivity assay similar to that described by McMillan *et al.* to monitor cell viability during morphogenesis checkpoint activation (432). Unfortunately, at latA concentrations that elicited a Swe1-dependent cell cycle delay in live cells, we were unable to consistently obtain a dose-dependent viability phenotype in *swe1Δ*, or *cdc55Δ* cells (data not shown). The prohibitive cost of latA prevented us from fully troubleshooting this assay. Regardless, these data together suggest that PP2A^{Cdc55}-dependent

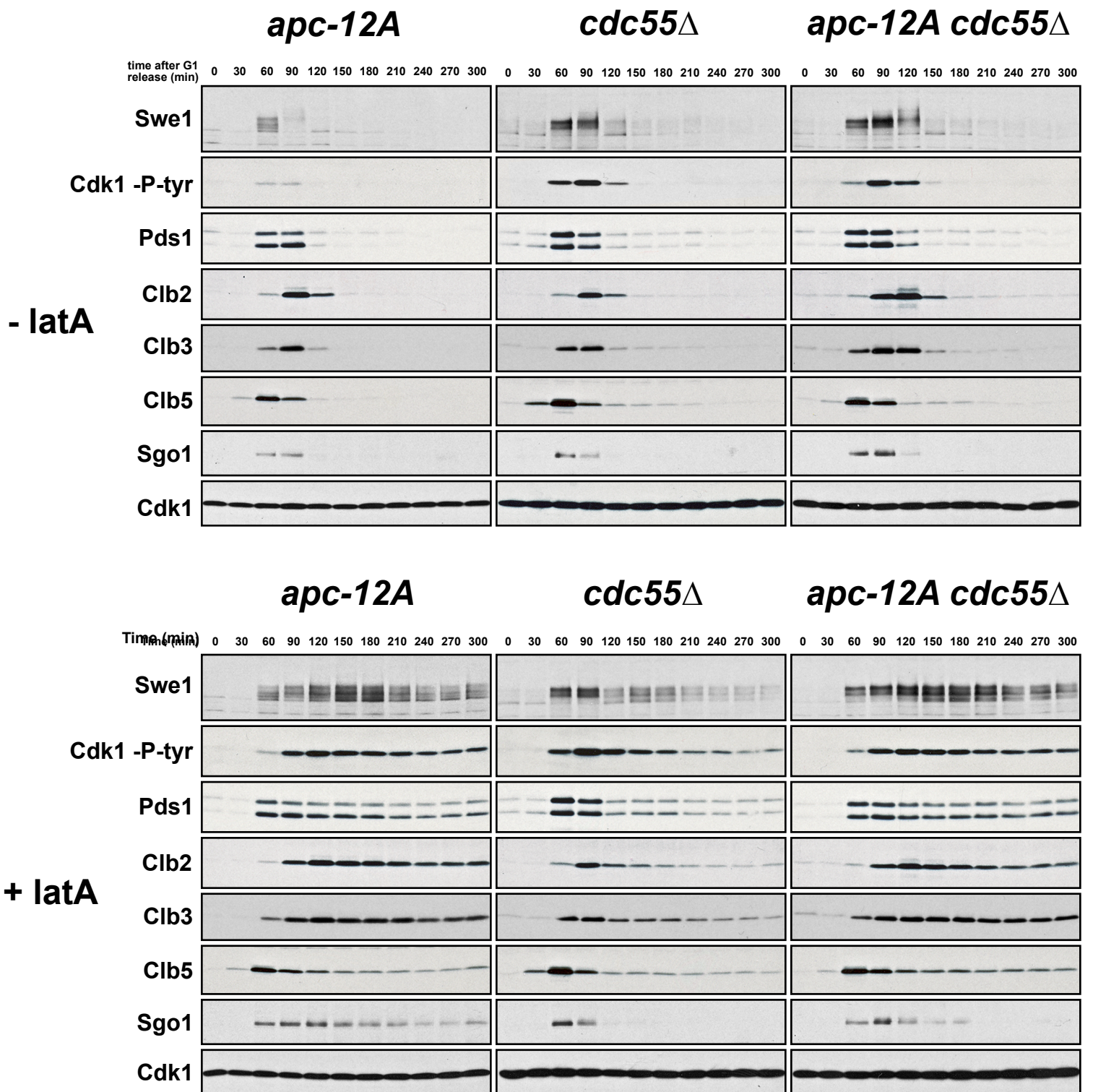


Figure 3-29

Figure 3-29. PP2A^{Cdc55} maintains morphogenesis checkpoint arrest by dephosphorylating the APC.

apc-12A (ADR4973), *cdc55Δ* (ADR4738) and *apc-12A cdc55Δ* (ADR4902) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints.

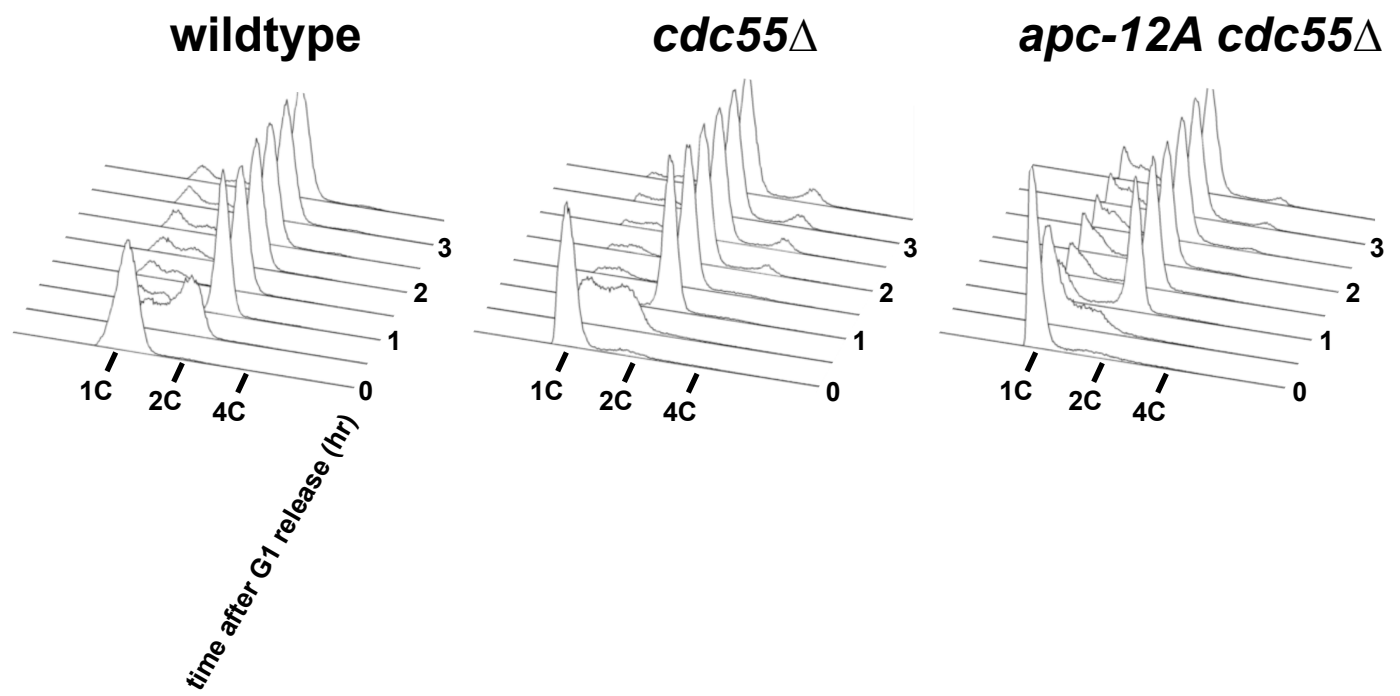


Figure 3-30

Figure 3-30. Preventing APC phosphorylation reduces DNA re-replication in *cdc55*Δ cells treated with latA.

apc-12A (ADR4973), *cdc55*Δ (ADR4738) and *apc-12A cdc55*Δ (ADR4902) cells were grown to log phase, arrested in G1 with α -factor, released into the cell cycle ($t = 0$) and treated with latA at $t = 25$ minutes. Samples for flow cytometry were taken at the indicated timepoints.

APC dephosphorylation helps maintain mitotic arrest during both morphogenesis and spindle checkpoint activation.

3.2.7 *cdc55Δ* rescues mutants with reduced APC activity

Since our model suggests that PP2A^{Cdc55} dephosphorylation of the APC lowers APC activity, we hypothesized that promoting APC phosphorylation by deleting *CDC55* could rescue mutants with reduced APC activity. *cdc16-1* is a mutant allele of *CDC16* that is lethal at restrictive temperatures (19). At the semi-restrictive temperature of 34°C, *cdc16-1* and *swe1Δ cdc16-1* cells are inviable but *cdc55Δ cdc16-1* cells exhibit near wildtype viability (Figure 3-31A). At the fully restrictive temperature of 37°C, *cdc55Δ* cannot rescue the viability of *cdc16-1* cells, demonstrating that *cdc55Δ* does not render the APC non-essential. Our model hypothesizes that *cdc16-1 cdc55Δ* cells are healthier than *cdc16-1* cells due to increased APC activity. If this were the case we would expect that if we monitored anaphase onset in these cells, *cdc16-1 cdc55Δ* cells would undergo anaphase onset more readily than *cdc16-1* cells at a semi-restrictive temperature. To test this, we monitored spindle lengths in synchronous populations of *cdc55Δ*, *cdc16-1* and *cdc16-1 cdc55Δ* cells incubated at the semi-restrictive temperature of 34°C and the restrictive temperature of 37°C (Figure 3-31B). 120 minutes after release from G1, many *cdc16-1 cdc55Δ* cells have begun to enter anaphase, as evidenced by a high proportion of cells with spindles greater than 3μm in length. In contrast, very few *cdc16-1* or *swe1Δ cdc16-1* cells had entered anaphase at that timepoint. Consistent with the inability of *cdc55Δ* to rescue *cdc16-1* viability at a fully restrictive temperature, *cdc16-1 cdc55Δ* cells do not display longer spindles than *cdc16-1* or *swe1Δ cdc16-1* controls when incubated at 37°C. *cdc23-1* is another mutation in an APC subunit that renders cells temperature-sensitive (19). *cdc55Δ* also partially rescues *cdc23-1*

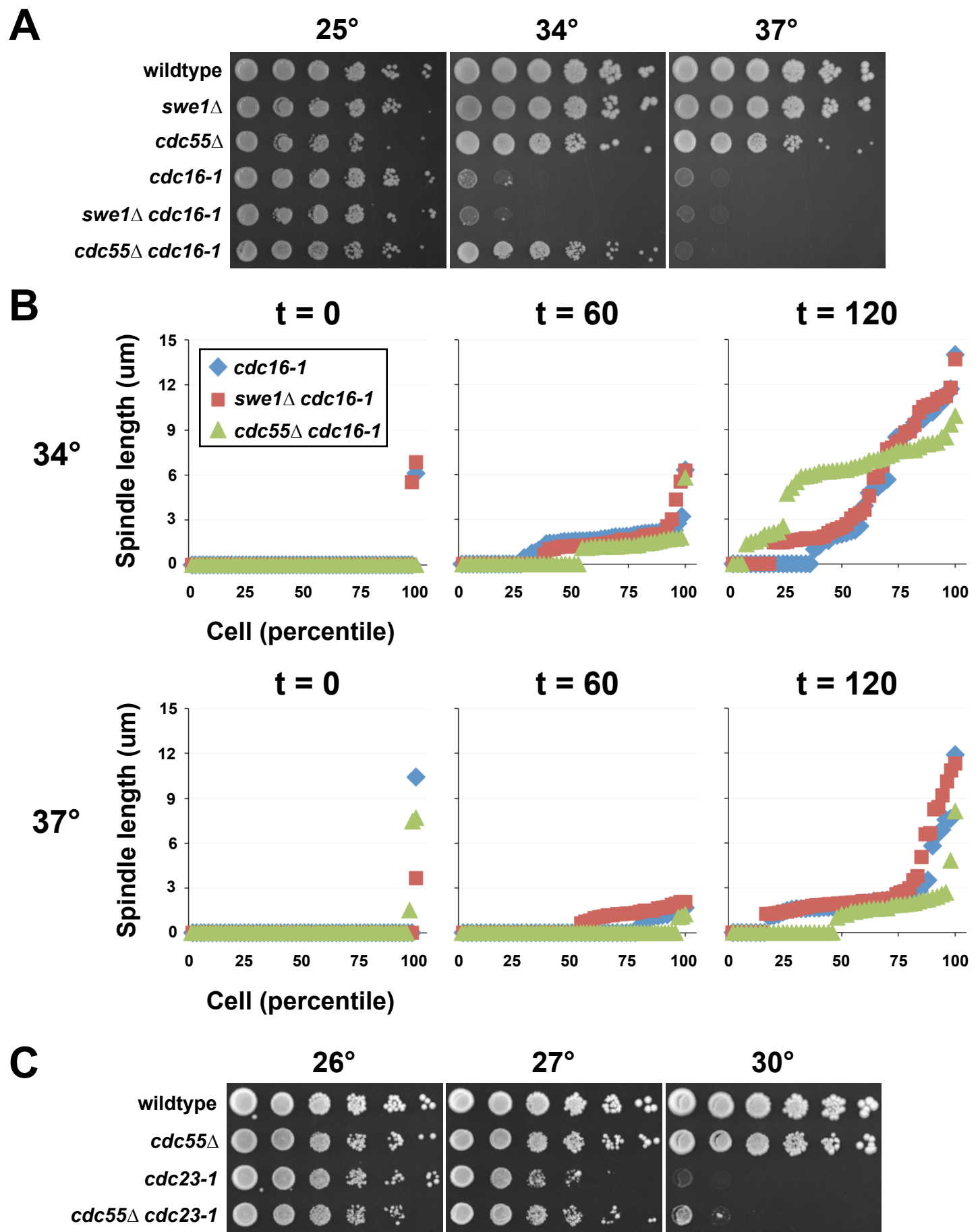


Figure 3-31

Figure 3-31. Deleting *CDC55* rescues the viability of cells with reduced APC activity.

(A) Deleting *CDC55* but not *SWE1* partially rescues the lethality of *cdc16-1* cells. Eight-fold serial dilutions of wildtype (ADR4009), *swe1Δ* (ADR4015), *cdc55Δ* (4738), *cdc16-1* (ADR4979), *swe1Δ cdc16-1* (ADR4984) and *cdc55Δ cdc16-1* (ADR5020) cells were spotted onto YPD plates and grown at the indicated temperatures.

(B) Deleting *CDC55* but not *SWE1* allows spindle elongation at a semi-restrictive temperature in *cdc16-1* cells. *cdc16-1* (ADR4979), *swe1Δ cdc16-1* (ADR4984) and *cdc55Δ cdc16-1* (ADR5020) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release cells were shifted to the indicated temperature. Cells were released from G1 arrest at $t = 0$ and samples were fixed for microscopy at the indicated timepoints.

(C) Deleting *CDC55* partially rescues the lethality of *cdc23-1* cells. Eight-fold serial dilutions of wildtype (ADR4009), *cdc55Δ* (ADR4738), *cdc23-1* (ADR5599) and *cdc55Δ cdc23-1* (ADR5623) cells were spotted onto YPD plates and grown at the indicated temperatures.

cells at a semi-restrictive temperature, though not to the same extent as it rescues *cdc16-1* (Figure 3-31C).

Since deleting *CDC55* causes spindle elongation in *cdc16-1* cells at a semi-restrictive temperature, we wondered whether we could detect earlier degradation of APC substrates in these cells as well. Therefore, we monitored APC substrate degradation in synchronous *cdc16-1*, *cdc16-1 swe1Δ* and *cdc16-1 cdc55Δ* cultures incubated at the semi-restrictive temperature of 34°C and the restrictive temperature of 37°C (Figure 3-32). Though there may be earlier degradation of Pds1 and Clb2 in *cdc16-1 cdc55Δ* cells compared to *cdc16-1* and *cdc16-1 swe1Δ* cells, this data is difficult to interpret due to poor cell synchrony in *cdc16-1 cdc55Δ* cells.

Finally, work from our lab has also shown that deleting *CDC55* increases APC phosphorylation in vivo and purified PP2A^{Cdc55} dephosphorylates the APC in vitro (425). Taken together, this data suggests that PP2A^{Cdc55} inhibits anaphase onset by dephosphorylating Cdk1^{Clb} phosphorylation sites on the APC.

3.3 Discussion

3.3.1 *Swe1 regulates anaphase onset*

In this work we demonstrated that Swe1 regulates anaphase onset in budding yeast cells by phosphorylating Cdk1 on tyrosine 19 (Figure 3-33). In the most thorough characterization to date of cells lacking *SWE1*, Harvey and Kellogg showed that *swe1Δ* cells formed mitotic spindles more quickly than wildtype cells after being released from a G1 arrest (102). Because they observed spindle formation in fixed cells they were unable to measure this phenotype quantitatively, though *swe1Δ* cells appear to form spindles on average ~10 minutes before wildtype cells. This is similar to our observation, in live cells,

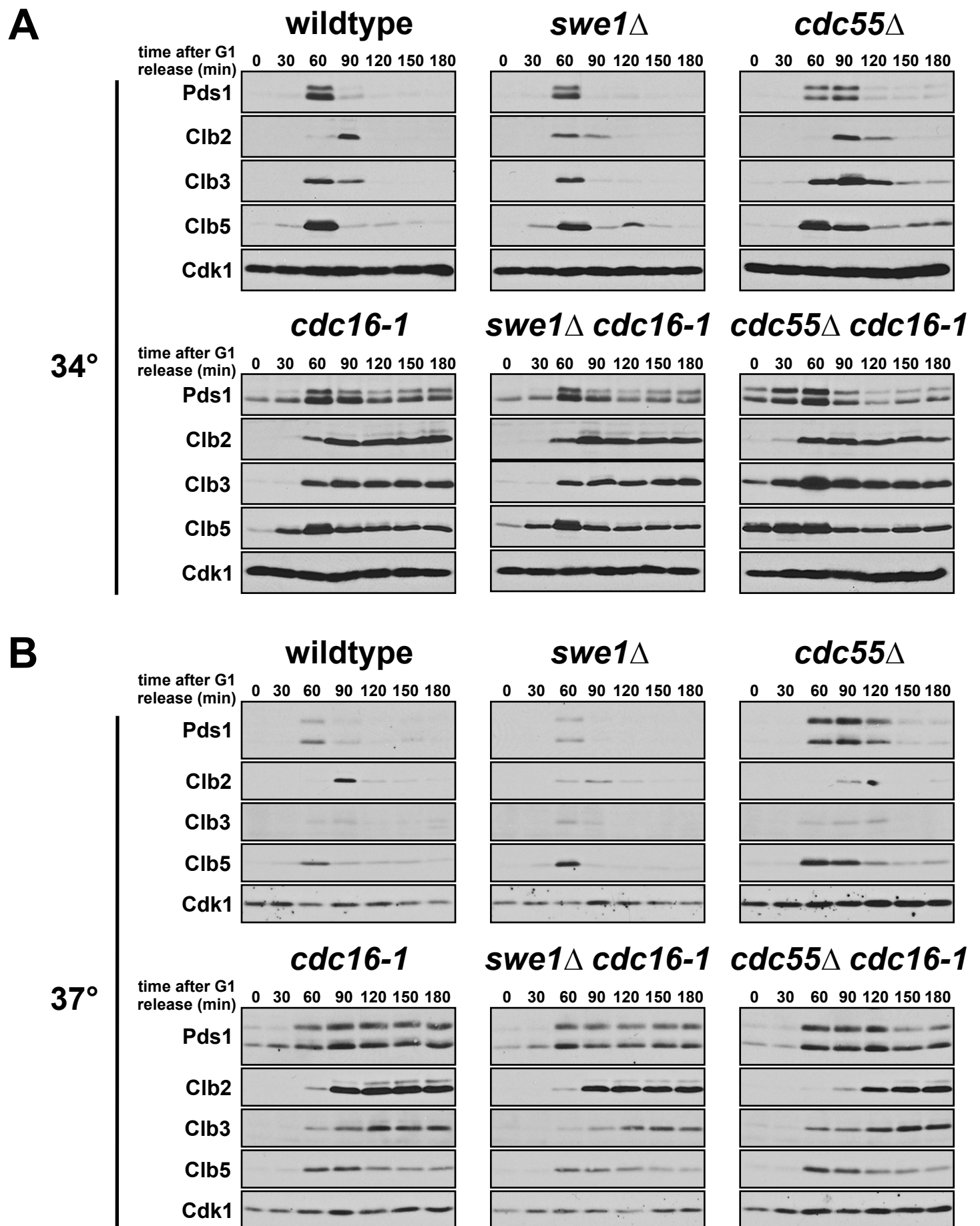


Figure 3-32

Figure 3-32. Deleting *CDC55* or *SWE1* does not destabilize APC substrates in cells with reduced APC activity

(A) Deleting *CDC55* or *SWE1* does not destabilize APC substrates in *cdc16-1* cells at a semi-restrictive temperature. Wildtype (ADR4009), *swe1Δ* (ADR4015), *cdc55Δ* (ADR4738), *cdc16-1* (ADR4979), *swe1Δ cdc16-1* (ADR4984) and *cdc55Δ cdc16-1* (ADR5020) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release cells were shifted to 34°C. Cells were released from G1 arrest at t = 0 and samples were taken for immunoblotting at the indicated timepoints.

(B) Deleting *CDC55* or *SWE1* does not destabilize APC substrates in *cdc16-1* cells at a restrictive temperature. Wildtype (ADR4009), *swe1Δ* (ADR4015), *cdc55Δ* (4738), *cdc16-1* (ADR4979), *swe1Δ cdc16-1* (ADR4984) and *cdc55Δ cdc16-1* (ADR5020) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release cells were shifted to 37°C. Cells were released from G1 arrest at t = 0 and samples were taken for immunoblotting at the indicated timepoints.

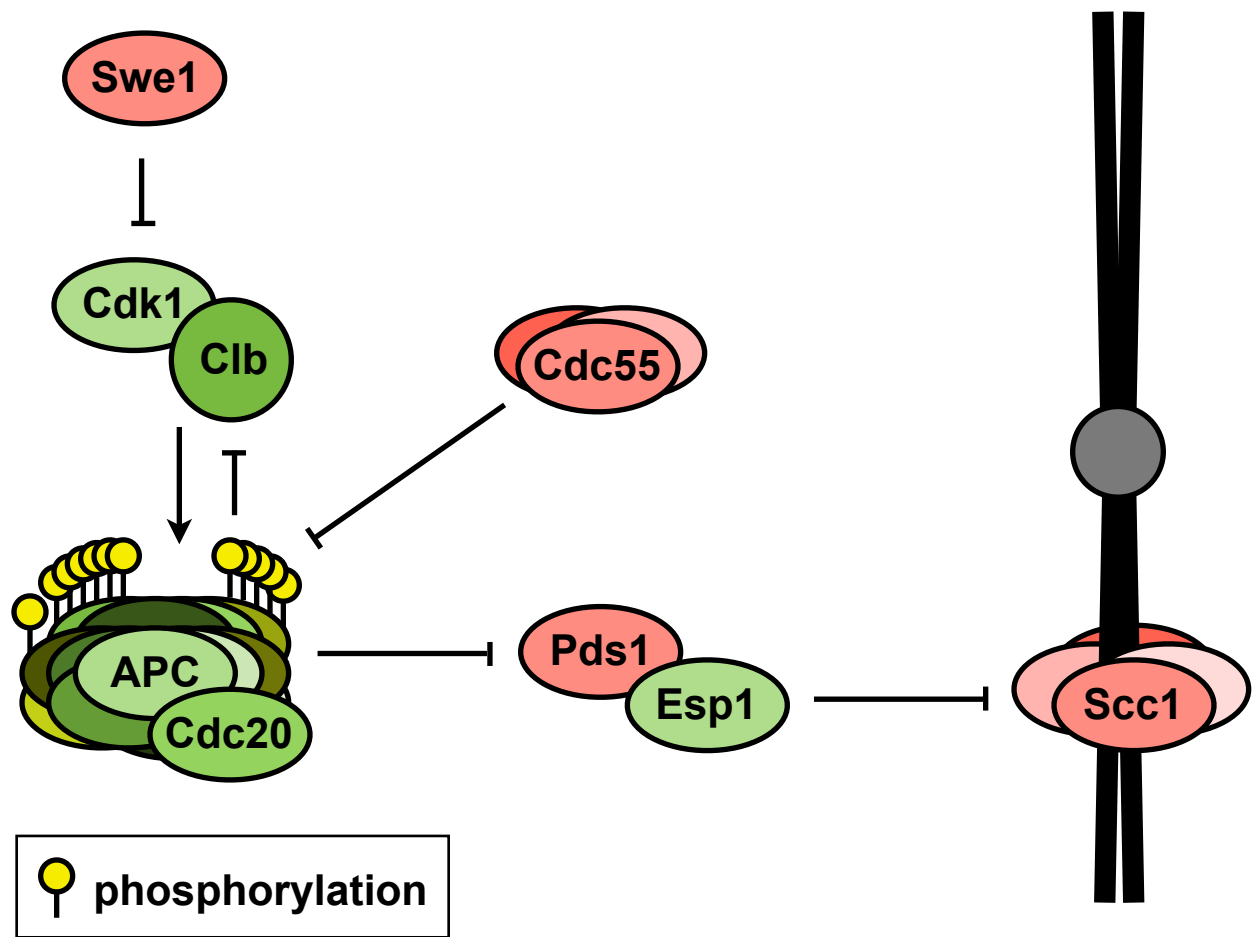


Figure 3-33

Figure 3-33. A new model for the regulation of anaphase onset.

Anaphase onset is triggered by the APC. Prior to anaphase Swe1 phosphorylation of Cdk1^{C1b} inhibits Cdk1^{C1b} and prevents Cdk1^{C1b} phosphorylation and activation of the APC. PP2A^{Cdc55} inhibits APC^{Cdc20} by opposing Cdk1^{C1b} phosphorylation of the APC^{Cdc20}. Upon activation by Cdk^{C1b}, APC^{Cdc20} targets mitotic cyclins and Pds1 for degradation. Cyclin degradation reduces Cdk1 activity and allows G1 re-entry. Pds1 degradation liberates Esp1 from inhibitory Pds1/Esp1 complexes. Esp1 cleaves the Scc1 subunit of cohesin. Scc1 cleavage allows sister chromatids to be pulled into daughter cells by the mitotic spindle.

that *swe1Δ* cells form spindles an average of 6.2 minutes earlier than wildtype cells (Figure 3-6D). Harvey and Kellogg also looked at the timing of anaphase onset in fixed *swe1Δ* cells released from a G1 arrest. They observed that, similar to spindle formation, spindle elongation at anaphase was advanced by ~10 minutes in *swe1Δ* cells. This data suggested that wildtype and *swe1Δ* cells spent an equivalent amount of time between spindle formation and anaphase onset and therefore that Swe1 did not regulate the timing of anaphase onset independently of spindle formation. In contrast to this data, we have observed that deleting *SWE1* causes cells to undergo anaphase ~3.5 minutes before wildtype cells, even after controlling for the timing of mitotic entry. Our ability to measure this small but significant difference can be attributed to the fact that we did our experiments in live as opposed to fixed cells. Deletion of *MIH1* has been shown to delay spindle formation following G1 release by ~ 10 minutes (103). We did not observe this delay in live cells (Figure 3-6D). However, when we monitored spindle formation in fixed *mih1Δ* cells, we also observed an ~10 minute delay in spindle formation (Figure 3-4B). It should be noted that the cell cycle perturbations observed by us and Harvey and Kellogg are quite small and measuring these phenotypes requires careful analysis. Therefore, it is not surprising that our measurements do not precisely coincide with theirs. However, taken together, it is clear that Mih1 and Swe1 have small but significant roles regulating both mitotic entry and anaphase onset in unperturbed cells.

Though Swe1 causes only a small cell cycle delay in unperturbed cells, activation of Swe1 using the actin-depolymerizing agent latrunculin A (latA) causes long delays before both spindle formation and anaphase onset (Figure 3-9B, D and F). Treating cells with latA immediately upon release from G1 arrest causes an increase from 2.6% to 31.5% in cells exhibiting delayed mitotic entry. Of the 68.5% of latA-treated cells that enter mitosis in a

timely fashion, 81.1% delay anaphase onset. This data suggests that, though Cdk1 inhibitory phosphorylation clearly inhibits mitotic entry, the majority of budding yeast cells with inhibited Cdk1 successfully enter mitosis and arrest cell cycle progression before anaphase onset. This is consistent with previous reports showing that cells lacking Clb1 and Clb2 activity arrest cell cycle progression with short mitotic spindles (72, 77, 78, 104). This data supports a model whereby spindle formation and anaphase onset are triggered sequentially by increasing Cdk1 activity (Figure 3-34). In *S. pombe* cells, tyrosine 15 phosphorylated Cdk1 has insufficient activity to signal either of these events. In *S. cerevisiae*, tyrosine 19 phosphorylated Cdk1 has sufficient activity to signal spindle formation but insufficient activity to signal anaphase onset.

3.3.2 Does Wee1 regulate anaphase onset in other organisms?

Treatment of fission yeast cells with low doses of latrunculin A and B has been shown to delay anaphase onset (443-445). It is thought that this is due to destabilization of the mitotic spindle, as 35% (11/28) of wildtype cells treated with 1.25uM latA exhibit spindle collapse during metaphase (445). It is clear that latA treatment of budding yeast cells delays anaphase onset through a different mechanism. Firstly, in our experiments, treatment of budding yeast cells with 2.5uM latA never resulted in spindle collapse before anaphase onset in wildtype (0/46), or any other cells. Secondly, treatment of budding yeast cells with 2.5uM latA results in a fully *SWE1*-dependent delay in cell cycle progression. In contrast, in fission yeast, though higher doses of latA delay mitotic entry by inhibiting Cdk1 through the cell size checkpoint, 1.25uM latA does not inhibit mitotic entry (444, 446).

It has recently been shown that Cdk1 phosphorylation may also be important for regulating anaphase onset in mammalian cells. Mutations in the human *FLNA* gene,

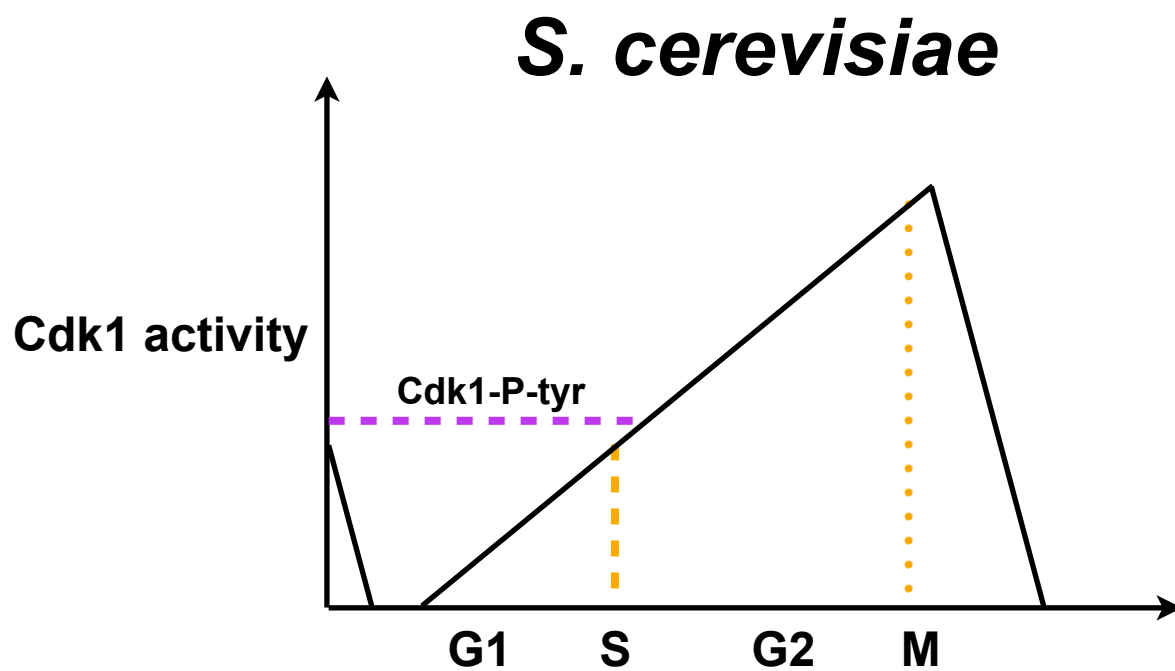
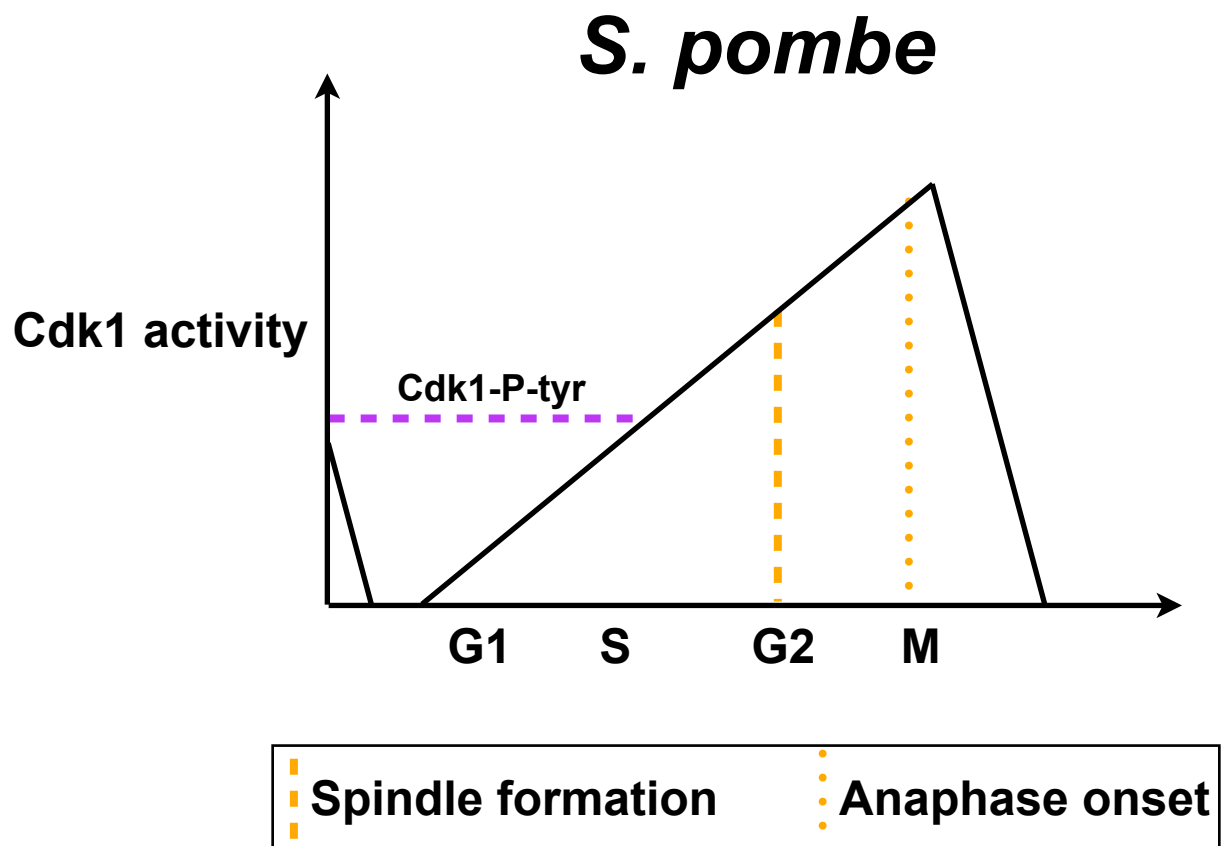


Figure 3-34

Figure 3-34. A model for spindle formation in yeasts.

In *S. pombe* spindle formation occurs relatively late in the cell cycle, after DNA replication is already complete and Cdk1 activity is quite high. Therefore Cdk1 inhibitory phosphorylation prevents spindle formation. In *S. cerevisiae* spindle formation occurs relatively early in the cell cycle, nearly concomitant with DNA replication, when Cdk1 activity is still relatively low. Therefore Cdk1 inhibitory phosphorylation does not prevent spindle formation.

encoding the filamin A protein, an actin-binding protein (reviewed in (447)), impaired cortical development and periventricular heterotopia (448, 449). Filamin A is essential for mouse embryogenesis. Filamin A depletion has recently been shown to cause microcephaly and increase cell cycle length by increasing the length of mitosis in *Dilp2* mice (450). Neuronal progenitor cells depleted of filamin A arrest in mitosis when treated with nocodazole, but upon release from mitotic arrest delay in mitosis with high levels of cyclin B, Cdh1, Cdc20 and tyrosine-phosphorylated Cdk1. Furthermore, filamin A physically interacts with Wee1 and Wee1 is mislocalized in cells depleted of filamin A. Taken together, this data demonstrates that, at least in this context, inhibitory phosphorylation of Cdk1 regulates anaphase onset in mammalian cells. Loss of filamin A has been shown to result in a decrease in overall body size (450), suggestive of a defect in cell cycle progression, not only in the brain but in other organs. Therefore, it is possible that filamin A dependent promotion of Cdk1 dephosphorylation and anaphase onset may be important in other mammalian organisms and cell types. Indeed, immunofluorescence of tyrosine 15-phosphorylated Cdk1 in HeLa cells demonstrates that Cdk1 inhibitory phosphorylation does not decrease until after centrosome separation (451). More research is required to determine if these are isolated cases or if Wee1-dependent regulation of anaphase onset is conserved more broadly.

There is also data suggesting that Cdk1 inhibitory phosphorylation regulates anaphase onset in *D. melanogaster* embryos. Treatment with hydroxyurea to prevent DNA synthesis or the topoisomerase inhibitor VM26, which interferes with chromosome condensation, causes an increase in the interval between nuclear envelope breakdown and anaphase onset in wildtype but not *dwee1*- (*Drosophila* homolog of *WEE1*) embryos (452).

Regardless of whether peak Cdk1 activity signals mitotic entry or anaphase onset, Cdk1 activity must be lowered for cells to transition from mitosis to the following G1. In

some situations it has been proposed that, in addition to APC-mediated cyclin degradation, Cdk1 inhibitory phosphorylation can assist in lowering Cdk1 activity during mitotic exit (422, 453, 454). If cells used phosphorylation to inhibit Cdk1 during mitotic exit, this would clearly demonstrate that Cdk1 inhibitory phosphorylation regulated processes distinct from mitotic entry. These situations, however, only seem to emerge when cells are genetically manipulated and may not reflect signaling pathways with general physiological relevance. One physiologically relevant situation in which cells may lower Cdk1 activity in the absence of APC activation is spindle checkpoint adaptation (424, 455, 456). In this process, spindle checkpoint activated cells undergo mitotic exit after prolonged spindle checkpoint arrest, despite maintaining spindle checkpoint signaling. In budding yeast, adaptation does not appear to be due to Cdk1 inhibitory phosphorylation, rather slow Clb2 accumulation and steady PP2A^{Cdc55} activity eventually promotes APC phosphorylation and Cdc20-associated activity (457). In vertebrate cells, adaptation appears to be caused by slow degradation of Clb2 by the APC^{Cdc20} and eventual passage into G1 (455).

Other work has shown that inhibition of Cdk1 inhibitory phosphorylation during G1 can cause mitotic re-entry in HeLa and *Xenopus* S3 cells (458). This data suggests that during G1, inhibitory phosphorylation of Cdk1 maintains low Cdk1 activity. This is likely not true in budding yeast as, in contrast to HeLa and S3 cells, Cdk1 inhibitory phosphorylation decreases as cells leave mitosis and enter G1 (Figure 3-1A). Also, in budding yeast, Swe1 protein levels diminish as cells exit mitosis and during nocodazole treatment (Figure 3-1A and Figure 3-26). The mechanism that leads to Wee1 inactivation and/or degradation has yet to be determined and may differ significantly between organisms. Differences in the timing of Wee1 degradation in different organisms may determine whether or not Wee1 is used to inhibit Cdk1 in G1.

Taken together, this data demonstrates that there may be considerable plasticity in how Cdk1 inhibitory phosphorylation is used by cells to regulate cell cycle transitions. Of particular importance will be to better characterize the upstream regulation of Wee1 and Cdc25 homologs. Our observation that in budding yeast Cdk1 inhibitory phosphorylation appears to primarily delay anaphase onset integrates well with our current understanding of how Wee1 and Cdc25 homologues regulate cell cycle progression. Cell cycle inhibition during G2 or mitosis achieves a similar function in the sense that, in both cases, DNA damage can be repaired using a replicated sister chromatid as a template. Budding yeast cells have relatively short G2 periods as compared to fission yeast cells. In fact, spindle formation coincides closely with completion of S-phase (459, 460). This suggests that relatively less Cdk1 activity is required in budding yeast to signal mitotic entry (Figure 3-34). If Cdk1 inhibitory phosphorylation inhibited total Cdk1 activity in fission yeast and budding yeast by a similar extent, then it is possible that this threshold would exceed that needed to enter mitosis in budding yeast but not be sufficient in fission yeast. It is also possible that, in budding yeast, if inhibitory phosphorylation reduced global Cdk1 activity to a level insufficient for spindle formation, cells would not have sufficient Cdk1 activity to signal origin firing during S-phase.

3.3.3 How does Cdk1 activity trigger anaphase?

Preventing phosphorylation of twelve sites on the APC subunits Cdc16, Cdc23 and Cdc27 does not cause a similar mitotic arrest as depleting Clb1 and Clb2, suggesting that phosphorylation of additional sites on the APC or another mitotic substrate are essential for anaphase onset. If the only essential function of Cdk1 in anaphase onset was APC activation, then we would have expected that deleting APC substrates would have allowed for anaphase

onset during morphogenesis checkpoint activation. However, depletion of Pds1, Clb5, Clb6 and Sgo1 individually or in various combinations does not bypass the morphogenesis checkpoint (Figure 3-16, Figure 3-18, Figure 3-19, Figure 3-20, Figure 3-21 and Figure 3-22). Though the possibility still exists that the essential function of Cdk1 in mitosis is solely APC phosphorylation and there is an additional APC substrate that we have not identified that is responsible for preventing anaphase during Cdk1 inhibition, we do not favour this model. Numerous other Cdk1 substrates have been identified that could be essential in promoting anaphase onset. Our characterization of one of them, Esp1, will be the main objective of Chapter 4.

3.3.4 Kip1 and Cin8 do not regulate spindle formation in budding yeast

Our conclusion that Cdk1 inhibitory phosphorylation primarily regulates anaphase onset directly contradicts past data claiming that Cdk1 dephosphorylation is required for spindle formation (99, 430). It had been proposed that spindle formation in budding yeast required Cdk1 dephosphorylation and activation (430). Specifically, cells with inactive Cdk1 were unable to inactivate APC^{Cdh1} and persistent APC^{Cdh1} activity targeted the microtubule-associated proteins Cin8, Kip1 and Ase1 for degradation. In the absence of Cin8, Kip1 and Ase1, spindles were unable to form. Our data does not support this model for several reasons: Firstly, in unperturbed cells, Crasta *et al.* observed Cdk1 tyrosine 19 phosphorylation disappear immediately before Clb2 and Cin8 accumulation(430). In contrast, we observed a large overlap in the presence of tyrosine-phosphorylated Cdk1 and Clb2 in unperturbed cells (Figure 3-1A). Secondly, Crasta *et al.* claimed that Cin8 protein levels increased with similar timing to Clb2, following the disappearance of tyrosine phosphorylated Cdk1. Our data agrees with theirs in so far as Clb2 and Cin8 are expressed

with similar timing, however peak expression of Cin8 coincides with peak Swe1 phosphorylation, timepoints characterized by high Cdk1 tyrosine phosphorylation (Figure 3-1B). We also saw very little evidence of cell cycle-dependent changes in Kip1 expression (Figure 3-1C). Thirdly, Crasta *et al.* did not observe spindle formation in any cells before Cdk1 tyrosine 19 phosphorylation had completely disappeared. In contrast, in wildtype cells we first observed Cdk1 inhibitory phosphorylation 55 minutes after G1 release (Figure 3-2A) and spindle formation began at precisely the same timepoints (Figure 3-4B). Finally, promoting Cdk1 phosphorylation by activating the morphogenesis checkpoint did not prevent spindle formation in at least 68.5% of cells (Figure 3-9D) and spindle formation in cells with phosphorylated Cdk1 appeared to be morphologically identical to spindle formation in unperturbed cells (Figure 3-10A).

In their analysis, Crasta *et al.* utilized cells expressing *cdc28-4* and four copies of the *cdc28Y19E* (*cdk1Y19E*) allele to make up for the low Cdk1 activity exhibited by the *cdk1Y19E* allele (430). It is possible that Cdk1-Y19E, in addition to exhibiting reduced activity, also exhibits altered activity and this is responsible for their observed phenotypes. Crasta *et al.* also use the *cdk1-as1* allele in some experiments. *cdk1-as1* encodes a protein that exhibits a ten-fold reduction in ATP-binding affinity and a sixfold reduction in maximum ATP turnover rate as compared to wildtype. Cells expressing Cdk1-as1 also exhibit reduced viability in media lacking the Cdk1-as1 inhibitor C3-1'-naphthylmethyl pyrazolo[3,4-*d*]pyrimidine (1NM-PP1) (424, 461). In contrast, our experiments used only the endogenously expressed, wildtype Cdk1 protein. Our observation that Cin8 and Kip1 protein expression is not regulated by Cdk1-inhibition has been verified by Chee and Haase, who found that inhibiting Cdk1 activity by deleting all of the B-type cyclins did not affect either *CIN8* or *KIP1* mRNA or protein levels (462). These authors also showed that Cdk1^{C1b2}

phosphorylates Cin8 and Kip1, but were unable to show that combining unphosphorylatable mutants in these motor proteins could prevent spindle formation. Therefore it does not appear that Cdk1 dephosphorylation is required for spindle pole body separation, either through Cin8/Kip1 stabilization or phosphorylation.

3.3.5 Swe1 and Mih1 regulate cell size

Similar to a previous report (102), our data clearly demonstrates that deleting *MIH1* or *SWE1* affects the size at which cells form mitotic spindles (Figure 3-8A and B). In addition, our data demonstrates that deleting *SWE1* affects the size at which cells enter anaphase. Consistent with previous reports (463) we observed that cell growth occurred primarily in the bud between spindle formation and anaphase onset. Wildtype, *mih1Δ* and *swe1Δ* mothers grew by an average of 5.1% +/- 2.6, 5.8% +/- 2.1% and -0.5% +/- 4.2% respectively and wildtype, *mih1Δ* and *swe1Δ* buds grew by an average of 325.4% +/- 41.1%, 188.7% +/- 25.7% and 278.2% +/- 52.5% (mean +/- SEM) respectively between spindle formation and anaphase onset. Consistent with Harvey and Kellogg, differences in size at spindle formation and anaphase onset were also primarily manifest in the bud. At spindle formation, buds in *swe1Δ* cells were significantly smaller than buds in wildtype and *mih1Δ* cells, and buds in *mih1Δ* cells were significantly larger than in wildtype cells (Figure 3-8C). At anaphase onset, buds in *swe1Δ* cells were significantly smaller than buds in wildtype and *mih1Δ* cells. Though Harvey and Kellogg measured cell size in fixed cells, and were therefore unable to calculate an average size at spindle formation, the bud size we measured in wildtype and *swe1Δ* cells at spindle formation closely resemble those in fixed cells with ~0.85 μm spindles, approximately the length of the spindle immediately after spindle formation. Differences in mother cell size at spindle formation and anaphase onset, which

were not reported by Harvey and Kellogg, were largely insignificant, with the only significant difference being that *swe1* Δ mothers are significantly smaller at anaphase onset than *mih1* Δ mothers. This data suggests that premature mitotic entry and anaphase onset in *swe1* Δ cells could be due to the absence of a biochemical checkpoint monitoring cell growth. Though we did not pursue this avenue of research further, recent work has begun to elucidate how budding yeast cells regulate Cdk1 inhibitory phosphorylation in response to cell growth (192). Our data suggests that this pathway is likely to regulate cell cycle progression by delaying anaphase onset.

3.3.6 Cdc20 is stabilized upon morphogenesis checkpoint activation

While this research was being conducted, it was shown that Cdk1 activity was required for recovery from spindle checkpoint activation (434). In one sense, this result was not surprising in that spindle checkpoint recovery requires anaphase onset and anaphase onset requires Cdk1 activity (72, 77, 78, 104). However, these authors showed that Cdk1 inhibition using the *cdk1-as1* allele during spindle checkpoint activation caused Cdc20 protein levels to fall dramatically. This suggested to us that perhaps Cdk1 inhibition by the morphogenesis checkpoint was preventing anaphase onset by lowering Cdc20 levels and thereby inhibiting the APC. We think that this is not the case for two reasons. First, supplying cells with a constant amount of Cdc20 does not allow spindle checkpoint recovery in *cdk1-as1* cells with inhibited Cdk1 (434). Second, we have shown that during morphogenesis checkpoint activation, Cdc20 is stabilized, not destabilized (Figure 3-15). Together, this data demonstrates that mitotic arrest in morphogenesis checkpoint activated cells appears not to be due to changes in Cdc20 protein levels.

3.3.7 Cells lacking CLB5 and CLB6 undergo abnormal spindle elongation

We observed that unperturbed *clb5Δ clb6Δ* cells entering mitosis undergo rapid spindle elongation upon spindle formation (Figure 3-18C and D). Liang *et al.* showed that inhibiting Cdk1 using the *cdk1-as1* allele after release from a nocodazole arrest resulted in abnormal spindle elongation in the absence of kinetochore capture (434). They also demonstrated that the premature elongation phenotype could be recapitulated in *ase1-7A* cells in which seven Cdk1 consensus sites on Ase1 have been mutated to alanine (140). This phenotype could be rescued by mimicking Ase1 phosphorylation using the *ase1-7D* allele. This data, along with in vitro kinase reactions, suggest that Ase1 is a specific target of Cdk1^{Clb5/Clb6} (464). Additionally, 70.5% of *clb5Δ clb6Δ* cells subsequently shorten their elongated mitotic spindle and undergo an apparently normal anaphase onset (Figure 3-18C). This could reflect delayed Ase1 phosphorylation by other Cdk1^{Clb} complexes, and subsequent kinetochore capture by spindle microtubules. Some simple experiments that would begin to address how Clb5 and Clb6 regulate spindle structure will be discussed in Chapter 5.

3.3.8 Cells lacking PDS1 and SGO1 have abnormal mitotic spindles

During this research we observed that mitotic spindles in *pds1Δ sgo1Δ* cells immediately elongate to a length greater than either *pds1Δ* or *sgo1Δ* cells (Figure 3-16 and Figure 3-20). Despite this increase in length, cells maintain a relatively constant metaphase spindle length following spindle formation and undergo a noticeable increase in spindle length at anaphase onset. This data suggest that *pds1Δ sgo1Δ* cells are capable of signaling anaphase relatively normally but are deficient in some aspect of metaphase spindle formation. In human cells shugoshin is responsible for maintaining centromeric sister

chromatid cohesion before mitotic anaphase (437). During the *prophase pathway*, unprotected cohesin is removed from chromosome arms by Wapl (465). Then at anaphase onset, the residual cohesin, which had been protected by Sgo1, is cleaved by separase (466). In budding yeast mitosis, shugoshin is involved in promoting sister kinetochore biorientation (467). In cells lacking *SGO1*, sister kinetochores that attach to the same spindle pole body remain improperly attached resulting in chromosome missegregation (468). During mitosis, sister chromatids attached to the same spindle pole would not oppose tension generated by the mitotic spindle. Therefore, it is possible that in *pds1Δ sgo1Δ* cells, if less sister chromatid pairs properly attach to the spindle, the spindle would have less force opposing it and would lengthen. Based on this simple model one would expect *sgo1Δ* cells to exhibit this phenotype, which is not the case. Perhaps in the absence of *PDS1*, Scc1 is partially destabilized and the reduction in properly cohesed sister chromatids results in abnormally long spindles. Though we did not further characterize this phenotype, visualizing centromeres and spindle pole bodies concurrently in live cells using a *pds1Δ sgo1Δ CENV-lacO lacI-GFP SPC42-mCherry* strain would elucidate whether co-depletion of Pds1 and Sgo1 causes increased *CENV* separation in some cells and no *CENV* separation in others, as would be predicted.

3.3.9 Deletion of CDC55 bypasses the morphogenesis checkpoint

While work on this project was ongoing, it was shown that morphogenesis checkpoint activation does not prevent anaphase onset in *cdc55Δ* cells (429). We reproduced this finding and extended it in two ways. Firstly, we demonstrated that *cdc55Δ* cells not only undergo anaphase spindle elongation, but also partially degrade APC substrates and enter a subsequent S-phase during morphogenesis checkpoint activation. Secondly, and more

importantly, we demonstrated that morphogenesis checkpoint-bypass in *cdc55Δ* cells is due, in part, to increased APC phosphorylation. The importance of these two findings will be discussed in turn.

Chiroli *et al.* claimed that morphogenesis checkpoint activated *cdc55Δ* cells entered anaphase but did not exit mitosis and re-enter the cell cycle. We showed that, in fact, *cdc55Δ* cells undergo an additional round of DNA replication during morphogenesis checkpoint activation (Figure 3-25). A key difference between our work and theirs is that Chiroli *et al.* looked at DNA re-replication in cells treated with 100μM latA, whereas we used only 2.5μM latA, a concentration at which all defects are Swe1- and Cdk1-Y19F-dependent. At higher concentrations we observed Swe1-independent delays in cell cycle progression. Chiroli *et al.* did not directly compare latA-treated versus untreated *swe1Δ* or *cdc55Δ* cells so it is impossible to determine if in their experiments latA is causing Swe1-independent cell cycle delays. *cdc55Δ* cells also delay APC substrate degradation in unperturbed cell cycles (Figure 3-24). Therefore it would be expected that *cdc55Δ* cells would not undergo a subsequent round of DNA re-replication with similar timing to wildtype cells. Taken together it seems likely that the reason that Chiroli *et al.* did not observe DNA re-replication in *cdc55Δ* cells treated with latA was a combination of Swe1-independent cell perturbation by latA and delayed cell cycle transitions due to deleting *CDC55*.

Although *cdc55Δ* cells maintain high levels of Swe1 and Cdk1-Y19 phosphorylation after latA treatment, we observed that these cells elongated their spindles, partially degraded APC substrates and initiated another round of DNA replication (Figure 3-23, Figure 3-24 and 3-25). These results suggested that PP2A^{Cdc55} regulates anaphase onset downstream of its effects modulating Cdk1 inhibitory phosphorylation. This hypothesis is supported by the

observations that *cdc55Δ* cells also bypass the spindle assembly checkpoint and separate sister chromatids at the centromeres during DNA damage checkpoint activation (422, 469).

We showed that the APC was one such important mitotic substrate of PP2A^{Cdc55}. This observation has since been confirmed independently (470). PP2A^{Cdc55} dephosphorylation of the APC appears to be fundamental in maintaining spindle checkpoint signaling, as preventing APC phosphorylation largely prevents APC substrate degradation and cell inviability in *cdc55Δ* cells treated with nocodazole. PP2A^{Cdc55} dephosphorylation of the APC may also maintain morphogenesis checkpoint signaling, as preventing APC phosphorylation in *cdc55Δ* cells treated with latA stabilizes APC substrates. Since preventing APC phosphorylation only partially rescues mitotic defects in *cdc55Δ* cells, it is clear that PP2A^{Cdc55} has other mitotic substrates that inhibit anaphase onset in response to PP2A^{Cdc55} dephosphorylation. We do not think that Scc1 phosphorylation plays an important role in bypassing the spindle checkpoint as preventing Scc1 phosphorylation does not rescue *cdc55Δ* cell sensitivity to nocodazole (Figure 3-27). As will be discussed in greater detail below, we think that Esp1 is another key PP2A^{Cdc55} mitotic substrate.

Chapter 4

Cdk1 phosphorylation of separase regulates spindle elongation

4.1 Purpose

Securin (Pds1) (Figure 1-4) is non-essential in both yeast and metazoans (257, 262, 339, 471, 472). Therefore in these cells Scc1 cleavage must be restrained by an additional mechanism for cells to remain viable. In metazoans, separase is inhibited by Cdk1 phosphorylation and binding (473, 474). In budding yeast, Cdc5 phosphorylation of Scc1 has been shown to promote anaphase onset (366, 413). Cdc5 phosphorylation of Scc1 may be opposed by PP2A^{Cdc55} (411). Therefore, in budding yeast, Scc1 cleavage may be inhibited during metaphase by active PP2A^{Cdc55} until Cdc5 phosphorylation of Scc1 promotes Scc1 cleavage. This model is supported by the observation that *CDC55* becomes essential in the absence of *PDS1* and it has been suggested that this is due to premature cleavage of Scc1 (436).

In this work we sought to determine how sister chromatid cohesion is regulated in the absence of Pds1. We show that Cdk1 phosphorylation and PP2A^{Cdc55} dephosphorylation of Esp1 regulate Esp1 function. Phosphorylated Esp1 appears to regulate spindle stability and sister separation through Slk19 in a FEAR-independent manner.

4.2 Results

4.2.1 Esp1 is activated independently of Pds1 degradation in cdc55Δ cells

To determine whether PP2A^{Cdc55} was bypassing morphogenesis checkpoint activation by regulating Esp1, we monitored Esp1 cleavage of its two known substrates, Scc1 and

Slk19, during latA treatment in wildtype, *swe1Δ* and *cdc55Δ* cells (Figure 4-1). In wildtype cells treated with latA, Scc1 and Slk19 degradation is reduced and delayed compared to untreated controls. Deletion of *SWE1* allows normal cleavage of Scc1 and Slk19 in latA treated cells. In *cdc55Δ* cells, latA treatment causes a very minor delay in Slk19 and Scc1 cleavage. Therefore, despite Pds1 being stabilized in *cdc55Δ* cells treated with latA, Esp1 appears to be activated relatively normally, suggesting that PP2A^{Cdc55} may directly inhibit Esp1 activity.

4.2.2 Depletion of Pds1 in cdc55Δ cells causes spindle defects

It has previously been hypothesized that the synthetic lethality observed in *pds1Δ cdc55Δ* cells is due to premature cleavage of cohesin (436). In order to study this we constructed an auxin-repressible Pds1-AID allele in cells expressing the *Oryza sativa* F-box protein Tir1 (417). Pds1 depletion using this method in otherwise wildtype cells results in reduced growth (Figure 4-2). Pds1 depletion in *cdc55Δ* cells results in complete loss of viability, demonstrating that this is a good system for studying the synthetic lethality of *cdc55Δ* and *pds1Δ*. We next monitored mitotic progression in cells lacking Pds1 and Cdc55 directly using live fluorescence microscopy of cells expressing Spc42-eGFP (Figure 4-3). *cdc55Δ PDS1-AID* cells treated with auxin demonstrate striking mitotic defects (Figure 4-3A). 45% of wildtype cells depleted of Pds1 display normal mitotic progression as determined by spindle morphology, however only 2% of *cdc55Δ* cells depleted of Pds1 undergo normal mitotic progression (Figure 4-3B). 28% of *cdc55Δ* cells depleted of Pds1 undergo immediate spindle elongation and 55% of cells undergo one or more rounds of spindle elongation, contraction and then a relatively normal anaphase. To measure the early spindle elongation phenotype, we determined average spindle lengths in the timepoints

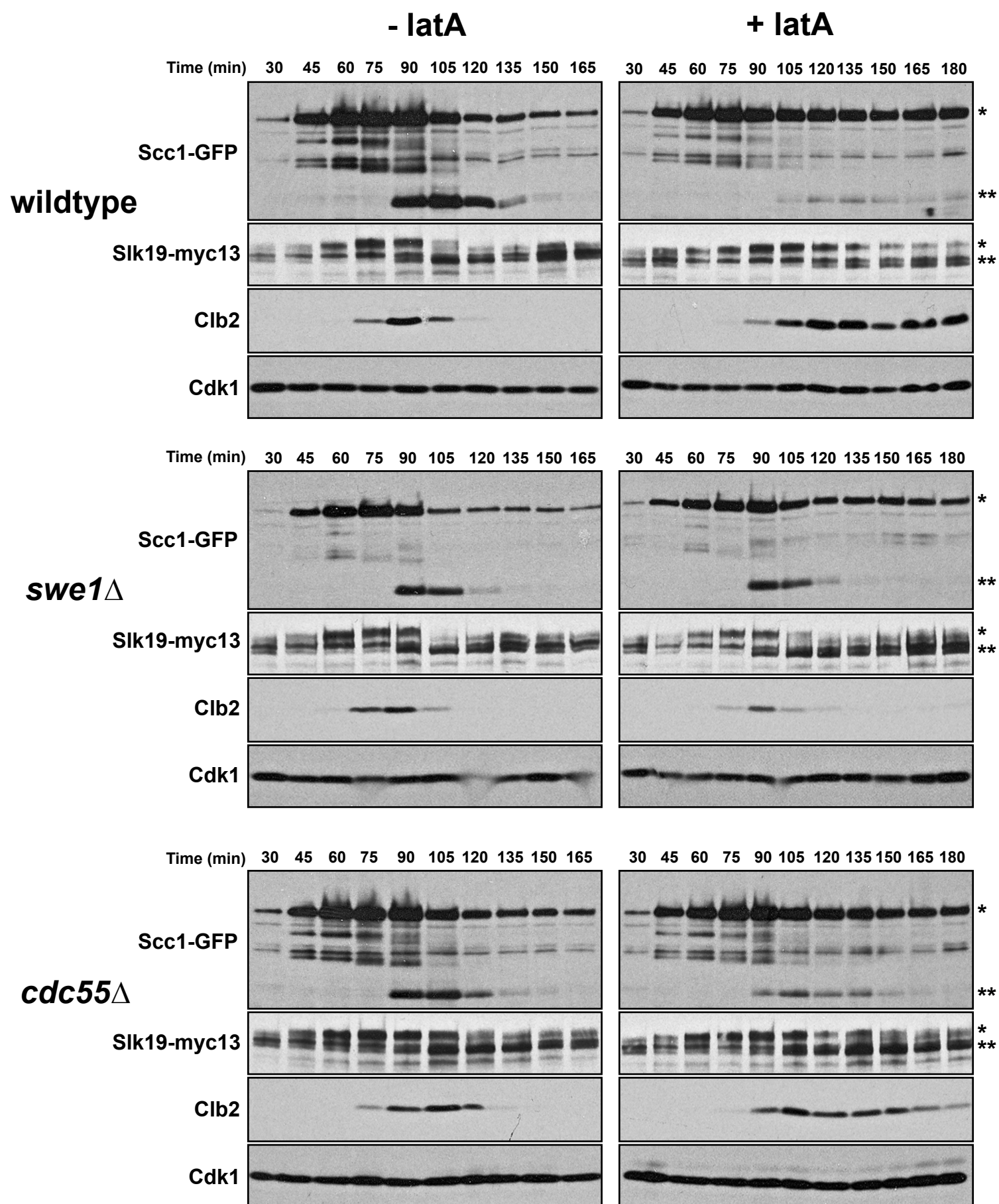


Figure 4-1

Figure 4-1. Esp1 substrates are degraded in *cdc55*Δ cells treated with latA.

SCC1-GFP SLK19-myc13 (ADR5123), *swe1*Δ *SCC1-GFP SLK19-myc13* (ADR5162) and *cdc55*Δ *SCC1-GFP SLK19-myc13* (ADR5154) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with +/- latA at $t = 25$ minutes and α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints. (* indicates full length protein, ** indicates a cleavage product).

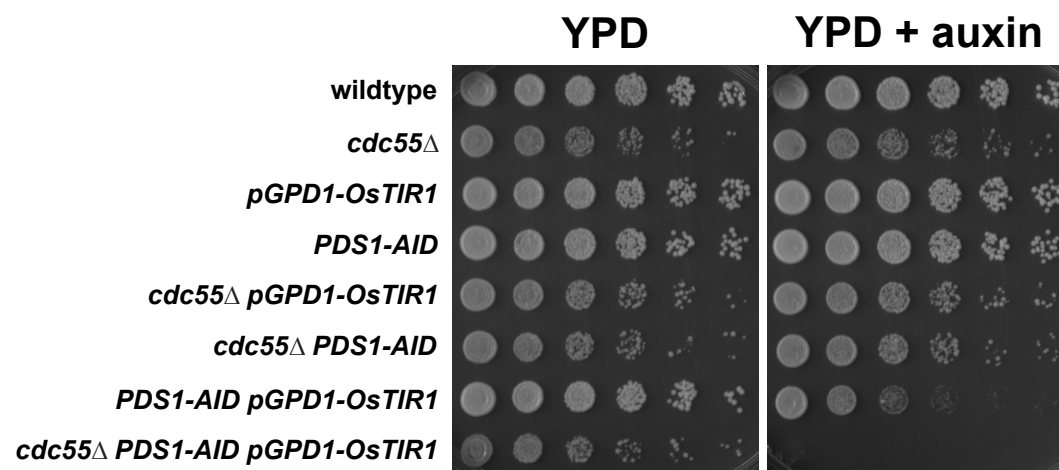


Figure 4-2

Figure 4-2. Pds1 depletion is lethal to *cdc55*Δ cells.

Eight-fold serial dilutions of wildtype (ADR22), *cdc55*Δ (ADR6436), *pGPD1-OsTIR1* (ADR6431), *PDS1-AID* (ADR6429), *cdc55*Δ *pGPD1-OsTIR1* (ADR6434), *cdc55*Δ *PDS1-AID* (ADR6432), *PDS1-AID pGPD1-OsTIR1* (ADR6428) and *cdc55*Δ *PDS1-AID pGPD1-OsTIR1* (ADR6426) cells were spotted onto the indicated plates and grown at 25°C.

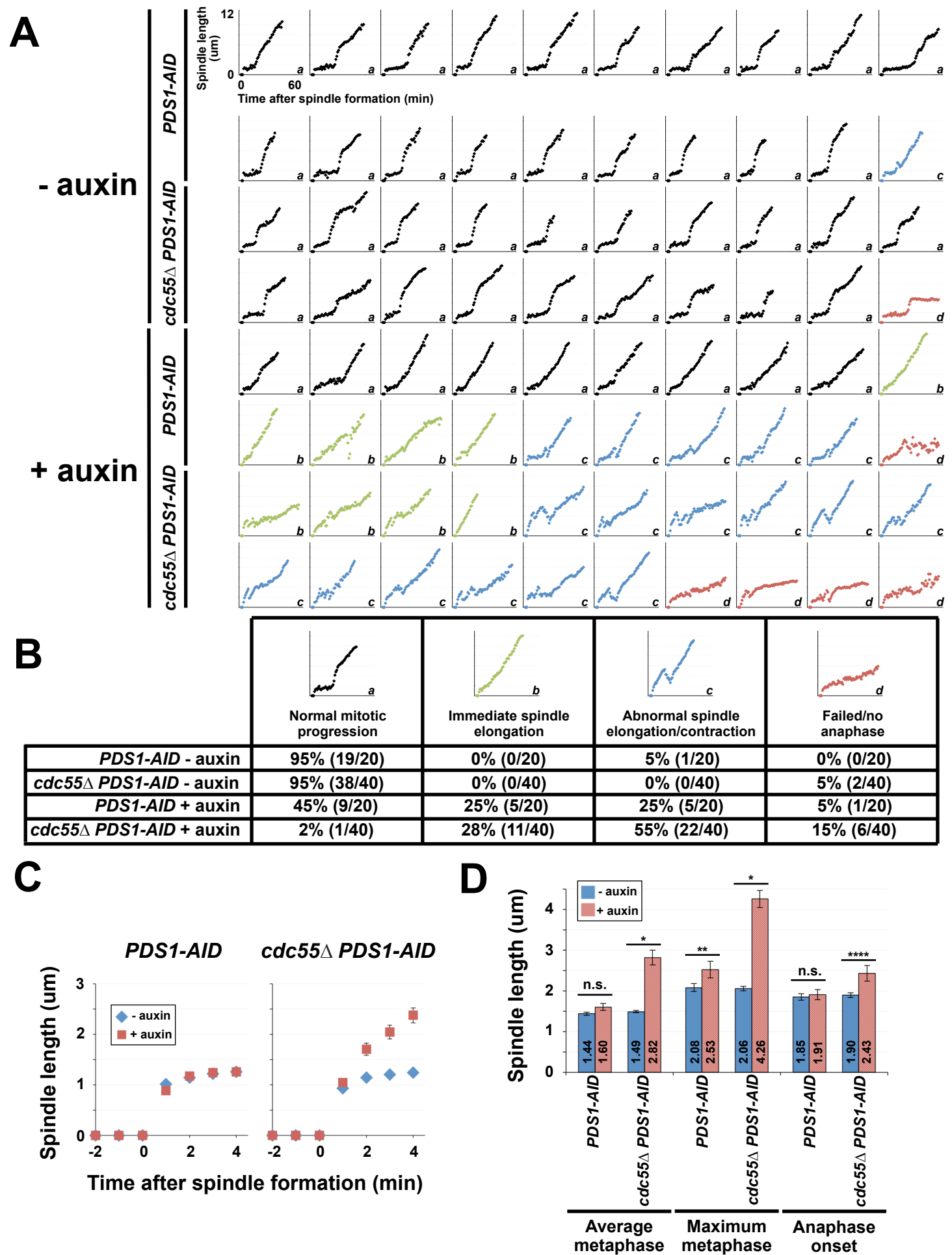


Figure 4-3

Figure 4-3. Pds1 depletion in *cdc55Δ* cells causes spindle defects.

(A-D) *PDS1-AID SPC42-eGFP* (ADR6389) and *cdc55Δ PDS1-AID SPC42-eGFP* (ADR6325) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. The *PDS1-AID SPC42-eGFP* strain was imaged twice in each condition. The *cdc55Δ PDS1-AID SPC42-eGFP* strain was imaged four times in each condition and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual wildtype and *cdc55Δ* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'. Cells from two experimental replicates of *cdc55Δ PDS1-AID* – auxin and *cdc55Δ PDS1-AID* + auxin are not shown in (A) due to space constraints but are included in the subsequent analysis.

(B) Pds1 depletion in *cdc55Δ* cells causes spindle defects. Population characteristics of each strain and treatment combination based upon spindle morphology.

(C) Pds1 depletion in *cdc55Δ* cells causes premature spindle elongation. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(D) Pds1 depletion in *cdc55Δ* cells significantly increases average and maximum metaphase spindle lengths and spindle length at anaphase onset. In cells that displayed both metaphase and anaphase, average and maximum spindle lengths during metaphase and spindle length at anaphase onset were determined. Displayed values are (mean +/- SEM). Student's t-tests were used to test for significant differences (* $p < 0.001$, ** $p = 0.042$, *** $p = 0.004$).

immediately after spindle formation in wildtype and *cdc55Δ* cells depleted of Pds1 (Figure 4-3C). Pds1 depletion does not cause any spindle formation defects on its own but in the absence of *CDC55* causes rapid elongation to a length greater than 2μm. To quantify the elongation and contraction phenotype of *cdc55Δ* cells depleted of Pds1 in an unbiased manner we determined; a) the average spindle length at anaphase onset; b) the average mitotic spindle length during metaphase; and c) the maximum spindle length during metaphase in *cdc55Δ* cells depleted of Pds1 with an identifiable point of anaphase onset (Figure 4-3D). Depletion of Pds1 alone causes a small but significant increase in the maximum length spindles reach during metaphase from 2.08 to 2.53μm. Pds1 depletion alone does not cause a significant change in either the length of spindles at anaphase onset or the average length of spindles during metaphase. Depletion of Pds1 in *cdc55Δ* cells, however, causes large statistically significant increases in spindle length at anaphase onset, average spindle length during metaphase and maximum spindle length during metaphase.

The early spindle elongation phenotype observed in *cdc55Δ* cells depleted of Pds1 suggested to us that these cells may be prematurely losing sister chromatid cohesion. If this were the case, we would expect that depleting cells of the cohesin subunit Scc1 would cause a similar phenotype. To test this, we depleted Scc1 using an auxin inducible degron (Figure 4-4). Unfortunately, AID-tagging Scc1 causes mitotic perturbation even in the absence of auxin (Figure 4-4A and B). These mitotic defects are accompanied by reduced cell viability (Figure 4-4C). This phenotype does not appear to be due to changes in Scc1 protein level, as Scc1-AID is expressed at levels similar to Scc1 in asynchronous cells (Figure 4-4D) and cells undergoing a synchronous cell cycle (Figure 4-4E).

To determine if early spindle elongation in *cdc55Δ* cells depleted of Pds1 is accompanied by premature sister separation, we integrated a *lacO* array on the arm of

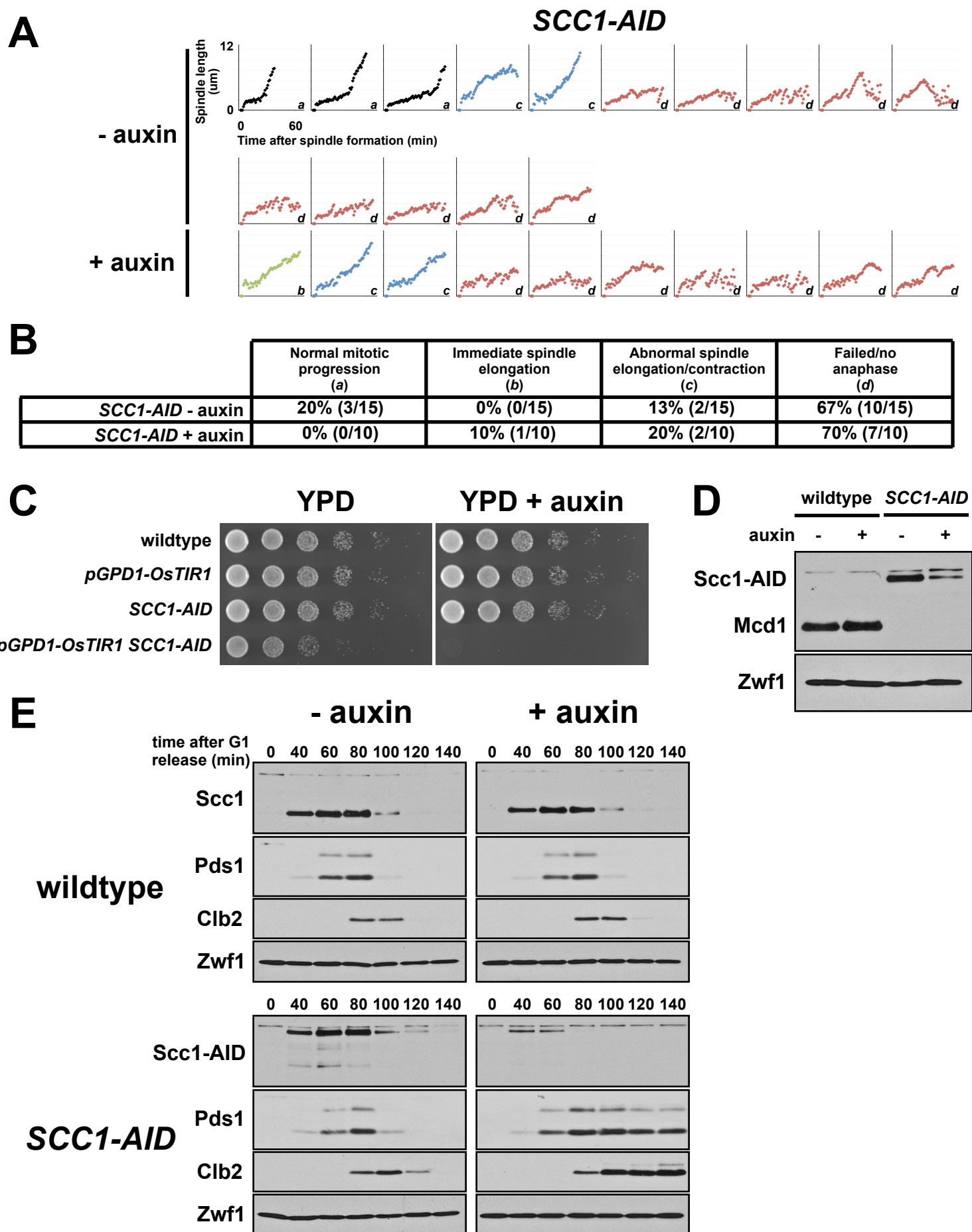


Figure 4-4

Figure 4-4. *SCC1-AID* cells display spindle defects in the absence of auxin.

(A-B) *SCC1-AID SPC42-eGFP* (ADR8091) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 25 minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Strains were imaged once in each condition (- auxin (n = 15), + auxin (n = 10)). Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual cells depleted of Scc1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(B) *SCC1-AID* cells display spindle defects in the absence of auxin. Population characteristics of both treatments based upon spindle morphology.

(C) *SCC1-AID* cells are sick in both the presence and absence of auxin. Eight-fold serial dilutions of wildtype (ADR22), *pGPD1-OsTIR1* (ADR6431), *SCC1-AID* (ADR8179) and *SCC1-AID pGPD1-OsTIR1* (ADR8180) cells were spotted onto the indicated plates and grown at 30°C.

(D) Scc1-AID is present at normal levels in asynchronous cells. Wildtype (ADR4006) and *SCC1-AID* (ADR6786) cells were grown to log phase and +/- auxin was added. Samples were taken for immunoblotting after 15 minutes.

(E) Scc1-AID is present at normal levels in synchronous cells. Wildtype (ADR4006) and *SCC1-AID* (ADR6786) cells were grown to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release cells were treated with +/- auxin. Cells were released from G1 arrest at t = 0 and samples were taken for immunoblotting at the indicated timepoints.

chromosome V at the *URA3* locus in cells expressing lacI-GFP and Spc42-mCherry. We used two-colour live imaging of these cells to measure the relative timing of spindle formation and sister chromatid separation in *cdc55Δ* cells depleted of Pds1 (Figure 4-5). Depletion of Pds1 alone causes a small but significant reduction in the amount of time between spindle formation and sister separation (Figure 4-5A). Pds1 depletion in *cdc55Δ* cells causes a striking decrease in the time between spindle formation and sister chromatid separation from 13.8 minutes in Pds1-depleted controls to an average of 6.7 minutes. Sister chromatid separation precedes spindle formation in 21% (3/14) of *cdc55Δ* cells depleted of Pds1, a phenotype not observed in wildtype cells depleted of Pds1 (0/26) (Figure 4-5B). To determine if premature sister chromatid separation in these cells is accompanied by premature Scc1 cleavage, we looked at cleavage of the Esp1 substrates Scc1 and Slk19 by immunoblot (Figure 4-5C). Interestingly, depletion of Pds1 in wildtype cells causes Scc1 and Slk19 to be cleaved earlier, though this only causes relatively minimal viability and spindle defects. Scc1 and Slk19 cleavage happens no earlier in *cdc55Δ* cells depleted of Pds1 than wildtype cells depleted of Pds1. This suggests that the spindle and sister separation defects observed in *cdc55Δ* cells depleted of Pds1 are not due solely to Scc1 misregulation.

4.2.3 Deleting SWE1 partially rescues cdc55Δ PDS1-AID spindle defects

We next sought to determine what PP2A^{Cdc55} substrate is responsible for preventing spindle defects in cells lacking Pds1. Some data suggests that Cdk1 inhibition causes metaphase spindle elongation defects in cells lacking sister chromatid cohesion (104). Deletion of *CDC55* has been shown to cause Cdk1 inhibitory phosphorylation through Swe1 activation (422, 440). Therefore, we asked whether deleting *SWE1* would rescue spindle defects in *cdc55Δ* cells depleted of Pds1 (Figure 4-6). *swe1Δ cdc55Δ* cells depleted of Pds1

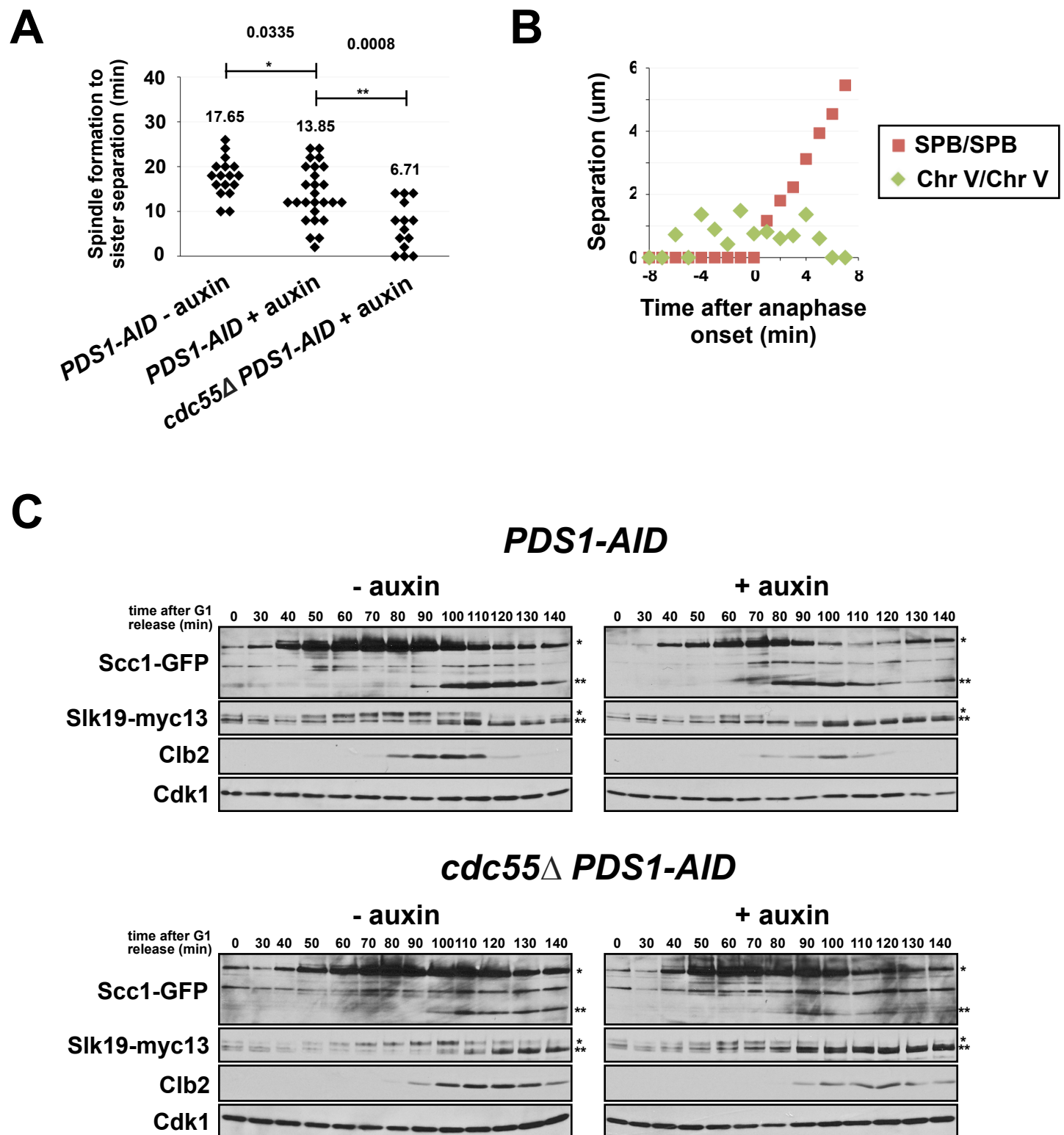


Figure 4-5

Figure 4-5. Pds1 depletion in *cdc55*Δ cells causes premature sister chromatid separation.

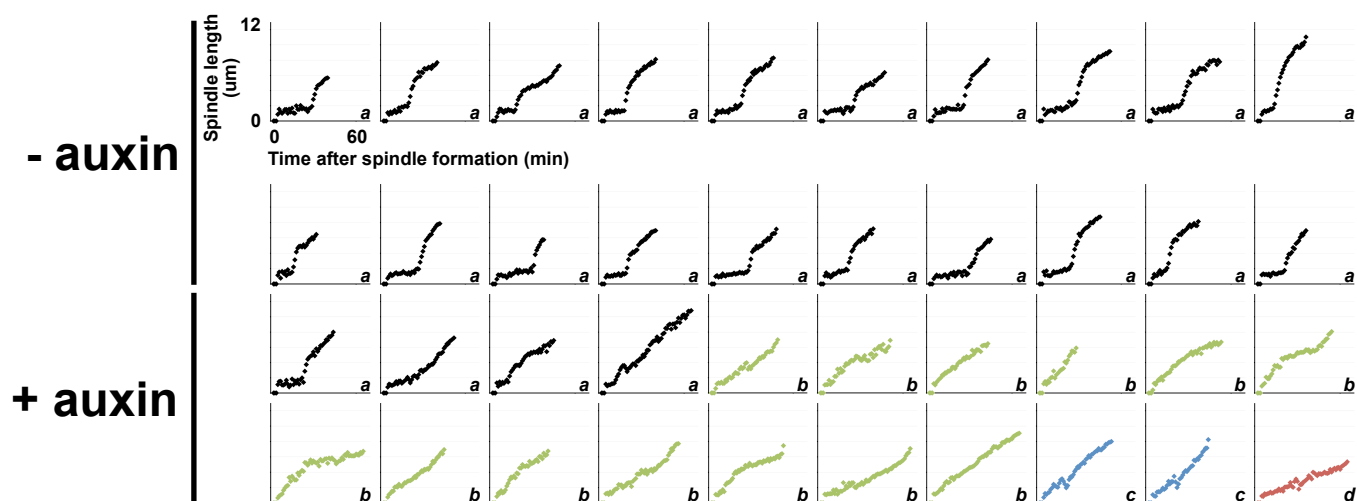
(A-B) *PDS1-AID pCUP1-eGFP-lacI ura3::lacO SPC42-mCherry* (ADR6724) and *cdc55*Δ *PDS1-AID ura3::lacO pCUP1-eGFP-lacI SPC42-mCherry* (ADR6722) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 25 minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Strains were imaged twice in each condition. Data from individual experiments were combined for subsequent analysis (*PDS1-AID* – auxin (n = 17), *PDS1-AID* + auxin (n = 26), *cdc55*Δ *PDS1-AID* – auxin (n = 14), *cdc55*Δ *PDS1-AID* + auxin (n = 19)).

(A) Pds1 depletion in *cdc55*Δ cells causes premature sister chromatid separation. The length of time between spindle formation and sister chromatid separation was determined for each imaged cell. If sister chromatid separation preceded spindle formation, this length of time was defined as 0. All measured times are shown along with the population means. Student's t-tests were used to test for significant differences (* p = 0.034, ** p = 0.001).

(B) A population of *cdc55*Δ cells depleted of Pds1 undergo sister chromatid separation before spindle formation. Inter-spindle pole body and inter-sister chromatid distance for an example cell in which sister chromatid separation preceded spindle formation.

(C) *CDC55* deletion in cells depleted of Pds1 does not cause premature Esp1 substrate cleavage. *PDS1-AID SCC1-GFP SLK19-myc13* (ADR6415) and *cdc55*Δ *PDS1-AID SCC1-GFP SLK19-myc13* (ADR6468) cells were grown to log phase at 25°C and arrested in G1 with α -factor. 30 minutes before α -factor release cells were treated with +/- auxin. Cells were released from G1 arrest at t = 0 and samples were taken for immunoblotting at the indicated timepoints (* indicates full length protein, ** indicates a cleavage product).

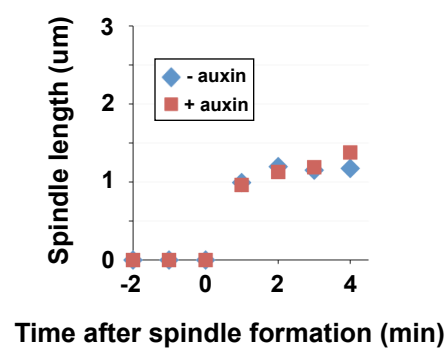
A *swe1* Δ *cdc55* Δ *PDS1-AID*



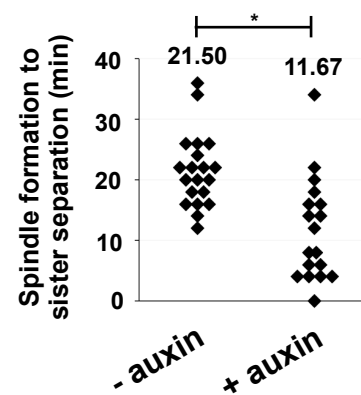
B

	Normal mitotic progression (a)	Immediate spindle elongation (b)	Abnormal spindle elongation/contraction (c)	Failed/no anaphase (d)
<i>swe1</i> Δ <i>cdc55</i> Δ <i>PDS1-AID</i> - auxin	100% (20/20)	0% (0/20)	0% (0/20)	0% (0/20)
<i>swe1</i> Δ <i>cdc55</i> Δ <i>PDS1-AID</i> + auxin	20% (4/20)	65% (13/20)	10% (2/20)	5% (1/20)

C



D



E

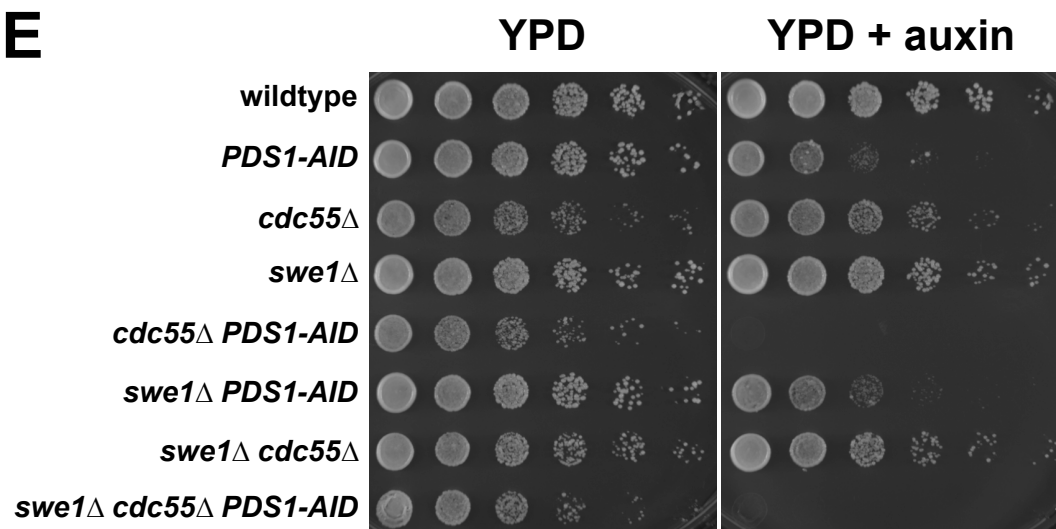


Figure 4-6

Figure 4-6. Rapid spindle elongation in *cdc55Δ* cells depleted of Pds1 requires Cdk1 inhibitory phosphorylation.

(A-C) *swe1Δ cdc55Δ PDS1-AID SPC42-eGFP* (ADR7043) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 25 minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Strains were imaged twice in each condition and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual *swe1Δ cdc55Δ* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(B) Pds1 depletion in *swe1Δ cdc55Δ* cells causes spindle defects. Population characteristics of each treatment based upon spindle morphology.

(C) Deleting *SWE1* prevents premature spindle elongation in *cdc55Δ* cells depleted of Pds1. The timepoint before spindle formation was defined as x = 0 for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(D) Depletion of Pds1 causes premature sister chromatid separation in *swe1Δ cdc55Δ* cells. *swe1Δ cdc55Δ PDS1-AID ura3::lacO pCUP1-eGFP-lacI SPC42-mCherry* (ADR7068) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 25 minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Strains were imaged twice in each condition. Data from individual experiments were combined for subsequent analysis (*swe1Δ cdc55Δ PDS1-AID* – auxin (n = 20), *swe1Δ cdc55Δ PDS1-AID* + auxin (n = 19)). The length of time between spindle formation and sister chromatid separation was determined for each imaged cell. If sister chromatid separation preceded spindle formation this length of time was defined as 0. All measured times are shown along with the population means. A student's t-test was used to test for a significant difference (* p = 0.002).

(E) Deleting *SWE1* does not rescue the sensitivity of *cdc55Δ* cells to Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *PDS1-AID* (ADR6428), *cdc55Δ* (ADR6436), *swe1Δ* (ADR7138), *cdc55Δ PDS1-AID* (ADR6426), *swe1Δ PDS1-AID* (ADR7143), *swe1Δ cdc55Δ* (ADR7140) and *swe1Δ cdc55Δ PDS1-AID* (ADR7145) cells were spotted onto the indicated plates and grown at 25°C.

display mitotic spindle morphology distinct from *cdc55Δ* cells depleted of Pds1 (Figure 4-6A). Most significantly, deleting *SWE1* causes a decrease in the number of cells displaying elongation and contraction of the mitotic spindle during metaphase (from 55% to 10%) and an increase in the number of cells that undergo immediate spindle elongation (from 28% to 65%) (Figure 4-6B). Thus Cdk1 inhibition appears to be largely responsible for the spindle elongation and contraction phenotype in *cdc55Δ* cells depleted of Pds1. The relative lack of cells displaying spindle elongation and contraction is likely due, at least in part, to the fact that *swe1Δ cdc55Δ* cells depleted of Pds1 do not undergo rapid spindle elongation immediately upon formation (Figure 4-6C). Instead, the majority of cells undergo relatively slow and steady spindle elongation that commences immediately upon spindle formation. To determine whether preventing Cdk1 phosphorylation also prevents premature sister chromatid separation, we depleted Pds1 in *swe1Δ cdc55Δ ura3::lacO* cells expressing LacI-GFP and Spc42-mCherry (Figure 4-6D). Sister chromatid separation occurs much earlier in *swe1Δ cdc55Δ* cells depleted of Pds1 as compared to untreated control cells. This demonstrates that the early spindle elongation phenotype of *cdc55Δ* cells depleted of Pds1 is not caused solely by lack of sister chromatid cohesion, as preventing Cdk1 inhibitory phosphorylation prevents early spindle elongation but not premature loss of sister chromatid cohesion. Clearly, PP2A^{Cdc55} must regulate spindle structure through additional substrates, as *swe1Δ cdc55Δ* cells depleted of Pds1 display striking mitotic spindle defects. To determine if Cdk1 inhibition contributes to the inviability of *cdc55Δ* cells depleted of Pds1, we asked whether *SWE1* deletion rescued the inviability of *cdc55Δ* cells depleted of Pds1 (Figure 4-6E). *swe1Δ cdc55Δ* are as sensitive to Pds1 depletion as *cdc55Δ* cells. From this data we conclude that Cdk1 inhibition causes rapid spindle elongation and spindle length contractions in *cdc55Δ* cells depleted of Pds1. However, an additional PP2A^{Cdc55} substrate must be

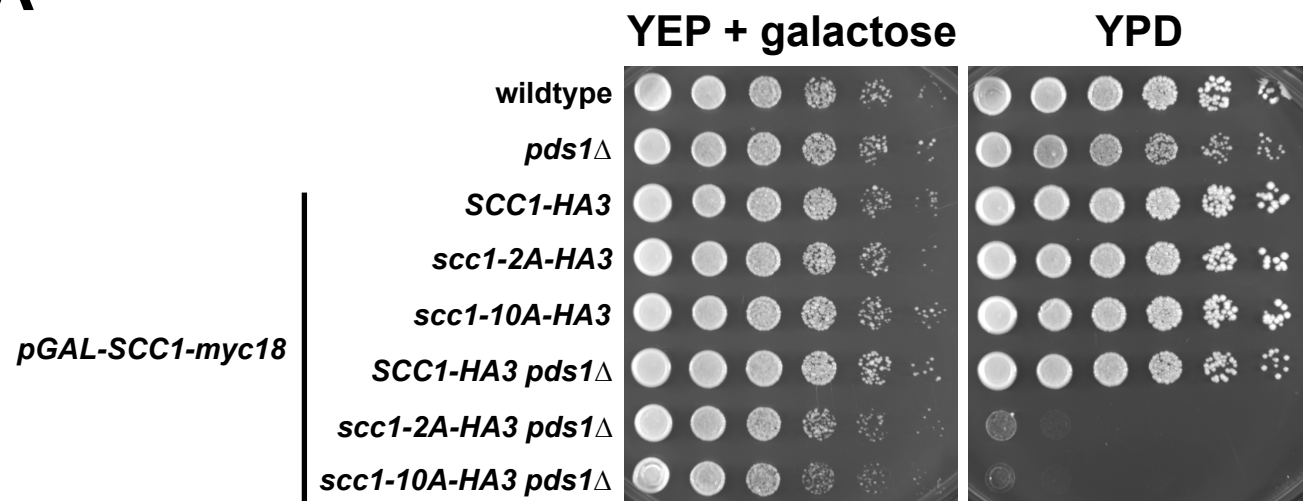
responsible for the steady spindle elongation, premature sister chromatid separation and inviability phenotypes of these cells.

It has previously been shown that Scc1 is phosphorylated by polo kinase (Cdc5) and this phosphorylation is responsible for anaphase regulation in the absence of *PDS1* (366). PP2A^{Cdc55} appears to be the phosphatase responsible for dephosphorylating these residues, suggesting that cells lacking Cdc55 and Pds1 could be inviable due to increased Scc1 phosphorylation (411). We found that, in agreement with Alexandru *et al.*, Scc1 phosphorylation is essential in the absence, but not the presence of *PDS1* (Figure 4-7A). In this assay *SCC1-HA3* rescues the slow growth phenotype of *pds1Δ* cells. We do not have an explanation for this, though adding HA tags could impact Scc1 stability. We next asked whether increased phosphorylation and destabilization of Scc1 in cells lacking *CDC55* could explain the mitotic defects seen in *cdc55Δ* cells depleted of Pds1 (Figure 4-7B). Surprisingly, we observed that preventing Scc1 phosphorylation using the *scc1-10A* allele does not cause inviability in cells depleted of Pds1. It is possible that in these experiments Pds1 is depleted enough to cause inviability in the absence of *CDC55*, but not enough to cause inviability in the absence of Scc1 phosphorylation. Regardless, preventing Scc1 phosphorylation does not rescue the lethality of *cdc55Δ* cells depleted of Pds1. This demonstrates that PP2A^{Cdc55} dephosphorylation of another substrate is required in the absence of Pds1.

4.2.4 *Esp1* is phosphorylated by *Cdk1^{Clb2}* and dephosphorylated by PP2A^{Cdc55}

Cdc55 has been shown to physically interact with Esp1 (331, 438). This suggests that in budding yeast, as in metazoan cells, Cdk1 may regulate Esp1 activity through phosphorylation and that PP2A^{Cdc55} may oppose this activity. Esp1 has six minimal Cdk1 sites (S/TP); two sites are found near the N-terminus (termed ‘N-terminal’), three sites near

A



B

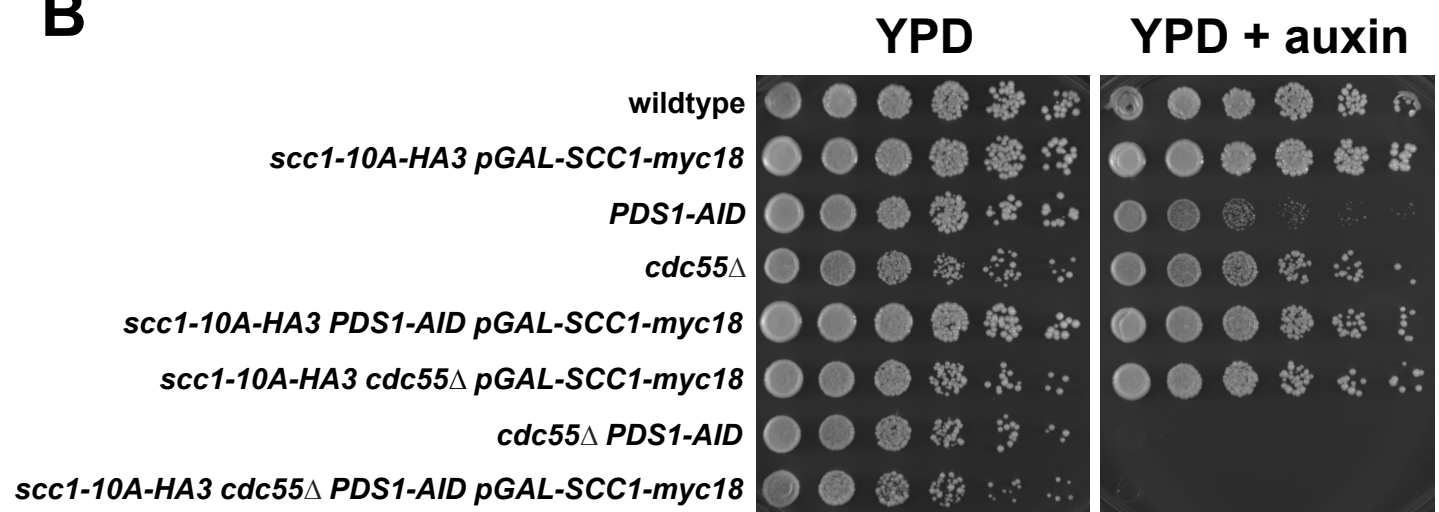


Figure 4-7

Figure 4-7. The lethality of Pds1 depletion to *cdc55*Δ cells is not due to Scc1 phosphorylation.

(A) Preventing Scc1 phosphorylation is lethal to *pds1*Δ cell. Eight-fold serial dilutions of wildtype (ADR4006), *pds1*Δ (ADR8339), *pGAL-SCC1* (ADR5013), *scc1-2A pGAL-SCC1* (ADR5015), *scc1-10A pGAL-SCC1* (ADR5014), *pds1*Δ *pGAL-SCC1* (ADR8336), *scc1-2A pds1*Δ *pGAL-SCC1* (ADR8341) and *scc1-10A pds1*Δ *pGAL-SCC1* (ADR8340) cells were spotted onto the indicated plates and grown at 25°C.

(B) *scc1-10A* does not rescue *cdc55*Δ cells depleted of Pds1. Eight-fold serial dilutions of wildtype (ADR22), *scc1-10A pGAL-SCC1* (ADR6635), *PDS1-AID* (ADR6428), *cdc55*Δ (ADR6436), *scc1-10A PDS1-AID pGAL-SCC1* (ADR6636), *scc1-10A cdc55*Δ *pGAL-SCC1* (ADR6639), *cdc55*Δ *PDS1-AID* (ADR6426) and *scc1-10A cdc55*Δ *PDS1-AID pGAL-SCC1* (ADR6642) cells were spotted onto the indicated plates and grown at 25°C.

the N-terminal end of the protease domain (termed ‘central’) and a single site close to the C-terminus (termed ‘C-terminal’) (Figure 4-8A). To determine if Esp1 undergoes Cdk1-dependent phosphorylation, we used in vivo metabolic labeling to look at the incorporation of ^{32}P phosphate into Esp1 in cells with both uninhibited and inhibited Cdk1 (Figure 4-8B). Wildtype cells incorporate ^{32}P phosphate and this incorporation is largely inhibited upon overexpression of the Cdk1 inhibitor Swe1. To identify the sites on which Esp1 is phosphorylated, we mutated each group of Cdk1 sites individually and in combination to unphosphorylatable alanine (A) or two phosphomimetic aspartic acid (DD) residues. All mutant Esp1 proteins constructed complement *ESP1* and are expressed at endogenous levels (Figure 4-8C). To determine which Cdk1 sites on Esp1 are phosphorylated in vivo we arrested cells expressing non-phosphorylatable Esp1 mutants in mitosis using benomyl and observed Esp1 migration on a polyacrylamide gel (Figure 4-8D). Preventing Esp1 phosphorylation on the three central residues results in a faster-migrating form of Esp1, suggesting that phosphorylation is prevented in this mutant. Mutation of the N-terminal cluster or C-terminal site has no effect on Esp1 mobility. To confirm that mutating the central sites on Esp1 prevents Esp1 phosphorylation, we used in vivo metabolic labeling of unphosphorylatable Esp1 mutants (Figure 4-8E). Mutants that are unable to be phosphorylated in the central region do not incorporate ^{32}P phosphate in vivo. This demonstrates that Esp1 is phosphorylated on one or more of these three residues. To determine directly if Cdk1 is responsible for phosphorylating these residues, we incubated purified wildtype Esp1 and unphosphorylatable Esp1 mutants with purified Cdk1^{C1b2} (Figure 4-8F). Esp1 is phosphorylated in vitro by Cdk1^{C1b2}, and mutating the central region prevents Cdk1 phosphorylation. Therefore we conclude that Cdk1^{C1b2} phosphorylates Esp1 in vivo and

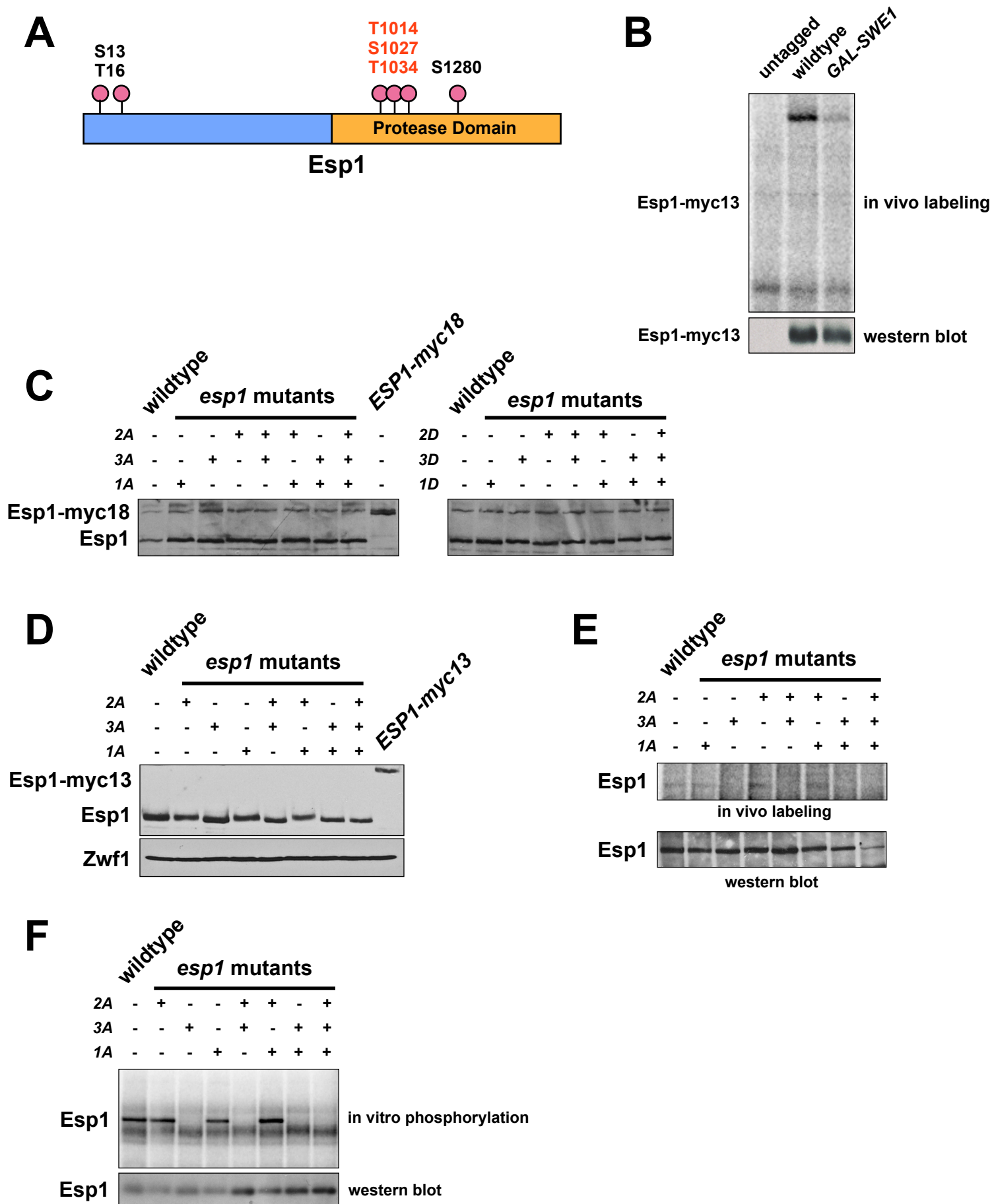


Figure 4-8

Figure 4-8. Cdk1^{Cib2} phosphorylates Esp1.

(A) Esp1 contains six minimal Cdk1 consensus sites (S/TP). S13 and T16 (termed *N-terminal*), T1014, S1027 and T1034 (termed *central*) and S1280 (termed *C-terminal*). The central sites are highlighted in red because they contain the phosphorylated residue(s) (see below).

(B) Esp1 is phosphorylated in vivo. In vivo labeling was used to incorporate ³²P phosphate into asynchronous log phase *ESP1* (ADR21), *ESP1-myc13* (ADR2146) and *pGAL-SWE1 ESP1-myc13* (ADR4854) cells. Esp1-myc13 was immunoprecipitated and run on a polyacrylamide gel. Esp1 phosphorylation was detected using a phosphorimager and Esp1 protein was detected by immunoblot.

(C) Mutating putative Cdk1 sites on Esp1 does not destabilize Esp1. Wildtype (ADR4006), *esp1-1A* (ADR6714), *esp1-3A* (ADR6715), *esp1-2A* (ADR6716), *esp1-2A + 3A* (ADR6717), *esp1-2A + 1A* (ADR6718), *esp1-3A + 1A* (ADR6774), *esp1-2A + 3A + 1A* (ADR6775), *ESP1-myc18* (ADR5509), *esp1-1D* (ADR5898), *esp1-3D* (ADR5909), *esp1-2D* (ADR5899), *esp1-2D + 3D* (ADR5901), *esp1-2D + 1D* (ADR5910), *esp1-3D + 1D* (ADR5900) and *esp1-2D + 3D + 1D* (ADR5635) cells were grown to log phase and samples were taken for immunoblotting.

(D) Esp1 is phosphorylated on the central region in vivo. Wildtype (ADR4006), *esp1-2A* (ADR6716), *esp1-3A* (ADR6715), *esp1-1A* (ADR6714), *esp1-2A + 3A* (ADR6717), *esp1-2A + 1A* (ADR6718), *esp1-3A + 1A* (ADR6774), *esp1-2A + 3A + 1A* (ADR6775) and *ESP1-myc18* (ADR5509) cells were grown to log phase at 25°C, arrested with nocodazole and samples were taken for immunoblotting.

(E) Mutating Esp1 central residues prevents Esp1 phosphorylation in vivo. In vivo labeling was used to incorporate ³²P phosphate into asynchronous log phase *ESP1* (ADR4006), *esp1-1A* (ADR6714), *esp1-3A* (ADR6715), *esp1-2A* (ADR6716), *esp1-3A + 2A* (ADR6717), *esp1-2A + 1A* (ADR6718), *esp1-3A + 1A* (ADR6774) and *esp1-2A + 3A + 1A* (ADR6775) cells. Esp1 was immunoprecipitated and run on a polyacrylamide gel. Esp1 phosphorylation was detected using a phosphorimager and Esp1 protein was detected by immunoblot.

(F) Cdk1^{Cib2} phosphorylates the central region of Esp1 in vitro. Esp1 was immunoprecipitated from wildtype (ADR4006), *esp1-2A* (ADR6716), *esp1-3A* (ADR6715), *esp1-1A* (ADR6714), *esp1-2A + 3A* (ADR6717), *esp1-2A + 1A* (ADR6718), *esp1-3A + 1A* (ADR6774) and *esp1-2A + 3A + 1A* (ADR6775) cells. Immunoprecipitated Esp1 was incubated with immunoprecipitated Cdk1^{Cib2} and samples were run on a polyacrylamide gel. Phosphorylation was detected using a phosphorimager and Esp1 protein levels was determined by immunoblot. *The experiments in (B) and (E) were completed by Dr. A. Rudner, the experiment in (C) was completed by C. Dore, the experiment in (F) was completed by C. Dore and L. Williams.

in vitro on one or more of the central residues. Attempts at mass spectrometric identification of sites have so far only detected two peptides with phosphorylated S1027 (data not shown).

We then asked whether PP2A^{Cdc55} is responsible for dephosphorylating Esp1 on these sites. Cells lacking PP2A^{Cdc55} activity display increased Esp1 phosphorylation, as more ³²P phosphate is incorporated into Esp1 purified from cells lacking *CDC55* than wildtype controls (Figure 4-9A). To determine if PP2A^{Cdc55} dephosphorylates these residues directly, we used purified PP2A^{Cdc55} in an in vitro dephosphorylation reaction with Cdk1^{Clb2} phosphorylated Esp1 as a substrate (Figure 4-9B). In this assay, PP2A^{Cdc55} is able to dephosphorylate Cdk1^{Clb2} phosphorylated Esp1. This dephosphorylation is due to PP2A^{Cdc55} activity, as dephosphorylation is inhibited by the PP2A inhibitor okadaic acid. Taken together, this data demonstrates that Esp1 is phosphorylated by Cdk1 and dephosphorylated by PP2A^{Cdc55} in vivo.

4.2.5 Mimicking phosphorylation on Esp1 recapitulates cdc55Δ phenotypes

The observation that PP2A^{Cdc55} dephosphorylates Esp1 suggests that perhaps the spindle defects observed in *cdc55Δ* cells depleted of Pds1 are due to increased Esp1 phosphorylation. If increased Esp1 phosphorylation in cells lacking *CDC55* were causing spindle defects, we would expect that depletion of Pds1 in cells expressing a phosphomimetic Esp1 mutant (Esp1-3D), in which all three sites in the central region are mutated to two aspartic acid residues (SP to DD), would cause similar spindle defects to depletion of Pds1 in *cdc55Δ* cells. We first determined if expressing Esp1-3D caused viability defects in cells depleted of Pds1 (Figure 4-10A). Indeed, *esp1-3D* cells display increased sensitivity to Pds1 depletion than wildtype cells, and *esp1-3A* cells display decreased sensitivity to Pds1 depletion than wildtype cells. To determine if the sensitivity of

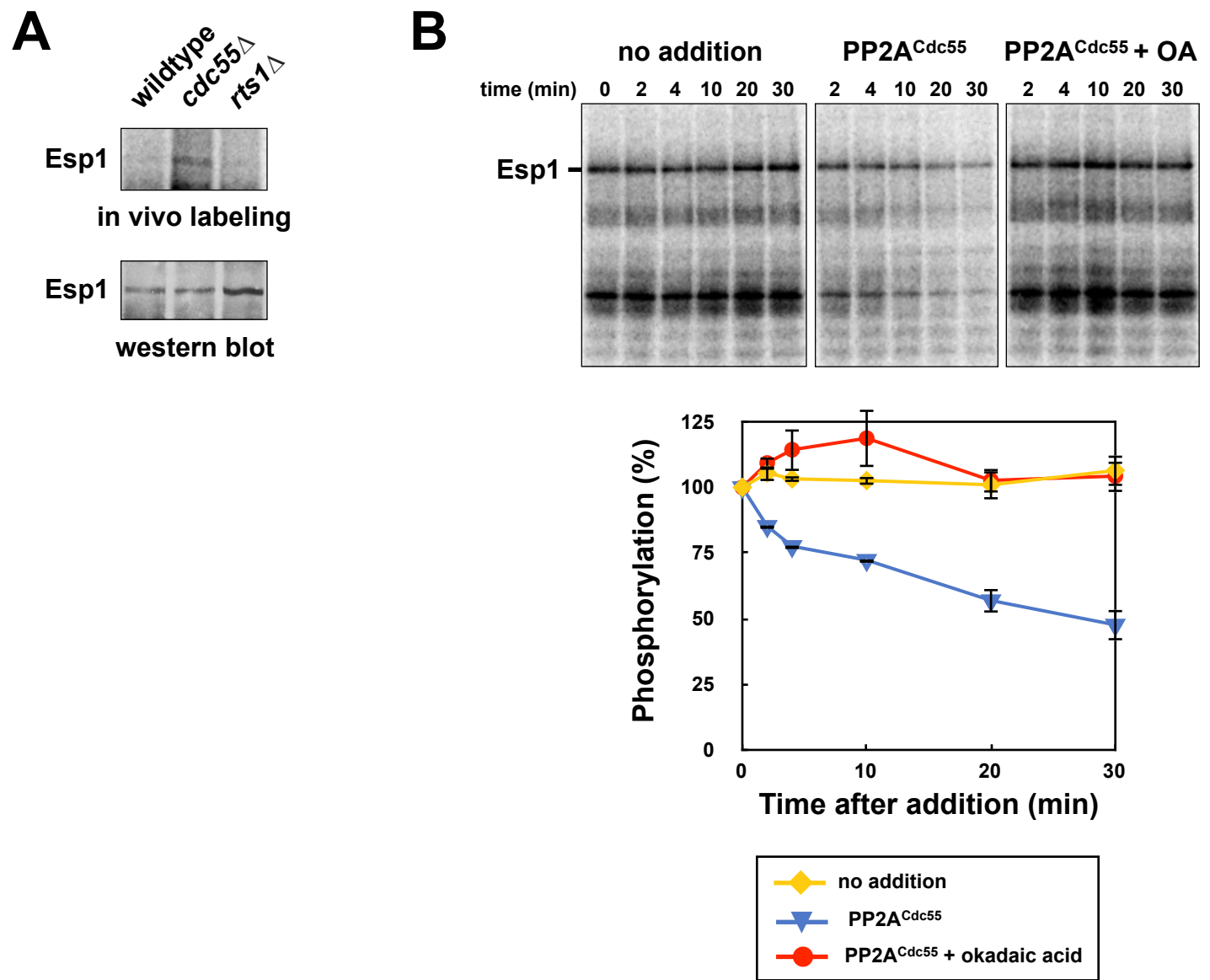


Figure 4-9

Figure 4-9. PP2A^{Cdc55} dephosphorylates Esp1.

(A) Deleting *CDC55* increases Esp1 phosphorylation in vivo. In vivo labeling was used to incorporate ³²P phosphate into asynchronous log phase wildtype (ADR4006), *cdc55Δ* (ADR6876) and *rtslΔ* (ADR6929) cells. Esp1 was immunoprecipitated and run on a polyacrylamide gel. Esp1 phosphorylation was detected using a phosphorimager and Esp1 protein was detected by immunoblot.

(B) PP2A^{Cdc55} dephosphorylates Esp1 in vitro. Immunoprecipitated Cdk1^{Clb2} was first used to phosphorylate immunoprecipitated Esp1 in vitro. Phosphorylated Esp1 was then incubated +/- immunoprecipitated PP2A^{Cdc55}. The phosphorylated Esp1 incubated with PP2A^{Cdc55} was incubated +/- okadaic acid (OA) concentration? Should be the same as in our paper.. Samples were taken at the indicated timepoints and run on a polyacrylamide gel. Esp1 phosphorylation was detected (top) and quantified (bottom) using a phosphorimager. *The experiment in (A) was completed by Dr. A. Rudner. The experiment in (B) was completed by C. Dore and L. Williams.

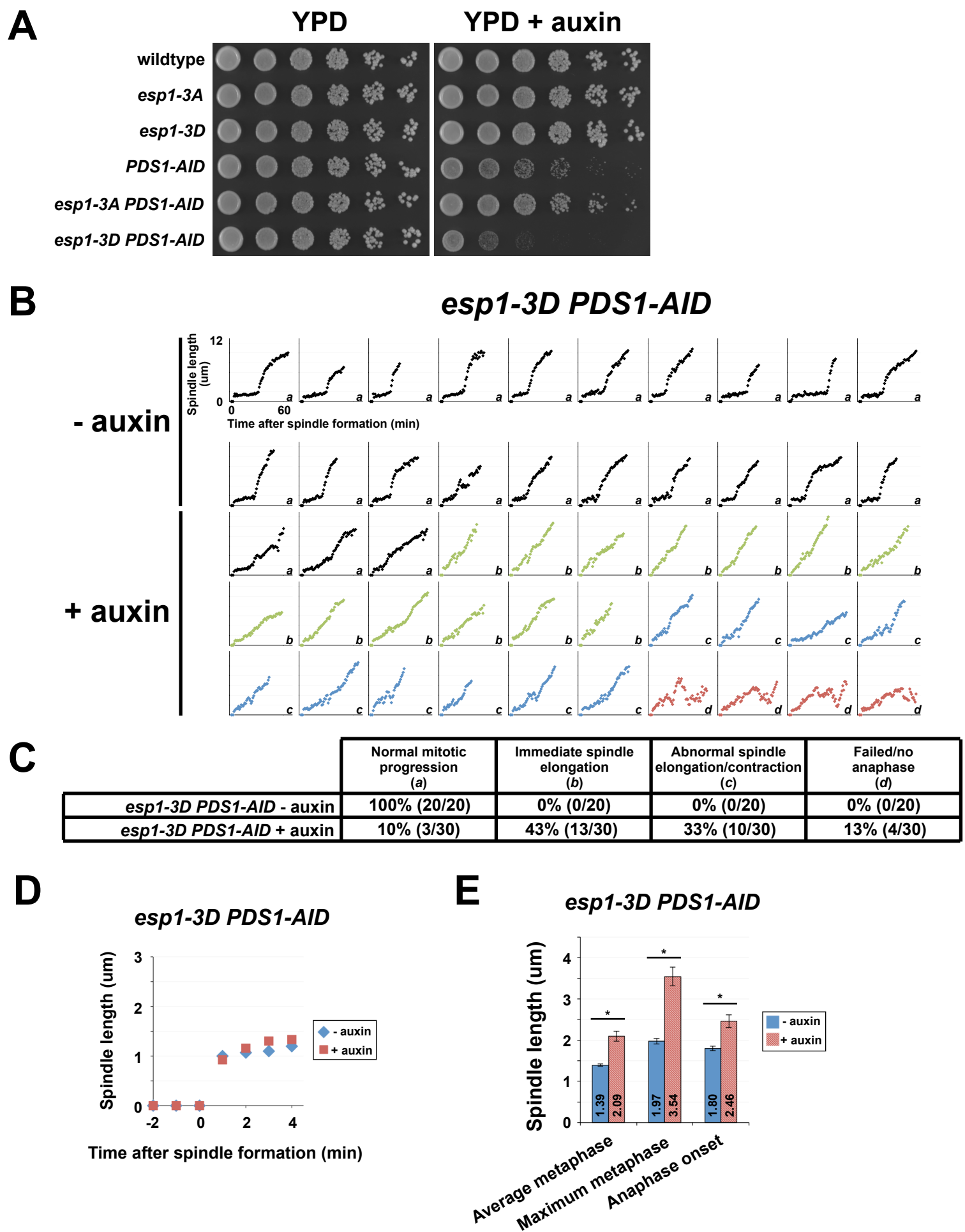


Figure 4-10

Figure 4-10. Pds1 depletion causes spindle defects in a phosphomimetic Esp1 mutant.

(A) Pds1 depletion causes synthetic sickness in *esp1-3D* cells. Eight-fold serial dilutions of wildtype (ADR4006), *esp1-3A* (ADR6715), *esp1-3D* (ADR6953), *PDS1-AID* (ADR6387), *esp1-3A PDS1-AID* (ADR6956) and *esp1-3D PDS1-AID* (ADR6448) cells were spotted onto the indicated plates and grown at 25°C. (B-E) *esp1-3D PDS1-AID SPC42-eGFP* (ADR6446) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. The *esp1-3D PDS1-AID SPC42-eGFP* strain was imaged twice – auxin and three times + auxin and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(B) Mitotic spindle morphology of individual *esp1-3D* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated ‘a’. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated ‘b’. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated ‘c’. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated ‘d’.

(C) Pds1 depletion in *esp1-3D* cells causes spindle defects. Population characteristics of each strain and treatment combination based upon spindle morphology.

(D) Pds1 depletion in *esp1-3D* cells does not cause premature spindle elongation. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(E) Pds1 depletion in *esp1-3D* cells significantly increases average and maximum metaphase spindle lengths and spindle length at anaphase onset. In cells that displayed both metaphase and anaphase, average and maximum spindle lengths during metaphase and spindle length at anaphase onset were determined. Displayed values are (mean +/- SEM). Student’s t-tests were used to test for significant differences (* $p < 0.001$).

esp1-3D cells to Pds1 depletion is correlated with spindle defects, we used live imaging to monitor mitotic progression in this mutant (Figure 4-10B). Mitotic progression in *esp1-3D* cells depleted of Pds1 is characterized by immediate spindle elongation in 43% of cells, as well as spindle elongation and contraction during metaphase in 33%, a phenotype reminiscent of *cdc55Δ* cells depleted of Pds1 (Figure 4-10C). *esp1-3D* cells depleted of Pds1 do not display the *SWE1*-dependent rapid spindle elongation phenotype of *cdc55Δ* cells depleted of Pds1 (Figure 4-10D). However, *esp1-3D* cells depleted of Pds1 do display increased average spindle length at anaphase onset and during metaphase, as well as increased maximum spindle length during metaphase (Figure 4-10E). Length at anaphase onset is similar in *esp1-3D* and *cdc55Δ* cells depleted of Pds1, whereas average and maximum length during metaphase is less in *esp1-3D* cells (compare Figure 4-10E and Figure 4-3D). This is likely due to the fact that spindles in *cdc55Δ* cells depleted of Pds1 elongate rapidly upon formation, while spindles in *esp1-3D* cells do not.

If phosphomimetic Esp1 is hypermorphic, cells expressing Esp1-3D should still be sensitive to Pds1 depletion in the presence wildtype Esp1 protein. Indeed, *esp1-3D* expressing Esp1 on a centromeric plasmid are just as sensitive to Pds1 depletion as cells carrying a vector alone (Figure 4-11A). Surprisingly, the *esp1-3A* allele is also dominant (Figure 4-11B).

To determine if spindle defects in *esp1-3D* cells depleted of Pds1 are associated with premature sister chromatid separation, we imaged live *esp1-3D ura3::lacO* cells expressing lacI-GFP and Spc42-mCherry. *esp1-3D* cells depleted of Pds1 do not display premature sister chromatid separation (Figure 4-12A). However, loss of sister chromatid cohesion in these cells may escape observation due to the absence of rapid spindle elongation in *esp1-3D* cells depleted of Pds1. Alternatively, the spindle defects observed in these cells could be

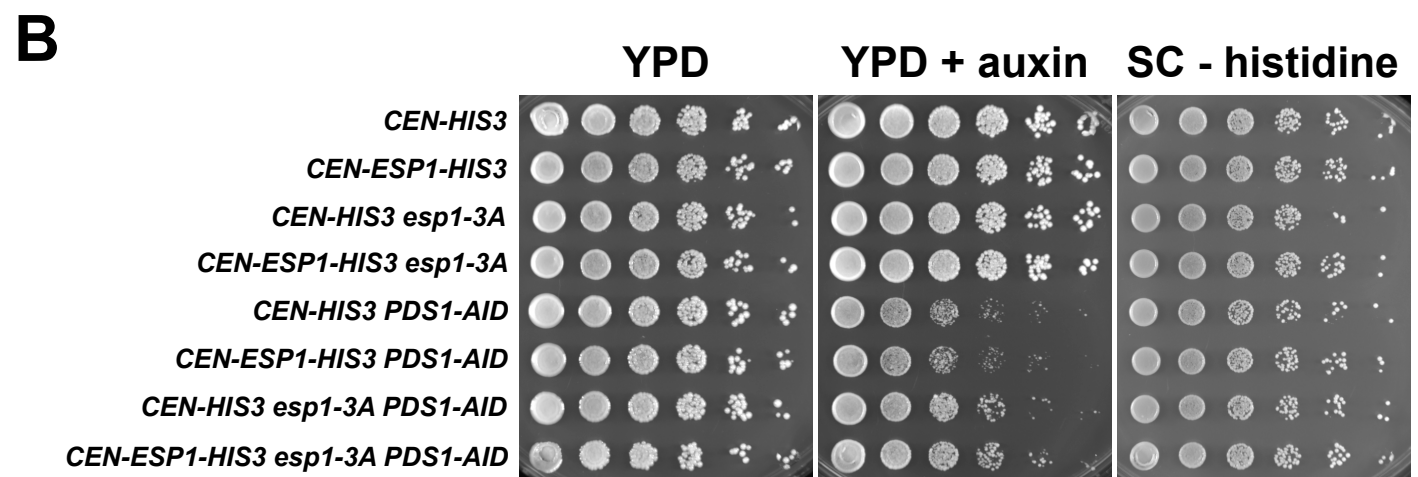
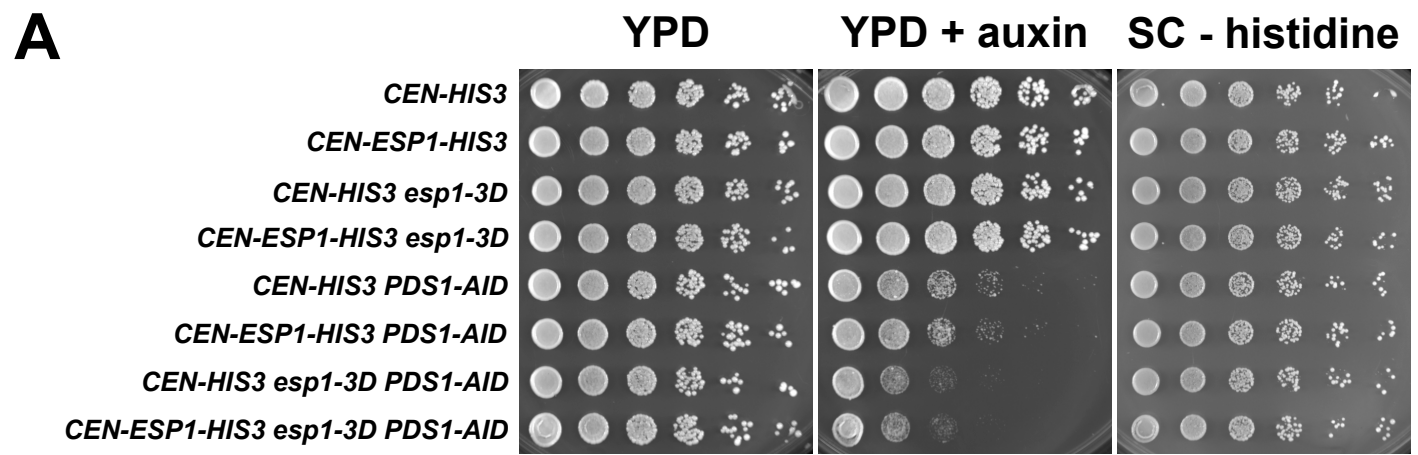


Figure 4-11

Figure 4-11. *esp1-3D* and *esp1-3A* are dominant.

(A) The *esp1-3D* allele is dominant. *CEN-HIS3* (ADR8635), *CEN-ESP1-HIS3* (ADR8534), *CEN-HIS3 esp1-3D* (ADR8636), *CEN-ESP1-HIS3 esp1-3D* (ADR8538), *CEN-HIS3 PDS1-AID* (ADR8640), *CEN-ESP1-HIS3 PDS1-AID* (ADR8536), *CEN-HIS3 esp1-3D PDS1-AID* (ADR8637) and *CEN-ESP1-HIS3 esp1-3D PDS1-AID* (ADR8539) cells were grown to saturation in YPD media, spotted onto the indicated plates and grown at 25°C.

(B) The *esp1-3A* allele is dominant. *CEN-HIS3* (ADR8635), *CEN-ESP1-HIS3* (ADR8534), *CEN-HIS3 esp1-3A* (ADR8638), *CEN-ESP1-HIS3 esp1-3A* (ADR8535), *CEN-HIS3 PDS1-AID* (ADR8640), *CEN-ESP1-HIS3 PDS1-AID* (ADR8536), *CEN-HIS3 esp1-3A PDS1-AID* (ADR8639) and *CEN-ESP1-HIS3 esp1-3A PDS1-AID* (ADR8537) cells were grown to saturation in YPD media, diluted eight-fold serially, spotted onto the indicated plates and grown at 25°C.

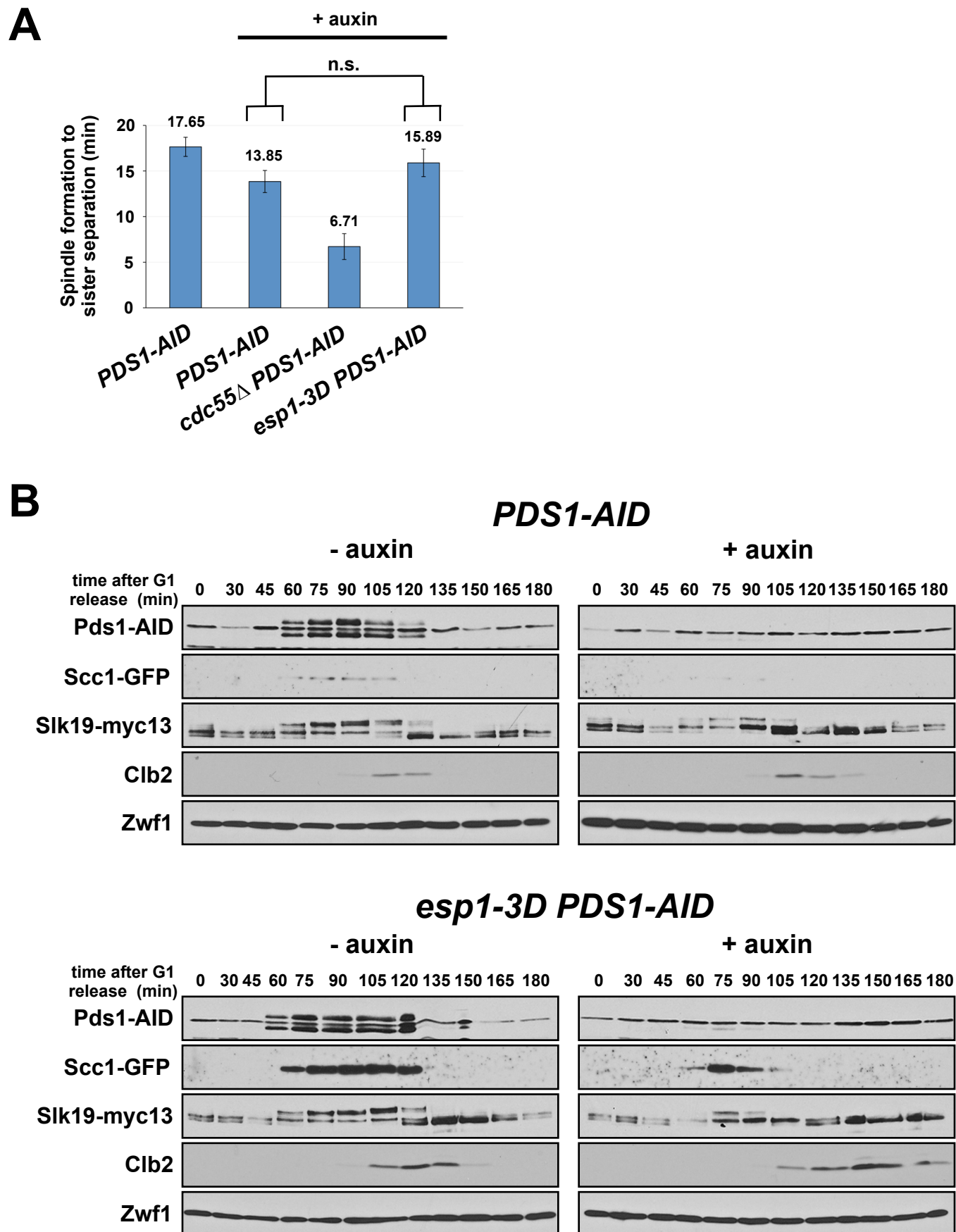


Figure 4-12

Figure 4-12. Pds1 depletion does not cause premature sister chromatid separation in a phosphomimetic Esp1 mutant.

(A) Pds1 depletion does not cause premature sister chromatid separation in a phosphomimetic Esp1 mutant. *esp1-3D PDS1-AID ura3::lacO pCUP1-eGFP-lacI SPC42-mCherry* (ADR6886) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads + auxin and imaged. Strains were imaged on two separate occasions. Data from individual experiments were combined for subsequent analysis ($n = 19$). The length of time between spindle formation and sister chromatid separation was determined for each imaged cell. *esp1-3D PDS1-AID* + auxin cells from this experiment are compared to the cells imaged in Figure 4-5A. Displayed values are (mean $\pm$ SEM).

(B) Mimicking Esp1 phosphorylation in cells depleted of Pds1 does not cause premature Esp1 substrate cleavage. *PDS1-AID SCC1-GFP SLK19-myc13* (ADR6415) and *esp1-3D PDS1-AID SCC1-GFP SLK19-myc13* (ADR6952) cells were grown to log phase at 25°C and arrested in G1 with α -factor. 30 minutes before α -factor release cells were treated with +/- auxin. Cells were released from G1 arrest at $t = 0$ and samples were taken for immunoblotting at the indicated timepoints. (* indicates full length protein, ** indicates a cleavage product).

unrelated to sister chromatid cohesion loss. Both Scc1 and Slk19 are degraded prematurely upon Pds1 depletion in both wildtype and *esp1-3D* cells and, as we saw for *cdc55Δ* cells, mimicking phosphorylation on Esp1 does not further advance Esp1 cleavage of Scc1 and Slk19 (Figure 4-12B). Scc1 appears to be present at significantly higher levels in *esp1-3D* cells regardless of Pds1 depletion. We are unsure of the reason for this, though it should be noted that *scc1-HA3* rescues the slow growth of *pds1Δ* cells (Figure 4-7A), so it is possible that C-terminal tagging of Scc1 may interfere with Scc1 function. Regardless, this data, in combination with that in Figure 4-3, strongly suggests that the spindle defects observed in *cdc55Δ* and *esp1-3D* cells depleted of Pds1 are not solely due to loss of sister chromatid cohesion through premature Scc1 cleavage but through an additional mechanism.

4.2.6 Preventing Esp1 phosphorylation does not rescue cell cycle defects in *cdc55Δ* cells

Since mimicking phosphorylation on Esp1 causes phenotypes similar to deleting *CDC55*, we asked whether phenotypes characteristic of deleting *CDC55* could be rescued by preventing phosphorylation of Esp1. Preventing phosphorylation on the central region of Esp1 using the *esp1-3A* allele does not rescue the sensitivity of *cdc55Δ* cells to Pds1 depletion (Figure 4-13A). We next examined if preventing Esp1 phosphorylation could rescue the sensitivity of *cdc55Δ* cells to the spindle poison benomyl (Figure 4-13B). Since we had shown that rapid spindle elongation at anaphase onset in *cdc55Δ* cells depleted of Pds1 was *SWE1*-dependent, we also determined whether deleting *SWE1* rescued *cdc55Δ* sensitivity to benomyl. *esp1-3A* and *swe1Δ*, either individually or in combination, do not rescue the sensitivity of *cdc55Δ* cells to benomyl. We have previously shown that preventing phosphorylation of the APC partially rescues *cdc55Δ* cells exposed to nocodazole (Figure 3-

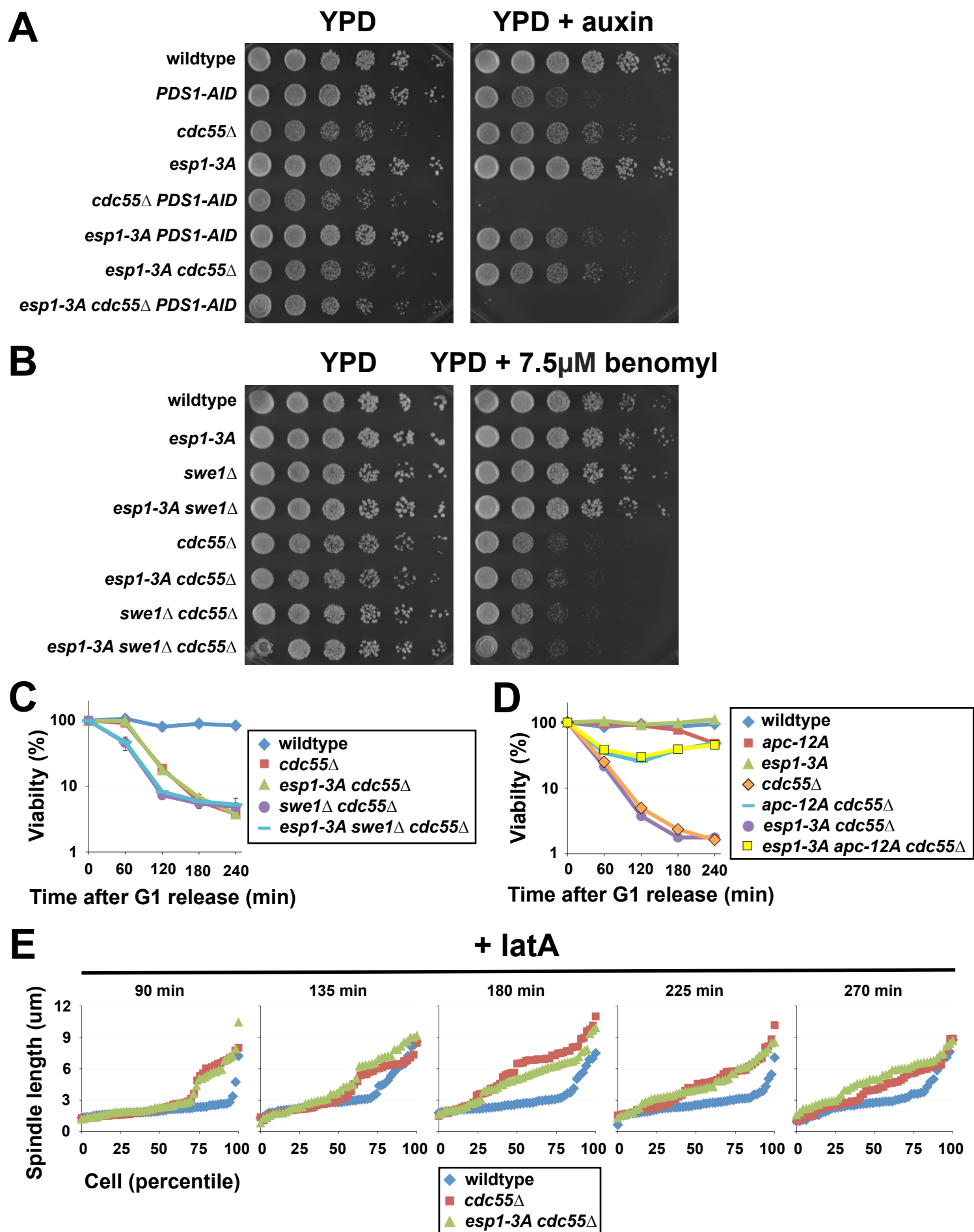


Figure 4-13

Figure 4-13. Preventing Esp1 phosphorylation does not rescue phenotypes associated with deleting *CDC55*.

(A) Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *PDS1-AID* (ADR6428), *cdc55Δ* (ADR6436), *esp1-3A* (ADR7195), *cdc55Δ PDS1-AID* (ADR6426), *esp1-3A PDS1-AID* (ADR6863), *esp1-3A cdc55Δ* (ADR6879) and *esp1-3A cdc55Δ PDS1-AID* (ADR6870) cells were spotted onto the indicated plates and grown at 25°C.

(B) Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to nocodazole. Eight-fold serial dilutions of wildtype (ADR4006), *esp1-3A* (ADR6715), *swe1Δ* (ADR7134), *esp1-3A swe1Δ* (ADR7135), *cdc55Δ* (ADR6876), *esp1-3A cdc55Δ* (ADR6748), *swe1Δ cdc55Δ* (ADR7136) and *esp1-3A swe1Δ cdc55Δ* (ADR7137) cells were spotted onto the indicated plates and grown at 25°C.

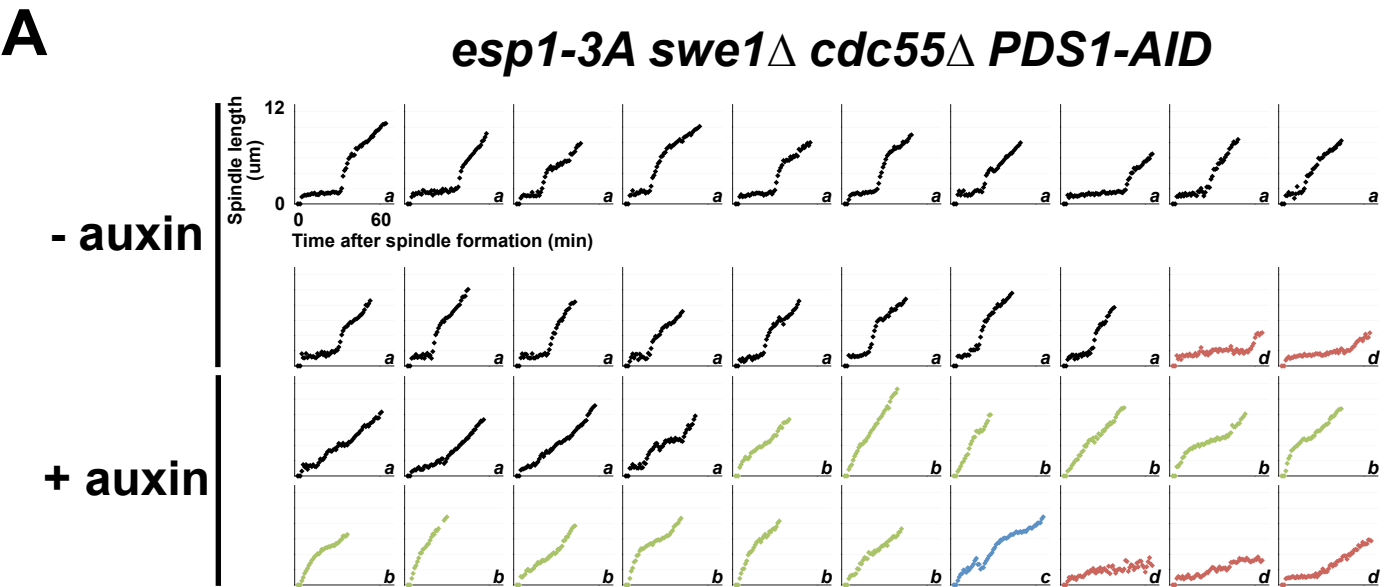
(C) Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to short-term nocodazole exposure. Wildtype (ADR4006), *cdc55Δ* (ADR6876), *esp1-3A cdc55Δ* (ADR6748), *swe1Δ cdc55Δ* (ADR7136) and *esp1-3A swe1Δ cdc55Δ* (ADR7137) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with nocodazole at $t = 35$ minutes and samples were taken for viability assays at the indicated timepoints. Viability assays were repeated twice. Displayed values are (mean $\pm$ SEM).

(D) Preventing Esp1 phosphorylation does not improve *apc-12A* rescue of *cdc55Δ* sensitivity to short-term nocodazole exposure. Wildtype (ADR4006), *apc-12A* (ADR6948), *esp1-3A* (ADR6715), *cdc55Δ* (ADR6876), *apc-12A cdc55Δ* (ADR6949), *esp1-3A cdc55Δ* (ADR6950) and *esp1-3A apc-12A cdc55Δ* (ADR6951) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). Cells were treated with nocodazole at $t = 35$ minutes and samples were taken for viability assays at the indicated timepoints. Displayed values are the result of a single experiment.

(E) Preventing Esp1 phosphorylation does not prevent *cdc55Δ* bypass of the morphogenesis checkpoint. *SPC42-eGFP* (ADR4009) *cdc55Δ SPC42-eGFP* (ADR4738) and *esp1-3A cdc55Δ SPC42-eGFP* (ADR7459) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). latA was added at $t = 25$ minutes, α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1 and samples were fixed for microscopy at the indicated timepoints.

27). In these experiments, preventing phosphorylation of the APC can rescue the sensitivity of *cdc55Δ* cells to nocodazole over short-term incubations. We therefore determined whether *swe1Δ* or *esp1-3A* rescued the sensitivity of *cdc55Δ* cells to short-term nocodazole exposure (Figure 4-13C). The *swe1Δ* and *esp1-3A* alleles, individually or in combination, do not improve the viability of *cdc55Δ* cells in nocodazole. Additionally, preventing Esp1 phosphorylation does not further increase the viability of *cdc55Δ* cells expressing unphosphorylatable APC subunits (Figure 4-13D). *cdc55Δ* cells also bypass the morphogenesis checkpoint (429). Therefore, we asked whether preventing Esp1 phosphorylation prevents bypass of this checkpoint in *cdc55Δ* cells. We activated the morphogenesis checkpoint in wildtype, *cdc55Δ* and *cdc55Δ esp1-3A* cells using the actin-depolymerizing agent latA and monitored spindle elongation in fixed cells (Figure 4-13E). Deleting *CDC55* allows spindles to elongate under latA treatment while the majority of wildtype cells arrest with short spindles. Preventing Esp1 phosphorylation does not prevent spindle elongation in *cdc55Δ* cells. Taken together, this data indicates that a) the *esp1-3A* allele impacts Esp1 function independent of phosphorylation prevention; and/or b) PP2A^{Cdc55} has additional mitotic substrates and *cdc55Δ* phenotypes are due to the misregulation of multiple pathways.

Since we have observed that a) rapid spindle elongation in *cdc55Δ* cells depleted of Pds1 can be rescued by deleting *swe1Δ* and b) spindle elongation and contraction can be recapitulated by mimicking phosphorylation of Esp1, we tested whether deleting *SWE1* and preventing Esp1 phosphorylation using the *esp1-3A* allele could rescue both of these phenotypes in *cdc55Δ* cells depleted of Pds1. Live imaging of *swe1Δ esp1-3A cdc55Δ* cells depleted of Pds1 demonstrated that these cells display similar spindle phenotypes to *swe1Δ cdc55Δ* cells depleted of Pds1 (Figure 4-14A). 60% of cells undergo immediate spindle



B

	Normal mitotic progression (a)	Immediate spindle elongation (b)	Abnormal spindle elongation/contraction (c)	Failed/no anaphase (d)
<i>esp1-3A swe1Δ cdc55Δ PDS1-AID</i> - auxin	90% (18/20)	0% (0/20)	0% (0/20)	10% (2/20)
<i>esp1-3A swe1Δ cdc55Δ PDS1-AID</i> + auxin	20% (4/20)	60% (12/20)	5% (1/20)	15% (3/20)

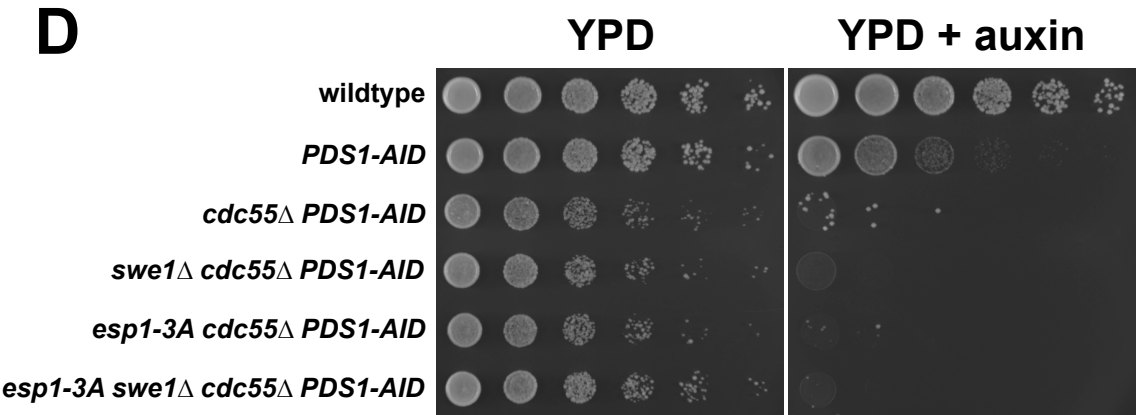


Figure 4-14

Figure 4-14. Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to Pds1 depletion.

(A-C) *esp1-3A swe1Δ cdc55Δ PDS1-AID SPC42-eGFP* (ADR7317) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 25 minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Each condition was imaged twice and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual *esp1-3A swe1Δ cdc55Δ* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(B) Preventing Esp1 phosphorylation does not rescue *cdc55Δ* sensitivity to Pds1 depletion. Population characteristics of each strain and treatment combination based upon spindle morphology.

(C) Spindles form normally in *esp1-3A swe1Δ cdc55Δ* cells depleted of Pds1. The timepoint before spindle formation was defined as x = 0 for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(D) Preventing Esp1 phosphorylation and Cdk1 inhibitory phosphorylation does not rescue the sensitivity of *cdc55Δ* cells to Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *PDS1-AID* (ADR6428), *cdc55Δ PDS1-AID* (ADR6426), *swe1Δ cdc55Δ PDS1-AID* (ADR7145), *esp1-3A cdc55Δ PDS1-AID* (ADR6870) and *esp1-3A swe1Δ cdc55Δ PDS1-AID* (ADR7149) cells were spotted onto the indicated plates and grown at 25°C.

elongation (Figure 4-14B), and immediate elongation upon spindle formation is not as rapid as in *cdc55Δ* cells depleted of Pds1 (Figure 4-14C). Deletion of *SWE1* and prevention of Esp1 phosphorylation in the same strain does not rescue the lethality of *cdc55Δ* cells depleted of Pds1 (Figure 4-14D). We next monitored Esp1 substrate cleavage in a synchronous population of unperturbed *esp1-3A* cells (Figure 4-15). *esp1-3A* cells cleave Scc1 and Slk19 with the same timing as wildtype controls while exiting a mitotic arrest. Furthermore, *esp1-3A* cells degrade Pds1 and Clb2 with similar timing. This data, taken together, demonstrates that, in addition to Swe1 and Esp1, at least one additional PP2A^{Cdc55} substrate is responsible for the mitotic defects in *cdc55Δ* cells depleted of Pds1.

4.2.7 Mimicking Esp1 phosphorylation causes subtle cell cycle defects

We next focused on better characterizing the *esp1-3D* allele. Since both *cdc55Δ* and *esp1-3D* cells depleted of Pds1 exhibit mitotic spindle defects, we wanted to know if the spindle checkpoint was activated under these conditions. Therefore, we imaged mitotic spindles in live *cdc55Δ* and *esp1-3D* cells depleted of Pds1 and lacking the checkpoint component Mad2 (Figure 4-16). Both *mad2Δ cdc55Δ* and *mad2Δ esp1-3D* cells depleted of Pds1 exhibit similar spindle defects during mitosis to *cdc55Δ* and *esp1-3D* cells depleted of Pds1 (compare Figure 4-3 and Figure 4-10 to Figure 4-16A and B). This demonstrates that either these cells a) do not activate the spindle checkpoint or b) activate the spindle checkpoint but also function downstream of the APC^{Cdc20} and subsequently bypass checkpoint inhibition of APC^{Cdc20} activity.

To determine if mimicking Esp1 phosphorylation perturbed cell cycle progression, we looked at mitotic markers in *esp1-3D* cells by immunoblot (Figure 4-17A). In cells released from a G1 arrest, Scc1 appears to be cleaved ~10 minutes earlier in *esp1-3D* cells

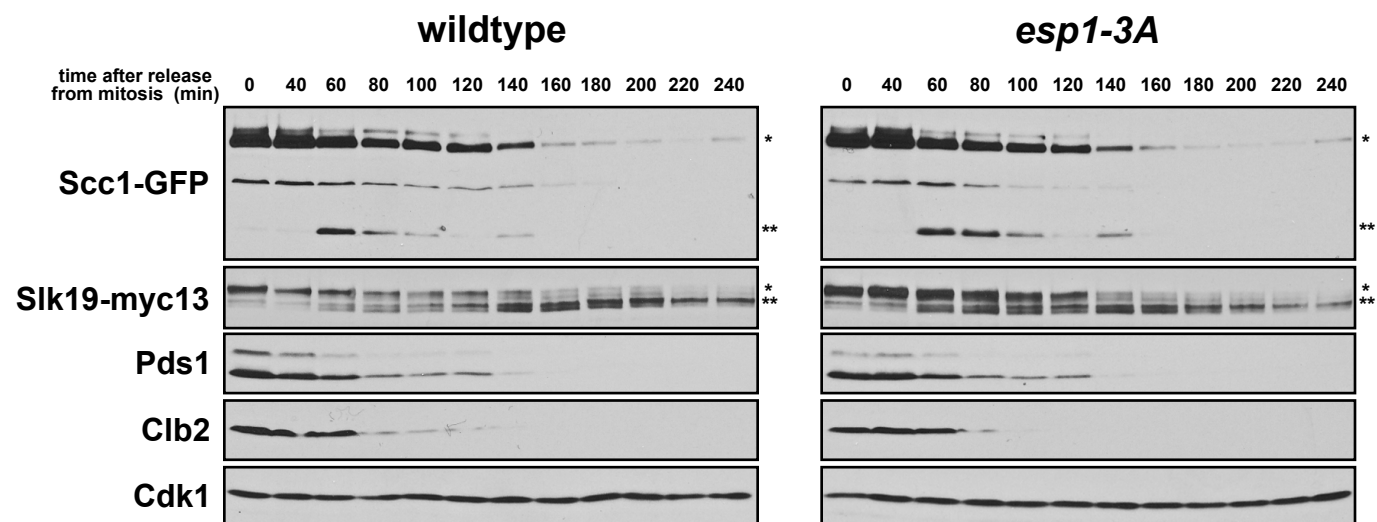
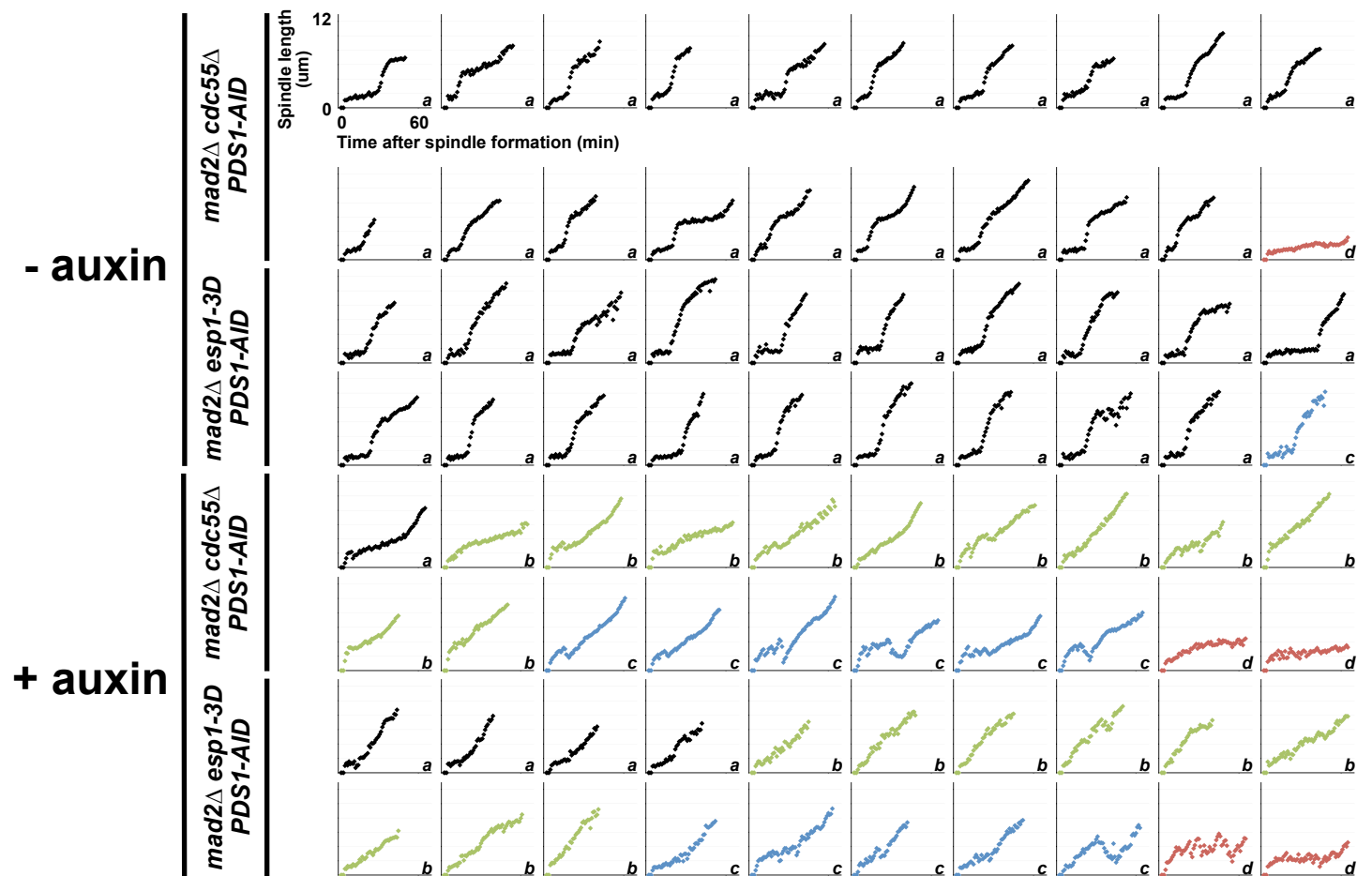


Figure 4-15

Figure 4-15. Preventing Esp1 phosphorylation does not perturb mitotic progression. *SCC1-GFP SLK19-myc13* (ADR5768) and *esp1-3A SCC1-GFP SLK19-myc13* (ADR6957) cells were grown to log phase, arrested in mitosis with nocodazole and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints. (* indicates full length protein, ** indicates a cleavage product).

A



B

	Normal mitotic progression	Immediate spindle elongation	Abnormal spindle elongation/contraction	Failed/no anaphase
<i>mad2Δ cdc55Δ PDS1-AID</i> - auxin	95% (19/20)	0% (0/20)	0% (0/20)	5% (1/20)
<i>mad2Δ esp1-3D PDS1-AID</i> - auxin	95% (19/20)	0% (0/20)	5% (1/20)	0% (0/20)
<i>mad2Δ cdc55Δ PDS1-AID</i> + auxin	5% (1/20)	55% (11/20)	30% (6/20)	10% (2/20)
<i>mad2Δ esp1-3D PDS1-AID</i> + auxin	20% (4/20)	45% (9/20)	25% (5/20)	10% (2/20)

Figure 4-16

Figure 4-16. **Spindle defects in *cdc55Δ* and *esp1-3D* cells depleted of Pds1 are not due to spindle checkpoint activation.**

(A-B) *mad2Δ cdc55Δ PDS1-AID SPC42-eGFP* (ADR6968) and *mad2Δ esp1-3D PDS1-AID SPC42-eGFP* (ADR7518) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Each strain and condition combination was imaged twice and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual *mad2Δ cdc55Δ* and *mad2Δ esp1-3D* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(B) Preventing spindle checkpoint signaling does not affect the spindle morphology of *cdc55Δ* and *esp1-3D* cells depleted of Pds1. Population characteristics of each strain and treatment combination based upon spindle morphology.

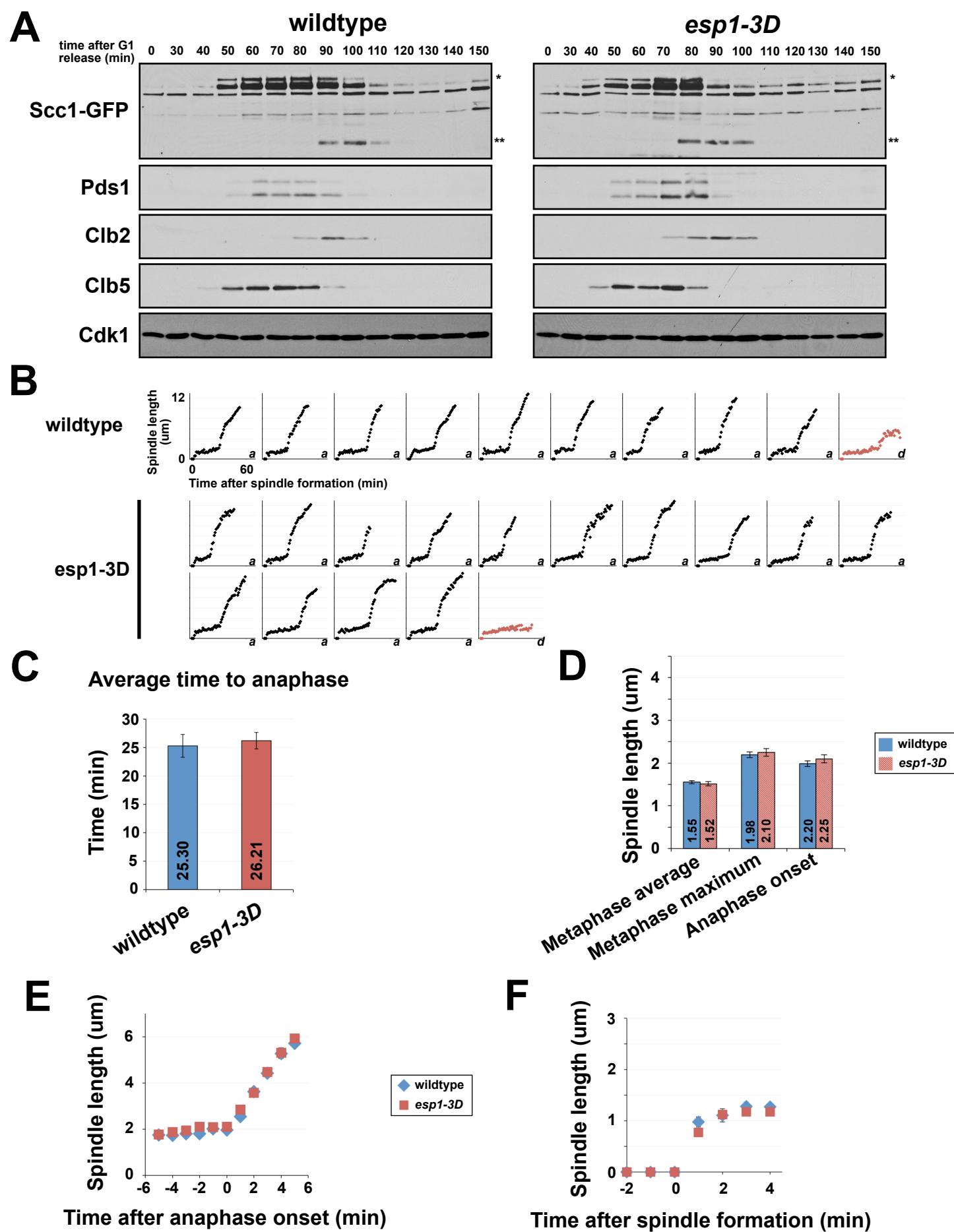


Figure 4-17

Figure 4-17. Mimicking Esp1 phosphorylation does not perturb mitotic progression.

(A) Scc1 is cleaved prematurely in cells expressing Esp1-3D. *SCC1-GFP* (ADR7691) and *esp1-3D SCC1-GFP* (ADR7680) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints. (* indicates full length protein, ** indicates a cleavage product).

(B-F) *SPC42-eGFP* (ADR4009) and *esp1-3D SPC42-eGFP* (ADR6454) cells were grown at 25°C to log phase and arrested in G1 with α -factor, released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads and imaged. The wildtype strain was imaged twice and the *esp1-3D* strain was imaged three times. Five cells were measured from each experiment. Data from individual experiments were combined for subsequent analysis.

(B) Mitotic spindle morphology of individual wildtype and *esp1-3D* cells. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(C) *esp1-3D* cells do not enter anaphase prematurely. The time spent between spindle formation and anaphase onset was determined for each cell. Displayed values are (mean $\pm$ SEM).

(D) *esp1-3D* cells do not exhibit spindle defects. Average and maximum spindle lengths during metaphase and spindle length at anaphase onset were determined. Displayed values are (mean $\pm$ SEM).

(E) Anaphase spindles elongate normally in *esp1-3D* cells. The timepoint before anaphase spindle elongation began was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after anaphase spindle elongation began were calculated. Displayed values are (mean $\pm$ SEM).

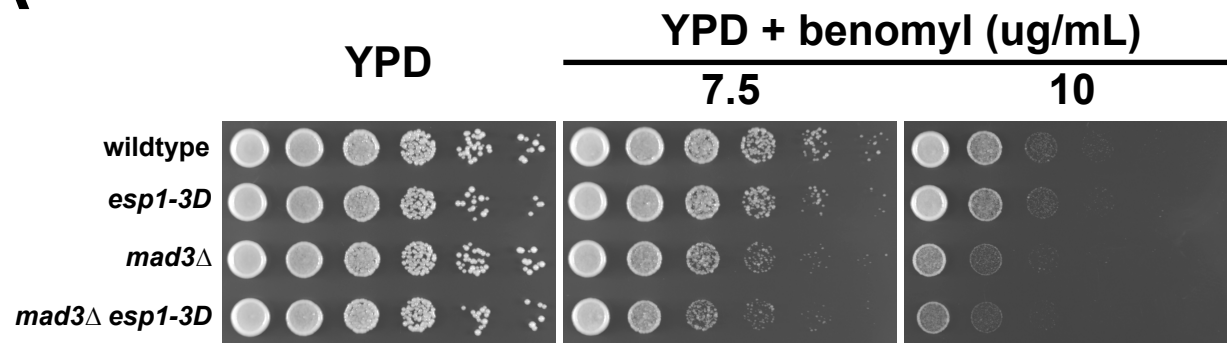
(F) Spindles form normally in *esp1-3D* cells. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean $\pm$ SEM).

compared to wildtype controls and Clb2 appears to be partially stabilized in these cells. To determine if early Scc1 cleavage caused earlier anaphase entry in *esp1-3D* cells, we imaged mitotic spindles in live *esp1-3D* cells. *esp1-3D* cells initiate anaphase with similar timing to wildtype controls (Figure 4-17B and C). We also observed no effect on spindle morphology in *esp1-3D* cells as reflected by average length at anaphase, average length during metaphase and maximum length during metaphase (Figure 4-17D). Spindle elongation at anaphase and spindle pole body separation at spindle formation also appeared normal in *esp1-3D* cells (Figure 4-17E and F). To determine if Clb2 stabilization in *esp1-3D* cells was due to spindle checkpoint activation, we assayed *esp1-3D* sensitivity to benomyl in the presence and absence of *MAD3* (Figure 4-18A). *esp1-3D* cells are not more sensitive to benomyl than wildtype cells. *esp1-3D mad3Δ* cells may be very slightly more sensitive to benomyl than *esp1-3D* or *mad3Δ* cells, suggesting that *esp1-3D* cells may partially activate the spindle checkpoint. Consistent with this observation, *esp1-3D* cells partially rescue the cell cycle delay caused by Mps1 overexpression (Figure 4-18B). Taken together, this data suggests that Esp1 phosphorylation has very little effect on cell cycle progression in unperturbed cells.

4.2.8 esp1-NLS rescues the lethality of Pds1 depletion in cdc55Δ cells

Esp1 interaction with Pds1 is important for Esp1 activity. Therefore, we immunoprecipitated Esp1, and determined whether preventing Esp1 phosphorylation affects Pds1 interaction (Figure 4-19A). Preventing Esp1 phosphorylation does not appear to affect Pds1 interaction. Pds1 acts as a chaperone for Esp1 and promotes Esp1 localization (258, 259). Therefore we sought to determine if Esp1 phosphorylation regulates its localization. We did this by examining the relative levels of nuclear and cytoplasmic Esp1-GFP in mitotic cells using fluorescence microscopy (Figure 4-19B). Mutating Cdk1 sites on Esp1 to be

A



B

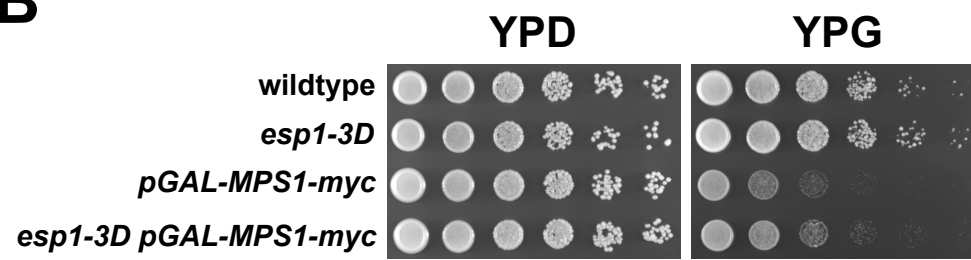


Figure 4-18

Figure 4-18. Mimicking Esp1 phosphorylation partially rescues sickness due to Mps1 overexpression.

(A) *esp1-3D* cells are not sensitive to spindle checkpoint activation. Eight-fold serial dilutions of wildtype (ADR4006), *esp1-3D* (ADR6953), *mad3Δ* (ADR7422) and *mad3Δ esp1-3D* (ADR7348) cells were spotted onto the indicated plates and grown at 25°C.

(B) Mimicking Esp1 phosphorylation partially rescues sickness due to Mps1 overexpression. Wildtype (ADR4006), *esp1-3D* (ADR6953), *pGAL-MPS* (ADR7345) and *esp1-3D pGAL-MPS1* (ADR7346) cells were spotted onto the indicated plates and grown at 30°C.

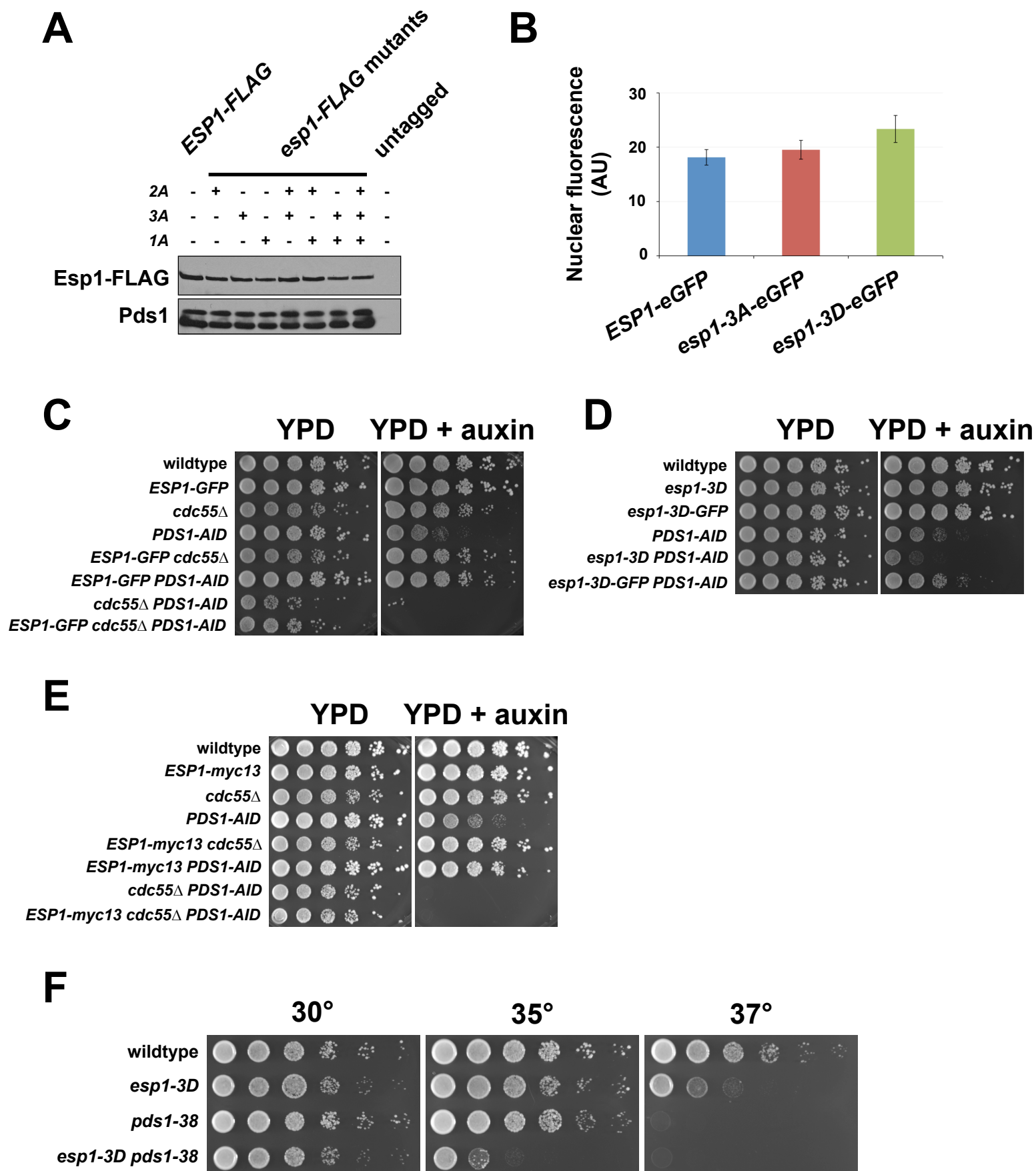


Figure 4-19

Figure 4-19. Mimicking Esp1 phosphorylation is lethal to cells deficient in Esp1 nuclear import.

(A) Preventing Esp1 phosphorylation does not affect Pds1/Esp1 interaction. *ESP1-FLAG* (ADR5307), *esp1-2A-FLAG* (ADR5491), *esp1-3A-FLAG* (ADR5494), *esp1-1A-FLAG* (ADR5482), *esp1-2A + 3A-FLAG* (ADR5481), *esp1-2A + 1A-FLAG* (ADR5488), *esp1-3A + 1A-FLAG* (ADR5492) and *esp1-2A + 3A + 1A-FLAG* (ADR5475) and untagged *ESP1* (ADR5308) cells were grown to log phase. Esp1-FLAG was immunoprecipitated and run on a polyacrylamide gel. Immunoprecipitated Esp1 and associated Pds1 were detected by immunoblot.

(B) Esp1 phosphorylation does not affect Esp1 mitotic localization. *ESP1-eGFP* (ADR7999), *esp1-3A-eGFP* (ADR8001) and *esp1-3D-eGFP* (ADR8003) cells were grown to log phase, arrested with nocodazole and relative nuclear fluorescence was measured in live cells. Fluorescence was measured in 10 cells in each of two experiments for each strain. Displayed values are (mean +/- SEM).

(C) C-terminally tagging Esp1 with GFP alters Esp1 function. Eight-fold serial dilutions of wildtype (ADR22), *ESP1-GFP* (ADR8075), *cdc55Δ* (ADR6436), *PDS1-AID* (ADR6428), *ESP1-GFP cdc55Δ* (ADR8076), *ESP1-GFP PDS1-AID* (ADR8077), *cdc55Δ PDS1-AID* (ADR6426) and *ESP1-GFP cdc55Δ PDS1-AID* (ADR8078) cells were spotted onto the indicated plates and grown at 25°C.

(D) *esp1-3D-GFP* cells are not sensitive to Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *esp1-3D* (ADR6409), *esp1-3D-GFP* (ADR8213), *PDS1-AID* (ADR6428), *esp1-3D PDS1-AID* (ADR6450) and *esp1-3D-GFP PDS1-AID* (ADR8209) cells were spotted onto the indicated plates and grown at 25°C.

(E) C-terminally tagging Esp1 with myc13 alters Esp1 function. Eight-fold serial dilutions of wildtype (ADR22), *ESP1-myc13* (ADR8183), *cdc55Δ* (ADR6436), *PDS1-AID* (ADR6428), *ESP1-myc13 cdc55Δ* (ADR8184), *ESP1-myc13 PDS1-AID* (ADR8185), *cdc55Δ PDS1-AID* (ADR6426) and *ESP1-myc13 cdc55Δ PDS1-AID* (ADR8186) cells were spotted onto plates and grown at 25°C. (F) Mimicking Esp1 phosphorylation is lethal to cells deficient in Esp1 nuclear import. Eight-fold serial dilutions of wildtype (ADR22), *esp1-3D* (ADR6409), *pds1-38* (ADR7551) and *esp1-3D pds1-38* (ADR7552) cells were spotted onto YPD plates and grown at the indicated temperatures. *The experiment in (A) was completed by C. Dore.

either unphosphorylatable or phosphomimetic caused no significant difference in the relative localization of Esp1. However, in independent experiments we determined that adding a GFP tag to either Esp1 or Esp1-3D partially rescues the lethality due to Pds1 depletion (Figure 4-19C and D), suggesting that tagging Esp1 with our GFP tag alters Esp1 function. We were therefore unable to draw any strong conclusions from the observed localization of Esp1-GFP. *ESP1-myc13* also rescued the slow growth phenotype of *PDS1-AID* cells on auxin, suggesting that any C-terminal tag on Esp1 may artificially perturb Esp1 function (Figure 4-19E). If Esp1 phosphorylation regulates its nuclear import we might expect that the *esp1-3D* allele would interact genetically with the *pds1-38* allele, which causes a defect in Esp1 nuclear import (259). Indeed *esp1-3D pds1-38* cells display increased temperature sensitivity at 35°C, suggesting that Esp1 phosphorylation may inhibit Esp1 nuclear import (Figure 4-19F). If this were the case, we would expect that forcing Esp1 into the nucleus should rescue phenotypes caused by Esp1 phosphorylation. To determine if the lethality of *cdc55Δ* cells depleted of Pds1 is due to an inability to localize Esp1 to the nucleus, we forced nuclear localization of Esp1 using a nuclear localization signal (NLS) (Figure 4-20A). Expression of *esp1-NLS* fully rescues the inviability of *cdc55Δ* cells depleted of Pds1. This rescue is not caused by a change in Esp1 expression level (Figure 4-20B). Nor is the rescue due to poor Pds1 depletion in these cells (Figure 4-20C). Forcing nuclear localization of either Esp1-3D (Figure 4-20D) or Esp1-3A (Figure 4-20E) also fully rescues the lethality of Pds1 depletion in cells lacking *CDC55*. To determine if the Esp1-NLS allele is hyper- or hypomorphic we assayed the sensitivity of *esp1-NLS cdc55Δ* cells containing a centromeric plasmid expressing endogenous Esp1 to Pds1 depletion (Figure 4-21). Endogenous Esp1 expression did not prevent Esp1-NLS from rescuing *cdc55Δ* cells depleted of Pds1, demonstrating that this allele is hypermorphic.

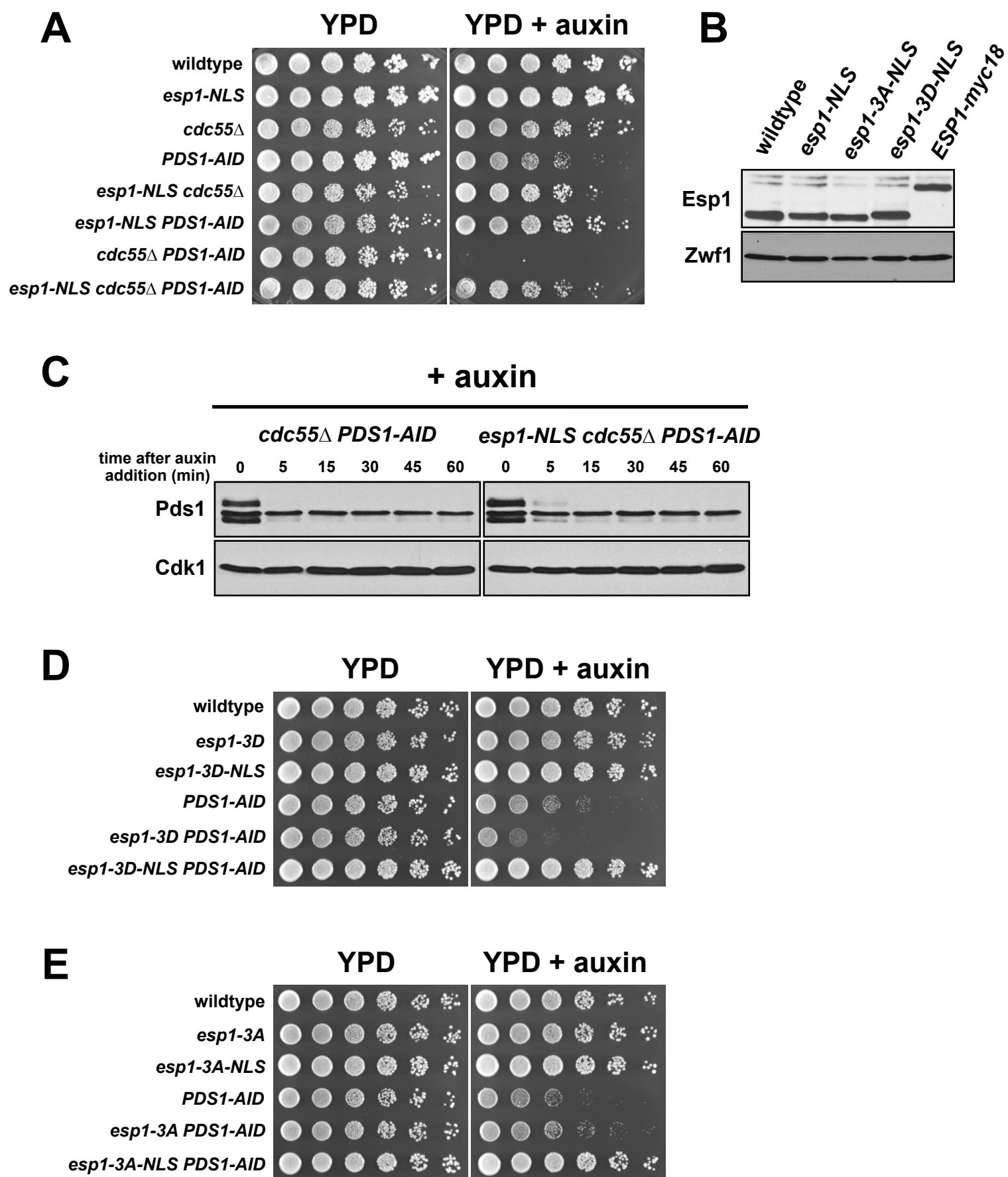


Figure 4-20

Figure 4-20. *esp1-NLS* rescues the lethality of *cdc55Δ* cells depleted of Pds1.

(A) Forcing Esp1 nuclear localization rescues the lethality of *cdc55Δ* cells depleted of Pds1. Eight-fold serial dilutions of wildtype (ADR22), *esp1-NLS* (ADR7592), *cdc55Δ* (ADR6436), *PDS1-AID* (ADR6428), *esp1-NLS cdc55Δ* (ADR7688), *esp1-NLS PDS1-AID* (ADR7687), *cdc55Δ PDS1-AID* (ADR6426) and *esp1-NLS cdc55Δ PDS1-AID* (ADR7689) cells were spotted onto the indicated plates and grown at 25°C.

(B) Esp1-NLS is expressed at endogenous levels. Wildtype (ADR22), *esp1-NLS* (ADR7592), *esp1-3A-NLS* (ADR7762), *esp1-3D-NLS* (ADR7765) and *ESP1-myc18* (ADR7984) cells were grown to log phase and samples were taken for immunoblotting.

(C) Pds1-AID is depleted in *esp1-NLS cdc55Δ PDS1-AID* cells treated with auxin. *cdc55Δ PDS1-AID* (ADR6426) and *esp1-NLS cdc55Δ PDS1-AID* (ADR7689) cells were grown to log phase and auxin was added. Samples were taken for immunoblotting at the indicated timepoints.

(D) Forcing Esp1-3D nuclear localization rescues the sickness caused by Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *esp1-3D* (ADR6409), *esp1-3D-NLS* (ADR7765), *PDS1-AID* (ADR6428), *esp1-3D PDS1-AID* (ADR6450) and *esp1-3D-NLS PDS1-AID* (ADR7858) cells were spotted onto the indicated plates and grown at 25°C.

(E) Forcing Esp1-3A nuclear localization rescues the sickness caused by Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *esp1-3A* (ADR7195), *esp1-3A-NLS* (ADR7762), *PDS1-AID* (ADR6428), *esp1-3A PDS1-AID* (ADR6863) and *esp1-3A-NLS PDS1-AID* (ADR7763) cells were spotted onto the indicated plates and grown at 25°C.

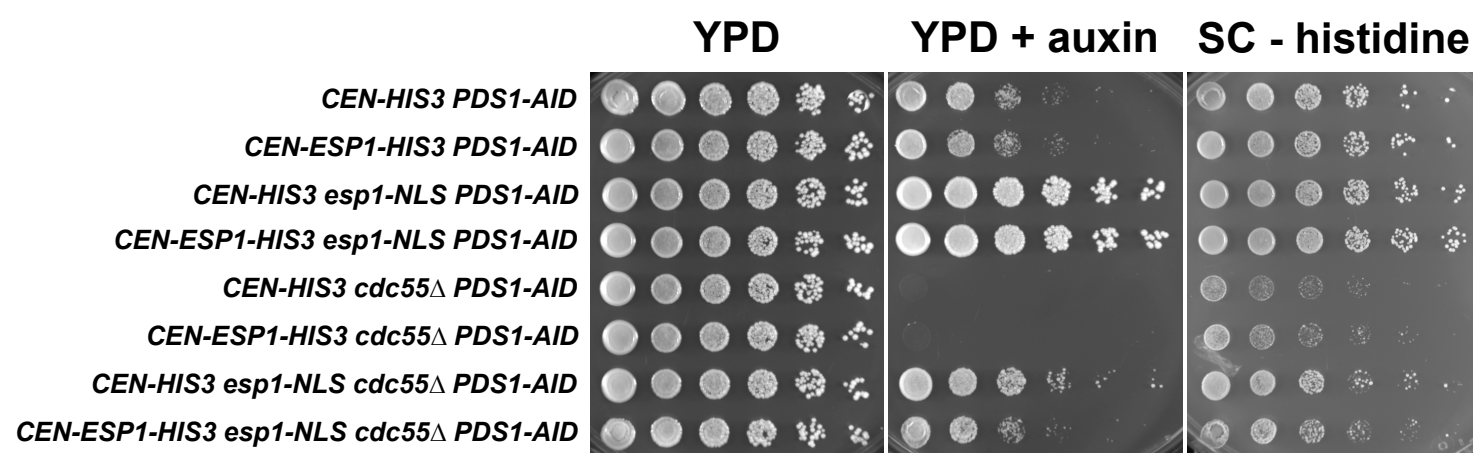


Figure 4-21

Figure 4-21. **The *esp1-NLS* allele is dominant.** *CEN-HIS3 PDS1-AID* (ADR8640), *CEN-ESP1-HIS3 PDS1-AID* (ADR8536), *CEN-HIS3 esp1-NLS PDS1-AID* (ADR8641), *CEN-ESP1-HIS3 esp1-NLS PDS1-AID* (ADR8632), *CEN-HIS3 cdc55Δ PDS1-AID* (ADR8642), *CEN-ESP1-HIS3 cdc55Δ PDS1-AID* (ADR8643), *CEN-HIS3 esp1-NLS cdc55Δ PDS1-AID* (ADR8644) and *CEN-ESP1-HIS3 esp1-NLS cdc55Δ PDS1-AID* (ADR8622) cells were grown to saturation in YPD media, diluted eight-fold serially, spotted onto the indicated plates and grown at 25°C.

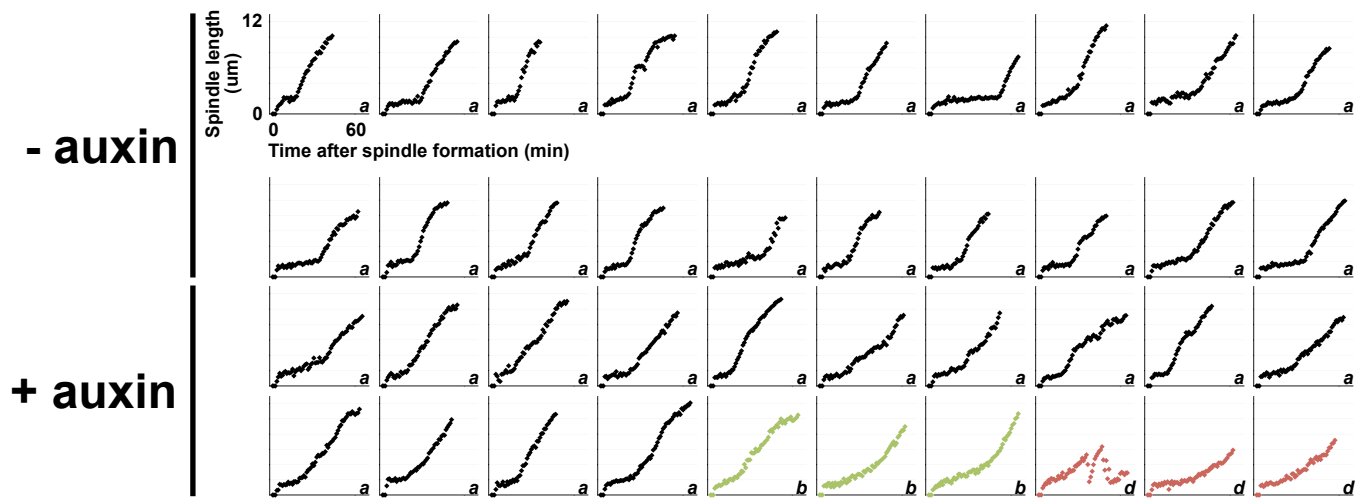
Since *esp1-NLS* rescued the viability of *cdc55Δ* cells depleted of Pds1, we wondered whether this allele also rescued the spindle defects displayed by these cells. Live imaging of *esp1-NLS cdc55Δ* cells depleted of Pds1 demonstrated that *esp1-NLS* fully rescues the spindle defects displayed by *cdc55Δ* cells depleted of Pds1 (Figure 4-22). 70% of *esp1-NLS cdc55Δ PDS1-AID* cells treated with auxin undergo a normal mitosis as opposed to 2% in *cdc55Δ PDS1-AID* cells (Figure 4-22A and B). Spindle formation is normal in these cells (Figure 4-22C), and spindle length at anaphase onset, average spindle length during metaphase and maximum spindle length during metaphase are all similar to untreated controls (Figure 4-22D). This rescue is not likely solely due to C-terminally tagging Esp1, as *esp1-NLS* fully rescues the inviability of *cdc55Δ* cells depleted of Pds1, whereas *ESPI-GFP* and *ESPI-myc13* are able to rescue poor growth due to Pds1 depletion but not inviability in the absence of both Cdc55 and Pds1 (Figure 4-19).

4.2.9 Depletion of Pds1 in *slk19Δ* cells causes spindle defects

Though Esp1 regulation of Scc1 is thought to be through Scc1 cleavage, Esp1 regulation of Slk19 is more complex. Although Esp1 cleaves Slk19, it is unclear if Slk19 cleavage affects Slk19 activity (475). We saw no difference in either Scc1 or Slk19 cleavage in either *cdc55Δ* or *esp1-3D* cells depleted of Pds1 compared to Pds1-depleted controls, suggesting that Esp1 phosphorylation may regulate Slk19 independently of its cleavage. Slk19, along with polo kinase (Cdc5), Esp1 and Spo12 are part of the FEAR pathway that releases Cdc14 during early anaphase (323). To determine if the lethality of *cdc55Δ* cells depleted of Pds1 is due to FEAR pathway activation we asked whether *SPO12* deletion could rescue *cdc55Δ* cells depleted of Pds1 (Figure 4-23). Though *spo12Δ* does appear to show a slight synthetic sickness with *PDS1-AID* in the presence of auxin, it does not affect the

A

esp1-NLS cdc55 Δ PDS1-AID

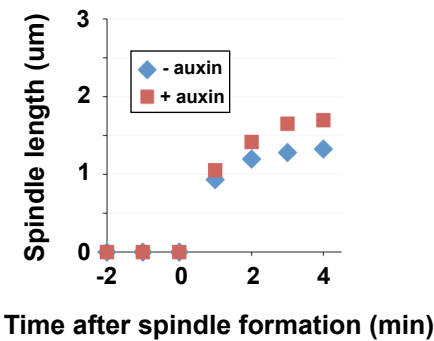


B

	Normal mitotic progression (a)	Immediate spindle elongation (b)	Abnormal spindle elongation/contraction (c)	Failed/no anaphase (d)
<i>esp1-NLS cdc55Δ PDS1-AID - auxin</i>	100% (20/20)	0% (0/20)	0% (0/20)	0% (0/20)
<i>esp1-NLS cdc55Δ PDS1-AID + auxin</i>	70% (14/20)	15% (3/20)	0% (0/20)	15% (3/20)

C

esp1-NLS cdc55 Δ PDS1-AID



D

esp1-NLS cdc55 Δ PDS1-AID

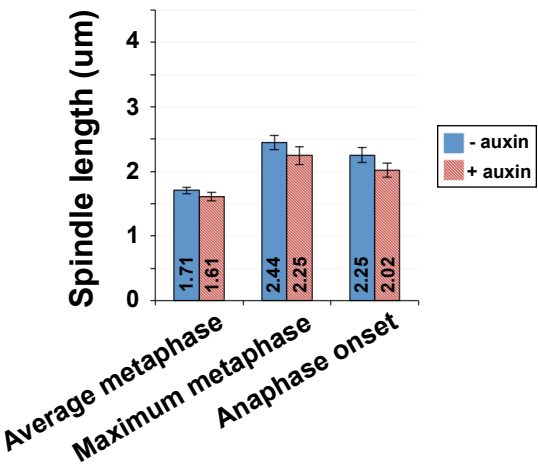


Figure 4-22

Figure 4-22. *esp1-NLS* rescues the spindle defects displayed by *cdc55Δ* cells depleted of Pds1.

(A-D) *esp1-NLS cdc55Δ PDS1-AID SPC42-eGFP* (ADR7750) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. Each condition was imaged twice and ten cells were measured in each experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual *esp1-NLS cdc55Δ* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated 'a'. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated 'b'. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated 'c'. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated 'd'.

(B) *esp1-NLS* rescues the spindle defects displayed by *cdc55Δ* cells depleted of Pds1. Population characteristics of each strain and treatment combination based upon spindle morphology.

(C) *esp1-NLS* rescues the early spindle elongation observed in *cdc55Δ* cells depleted of Pds1. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(D) *esp1-NLS* rescues the spindle defects observed in *cdc55Δ* cells depleted of Pds1. Average and maximum spindle lengths during metaphase and spindle length at anaphase onset were determined. Displayed values are (mean +/- SEM).

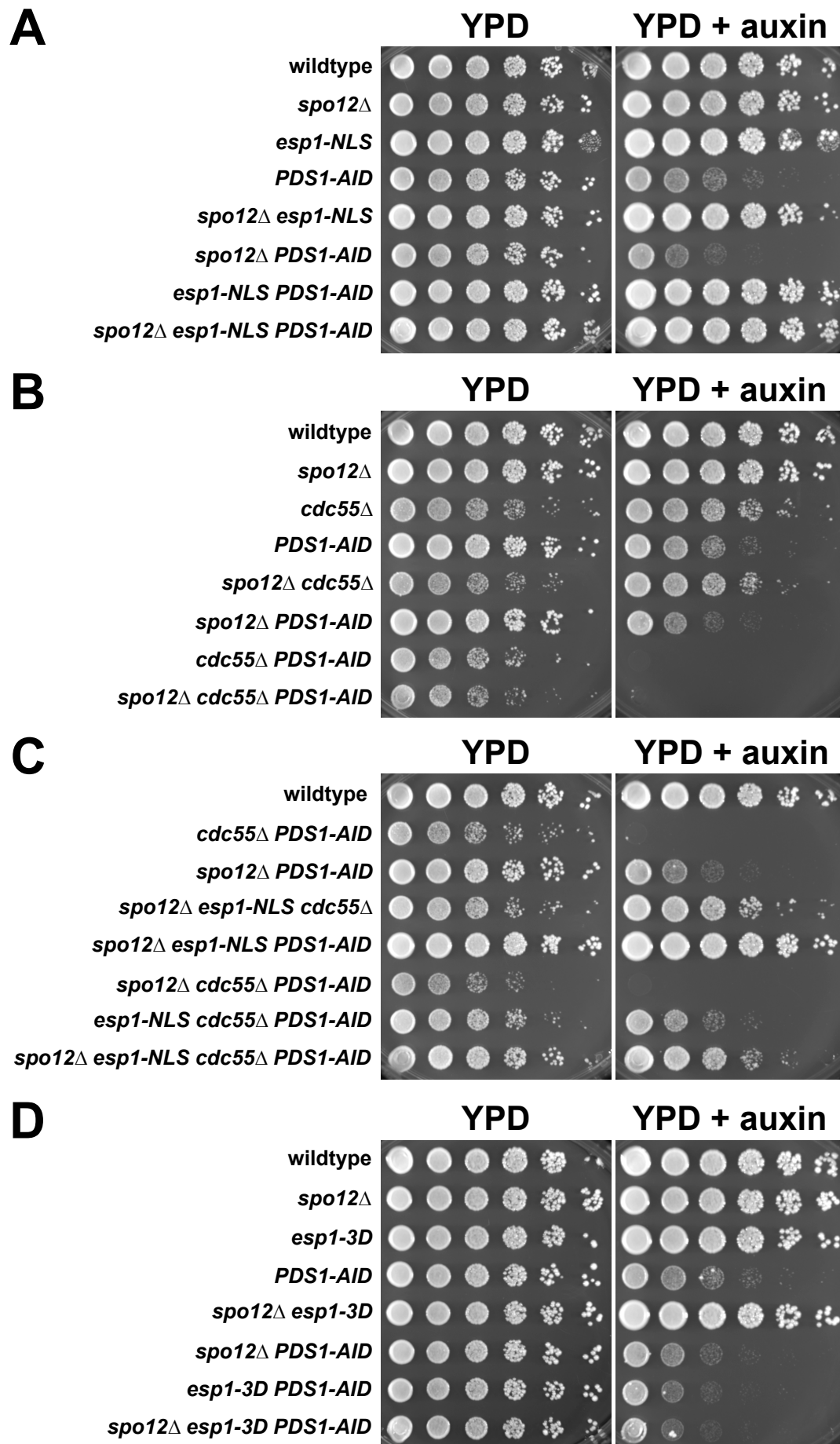


Figure 4-23

Figure 4-23. The lethality of Pds1 depletion in *cdc55Δ* cells is not due to FEAR pathway activation.

(A) Deleting *SPO12* does not prevent *ESP1-NLS* rescue of the sensitivity displayed by *cdc55Δ* cells to Pds1 depletion. Eight-fold serial dilutions of wildtype (ADR22), *spo12Δ* (ADR8097), *esp1-NLS* (ADR7592), *PDS1-AID* (ADR6428), *spo12Δ esp1-NLS* (ADR8108), *spo12Δ PDS1-AID* (ADR8109), *esp1-NLS PDS1-AID* (ADR7593) and *spo12Δ esp1-NLS PDS1-AID* (ADR8111) cells were spotted onto the indicated plates and grown at 25°C.

(B) Deleting *SPO12* does not rescue the lethality of *cdc55Δ* cells depleted of Pds1. Eight-fold serial dilutions of wildtype (ADR22), *spo12Δ* (ADR8097), *cdc55Δ* (ADR6436), *PDS1-AID* (ADR6428), *spo12Δ cdc55Δ* (ADR8110), *spo12Δ PDS1-AID* (ADR8109), *cdc55Δ PDS1-AID* (ADR6426) and *spo12Δ cdc55Δ PDS1-AID* (ADR8113) cells were spotted onto the indicated plates and grown at 25°C.

(C) *SPO12* deletion improves *esp1-NLS* rescue of *cdc55Δ* cells depleted of Pds1. Eight-fold serial dilutions of wildtype (ADR22), *cdc55Δ PDS1-AID* (ADR6426), *spo12Δ PDS1-AID* (ADR8109), *spo12Δ esp1-NLS cdc55Δ* (ADR8112), *spo12Δ esp1-NLS PDS1-AID* (ADR8111), *spo12Δ cdc55Δ PDS1-AID* (ADR8113), *esp1-NLS cdc55Δ PDS1-AID* (ADR7689) and *spo12Δ esp1-NLS cdc55Δ PDS1-AID* (ADR8114) cells were spotted onto the indicated plates and grown at 25°C.

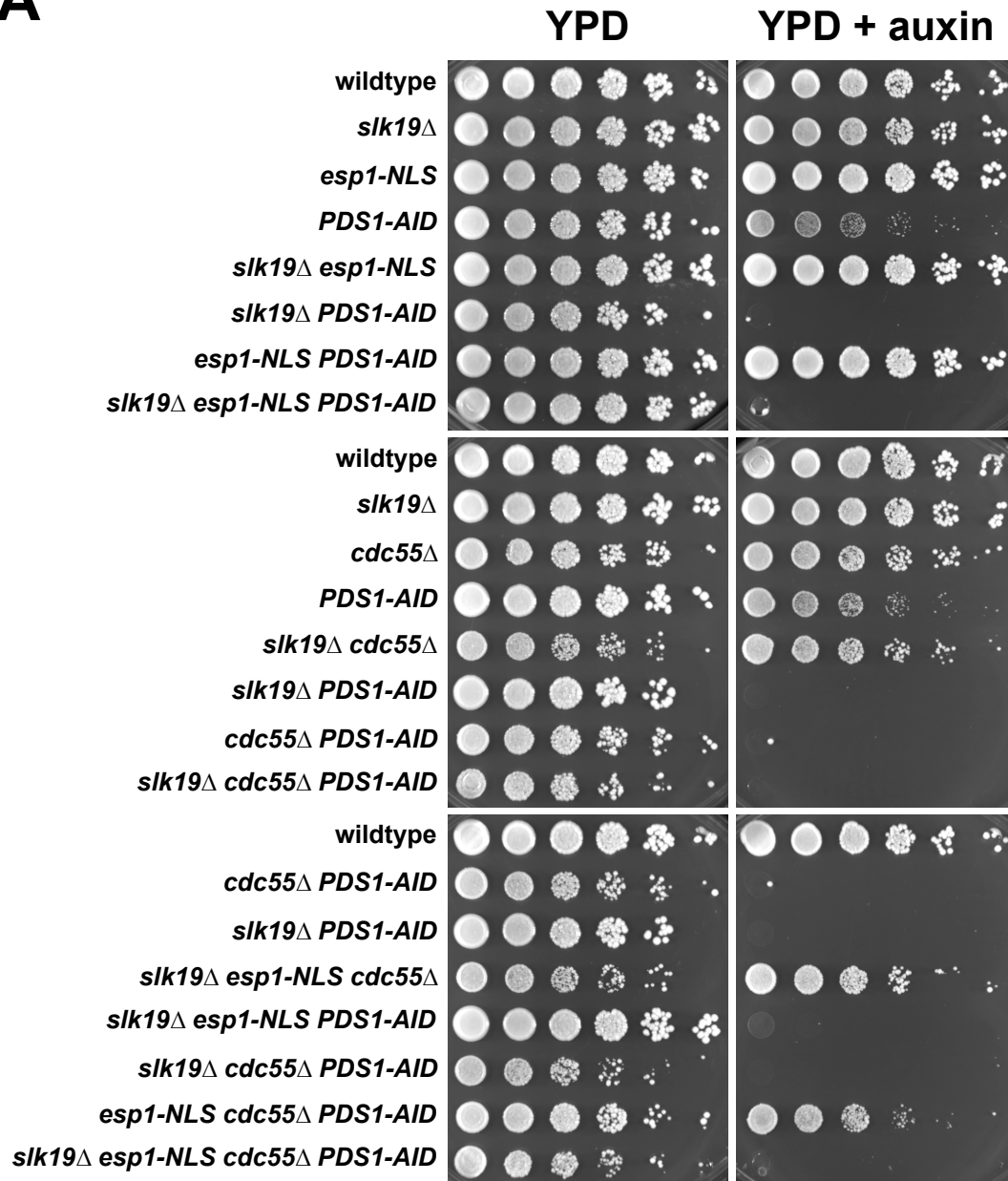
(D) Deleting *SPO12* does not rescue the sickness displayed by *esp1-3D* cells depleted of Pds1. Eight-fold serial dilutions of wildtype (ADR22), *spo12Δ* (ADR8097), *esp1-3D* (ADR6409), *PDS1-AID* (ADR6428), *spo12Δ esp1-3D* (ADR8223), *spo12Δ PDS1-AID* (ADR8109), *esp1-3D PDS1-AID* (ADR6450) and *spo12Δ esp1-3D PDS1-AID* (ADR8224) cells were spotted onto the indicated plates and grown at 25°C.

rescue of *PDS1-AID* by *esp1-NLS* on auxin (Figure 4-23A). This suggests that *esp1-NLS* does not rescue sickness due to Pds1 depletion by activating the FEAR pathway. *spo12Δ* also does not rescue *cdc55Δ* cells depleted of Pds1 (Figure 4-23B), suggesting that the inviability of *cdc55Δ PDS1-AID* cells on auxin is not due to FEAR pathway activation. *spo12Δ* also does not prevent the rescue of *cdc55Δ PDS1-AID* on auxin by *esp1-NLS*, suggesting that increased FEAR pathway signaling is not responsible for this rescue (Figure 4-23C). Similar to *spo12Δ* and *cdc55Δ*, *spo12Δ* and *esp1-3D* do not interact genetically either in the presence or absence of Pds1 (Figure 4-23D). Taken together, this data suggests that FEAR pathway signaling is not involved in the phenotypes displayed by *cdc55Δ* cells depleted of Pds1.

We next asked whether the phenotypes displayed by *cdc55Δ* cells depleted of Pds1 depended on *SLK19*, which, though a component of the FEAR pathway, also has FEAR-independent functions (476). It has previously been shown that, similar to *CDC55*, *SLK19* becomes essential in the absence of *PDS1* (477). This prevented us from directly testing whether *SLK19* is required for *cdc55Δ* lethality upon Pds1 depletion or whether *SLK19* is required for the rescue of this lethality by *esp1-NLS* (Figure 4-24). Interestingly, though *esp1-NLS* was able to rescue the lethality of *cdc55Δ* cells depleted of Pds1, *esp1-NLS* was unable to rescue the lethality of *slk19Δ* cells depleted of Pds1 (Figure 4-24A). *slk19Δ*, similar to *cdc55Δ*, does not interact genetically with *esp1-3D* (Figure 4-24B).

The observation that *slk19Δ* cells are inviable in the absence of Pds1 suggested the possibility that *cdc55Δ* sensitivity to Pds1 depletion is due to Slk19 inactivation. To address this, we did live imaging of *slk19Δ* mutants depleted of Pds1 (Figure 4-25). These cells exhibit similar spindle phenotypes to *cdc55Δ* cells depleted of Pds1 and *esp1-3D* cells depleted of Pds1 (Figure 4-25A and B). 40% of these cells undergo spindle elongation and contraction during mitosis. Upon spindle formation, spindles in these cells elongate more

A



B

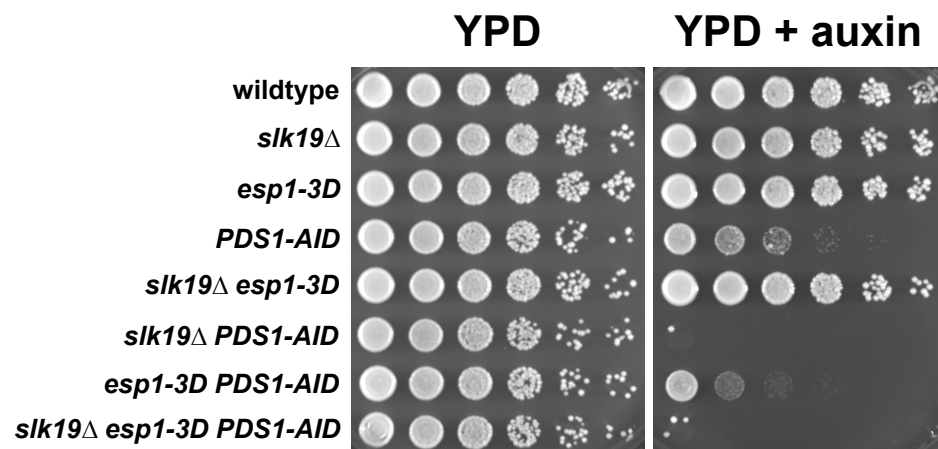


Figure 4-24

Figure 4-24. ***slk19Δ* exhibits different genetic interactions than *spo12Δ*.**

(A) Depletion of Pds1 in *slk19Δ* cells is lethal. Eight-fold serial dilutions of wildtype (ADR22), *slk19Δ* (ADR7678), *esp1-NLS* (ADR7592), *PDS1-AID* (ADR6428), *cdc55Δ* (ADR6436), *slk19Δ esp1-NLS* (ADR8050), *slk19Δ PDS1-AID* (ADR8052), *esp1-NLS PDS1-AID* (ADR7593), *slk19Δ cdc55Δ* (ADR8051), *cdc55Δ PDS1-AID* (ADR6426), *slk19Δ esp1-NLS PDS1-AID* (ADR8054), *slk19Δ cdc55Δ PDS1-AID* (ADR8055), *slk19Δ esp1-NLS cdc55Δ* (ADR8053), *esp1-NLS cdc55Δ PDS1-AID* (ADR7689) and *slk19Δ esp1-NLS cdc55Δ PDS1-AID* (ADR8056) cells were spotted onto the indicated plates and grown at 25°C.

(B) *slk19Δ* does not interact genetically with *esp1-3D*. Eight-fold serial dilutions of wildtype (ADR6431), *slk19Δ* (ADR8230), *esp1-3D* (ADR8229), *PDS1-AID* (ADR6428), *slk19Δ esp1-3D* (ADR8231), *slk19Δ PDS1-AID* (ADR8233), *esp1-3D PDS1-AID* (ADR6450) and *slk19Δ esp1-3D PDS1-AID* (ADR8232) cells were spotted onto the indicated plates and grown at 25°C.

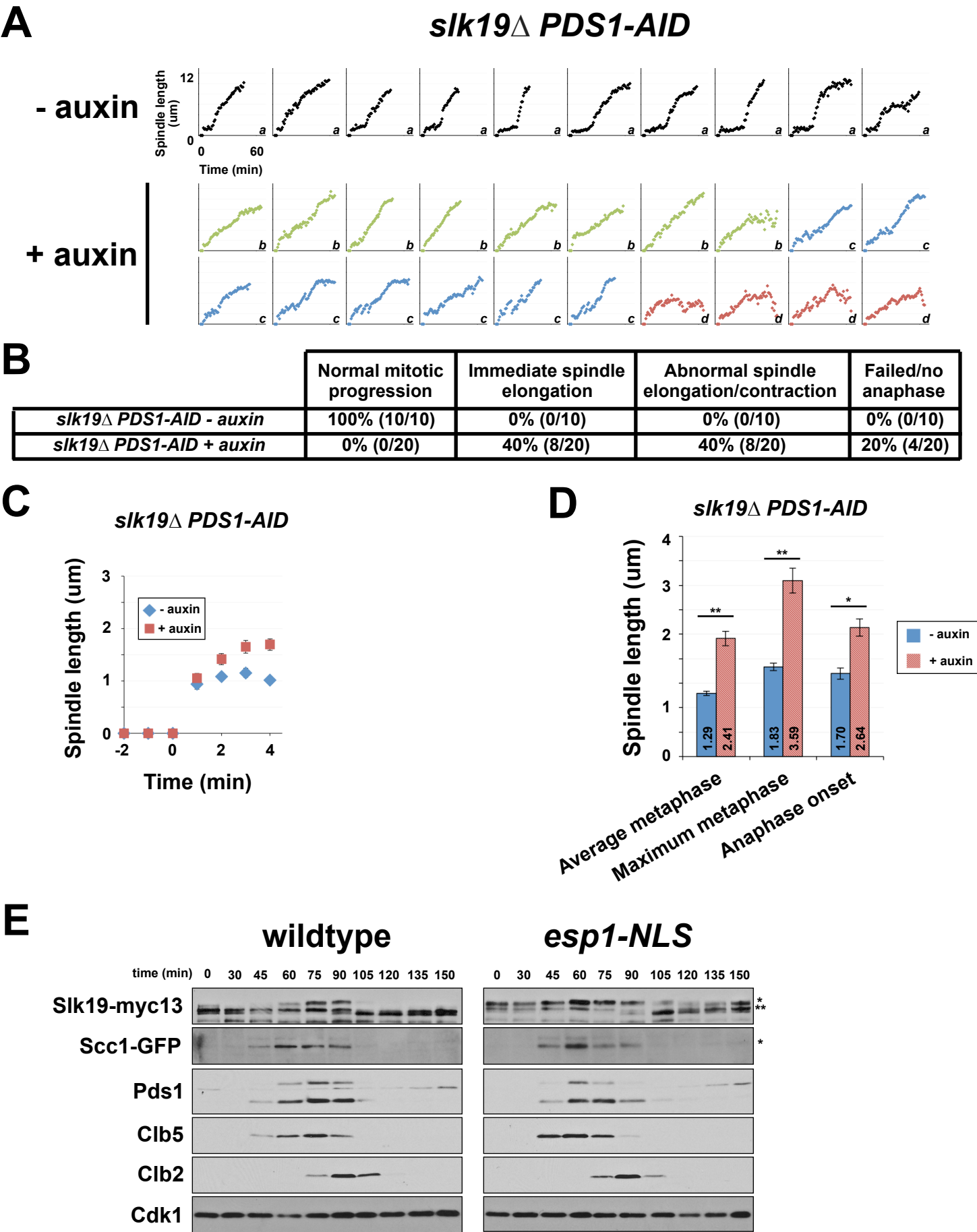


Figure 4-25

Figure 4-25. Pds1 depletion causes spindle defects in cells lacking *SLK19*.

(A-D) *slk19Δ PDS1-AID SPC42-eGFP* (ADR8104) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at $t = 0$ and at $t = 25$ minutes cells were plated onto YPD live microscopy pads +/- auxin and imaged. The *slk19Δ PDS1-AID SPC42-eGFP* strain was imaged twice in each condition. Five cells were measured in each – auxin experiment and ten cells were measured in each + auxin experiment. Data from individual experiments were combined for subsequent analysis.

(A) Mitotic spindle morphology of individual *slk19Δ* cells depleted of Pds1. Cells that undergo *normal mitotic progression* are shown in black and annotated ‘a’. Cells that undergo *immediate spindle elongation* upon spindle formation are shown in green and annotated ‘b’. Cells that undergo *abnormal spindle elongation and contraction* during metaphase are shown in blue and annotated ‘c’. Cells that exhibit *failed or no anaphase* spindle elongation are shown in red and annotated ‘d’.

(B) Pds1 depletion in *slk19Δ* cells causes spindle defects. Population characteristics of each strain and treatment combination based upon spindle morphology.

(C) Pds1 depletion in *slk19Δ* cells causes premature spindle elongation. The timepoint before spindle formation was defined as $x = 0$ for each cell. Average spindle lengths in the timepoints before and after spindle formation were calculated. Displayed values are (mean +/- SEM).

(D) Pds1 depletion in *slk19Δ* cells significantly increases average and maximum metaphase spindle lengths and spindle length at anaphase onset. In cells that displayed both metaphase and anaphase, average and maximum spindle lengths during metaphase and spindle length at anaphase onset were determined. Displayed values are (mean +/- SEM). Student’s t-tests were used to test for significant differences (* $p < 0.001$, ** $p = 0.003$).

(E) Slk19 protein is modified in cells expressing Esp1-NLS. *SCC1-GFP SLK19-myc13* (ADR5768) and *esp1-NLS SCC1-GFP SLK19-myc13* (ADR8072) cells were grown to log phase, arrested in G1 with α -factor, and released into the cell cycle ($t = 0$). α -factor was re-added at $t = 55$ minutes to arrest cells in the following G1. Samples were taken for immunoblotting at the indicated timepoints. (* indicates full length protein, ** indicates a cleavage product).

rapidly than control cells, though not to the extent of *cdc55Δ* cells depleted of Pds1 (Figure 4-25C). Spindle elongation and contraction during metaphase in these cells is reflected in a greater length at anaphase onset as well as greater average and maximum lengths during metaphase (Figure 4-25D). The observations that depletion of Pds1 causes lethality in both *cdc55Δ* and *slk19Δ* cells and that spindle morphology is affected in a similar manner suggests that PP2A^{Cdc55} and Slk19 function in the same pathway to regulate spindle morphology. *esp1-NLS* cannot rescue *slk19Δ* sensitivity to Pds1 depletion as it can *cdc55Δ* sensitivity to Pds1 depletion, suggesting that Esp1 functions downstream of PP2A^{Cdc55} but upstream of Slk19 in this pathway. Though it is presently unclear exactly how Esp1 regulates Slk19, it is interesting that in unperturbed *esp1-NLS* cells, Scc1 appears to undergo normal cleavage, whereas Slk19 protein species' appear to be perturbed (Figure 4-25E).

The spindle defects displayed by *cdc55Δ esp1-3D* and *slk19Δ* cells depleted of Pds1 suggest that sister chromatid segregation may be impaired in these strains. Indeed, though 12.9% of Pds1-depleted control cells displayed sister chromatid missegregation, 26.8% and 43.9% respectively of *cdc55Δ* and *slk19Δ* cells depleted of Pds1 display missegregation of a GFP-labeled chromosome (Figure 4-26). *esp1-3D* cells depleted of Pds1 displayed a similar rate of chromosome missegregation to Pds1-depleted controls (10.3%).

Esp1 and Slk19 cooperate with the microtubule organizer Ase1 to organize the spindle midzone, and Slk19, Esp1 and Ase1 recruit the kinesin-5 motor protein Cin8 to the spindle midzone, thereby promoting spindle elongation (140, 141). If *cdc55Δ* and *slk19Δ* lethality in the absence of Pds1 were due to spindle midzone misregulation, we would expect that cells lacking *CIN8* or *ASE1* would also be sensitive to Pds1 depletion. Indeed *CIN8* and *ASE1* are both essential in the absence of Pds1 (Figure 4-27A and B). Moreover, in both cases, expression of *esp1-NLS* rescues this lethality. *cin8Δ* and *ase1Δ* also display synthetic

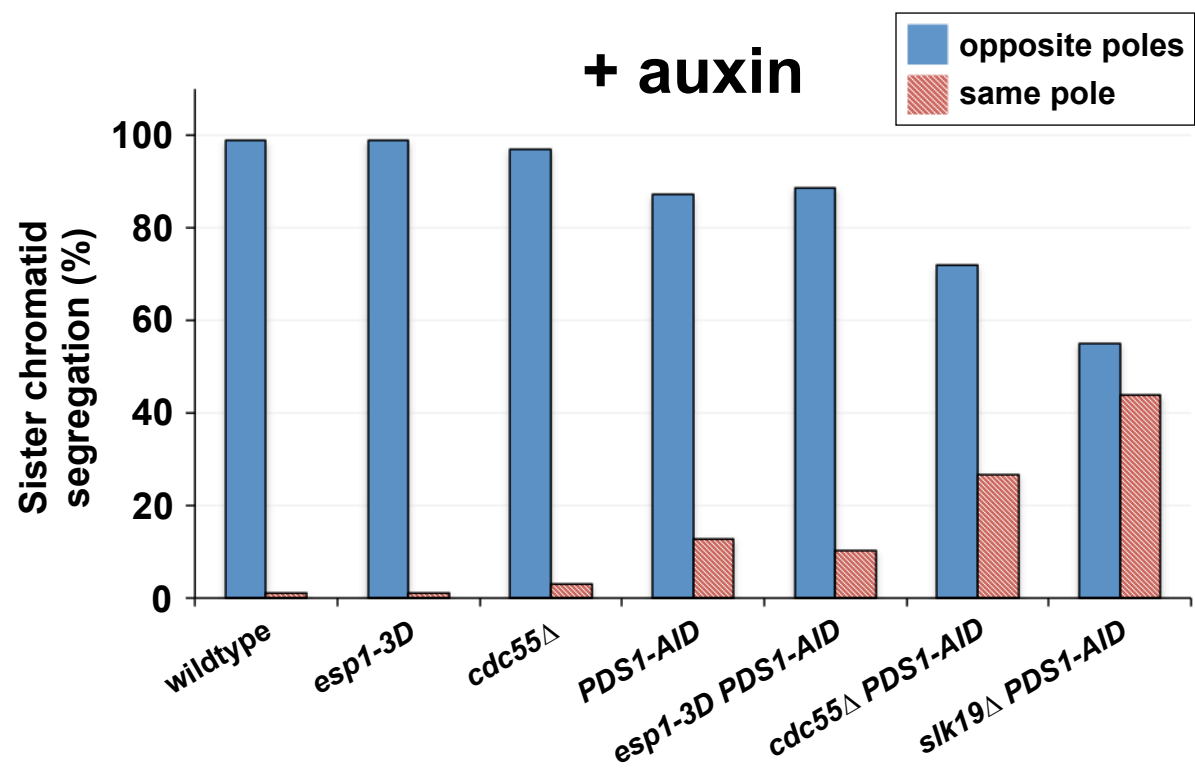


Figure 4-26

Figure 4-26. *cdc55* Δ and *slk19* Δ cells depleted of Pds1 co-segregate sister chromatids. *pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR6464), *esp1-3D pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR6887), *cdc55* Δ *pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR7252), *PDS1-AID pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR6724), *esp1-3D PDS1-AID pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR6886), *cdc55* Δ *PDS1-AID pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR6722) and *slk19* Δ *PDS1-AID pCUP1-eGFP12-lacI12 ura3::lacO-URA3 SPC42-mCherry* (ADR8421) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release auxin was added. Cells were released at t = 0 and at t = 120 minutes samples were fixed for microscopy. Telophase cells were identified using the SPC42-mCherry signal. The presence of GFP-lacI foci at either both spindle poles or at a single spindle pole was scored in these telophase cells. Strains were imaged once (n = 100 for each strain).

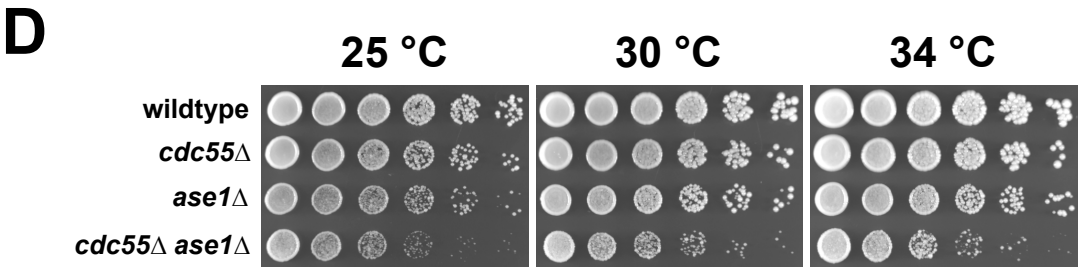
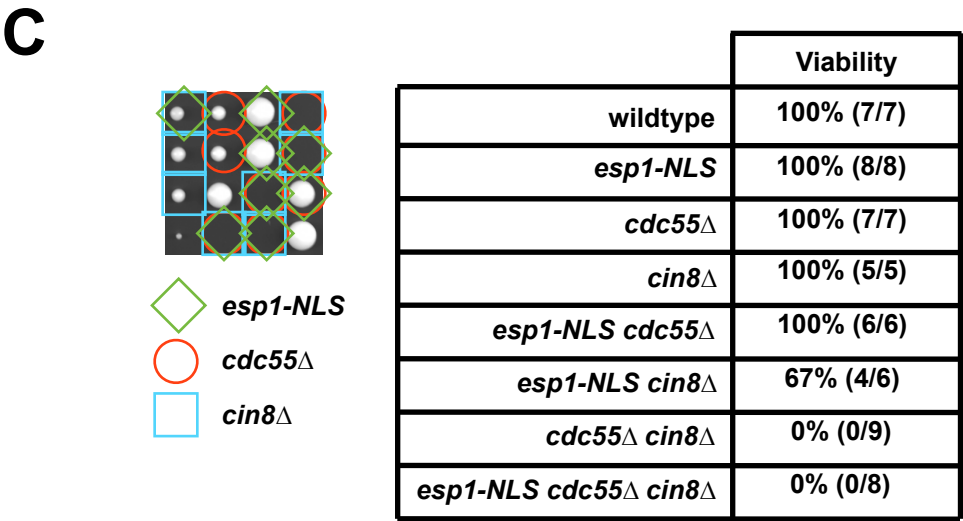
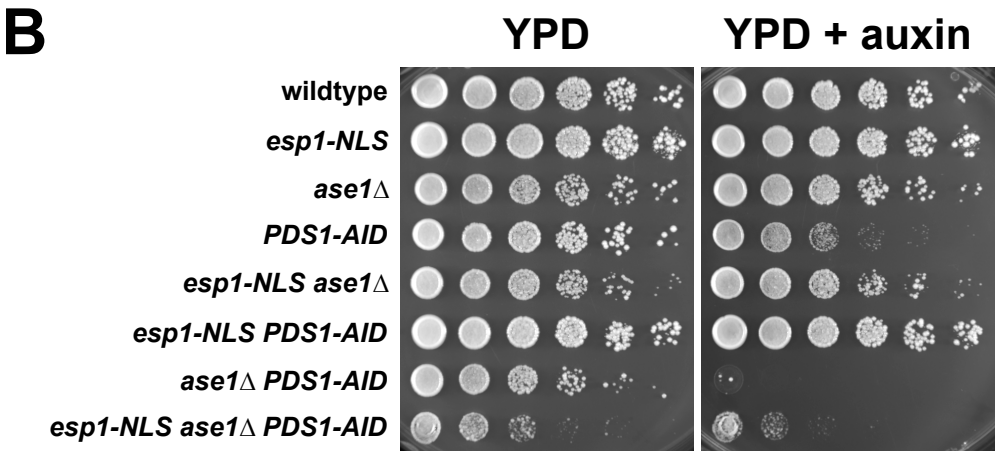
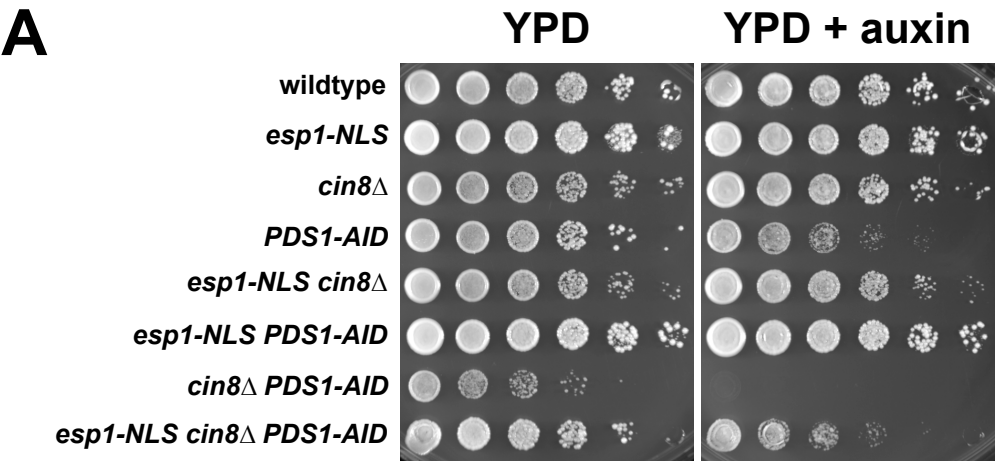


Figure 4-27

Figure 4-27. Pds1 depletion is lethal to cells lacking spindle midzone proteins.

- (A) Pds1 depletion is lethal to *cin8Δ* cells. Eight-fold serial dilutions of wildtype (ADR22), *esp1-NLS* (ADR7592), *cin8Δ* (ADR1172), *PDS1-AID* (ADR6428), *esp1-NLS cin8Δ* (ADR8556), *esp1-NLS PDS1-AID* (ADR7687), *cin8Δ PDS1-AID* (ADR8606) and *esp1-NLS cin8Δ PDS1-AID* (ADR8558) cells were spotted onto the indicated plates and grown at 25°C.
- (B) Pds1 depletion is lethal to *ase1Δ* cells. Eight-fold serial dilutions of wildtype (ADR22), *esp1-NLS* (ADR7592), *ase1Δ* (ADR8497), *PDS1-AID* (ADR6428), *esp1-NLS ase1Δ* (ADR8564), *esp1-NLS PDS1-AID* (ADR7687), *ase1Δ PDS1-AID* (ADR8560) and *esp1-NLS ase1Δ PDS1-AID* (ADR8610) cells were spotted onto the indicated plates and grown at 25°C.
- (C) Cells lacking *CDC55* and *CIN8* are inviable. *MATα cin8Δ* (ADR1172) and *MATα esp1-NLS cdc55Δ* (ADR8645) cells were mated, sporulated and the resultant tetrads dissected. A portion of the dissection is shown (left) with the genotype of each spore. Tetrads in which the genotype of each spore could be determined unequivocally were used to determine the viability of each genotype (right). A total of 56 spores were scored.
- (D) Cells lacking *CDC55* and *ASE1* exhibit reduced viability. Eight-fold serial dilutions of wildtype (ADR22), *cdc55Δ* (ADR7592), *ase1Δ* (ADR8497), and *cdc55Δ ase1Δ* (ADR8563) cells were spotted onto the indicated plates and grown at 25°C.

interactions with *cdc55Δ* (Figure 4-27C and D). *cdc55Δ cin8Δ* cells are inviable and *cdc55Δ ase1Δ* cells are synthetically sick.

PP2A^{Cdc55} has been shown to be important in maintaining Cdc14 in the nucleolus during mitosis (478). We therefore wondered whether early Cdc14 release is responsible for the lethality of *cdc55Δ* cells depleted of Pds1. If this were the case we would expect to see earlier Cdc14 release in cells that exhibit spindle defects than those that do not. Correlating spindle length to Cdc14 release could lead to confusing results in *cdc55Δ PDS1-AID* cells treated with auxin, as these cells elongate and then contract their mitotic spindles during metaphase. Therefore we looked at Cdc14 release in *swe1Δ cdc55Δ* cells depleted of Pds1 (Figure 4-28), which generally exhibit relatively slow and consistent spindle elongation upon spindle formation. In untreated control cells, Cdc14 is unreleased in 94.7% of cells without a spindle and 85.7% of cells with short spindles less than or equal to 2μm in length (Figure 4-28A and B). In cells with spindles greater than 2μm in length, 90.9 % of cells have released Cdc14. This data suggests that in *cdc55Δ swe1Δ* cells Cdc14 is released at a spindle length of ~ 2μm. In auxin-treated *swe1Δ cdc55Δ PDS1-AID* cells, which exhibit spindle defects, 89.3% of cells with a single Spc42-mCherry signal and 100% of cells with spindles less than or equal to 2μm in length display nucleolar Cdc14 (Figure 4-28A and B). In auxin-treated cells with spindles greater than 2μm in length, 81.8% of cells contain released Cdc14. This data suggests that premature Cdc14 release is likely not responsible for the spindle defects observed in this strain.

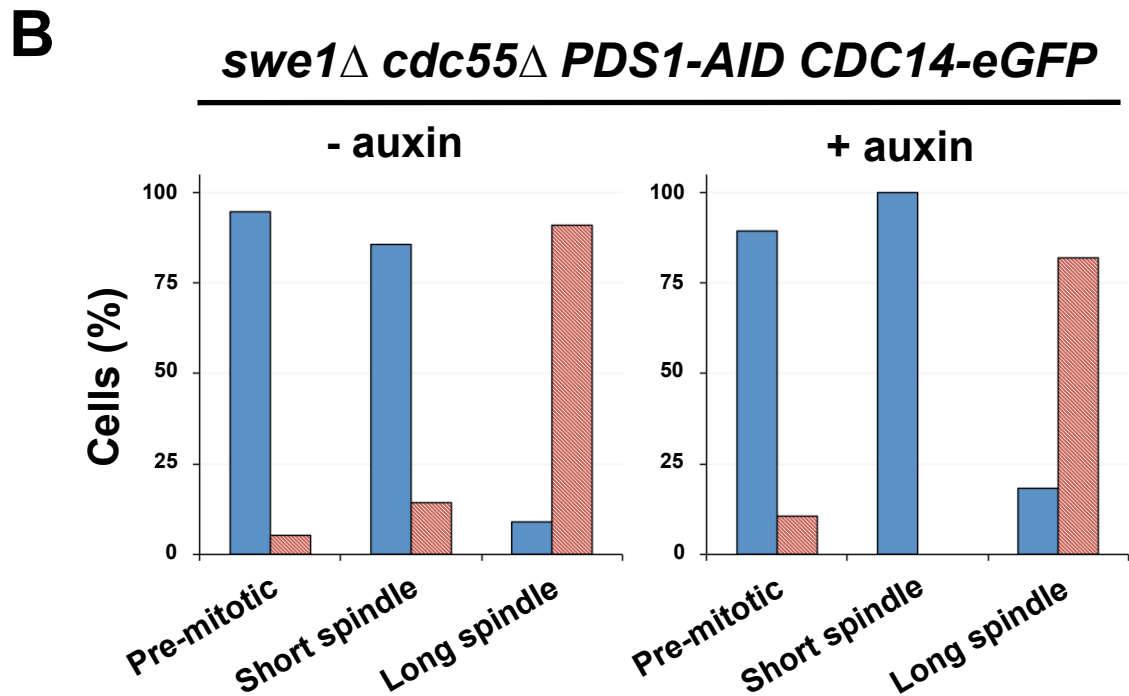
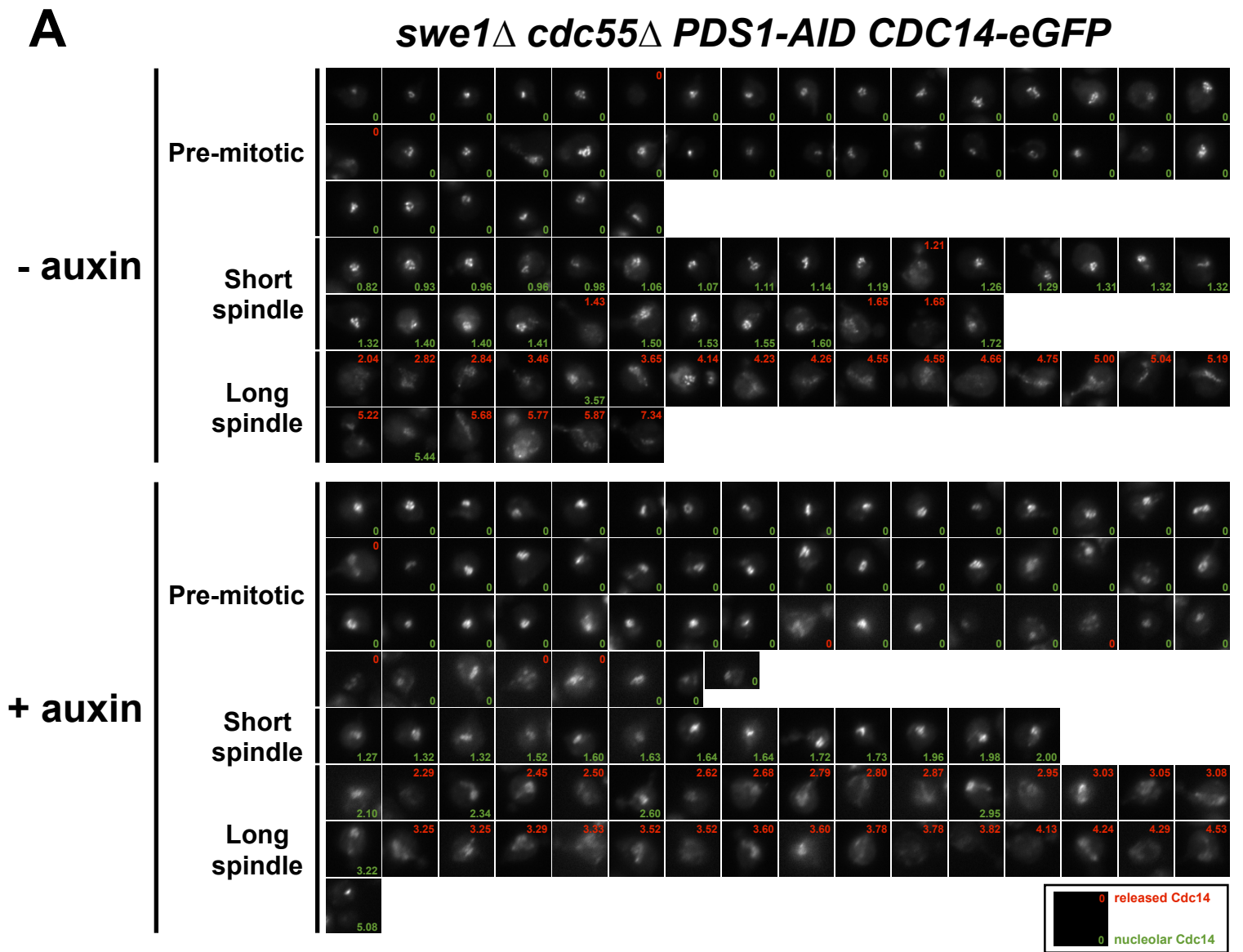


Figure 4-28

Figure 4-28. Cdc14 is not released from the nucleolus prematurely in *swe1Δ cdc55Δ* cells depleted of Pds1.

(A-B) *swe1Δ cdc55Δ PDS1-AID CDC14-eGFP SPC42-mCherry* (ADR7205) cells were grown at 25°C to log phase and arrested in G1 with α -factor. 30 minutes before α -factor release +/- auxin was added. Cells were released at t = 0 and at t = 90 minutes samples were fixed for microscopy. The distance between spindle pole bodies was measured in each cell.

(A) Images of Cdc14-eGFP. Each cell was categorized as *pre-mitotic* (one Spc42-mCherry focus), *short spindle* (Spc42-mCherry foci separated by < 2 μ m) or *long spindle* (Spc42-mCherry foci separated by > 2 μ m). In each cell Cdc14 was characterized as *nucleolar* or *released* qualitatively.

(B) Cdc14 is released at a spindle length of approximately 2 μ m in *swe1Δ cdc55Δ* cells regardless of Pds1 depletion. The proportion of cells displaying nucleolar and released Cdc14 at each cell cycle stage as determined by spindle length was calculated.

4.3 Discussion

4.3.1 Depletion of *Pds1* causes premature *Scc1* cleavage

We observed that cells depleted of Pds1 exhibited relatively normal spindle dynamics (Figure 4-3). Additionally, cells depleted of Pds1 initiated sister chromatid separation earlier than controls (13.85 +/- 1.21 minutes versus 17.65 +/- 1.05 minutes). This data is consistent with previous reports that *PDS1* deletion is not lethal and does not cause major mitotic defects in unperturbed cells (257, 366). However, contrary to previously published reports, our data suggests that Esp1 protease activity is activated ~ 20 minutes prematurely in cells depleted of Pds1, as demonstrated by the premature cleavage of Scc1 and Slk19 (Figures 4-5 and 4-12) (366, 436). This striking difference is difficult to explain as both Alexandru *et al.* and Clift *et al.* looked at Scc1 cleavage in unperturbed *pds1Δ* cells undergoing a synchronous mitosis and observed that Scc1 (339) and Slk19 (436) were cleaved with wildtype timing. A likely explanation for this observation is that depletion of Pds1-AID using auxin does not fully deplete Pds1 protein. Enough Pds1 is left to promote Esp1 import into the nucleus but not enough to effectively inhibit Esp1 proteolytic activity. Our data therefore suggests that the relatively comparable timing of Scc1 cleavage in wildtype and *pds1Δ* cells is due to less Esp1 entering the nucleus in *pds1Δ* as compared to wildtype cells, but this Esp1 having greater activity due to the lack of Pds1. It is surprising that these effects would balance so closely that *pds1Δ* cells maintain nearly full viability. However, this seems like the simplest explanation for why cells depleted of Pds1 using the Pds1-AID allele cleave Esp1 substrates prematurely.

4.3.2 Independent regulation of spindle elongation and sister separation

Our observation that depletion of Pds1 results in significantly earlier Scc1 cleavage, yet relatively normal anaphase onset, suggests that spindle elongation at anaphase onset in these cells is triggered by either a) dissolution of cohesin-independent sister chromatid linkages or b) regulated spindle elongation, independently of sister chromatid cohesion. I will discuss each of these possibilities in turn. Though cohesin-independent sister chromatid cohesion has been demonstrated, it seems unlikely to be responsible for delayed sister chromatid separation in Pds1-depleted cells that have cleaved Scc1. Following DNA replication, sister chromatids are wound around each other in topological linkages termed catenanes. Resolution of these linkages by DNA topoisomerase II is necessary for anaphase onset, though it is unclear if chromosome decatenation is actively regulated to delay anaphase onset (479). The condensin protein complex has been shown to mediate sister chromatid linkages in arm regions (480). However, condensin-mediated linkages appear not to link centromeric or telomeric regions. In stark contrast to this observation, Cdc14-dependent recruitment of condensin complexes to the telomeres and rDNA locus appears to be necessary for proper segregation of these DNA regions (481-483). Mutants in the gene *SMT4*, which encodes a SUMO isopeptidase, have been shown to have centromeric cohesion defects, and this defect appears to be mediated by DNA topoisomerase II (484, 485). Orc2, a component of the origin recognition complex has also been implicated in sister chromatid cohesion independently of cohesin (486).

Cells depleted of Pds1, though exhibiting defects in mitotic spindle structure, in general display a discernible transition between metaphase and anaphase (Figure 4-3). This suggests that even in the absence of Scc1, a regulated process governs anaphase spindle elongation. Though cohesin-independent sister chromosome linkages undoubtedly exist, it is

not known if cohesin-independent dissolution of these linkages result in a morphologically normal anaphase. Indeed, in *D. melanogaster* embryos and yeast, artificial cleavage of the alpha-kleisin subunit of cohesin (Scc1 in yeast, Rad21 in *D. melanogaster*) at metaphase leads to sister chromatid disjunction (248, 487). This strongly argues that the majority of cohesion during mitosis is mediated by the cohesin complex and that regulated cohesion loss is signaled through Scc1 cleavage. We therefore favour the model that, in cells depleted of Pds1, spindle elongation is delayed, not through cohesive linkages between sister chromatids but by a delay in triggering spindle elongation.

It is clear that cohesin cleavage alone is not sufficient for morphologically normal anaphase onset. In yeast, when Scc1 is artificially cleaved in the absence of separase activation, sister chromatids separate but anaphase spindles are unstable (248). In *D. melanogaster* embryos, similar experiments have demonstrated that when the *D. melanogaster* Scc1 homologue Rad21 is artificially cleaved in metaphase arrested cells, sister chromatids disjoin but fail to synchronously segregate to opposite poles (487). This phenotype can be rescued by inhibiting Cdk1 activity. This suggests that proper anaphase progression requires not only Scc1 cleavage but also dephosphorylation of a Cdk1 substrate that inhibits mitotic spindle elongation. Experiments in rat kidney epithelial (NRK) cells have demonstrated that maintaining Cdk1 activity using a non-degradable cyclinB mutant disrupts spindle elongation (488). However, in these experiments, maintaining Cdk1 activity during anaphase resulted in an increased rate of spindle elongation. In budding yeast it has been shown that inhibiting the mitotic cyclins Clb1 and Clb2 causes spindle elongation defects at anaphase independent of sister chromatid cohesion, suggesting a positive role for Cdk1^{Clb2} in spindle elongation (104). A full understanding of how Cdk1 regulates mitotic spindle elongation at anaphase remains to be achieved. In this context, it is interesting to note

that in yeast the APC regulatory subunit Cdc20, which is required for signaling anaphase onset can be rendered non-essential by deleting *PDS1* and *CLB5*, suggesting that the fundamental importance of Scc1 cleavage and Cdk1 inhibition in anaphase onset may be evolutionarily conserved (225).

4.3.3 CDC55 deletion is lethal to Pds1-depleted cells independent of

Scc1 phosphorylation

It has previously been proposed that in the absence of Pds1, PP2A^{Cdc55} inhibition of Esp1 maintains cell viability (436). In support of this, Clift *et al.* showed that cells lacking *PDS1* and *CDC55* are inviable, and co-depletion of these proteins leads to premature Scc1 cleavage and sister chromatid separation. Since PP2A^{Cdc55} and Esp1 interact physically (331), Clift *et al.* hypothesized that PP2A^{Cdc55} dephosphorylated and inhibited Esp1 directly, though they were unable to provide any direct evidence of this. Consistent with Clift *et al.*, we, and others, have demonstrated that co-depletion of Pds1 and Cdc55 is lethal to cells (Figure 4-2) (429, 436, 469).

A potential mechanism through which PP2A^{Cdc55} could delay anaphase in the absence of Pds1 could be by preventing premature Scc1 phosphorylation. Past work has proposed that PP2A^{Cdc55} dephosphorylates Cdc5 sites on Scc1 thereby preventing Scc1 phosphorylation and delaying Scc1 cleavage (411). Our data suggests that this is not the case for two reasons: 1) Scc1 cleavage does not appear to be further advanced in Pds1-depleted *cdc55Δ* cells as compared to Pds1-depleted controls. And 2) we observed that preventing Scc1 phosphorylation on ten Cdc5 sites does not rescue the sensitivity of *cdc55Δ* cells to Pds1 depletion (Figure 4-7B). This experiment was made possible by the surprising observation that preventing Scc1 phosphorylation is lethal to *pds1Δ* but not cells depleted of

Pds1-AID (Figure 4-7A and B). The simplest explanation for this is that cells depleted of Pds1-AID express enough Pds1 to maintain viability in the absence of Scc1 phosphorylation but not maintain viability in the absence of *CDC55*. It should be noted that expression of Scc1-HA3, Scc1-2A-HA3 or Scc1-10A-HA3 all rescued the growth defect of *pds1Δ* cells and expression of Scc1-10A rescued the growth defect of *PDS1-AID* cells on auxin plates. We were therefore cautious in drawing strong conclusions from these experiments. Regardless, these experiments demonstrate that PP2A^{Cdc55} regulates anaphase spindle elongation independently of Scc1 phosphorylation.

4.3.4 Cdk1 phosphorylates and PP2A^{Cdc55} dephosphorylates Esp1

We showed that Cdk1 phosphorylates Esp1 on at least one of three Cdk1 consensus sites found towards the N-terminal end of the Esp1 protease domain (Figure 4-8). We also showed that PP2A^{Cdc55} dephosphorylates Cdk1-phosphorylated Esp1 (Figure 4-9). Esp1 appears to be phosphorylated during mitosis, as Esp1 harvested during a nocodazole arrest migrates more slowly than Esp1-3A. Though this mobility shift could be due to mutating the Cdk1-sites and not to preventing phosphorylation per se, the observation that mutating the central sites also prevents Cdk1^{Cib2} phosphorylation of Esp1 in vitro and in vivo strongly suggest that the mobility shift is due to preventing phosphorylation. Vertebrate separase has also been shown to be phosphorylated and is inhibited by Cdk1^{cyclinB1} phosphorylation (473, 474).

Similar to the situation in vertebrate cells, Esp1 phosphorylation appears to be important in the absence of *PDS1*. However, though phosphorylation of vertebrate separase is inhibitory, phosphorylation of yeast Esp1 may not be. Our data suggest that the *esp1-3D* and *esp1-3A* alleles likely affect Esp1 non-proteolytic function. Until the non-proteolytic

activity of Esp1 is better understood, the function of Esp1 phosphorylation may be difficult to discern. Many eukaryotic separases contain minimal Cdk1 sites, though it is not obvious that specific Cdk1 sites have been conserved through evolution, (Figure 4-29). It has been shown that this is often the case with Cdk1 phospho-sites (69). Our data suggests that Cdk1 only phosphorylates Esp1 within the central three Cdk1 sites, and we have been unable to identify any other sites that are phosphorylated in vivo. It will be interesting to find out if Cdk1 does indeed phosphorylate other eukaryotic separases.

4.3.5 How does *PP2A^{Cdc55}* regulate spindle structure?

cdc55Δ cells depleted of Pds1 display striking spindle defects and are inviable (Figure 4-2 and 4-3). Though we cannot conclusively state that the spindle defects are causing inviability, the severity of spindle defects exhibited by *cdc55Δ*, *esp1-3D* and *slk19Δ* cells depleted of Pds1 and their relative viabilities shows strong correlation (Figure 4-2, Figure 4-3, Figure 4-10 and Figure 4-25). Moreover, the *esp1-NLS* allele, which rescues the lethality of *cdc55Δ* cells depleted of Pds1, also rescues the spindle defects (Figure 4-20). Finally, spindle defects in the different strains we have imaged also correlate strongly with sister chromatid missegregation (Figure 4-26). Taken together, this data strongly suggests that key to understanding why Cdc55 is required in the absence of Pds1 is understanding how *PP2A^{Cdc55}* regulates spindle structure.

It is clear that part of the answer is Cdk1 inhibition in the absence of Cdc55. It is known that Cdk1 is phosphorylated and inhibited in cells lacking *CDC55*, and this seems to be caused by both upregulation of Swe1 and downregulation of Mih1 (103, 186, 422, 440). This cannot be the entire mechanism, though, because deleting *SWE1* does not rescue *cdc55Δ* cells depleted of Pds1 and *swe1Δ cdc55Δ* cells depleted of Pds1 still display

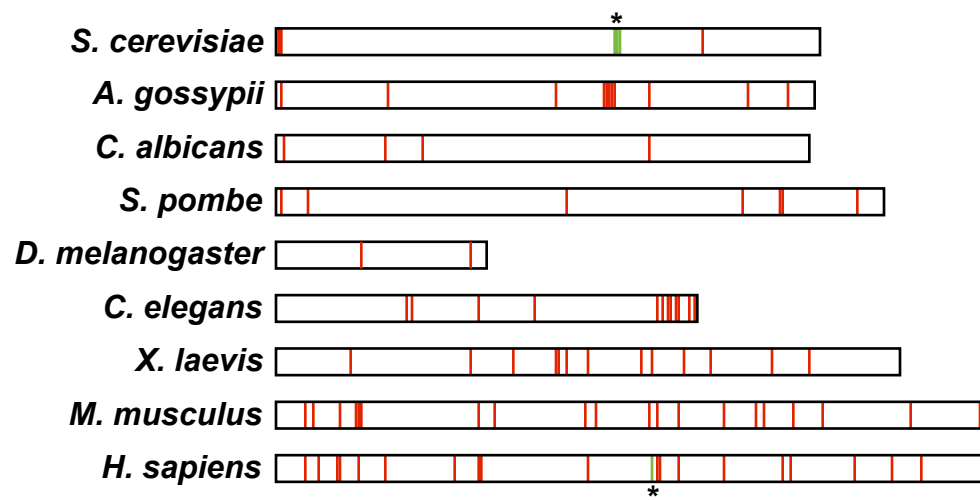


Figure 4-29

Figure 4-29. Cdk1 sites in eukaryotic separases.

The relative positions and distributions of minimal Cdk1 phosphorylation sites (S/TP) in various eukaryotic separases. The sites highlighted in green and marked with ‘*’ are Cdk1 sites that have been shown to be phosphorylated.

significant spindle defects (Figure 4-6). Part of the mechanism may also be Esp1 phosphorylation, as the phenotype of *esp1-3D* cells depleted of Pds1 is reminiscent of *cdc55Δ* cells depleted of Pds1 (Figure 4-10). However, this cannot fully explain the phenotype of *cdc55Δ* cells depleted of Pds1, as *esp1-3D* spindle defects in the absence of Pds1 are much smaller than those of *cdc55Δ*, and preventing Esp1 phosphorylation neither rescues the viability nor the spindle defects of *cdc55Δ* cells depleted of Pds1. Therefore, additional mechanism must account for this lethality.

The *esp1-NLS* allele rescue of *cdc55Δ* cells depleted of Pds1 is likely to prove crucial in understanding this phenotype. This rescue suggests that either Esp1 itself or a direct Esp1 target is misregulated in *cdc55Δ* cells lacking Pds1. Since Scc1 cleavage appears similar in *cdc55Δ* or wildtype cells depleted of Pds1, then the next most likely option appears to be Slk19. Slk19 is one of the few proteins that has been shown to physically interact with Esp1 (104, 328). Interestingly, *esp1-NLS* cannot rescue *slk19Δ* cells depleted of Pds1, suggesting that Slk19 functions downstream of Esp1 in a pathway regulating spindle structure. A simple genetic model that could explain all the pertinent genetic and spindle morphology data would then have to place Cin8 and Ase1 upstream of Slk19 as well (Figure 4-30). The phenotype of *slk19Δ* cells depleted of Pds1 suggests that this pathway may act to stabilize the metaphase spindle, and in the absence of any one of Ase1, Cin8, Esp1 or Slk19, the spindle is destabilized. In this model, Ase1 and Cin8 would promote Slk19-dependent spindle-stabilizing activity either through direct regulation of Esp1 or regulation of Esp1/Slk19 interaction. A major strength of this model is that it can easily explain the genetic interaction data we observed between the *esp1-NLS* allele and mutations in midzone proteins. If Esp1-NLS promoted Slk19 metaphase spindle-stabilizing activity and was refractory to Ase1 and

Figure 4-30. **A model for Esp1/Slk19 regulation by spindle midzone proteins.** Esp1 binds Slk19 and promotes Slk19 stabilization of the mitotic spindle. Cdk1^{Clb2} phosphorylation destabilizes Esp1/Slk19 complexes, whereas PP2A^{Cdc55} dephosphorylation and Ase1/Cin8 activity stabilize Esp1/Slk19 complexes.

Cin8 regulation, then *esp1*-NLS would be expected to rescue *ase1* Δ *PDS1-AID* and *cin8* Δ *PDS1-AID*, but not *slk19* Δ *PDS1-AID* cells treated with auxin.

As mentioned previously, Slk19 and Esp1 have been shown to physically interact, suggesting a biochemical mechanism through which Esp1 could promote Slk19 metaphase spindle-stabilizing activity. In this context, the phenotypes exhibited by strains expressing the *esp1-3D* and *esp1-3A* alleles could be explained by modulation of the affinity between Esp1 and Pds1. Cin8, Ase1, Esp1 and Slk19 all localize to the spindle midzone, though the dependency relationships between them appear to be complicated and disputed (140, 475). It is thought that Cin8 recruitment to the spindle midzone is dependent on Slk19, Esp1 and Ase1 (140, 141). It therefore seems especially problematic to postulate a model that places Cin8 upstream of Slk19.

In this context, it is interesting to note that there are striking similarities between both the localization of Slk19 and Cin8 proteins and the phenotypes of cells lacking these respective proteins. Slk19 and Cin8 both localize to the kinetochore during metaphase and then relocate to the spindle midzone at anaphase onset (380, 489). *slk19* Δ and *cin8* Δ cells display kinetochore clustering defects (490, 491). During metaphase, kinetochores on sister chromatids come under tension through attachment to opposite poles of the mitotic spindle. This results in enough physical separation of sister chromatids that the cluster of kinetochores pulled towards each spindle pole body can be easily identified using fluorescence microscopy of kinetochore proteins. In cells lacking *CIN8*, rather than exhibiting a bilobed localization with a cluster of kinetochores pulled towards each spindle pole body, kinetochores become unclustered and more than two kinetochore foci are apparent (491). Cin8 appears to promote kinetochore clustering by preferentially destabilizing longer kinetochore microtubules (491). It has recently been shown that cells

lacking *SLK19* also display a kinetochore clustering defect (490). This defect is apparent in cells released from a nocodazole arrest, but not in unperturbed cycle cells as is the kinetochore clustering defect seen in *cin8Δ* cells. There is no clear biochemical data suggesting a mechanism through which Slk19 could regulate kinetochore clustering, though two possible mechanisms are a) Cooperation with Cin8 in destabilizing relatively long kinetochore microtubules, b) stabilizing pericentromeric cohesion. Support for the former mechanism stems largely from the fact that Cin8 and Slk19 are both clearly regulators of spindle dynamics and they both localize to kinetochores. A potential role for Slk19 regulating the stability of pericentromeric cohesin is supported by the observation that Slk19 physically interacts with the Scc1 component of cohesin (492), the Smc2 component of condensin (493) and separase (104, 328). Cells lacking *SLK19* or *CIN8* also display reduced mitotic spindle length (380, 489). Interestingly, both *slk19Δ* and *cin8Δ* cells cause a reduction in metaphase spindle length to approximately 1μm.

Deletion of *CIN8* or *SLK19* also cause defects in astral microtubules and nuclear positioning (494, 495). *slk19Δ* cells have been shown to display an increase in the number of astral microtubules extending into the cytoplasm (494). *cin8Δ* cells have been shown to display the same phenotype (495). Perhaps the most striking phenotype shared by cells lacking *SLK19* or *CIN8* is the so-called ‘daughterly’ phenotype (494, 495). When cohesin cleavage is inhibited using an uncleavable Scc1 subunit, wildtype cells delay cell cycle progression with large buds and the nucleus positioned in the mother or mother-bud neck. Cells lacking *SLK19* or *CIN8* arrest with a high proportion of nuclei in the daughter cell (494, 495). Interestingly, both Esp1-inhibition and *ASE1* deletion also result in a ‘daughterly’ phenotype and an increased number of astral microtubules (494, 495). This suggests that in wildtype cells Slk19, Cin8, Esp1 and Ase1 all promote a mother-directed force acting on the

nucleus. Though it seems likely that this force will be found to be mediated by astral microtubules, the mechanism has not yet been elucidated.

Together, these data would suggest that Slk19 and Cin8 (and perhaps Ase1 and Esp1) may directly cooperate to regulate microtubule dynamics in a variety of cellular contexts. To our knowledge, a direct physical interaction between Slk19 and Cin8 has not yet been observed. Though this model is highly speculative it is easily testable and regardless of its validity, pursuing the questions that emerge from it are likely to provide insight into spindle regulation by midzone proteins.

Regulation of the spindle midzone structure is well conserved in higher eukaryotes (reviewed in (496)). The Ase1 homolog PRC-1 (protein required for cytokinesis) plays a central role in organizing the spindle midzone (497). Similar to Ase1, in the absence of PRC-1, the spindle midzone fails to form. The kinesin-5 Eg5 (vertebrate Cin8 homologue) localizes to the spindle midzone and has a conserved role exerting poleward force on spindle microtubules (498). It does not appear that separase has conserved functions at the spindle midzone. There is also some question as to whether Slk19 is the budding yeast homolog of CENP-F, a protein that also relocalizes at anaphase onset from the kinetochore to the spindle midzone (499, 500).

4.3.6 Cdk1 regulation of mitotic proteins

In this work we have identified Esp1 as an in vivo target of PP2A^{Cdc55} and Cdk1^{Cib2}. In work not presented here, our lab showed that PP2A^{Cdc55} could also dephosphorylate the APC in vivo and in vitro (425). The growing number of studies identifying specific targets of Cdk1 complexes and phosphatases may be starting to provide significant insight into the architecture of phosphorylation signaling during yeast mitosis (Figure 4-31).

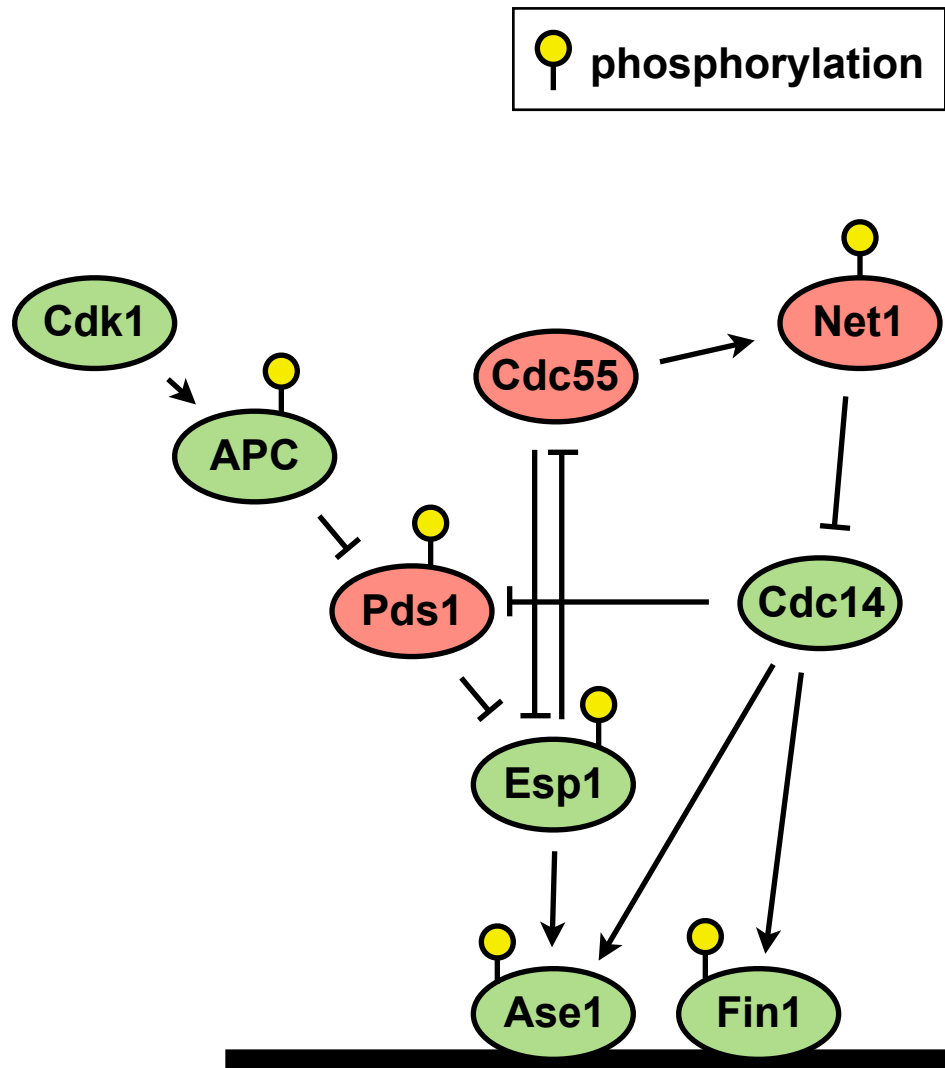


Figure 4-31

Figure 4-31. A model for cohesin cleavage and spindle stabilization.

Increasing APC activity promotes Pds1 degradation and Esp1 activation. Esp1 cleaves cohesin and inhibits PP2A^{Cdc55}, thereby promoting Net1 phosphorylation. This releases Cdc14 which further inhibits Pds1 and promotes Esp1 activity. Cdc14 also dephosphorylates and stabilizes the spindle associated proteins Fin1 and Ase1. Ase1 dephosphorylation promotes Esp1 localization to the spindle midzone and activation of Ase1.

Numerous spindle-associated proteins have been found to be phosphorylated by Cdk1, and interestingly, at least two of them appear to be phosphorylated specifically by Cdk1^{Clb5}. Fin1 is phosphorylated from S-phase to metaphase and phosphorylation inhibits Fin1 association with the mitotic spindle (136). At anaphase onset Cdc14 dephosphorylates Fin1 and promotes Fin1 spindle association. Fin1 association with the spindle contributes to spindle integrity. Another spindle associated protein, Ase1, also appears to be phosphorylated by Cdk1^{Clb5}. Ase1 dephosphorylation during anaphase promotes Ase1 localization of Slk19 and Esp1 to the spindle midzone (140). Phosphomimetic Ase1 mutants are defective in spindle midzone assembly and have unstable anaphase spindles. Thus, the switch from high Cdk1^{Clb5} activity to high Cdc14 activity results in spindle stabilization through Fin1 and Ase1.

The kinesin-5 motor protein Cin8 is also dephosphorylated by Cdc14, however it appears to be specifically phosphorylated by Cdk1^{Clb2} complexes in vitro and exhibits peak phosphorylation during anaphase (501). Phosphorylation of Cin8 appears to promote dissociation of Cin8 from the spindle and Cin8 dissociation perturbs spindle elongation. Thus, as cells progress through late anaphase, Cin8 phosphorylation slows spindle elongation.

Many proteins that are not associated with the mitotic spindle are also regulated by Cdk1 phosphorylation during mitosis. Net1 binds Cdc14 in inhibitory complexes in the nucleolus until Cdk1^{Clb2} phosphorylation promotes Net1 release of Cdc14 (320, 333). Net1 is maintained in a dephosphorylated state by PP2A^{Cdc55} until Esp1 inhibits PP2A^{Cdc55} activity at anaphase onset. (331).

In this study we showed that Esp1 is phosphorylated by Cdk1^{Clb2} and dephosphorylated by PP2A^{Cdc55}. This suggests that perhaps Esp1 is phosphorylated with

similar timing as Net1. Though we have not determined if this is the case, it seems likely given that Esp1 and Cdc55 physically interact (331). Thus Esp1 inhibition of PP2A^{Cdc55} as proposed by Queralt *et al.* would result in less PP2A^{Cdc55} dephosphorylation of Esp1 and a coincident increase in Net1 and Esp1 phosphorylation. We have also shown that PP2A^{Cdc55} and Cdk1^{Cib2} directly regulate the phosphorylation status of the APC. It is tempting to speculate that a shift in balance between the relative activities of Cdk1^{Cib2} and PP2A^{Cdc55} activates the APC, which targets Pds1 for degradation, thereby liberating Esp1, promoting Net1 release and concomitant Esp1 activation.

The APC substrate Pds1 is also a Cdk1 substrate, though there is some evidence that it is targeted by both Cdk1^{Cib2} and Cdk1^{Cib5} (263). Pds1 dephosphorylation has been shown to lead to its ubiquitination. Therefore, Cdc14 release promotes Pds1 degradation, further Esp1 release and further Cdc14 activation.

There are a number of interesting aspects to this model. When Holt *et al.* identified that Cdc14 dephosphorylated Pds1, thereby promoting its degradation, this implicated Cdc14 in a feedback loop promoting its own continued activation (263). In short, Cdc14 dephosphorylation would liberate Esp1 from Pds1/Esp1 complexes, Esp1 would then inhibit PP2A^{Cdc55} and thereby promote Net1 phosphorylation and further Cdc14 release. Our identification of PP2A^{Cdc55} as the phosphatase that dephosphorylates Esp1 identifies another feedback loop that could potentially lead to amplification of Esp1 activity. Though we have not provided any direct evidence that modulating Esp1 phosphorylation affects this feedback loop, it would be interesting to test experimentally.

Another interesting aspect of this model is that, from the network topology, it is apparent that spindle stabilization through Ase1 and Fin1 would likely occur temporally later than Scc1 cleavage upon APC activation. Cleavage of Scc1 is only one step removed from

Esp1 activation. Spindle stabilization though, requires many steps before Cdc14-dependent dephosphorylation of Ase1 allows direct Esp1 promotion of Ase1 midzone localization (140). This could have evolved so that spindle elongation didn't precede cohesion cleavage, thereby threatening chromosomal integrity.

Finally, this model neatly outlines how rising Cdk1 activity could shift the balance of phosphatase activity from PP2A^{Cdc55} to Cdc14, thereby ensuring its own inactivation. Simply by increasing in activity, Cdk1 ensures that (as long as the relevant pools of PP2A^{Cdc55} and Esp1 are of appropriate size) eventually enough Esp1 will become activated to overcome PP2A^{Cdc55} inhibition and bring about the events of mitotic exit. Despite the limited phenotypes observed for the *esp1-3A* mutant thus far, perhaps preventing Esp1 phosphorylation will yet result in some more interesting phenotypes.

Chapter 5

Conclusions and Future Directions

In this work we have demonstrated that the budding yeast Wee1 (Swe1) kinase regulates anaphase onset by inhibiting Cdk1 and thereby preventing APC activation. This work is a major step in identifying the downstream pathways that Swe1 regulates. We think that regulation of anaphase onset by Wee1 kinases may be more common than previously appreciated as numerous studies are consistent with a model that Wee1 kinases may regulate multiple cell cycle transitions. We showed that the spindle checkpoint and morphogenesis checkpoint bypass displayed by cells lacking the regulatory subunit of PP2A, *CDC55* was due to increased phosphorylation and activation of the APC. This work identified an important mitotic substrate of PP2A^{Cdc55} and helped elucidate how eukaryotic cells signal mitotic transitions. In the second part of this project we showed that Cdk1^{Clb2} and PP2A^{Cdc55} regulate the phosphorylation status of a conserved protease separase. Phosphoregulation of yeast separase had not previously been reported, though vertebrate separase has been shown to be phosphorylated by Cdk1, suggesting that perhaps separase phosphorylation is conserved evolutionarily. Finally, we showed that phosphorylated separase likely regulates mitotic spindle structure through a set of proteins residing at the spindle midzone. This set of proteins include the yeast homologues of vertebrate PRC-1, which organizes the spindle midzone, and the kinesin-5 motor Eg5 which is important for maintaining spindle bipolarity.

Though we were able to provide some answers over the course of this research, there are many questions that remain. Experiments I have done over the course of many years have hinted at the importance of the mitotic cyclins Clb5 and Clb6 in regulating spindle structure.

Upon formation of mitotic spindles in cells lacking CLB5 and CLB6, spindle pole bodies immediately separate to a length of ~3 μ m, some reaching as far as 5 μ m, before quickly returning to a more normal length and undergoing a relatively normal anaphase onset. Some very simple experiments would provide very useful insight into why this process is taking place. By imaging Ndc80-eGFP or another kinetochore component we could determine whether kinetochores are remaining bound to a single spindle pole or are being separated by the spindle at this early stage. Conversely, do these spindles return to a normal length because as the spindle captures kinetochores, the force opposing spindle elongation gradually increases? Similar experiments using lacI-GFP labeled chromosomes would also likely provide insight into the events that are leading to this aberrant spindle morphology. Cdk1^{Clb5} has been implicated in phosphorylating the microtubule associated proteins Fin1 and Ase1, and it would be interesting if mimicking phosphorylation of one of these proteins could rescue the spindle defects displayed by *clb5 Δ clb6 Δ* cells.

Given that Fin1 and Ase1 have been shown to be Cdk1^{Clb5} substrates, it would be interesting to systematically purify proteins that have been shown to be phosphorylated in mitosis and determine if they are preferentially phosphorylated by a specific Cdk1 complex. A previous study (69) that sought to address this question used the *cdk1-as1* allele that displays ~50-fold lower activity than the wildtype protein. Additionally, many Cdk1 substrates have been identified since that study. During mitosis, PP2A^{Cdc55} activity is relatively high before it drops as Cdc14 activity rises during mitotic exit. This shift in phosphatase activity affects the phosphorylation status of many mitotic proteins. Clb5 levels fall before those of Clb2 as cells transit mitosis, so cells are also exposed to a similar shift in overall kinase activity during mitosis. Shifts in kinase and phosphatase activities could play an important role in signaling major cell cycle transitions. An important first step in

answering this question is to identify the specificity of mitotic kinases in a more systematic manner.

The most exciting potential avenue of research that this work opened up concerns the regulation of spindle elongation at anaphase onset. Our data makes a strong case that sister chromatid cohesion is lost in cells depleted of Pds1, yet spindle elongation occurs relatively normally. If, as I believe, the key to understanding this phenomenon lies at the spindle midzone, then a few simple experiments may provide a great deal of knowledge about how this structure is regulated. The first experiments I would undertake would be to do live imaging of mitosis in *cin8Δ* and *ase1Δ* cells depleted of Pds1. If these cells resemble *cdc55Δ* and *slk19Δ* cells depleted of Pds1 then that would implicate these proteins in regulating some cellular function that becomes critically important in the absence of sister chromatid cohesion. Another way of addressing this issue would be to attempt to detect protein/protein interactions between midzone proteins. Slk19 and Esp1 have already been shown to physically interact. I have already attempted to look at Slk19/Esp1 interaction in *cdc55Δ* *esp1-NLS*, *esp1-3D* and *esp1-3A* cells by co-immunoprecipitation. Thus far my attempts have been unsuccessful. One problem may be that I have been immunoprecipitating a tagged Slk19 and then attempting to detect Esp1 with our lab-generated rabbit polyclonal Esp1 antibody. We have used this approach because all of the C-terminal tagged Esp1-alleles that we have periodically used to detect Esp1 protein display genetic interactions with the *PDS1-AID* allele. I am confident that with a bit of troubleshooting these problems could be solved and protein/protein interactions between Esp1 and Slk19 will be observed. My simple model predicts that in the absence of *CIN8* or *ASE1*, Esp1 interaction with Slk19 is lost or reduced, and the *esp1-NLS* allele restores Slk19 binding. My model would also predict that the Esp1-3D would bind less tightly to Slk19 and Esp1-3A would bind more tightly.

Years ago I spent many months imaging strains with different APC substrates deleted to try to find one that allowed bypass of the morphogenesis checkpoint. This would have been the key experiment that conclusively showed that the morphogenesis checkpoint signal was mediated by the APC. Unfortunately, those experiments were unsuccessful and I never identified a single APC substrate or combination of substrates that bypassed the checkpoint. It would be interesting to attempt these experiments again but instead of using the *pds1* Δ allele use the *PDS1-AID* allele. It is possible that *pds1* Δ cells do not bypass the morphogenesis checkpoint partially because of a) additional targets, but also b) they don't effectively degrade cohesin due to inefficient Esp1 import. In the likely scenario that *PDS1-AID* cells depleted of Pds1 do not bypass the morphogenesis checkpoint I would like to image *slk19* Δ *PDS1-AID* cells. Given that deletion of *CDC55* on its own bypasses the morphogenesis checkpoint, it seems likely that depletion of both Slk19 and Pds1 would achieve a similar result.

By addressing questions such as those I have just raised and undertaking experiments such as those I have proposed, I believe that some interesting things will be learned about cell cycle progression and mitotic signaling in yeast. Given the high degree of evolutionary conservation in the pathways we study it seems quite likely that this work and work like this will inform our understanding of all eukaryotic cells.

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Contributions of Collaborators

All data obtained by others that I have included in this thesis are attributed in the appropriate figure legend. The in vivo labeling experiments in Figure 4-8B, 4-8E and 4-9A were done by Dr. Adam Rudner. The immunoblot experiment in Figure 4-8C was done by Carole Dore. The in vitro phosphorylation reaction in Figure 4-8F and in vitro dephosphorylation reaction in Figure 4-9B were done by Elizabeth Williams and Carole Dore.

Appendix 1

Yeast strains used in this study

All strains used in this study are derivatives of W303 (*ura3-1 leu2-3,112 trp1-1 his3-11 ase2-1 can1-100*)

Strain	Mating type	Genotype
ADR21	<i>MATa</i>	
ADR22	<i>MATa</i>	
ADR1172	<i>MATa</i>	<i>cin8Δ::TRP1</i>
ADR1555	<i>MATa</i>	<i>clb5Δ::hisG-URA3-hisG</i>
ADR2146	<i>MATa</i>	<i>esp1-myc13-KAN^R</i>
ADR4006	<i>MATa</i>	<i>bar1Δ</i>
ADR4009	<i>MATa</i>	<i>SPC42-eGFP-KAN^R bar1Δ</i>
ADR4012	<i>MATa</i>	<i>mih1Δ::LEU2 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4015	<i>MATa</i>	<i>swe1Δ::TRP1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4099	<i>MATa</i>	<i>mad2Δ::URA3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4100	<i>MATa</i>	<i>swe1Δ::TRP1 bar1Δ</i>
ADR4191	<i>MATa</i>	<i>KIP1-13myc-NAT^R CIN8-GFP-HYG^R bar1Δ</i>
ADR4193	<i>MATa</i>	<i>PDS1-18myc-leu2::HIS3 CIN8-GFP-HYG^R bar1Δ</i>
ADR4313	<i>MATa</i>	<i>KAN^R-cdk1-Y19F SPC42-eGFP-HYG^R bar1Δ</i>
ADR4381	<i>MATa</i>	<i>clb5Δ::hisG-URA3-hisG SPC42-eGFP-KAN^R bar1Δ</i>
ADR4403	<i>MATa</i>	<i>clb5Δ::hisG-URA3-hisG clb6Δ::LEU2 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4486	<i>MATa</i>	<i>clb5Δ::hisG-URA3-hisG pds1Δ::HYG^R SPC42-eGFP-KAN^R bar1Δ</i>
ADR4491	<i>MATa</i>	<i>pds1Δ::HYG^R SPC42-eGFP-KAN^R bar1Δ</i>
ADR4502	<i>MATa</i>	<i>sgo1Δ::HYG^R SPC42-eGFP-KAN^R bar1Δ</i>
ADR4517	<i>MATa</i>	<i>SGO1-13myc-HIS3 bar1Δ</i>
ADR4533	<i>MATa</i>	<i>pds1Δ::NAT^R sgo1Δ::HYG^R SPC42-eGFP-KAN^R bar1Δ</i>
ADR4647	<i>MATa</i>	<i>pMET-CLB5-KAN^R pds1Δ::NAT^R sgo1Δ::HYG^R SPC42-eGFP-Sphis5⁺ bar1Δ</i>
ADR4738	<i>MATa</i>	<i>cdc55Δ::HIS3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4854	<i>MATa</i>	<i>swe1::pGAL-SWE1-LEU2 ESP1-myc13-KAN^R mihΔ::HYG^R bar1Δ</i>
ADR4902	<i>MATa</i>	<i>cdc16-6A-trp1::LEU2 cdc27-5A-KAN^R cdc23-A-HA cdc55Δ::LEU2 SPC42-eGFP-HYG^R bar1Δ</i>
ADR4973	<i>MATa</i>	<i>cdc16-6A-trp1::LEU2 cdc27-5A-KAN^R cdc23-A-HA SPC42-eGFP-HYG^R bar1Δ</i>
ADR4979	<i>MATa</i>	<i>cdc16-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR4984	<i>MATa</i>	<i>swe1Δ::TRP1 cdc16-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5020	<i>MATa</i>	<i>cdc55Δ::HIS3 cdc16-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5026	<i>MATa</i>	<i>NDC80-eGFP-Sphis5⁺ bar1Δ</i>
ADR5044	<i>MATa</i>	<i>cdc55Δ::HIS3 trp1::pSCC1-SCC1-HA3-TRP1 scc1Δ::pGAL-SCC1-myc18-URA3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5049	<i>MATa</i>	<i>cdc55Δ::HIS3 trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-myc18-URA3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5056	<i>MATa</i>	<i>cdc55Δ::HIS3 leu2::pSCC1-scc1-2A-HA3-LEU2 scc1Δ::pGAL-SCC1-myc18-URA3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5123	<i>MATa</i>	<i>SLK19-13myc-KAN^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR5154	<i>MATa</i>	<i>cdc55Δ::LEU2 SLK19-13myc-KAN^R SCC1-GFP-Sphis5⁺ bar1Δ</i>

ADR5162	<i>MATa</i>	<i>swe1Δ::TRP1 SLK19-13myc-KAN^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR5274	<i>MATa</i>	<i>ESP1-18myc-TRP1</i>
ADR5300	<i>MATa</i>	<i>pds1Δ-HYG^R ESP1-18myc-TRP1</i>
ADR5307	<i>MATa</i>	<i>CEN-pESP1-ESP1-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5308	<i>MATa</i>	<i>CEN-pESP1-ESP1-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5410	<i>MATa</i>	<i>clb5Δ::hisG-URA3-hisG clb6Δ::LEU2 SPC42-eGFP-NAT^R bar1Δ</i>
ADR5465	<i>MATa</i>	<i>CDC55-TAP-KITRP1 bar1Δ</i>
ADR5417	<i>MATa</i>	<i>cdc16-6A-trp1::LEU2 cdc27-5A-KANR cdc23-A-HA trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-myc18-URA3 cdc55Δ::HIS3 SPC42-eGFP-NATR bar1Δ</i>
ADR5475	<i>MATa</i>	<i>CEN-pESP1-esp1-2A + 3A + 1A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5481	<i>MATa</i>	<i>CEN-pESP1-esp1-2A + 3A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5482	<i>MATa</i>	<i>CEN-pESP1- esp1-1A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5488	<i>MATa</i>	<i>CEN-pESP1- esp1-2A + 1A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5491	<i>MATa</i>	<i>CEN-pESP1- esp1-2A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5492	<i>MATa</i>	<i>CEN-pESP1- esp1-3A + 1A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5494	<i>MATa</i>	<i>CEN-pESP1- esp1-3A-3FLAG-KAN^R-LEU2 CEN-pESP1-ESP1-URA3 esp1Δ-HYG^R</i>
ADR5504	<i>MATa</i>	<i>pMET-PDS1-KAN^R clb5Δ::hisG-URA3-hisG clb6Δ::LEU2 SPC42-eGFP-NAT^R bar1Δ</i>
ADR5509	<i>MATa</i>	<i>ESP1-18myc-TRP1 bar1Δ</i>
ADR5599	<i>MATa</i>	<i>cdc23-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5604	<i>MATa</i>	<i>swe1Δ::TRP1 cdc23-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5623	<i>MATa</i>	<i>cdc55Δ::HIS3 cdc23-1 SPC42-eGFP-KAN^R bar1Δ</i>
ADR5635	<i>MATa</i>	<i>esp1-2D + 3D + 1D-NAT^R</i>
ADR5768	<i>MATa</i>	<i>SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR5898	<i>MATa</i>	<i>esp1-1D-NAT^R</i>
ADR5899	<i>MATa</i>	<i>esp1-2D-NAT^R</i>
ADR5900	<i>MATa</i>	<i>esp1-3D + 1D-NAT^R</i>
ADR5901	<i>MATa</i>	<i>esp1-3D + 2D-NAT^R</i>
ADR5909	<i>MATa</i>	<i>esp1-3D-NAT^R</i>
ADR5910	<i>MATa</i>	<i>esp1-2D + 1D-NAT^R</i>
ADR6325	<i>MATa</i>	<i>cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-NAT^R bar1Δ</i>
ADR6387	<i>MATa</i>	<i>PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR6389	<i>MATa</i>	<i>PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-Sphis5⁺ bar1Δ</i>
ADR6409	<i>MATa</i>	<i>esp1-3D-HYG^R</i>
ADR6415	<i>MATa</i>	<i>PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR6426	<i>MATa</i>	<i>cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6428	<i>MATa</i>	<i>PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6429	<i>MATa</i>	<i>PDS1-AID-KAN^R</i>
ADR6431	<i>MATa</i>	<i>leu2::pGPD1-OsTIR1-LEU2</i>
ADR6432	<i>MATa</i>	<i>cdc55Δ::HYG^R PDS1-AID-KAN^R</i>
ADR6434	<i>MATa</i>	<i>cdc55Δ::HYG^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6436	<i>MATa</i>	<i>cdc55Δ::HYG^R</i>

ADR6446	<i>MATa</i>	<i>esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-Sphis5⁺ bar1Δ</i>
ADR6448	<i>MATa</i>	<i>esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR6450	<i>MATa</i>	<i>esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6454	<i>MATa</i>	<i>esp1-3D-HYG^R SPC42-eGFP-Sphis5⁺ bar1Δ</i>
ADR6464	<i>MATa</i>	<i>his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR6468	<i>MATa</i>	<i>cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR6635	<i>MATa</i>	<i>trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-18myc-URA3</i>
ADR6636	<i>MATa</i>	<i>trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-18myc-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6639	<i>MATa</i>	<i>trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-18myc-URA3 cdc55Δ::HYG^R</i>
ADR6642	<i>MATa</i>	<i>cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-18myc-URA3</i>
ADR6714	<i>MATa</i>	<i>esp1-1A-NAT^R bar1Δ</i>
ADR6715	<i>MATa</i>	<i>esp1-3A-NAT^R bar1Δ</i>
ADR6716	<i>MATa</i>	<i>esp1-2A-NAT^R bar1Δ</i>
ADR6717	<i>MATa</i>	<i>esp1-2A + 3A-NAT^R bar1Δ</i>
ADR6718	<i>MATa</i>	<i>esp1-1A + 2A-NAT^R bar1Δ</i>
ADR6722	<i>MATa</i>	<i>cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR6724	<i>MATa</i>	<i>PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR6748	<i>MATa</i>	<i>esp1-3A-NAT^R cdc55Δ::HYG^R bar1Δ</i>
ADR6774	<i>MATa</i>	<i>esp1-3A + 1A-NAT^R bar1Δ</i>
ADR6775	<i>MATa</i>	<i>esp1-2A + 3A + 1A-NAT^R bar1Δ</i>
ADR6786	<i>MATa</i>	<i>SCC1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR6863	<i>MATa</i>	<i>esp1-3A-NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6870	<i>MATa</i>	<i>esp1-3A-NAT^R cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR6876	<i>MATa</i>	<i>cdc55Δ::HYG^R bar1Δ</i>
ADR6879	<i>MATa</i>	<i>esp1-3A-NAT^R cdc55Δ::HYG^R</i>
ADR6886	<i>MATa</i>	<i>esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR6887	<i>MATa</i>	<i>esp1-3D-HYG^R his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR6929		<i>rts1Δ::HYG^R bar1+</i>
ADR6948	<i>MATa</i>	<i>cdc16-6A-trp1::LEU2 cdc27-5A-KAN^R cdc23-A-HA bar1Δ</i>
ADR6949	<i>MATa</i>	<i>cdc16-6A-trp1::LEU2 cdc27-5A-KAN^R cdc23-A-HA cdc55Δ::HIS3 bar1Δ</i>
ADR6950	<i>MATa</i>	<i>esp1-3A-NAT^R cdc55Δ::HIS3 bar1Δ</i>
ADR6951	<i>MATa</i>	<i>esp1-3A-NAT^R cdc16-6A-trp1::LEU2 cdc27-5A-KAN^R cdc23-A-HA cdc55Δ::HIS3 bar1Δ</i>
ADR6952	<i>MATa</i>	<i>esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR6953	<i>MATa</i>	<i>esp1-3D-NAT^R bar1Δ</i>
ADR6956	<i>MATa</i>	<i>esp1-3A-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 bar1Δ</i>
ADR6957	<i>MATa</i>	<i>esp1-3A-HYG^R SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR6968	<i>MATa</i>	<i>mad2Δ::URA3 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-NAT^R bar1Δ</i>

ADR7043	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-NAT^R bar1Δ</i>
ADR7068	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR7134	<i>MATa</i>	<i>swe1Δ::TRP1 bar1Δ</i>
ADR7135	<i>MATa</i>	<i>esp1-3A-HYG^R swe1Δ::TRP1 bar1Δ</i>
ADR7136	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HIS3 bar1Δ</i>
ADR7137	<i>MATa</i>	<i>esp1-3A-HYG^R swe1Δ::TRP1 cdc55Δ::HIS3 bar1Δ</i>
ADR7138	<i>MATa</i>	<i>swe1Δ::TRP1</i>
ADR7140	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HIS3</i>
ADR7143	<i>MATa</i>	<i>swe1Δ::TRP1 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7145	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7149	<i>MATa</i>	<i>esp1-3A-HYG^R swe1Δ::TRP1 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7195	<i>MATa</i>	<i>esp1-3A-NAT^R</i>
ADR7205	<i>MATa</i>	<i>swe1Δ::TRP1 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 CDC14-eGFP-Sphis5⁺ SPC42-mCherry-NAT^R bar1Δ</i>
ADR7252	<i>MATa</i>	<i>cdc55Δ::HYG^R his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-KAN^R bar1Δ</i>
ADR7317	<i>MATa</i>	<i>esp1-3A-HYG^R swe1Δ::TRP1 cdc55Δ::HIS3^R PDS1-AID-NAT^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-KAN^R bar1Δ</i>
ADR7345	<i>MATa</i>	<i>ura3::pGAL-MPS1-myc-URA3 bar1Δ</i>
ADR7346	<i>MATa</i>	<i>esp1-3D-HYG^R ura3::pGAL-MPS1-myc-URA3 bar1Δ</i>
ADR7348	<i>MATa</i>	<i>mad3Δ::LEU2 esp1-3D-HYG^R bar1Δ</i>
ADR7422	<i>MATa</i>	<i>mad3Δ::LEU2 bar1Δ</i>
ADR7432	<i>MATa</i>	<i>swe1Δ::TRP1 2μ-pGAL-CLB2-TAP-URA3 bar1Δ</i>
ADR7459	<i>MATa</i>	<i>esp1-3A-ADE2 cdc55Δ::HIS3 SPC42-eGFP-KAN^R bar1Δ</i>
ADR7518	<i>MATa</i>	<i>mad2Δ::URA3 esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-Sphis5⁺ bar1Δ</i>
ADR7551	<i>MATa</i>	<i>pds1-38-URA3</i>
ADR7552	<i>MATa</i>	<i>pds1-38-URA3 esp1-3D-HYG^R</i>
ADR7592	<i>MATa</i>	<i>esp1-NLS-URA3</i>
ADR7593	<i>MATa</i>	<i>esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7678	<i>MATa</i>	<i>slk19Δ::NAT^R</i>
ADR7680	<i>MATa</i>	<i>esp1-3D-ADE2 SCC1-eGFP-Sphis5⁺ bar1Δ</i>
ADR7687	<i>MATa</i>	<i>esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7688	<i>MATa</i>	<i>esp1-NLS-URA3 cdc55Δ::HIS3</i>
ADR7689	<i>MATa</i>	<i>esp1-NLS-URA3 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7691	<i>MATa</i>	<i>SCC1-eGFP-Sphis5⁺ bar1Δ</i>
ADR7750	<i>MATa</i>	<i>esp1-NLS-URA3 cdc55Δ::HIS3 PDS1-AID- KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-NAT^R bar1Δ</i>
ADR7762	<i>MATa</i>	<i>esp1-3A-NLS-URA3-NAT^R</i>
ADR7763	<i>MATa</i>	<i>esp1-3A-NLS-URA3-NAT^R PDS1-AID- KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7765	<i>MATa</i>	<i>esp1-3D-NLS-URA3-HYG^R</i>
ADR7858	<i>MATa</i>	<i>esp1-3D-NLS-URA3-HYG^R PDS1-AID- KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR7984	<i>MATa</i>	<i>ESP1-18myc-TRP1 cdc20Δ::LEU2 trp1::pGAL-CDC20-TRP1 bar1Δ</i>

ADR7999	<i>MATa</i>	<i>ESP1-eGFP-Sphis5⁺ bar1Δ</i>
ADR8001	<i>MATa</i>	<i>esp1-3A-eGFP-Sphis5⁺-NAT^R bar1Δ</i>
ADR8003	<i>MATa</i>	<i>esp1-3D-eGFP-Sphis5⁺-NAT^R bar1Δ</i>
ADR8050	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-NLS-URA3</i>
ADR8051	<i>MATa</i>	<i>slk19Δ::NAT^R cdc55Δ::HIS3</i>
ADR8052	<i>MATa</i>	<i>slk19Δ::NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8053	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-NLS-URA3 cdc55Δ::HIS3</i>
ADR8054	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8055	<i>MATa</i>	<i>slk19Δ::NAT^R cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8056	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-NLS-URA3 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8072	<i>MATa</i>	<i>esp1-NLS-URA3 SLK19-13myc-NAT^R SCC1-GFP-Sphis5⁺ bar1Δ</i>
ADR8075	<i>MATa</i>	<i>ESP1-GFP-TRP1</i>
ADR8076	<i>MATa</i>	<i>ESP1-GFP-TRP1 cdc55Δ::HYG^R</i>
ADR8077	<i>MATa</i>	<i>ESP1-GFP-TRP1 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8078	<i>MATa</i>	<i>ESP1-GFP-TRP1 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8091	<i>MATa</i>	<i>SCC1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-eGFP-HYG^R bar1Δ</i>
ADR8097	<i>MATa</i>	<i>spo12Δ::NAT^R</i>
ADR8104	<i>MATa</i>	<i>slk19Δ::NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 SPC42-GFP-Sphis5⁺ bar1Δ</i>
ADR8108	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-NLS-URA3</i>
ADR8109	<i>MATa</i>	<i>spo12Δ::NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8110	<i>MATa</i>	<i>spo12Δ::NAT^R cdc55Δ::HIS3</i>
ADR8111	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8112	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-NLS-URA3 cdc55Δ::HIS3</i>
ADR8113	<i>MATa</i>	<i>spo12Δ::NAT^R cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8114	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-NLS-URA3 cdc55Δ::HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8179	<i>MATa</i>	<i>SCC1-AID-KAN^R</i>
ADR8180	<i>MATa</i>	<i>SCC1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8183	<i>MATa</i>	<i>ESP1-13myc-KAN^R</i>
ADR8184	<i>MATa</i>	<i>ESP1-13myc-KAN^R cdc55Δ::HYG^R</i>
ADR8185	<i>MATa</i>	<i>ESP1-13myc-KAN^R PDS1-AID-NAT^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8186	<i>MATa</i>	<i>ESP1-13myc-KAN^R cdc55Δ::HYG^R PDS1-AID-NAT^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8209	<i>MATa</i>	<i>esp1-3D-eGFP-Sphis5⁺-NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8213	<i>MATa</i>	<i>esp1-3D-eGFP-Sphis5⁺-NAT^R</i>
ADR8223	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-3D-HYG^R</i>
ADR8224	<i>MATa</i>	<i>spo12Δ::NAT^R esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8229	<i>MATa</i>	<i>esp1-3D-HYG^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8230	<i>MATa</i>	<i>slk19Δ::NAT^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8231	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-3D-HYG^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8232	<i>MATa</i>	<i>slk19Δ::NAT^R esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8233	<i>MATa</i>	<i>slk19Δ::NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8336	<i>MATa</i>	<i>pds1Δ::HYG^R trp1::pSCC1-SCC1-HA3-TRP1 scc1Δ::pGAL-SCC1-myc18-URA3 bar1Δ</i>

ADR8339	<i>MATa</i>	<i>pds1Δ::HYG^R bar1Δ</i>
ADR8340	<i>MATa</i>	<i>pds1Δ::HYG^R trp1::pSCC1-scc1-10A-HA3-TRP1 scc1Δ::pGAL-SCC1-myc18-URA3 bar1Δ</i>
ADR8341	<i>MATa</i>	<i>pds1Δ::HYG^R leu2::pSCC1-scc1-2A-HA3-LEU2 scc1Δ::pGAL-SCC1-myc18-URA3 bar1Δ</i>
ADR8421	<i>MATa</i>	<i>slk19Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2 his3::pCUP1-eGFP12-lacI12-HIS3 ura3::lacO-URA3 SPC42-mCherry-NAT^R bar1Δ</i>
ADR8534	<i>MATa</i>	<i>CEN-ESP1-HIS3</i>
ADR8535	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-3A-NAT^R</i>
ADR8536	<i>MATa</i>	<i>CEN-ESP1-HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8537	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-3A-NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8538	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-3D-HYG^R</i>
ADR8539	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8622	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-NLS-URA3 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8623	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8497	<i>MATa</i>	<i>ase1Δ::HYG^R</i>
ADR8556	<i>MATa</i>	<i>esp1-NLS-URA3 cin8Δ::TRP1</i>
ADR8558	<i>MATa</i>	<i>esp1-NLS-URA3 cin8Δ::TRP1 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8560	<i>MATa</i>	<i>ase1Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8564	<i>MATa</i>	<i>esp1-NLS-URA3 ase1Δ::HYG^R</i>
ADR8606	<i>MATa</i>	<i>cin8Δ::TRP1 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8610	<i>MATa</i>	<i>esp1-NLS-URA3 ase1Δ::KAN^R PDS1-AID-HYG^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8622	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-NLS-URA3 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8632	<i>MATa</i>	<i>CEN-ESP1-HIS3 esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8635	<i>MATa</i>	<i>CEN-HIS3</i>
ADR8636	<i>MATa</i>	<i>CEN-HIS3 esp1-3D-HYG^R</i>
ADR8637	<i>MATa</i>	<i>CEN-HIS3 esp1-3D-HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8638	<i>MATa</i>	<i>CEN-HIS3 esp1-3A-NAT^R</i>
ADR8639	<i>MATa</i>	<i>CEN-HIS3 esp1-3A-NAT^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8640	<i>MATa</i>	<i>CEN-HIS3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8641	<i>MATa</i>	<i>CEN-HIS3 esp1-NLS-URA3 PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8642	<i>MATa</i>	<i>CEN-HIS3 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8643	<i>MATa</i>	<i>CEN-ESP1-HIS3 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8644	<i>MATa</i>	<i>CEN-HIS3 esp1-NLS-URA3 cdc55Δ::HYG^R PDS1-AID-KAN^R leu2::pGPD1-OsTIR1-LEU2</i>
ADR8645	<i>MATa</i>	<i>esp1-NLS-URA3 cdc55Δ::HYG^R</i>

Noel Lianga – Curriculum Vitae

Education

- PhD. 2007-present Program in Biochemistry, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON.
- BSc. 2002-2007 Majors in Molecular Biology/Genetics and Political Science, Departments of Molecular and Cellular Biology and Political Science, University of Guelph, Guelph, ON.

Research Experience

- 2007-present Graduate Student, Program in Biochemistry, Department of Biochemistry, Microbiology and Immunology University of Ottawa, Ottawa, ON.
Advisor: Dr. Adam Rudner
Swe1 regulation of the metaphase to anaphase transition.
- 2006-2007 4th year project student
Departments of Molecular and Cellular Biology and Political Science, University of Guelph, Guelph, ON.
Advisor: Dr. Steffen Graether
Expression and detection of a type II antifreeze protein.
- Summer 2006 Student Research Assistant
Departments of Molecular and Cellular Biology and Political Science, University of Guelph, Guelph, ON.
Advisor: Dr. Steffen Graether
Isolation and expression of domains of the insulin receptor protein.

Papers

Lianga, N., E.C. Williams, E.K. Kennedy, C. Doré, S. Pilon, S.L. Girard, J.S. Deneault, A.D. Rudner. 2013. A Wee1 checkpoint inhibits anaphase onset. *J. Cell Biol.* 201:843–862

Talks

Lianga, N., Doré, C., Williams, L. and Rudner, A.D. How does PP2A^{Cdc55} dephosphorylation of Separase regulate anaphase onset? Cell Dynamics Workshop, Keene, ON. May 2013.

Lianga, N., Williams, L., Redmond, E. and Rudner, A.D. A new role for Swe1 regulating anaphase onset. Salk Institute Cell Cycle Symposium, La Jolla, CA. July 2011.

Lianga, N., Rudner, A.D. New roles for Cdc55 regulating anaphase onset. Ottawa Institute of Systems Biology Symposium, Montebello, QC. June 2011.

Liang, N., Rudner, A.D. Swe1 regulation of the metaphase to anaphase transition. Ottawa Regional Microbiology Symposium, Ottawa, ON. April 2010.

Posters

Liang, N., Doré, C., Williams, E., Rudner, A.D. Does Cdk1 promote anaphase onset by phosphorylating Separase? ASCB meeting, San Francisco, CA. December 2012.

Liang, N., Doré, C., Williams, E., Pilon, S. and Rudner, A.D. PP2A^{Cdc55} opposes activation of anaphase onset by inhibiting APC^{Cdc20} and Separase. FASEB meeting, Steamboat Springs, CO. July 2012.

Liang, N., Rudner, A.D. Cdk1 and PP2A^{Cdc55} play opposing roles in anaphase promoting complex regulation. ASCB meeting, Denver, CO. December 2011.

Liang, N., Dodd-Moher, E., Redmond, E. and Rudner, A.D. The budding yeast Wee1 kinase inhibits the metaphase to anaphase transition. Ottawa Institute of Systems Biology Symposium, Ottawa, ON. April 2009.

Liang, N., Dodd-Moher, E., Redmond, E. and Rudner, A.D. The budding yeast Wee1 kinase inhibits the metaphase to anaphase transition. ASCB meeting, San Francisco, CA. December 2008.

Liang, N., Dodd-Moher, E., Antoniuk, E. and Rudner, A.D. Does Swe1 regulate the metaphase to anaphase transition? FASEB meeting, Carefree, AZ. June 2008.

Awards

2009 to 2012	CIHR Canadian Graduate Scholarship
2008 to 2012	University of Ottawa Excellence Scholarship
2008 to 2009	Ontario Graduate Scholarship in Science and Technology
2007 to 2008	University of Ottawa Admission Scholarship
2005	University of Guelph Deans' Scholarship
2004	CSAHS Alumni Heritage Scholarship
2002	University of Guelph Entrance Scholarship