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CDC7-DBF4 REGULATES MITOTIC EXIT BY INHIBITION OF THE BUDDING YEAST POLO KINASE CDC5

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CDC7-DBF4 REGULATES MITOTIC EXIT BY INHIBITION OF THE BUDDING YEAST POLO KINASE CDC5

By

Charles Thomas Miller

A DISSERTATION

Submitted to
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ABSTRACT

CDC7-DBF4 REGULATES MITOTIC EXIT BY INHIBITON OF THE BUDDING YEAST POLO KINASE CDC5

By

Charles Thomas Miller

The Dbf4-dependent kinase (DDK) is essential for DNA replication initiation and N-terminal Dbf4 sequences are important for a robust response to replication stress in S-phase. I identified a novel function for Dbf4 in regulating events late in mitosis. This function is mediated by an interaction between N-terminal Dbf4 sequences and the budding yeast polo kinase Cdc5. The Dbf4-Cdc5 interaction requires a functional Cdc5 polo box domain (PBD), but is independent of protein phosphorylation. Although Dbf4 mutants proceed normally through S-phase, loss of Dbf4-dependent regulation of Cdc5 causes premature nuclear segregation and mitotic exit in the presence of a mispositioned spindle. Dbf4 does not regulate Cdc5 localization to spindle pole bodies or kinase activity, raising the possibility that Dbf4 inhibits Cdc5 by regulating kinase targeting to specific substrates. The Cdc5 polo box domain is an *in vitro* substrate of DDK and alanine substitution of conserved PBD residues eliminates PBD phosphorylation. These studies raise the possibility that Dbf4 may regulate Cdc5-dependent mitotic exit by phosphorylation of the PBD.

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ABBREVIATIONS

3AT	3-aminotriazole
AA	Amino Acid
APC	Anaphase Promoting Complex
ASE1	Anaphase Spindle Elongation 1
ASK	Activator of S-phase Kinase
ASK1	Associated with Spindles and Kinetechores
Asy	Asynchronous
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine TriPhosphate
ATR	ATM and Rad3 related
ATRIP	ATR Interacting Protein
BCK2	Bypass of C Kinase 2
BFA1	Byr 4 Alike 1
BRCA1	BReast CAncer 1
BRCT	BRCA1 C-Terminal domain
BUB	Budding Uninhibited by Benzimidizoles
BUBR1	Budding Uninhibited by Benzimidizoles Related 1
C	Celsius

CDC	Cell Division Cycle
CDE	Cell cycle Dependent Element
CDH1	Cdc20 Homolog 1
CDK	Cyclin Dependent Kinase
CDT1	Cdc10 Dependent Transcript 1
CFI1	Cdc14 Inhibitor 1
CHK1	Checkpoint Kinase 1
CHR	Cell cycle genes Homology Region
CIP1	CDK Interacting Protein 1
CLA4	CLn Activity dependent 4
CLB	Cyclin B-type
CLN	CycLiN
C-terminal	Carboxy-terminal
Δ	Deletion
DAPI	4',6-DiAmidino-2-PhenylIndole
DBF2	Dumbell Former 2
DBF4	Dumbell Former 4
DDC2	DNA Damage Checkpoint 2
DDK	Dbf4-Dependent Kinase

DNA	DeoxyriboNucleic Acid
DPB11	DNA Polymerase B 11
DSB	Double Strand Break
dsDNA	Double Stranded DNA
DTT	DiThioThreitol
DYN1	Dynein 1
E2F	E2 promoter (adenovirus) Factor
EDTA	Ethylene Diamine Tetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Estrogen Receptor
ESP1	Extra Spindle Pole Bodies 1
FAR1	Factor Arrest 1
FEAR	Cdc14 Early Anaphase Release
FHA	Fork Head Associated (domain)
FIN1	Filaments in between Nuclei 1
FITC	Fluorescein IsoThioCyanate
FOB1	FOrk Blocking less 1
G0	Gap 0

G1	Gap 1
G2	Gap 2
GEF	Guanine nucleotide Exchange Factor
GINS	Go Ichi Nii San (Japanese; Sld5, Psf1, Psf2 and Psf3)
GST	Glutathione-S-transferase
GTP	Guanosine Triphosphate
H	Histidine
H1	Histone 1
HA	HemAgglutinin
HCl	HydroChloric Acid
HeLa	Henrietta Lacks
HSK1	Homolog of Cdc7 Kinase 1
HSL	Histone Synthetic Lethal
HU	HydroxyUrea
INK4	INhibitor of CDK4
IP	ImmunoPrecipitation
K	lysine
KAR9	KARyogamy 9

KIN4	KINase 4
KIP	KInesin related Protein
KOH	Potassium Hydroxide
L	Leucine
LTE1	Low Temperature Essential 1
M	Mitotic
MAD	Mitotic Arrest Deficient
MAPK	Mitogen Activated Protein Kinase
MCB	*MluI* Cell cycle Box
MCC	Mitotic Checkpoint Complex
MCM	MiniChromosome Maintenance
MEC1	Mitosis Entry Checkpoint 1
MEN	Mitotic Exit Network
mg	milligrams
MgCl2	Magnesium Chloride
MIH1	Mitotic Inducer Homolog 1
Min	Minutes
MMS	Methyl Methane Sulfonate
MOB1	Mps One Binder

MPF	Mitotic Promoting Factor
MPS1	Multipolar Spindle 1
MRC1	Mediator of Replication Checkpoint 1
MTOCs	MicroTubule Organizing Centers
MYO2	Myosin 2
MYPT1	Myosin Phosphatase Targeting Subunit 1
NaCl	Sodium Chloride
NET1	Nucleolar silencing Establishing factor and Telophase
ng	nanograms
Ni	Nickel
NIH	National Institutes of Health
NP-40	Nonidet P-40
NPT	Non Permissive Temperature
N-terminal	Amino-terminal
ORC	Origin Recognition Complex
p	protein
P	Proline
PAGE	Poly Acrylamide Gel Electrophoresis

PBD	Polo Box Domain
PCNA	Proliferating Cell Nuclear Antigen
PDS1	Precocious Disassociation of Sister Chromatids
PI3K	Phosphatidyl-inositol 3 Kinase
PIR	Polo box Interacting Region
PLK1	Polo-Like Kinase 1
POL	Polymerase
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PP4	Protein Phosphatase 4
PPH21	Protein PHosphatase 21
PPH22	Protein PHosphatase 22
pre-RC	pre-Replicative Complex
RAD	RADiation sensitive
RB	RetinoBlastoma
rDNA	Ribosomal DNA
RPA	Replication Protein A
RTS1	Suppressor of Rox3 Temperature Sensitive allele 1

S	Synthesis
SAC	Spindle Assembly Checkpoint
SCC	Sister Chromatid Cohesion 1
SCC3	Sister Chromatid Cohesion 3
SF9	*Spodoptera frugiperda* 9
SIC1	Subunit Inhibitor of CDK
SIN	Septation Initiation Network
SLD2	Synthetic Lethal with Dpb11-2
SLD3	Synthetic Lethal with Dpb11-3
SLI15	Synthetic Lethal with Ipl15
SLK19	Synthetic Lethal with Kar3 19
SMC1	Structural Maintenance of Chromosomes 1
SMC3	Structural Maintenance of Chromosomes 3
SPB	Spindle Pole Body
SPO12	SPOrulation 12
SPOC	Spindle Position Checkpoint
ssDNA	Single Stranded DNA
SWE1	Saccharomyces WEe1
SWI5	SWItching Deficient 5

T	Threonine
t	Time
TEL1	TELomere maintenance 1
TEM1	TErmination of M phase 1
Thr	Threonine
TPD3	tRNA Processing Deficient 3
TPL	Increase in Ploidy 1
tRNA	transfer RNA
ts	Temperature Sensitive
ug	micrograms
W	Tryptophan
WT	Wild type
YPD	Yeast extract Peptone Dextrose

INTRODUCTION

CELL CYCLE OVERVIEW

Fundamentals of the Eukaryotic Cell Cycle

Duplication and partitioning of the genome into two daughter cells is a fundamental process of life. Cells without a nucleus (prokaryotes) replicate by binary fission, a process where cells physically separate into two daughter cells, each with a genetic complement. Organisms containing nucleated DNA (eukaryotes) by contrast separate replication and chromosome division into the synthesis (S)-phase and mitotic (M)-phase respectively. These phases are divided by two gap (G) phases: one preceding DNA synthesis (G1) and another (G2), which separates replication from mitosis (Figure 1). The length of gap phases varies considerably between species. In general, G phases enhance cell cycle regulation and maintain genome fidelity. In multicellular organisms many differentiated cells exit the cell cycle, stop dividing or exist in a reversible state of senescence or self-induced quiescence (G0-phase). In addition, cells that sustain irreversible genetic damage can permanently exit the cell cycle as an alternative to programmed cell death (apoptosis).

In the first phase of interphase (G1), cells resume normal biosynthesis after completion of the preceding cell cycle and disassemble the mitotic machinery (Figure 1). During this phase, cells grow in size and respond to external stimuli using receptors on the cell surface (i.e. receptor tyrosine kinases and G-protein coupled receptors) or within the cytoplasm (i.e. nuclear hormone

receptors). In unicellular organisms, the decision to remain in G1 or to enter the cell cycle is primarily determined by the nutritional status of the environment [1, 2]. Metazoan cells, by contrast, must integrate myriad signals that influence cell fate [3]. Synthesis of enzymes required for S-phase also occurs in G1 in preparation for the highly anabolic phase of DNA replication.

Transition through the restriction point/START (G1-phase) is a highly regulated and irreversible process requiring faithful replication and segregation of chromosomes. Eukaryotic S-phase commences at the initiation of DNA replication and is complete once the entire genome has been duplicated. In general, genomic replication occurs outside actively transcribed regions [4]. Successful completion of DNA synthesis produces a homologous pair of intact sister chromatids, which are connected at the centromere and along the chromosome arms until mitosis. Critical to the success of DNA replication is a collection of S-phase checkpoint proteins (discussed in more detail below), which respond to problems during DNA replication. After replication, cells enter G2 until the beginning of mitosis. During G2, cells synthesize proteins required for assembly of the mitotic spindle in preparation for chromosome segregation and monitor the genome for the presence of DNA damage. Damage is corrected (as discussed below) prior to the beginning of mitosis and sister chromatid separation.

Mitosis is the most physically dynamic phase of the cell cycle. In mammalian cells, mitosis commences in prophase with the rising activity of mitotic kinases, inducing nuclear envelope disassembly, chromosome

condensation and separation of sister chromatid arms. As chromosomes condense they attach to bipolar centrosomes and align along the metaphase plate. After cells ensure that all sister chromatids are properly attached to the mitotic spindle, rapid degradation of cohesion proteins during anaphase permits the segregation of chromatids to opposite spindle poles. In telophase, chromatids decondense and the nuclear envelope reforms. Cells physically separate by the formation of an actin cleavage furrow during cytokinesis. Constriction of the actin contractile ring minimizes the cytoplasmic volume between daughter cells and forms a cytoplasmic bridge in preparation for abscission.

Molecular Regulation of the Eukaryotic Cell Cycle

Faithful transmission of the genetic material is an absolute requirement of life. As a consequence, all organisms have developed a sophisticated and tightly regulated system of signaling pathways and mechanisms to ensure accurate transmission of genetic material to progeny. To understand the biochemical nature of cell cycle progression in both normal and malignant cells, considerable effort has been invested in the last four decades to identify and characterize proteins involved in regulating cell division. Seminal discoveries by Tim Hunt and Paul Nurse in sea urchins and fission yeast, respectively, identified crucial proteins regulating entry into mitosis [5, 6]. Hunt and colleagues observed cyclical expression of proteins (cyclins) during every round of mitosis in sea urchin embryos. In the laboratory of Paul Nurse, fission yeast *wee1* mutants were shown to enter M-phase prematurely [6]. These and subsequent studies led to

the conclusion that the activity of cyclin-dependent kinases is required for entry into mitosis and progression through the cell cycle [7-10].

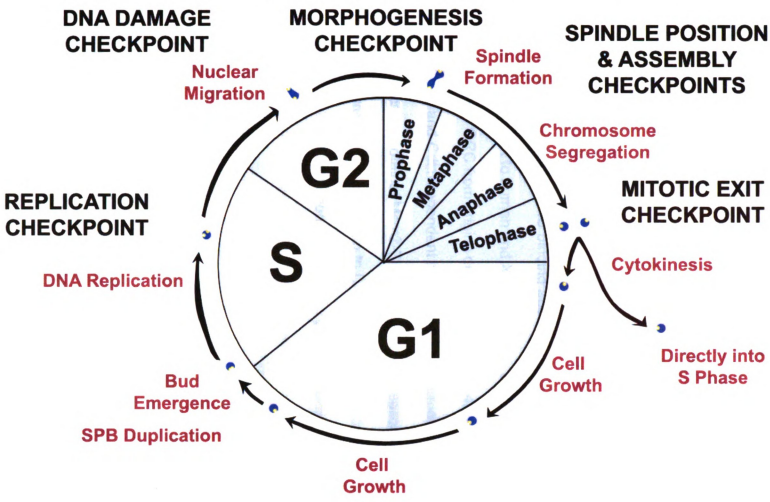
A family of cyclins and cyclin-dependent kinases (CDKs) control the progression through G1 and initiation of DNA replication in mammalian cells [11]. Two broad classes of CDK inhibitors, which are regulated by growth factors, target G1 CDKs and suppress their activity [12]. The INK4A family (p15, p16 and p19) appears to exclusively regulate Cdk4-cyclin D activity [12]. Cip1 and Kip1 (p21 and p27) are more promiscuous and inhibit the activity of multiple CDKs [13, 14]. In humans, as cells exit quiescence (G0) and enter G1 phase, expression and binding of D-type cyclins (D1, D2, and D3) to Cdk4 and Cdk6 initiates a phosphorylation cascade targeting the retinoblastoma (Rb) family of transcriptional repressors [15, 16]. Hyperphosphorylation of the Rb family of 'pocket proteins' facilitates their release from DNA-bound E2F transcriptional activators, which initiate the coordinated expression of cell cycle specific genes [17]. In addition, CDK phosphorylates and inactivates the Cdc20 homolog 1 (Cdh1) subunit of the anaphase promoting complex (APC), inhibiting its ability to ubiquitinate cell cycle proteins and target them for proteosomal degradation [18, 19]. Late in G1, Cdk2-cyclin E complexes phosphorylate Rb on additional sites, reinforcing progression through the restriction point and into S-phase [20]. At this moment, cells are irreversibly committed to replicating their DNA and segregating their chromosomes into two daughter cells.

The unicellular fungus *Saccharomyces cerevisiae* has a single cyclin-dependent kinase (cell division cycle 28) that regulates entry into S-phase and

progression through mitosis [21]. Activation of CDK in budding yeast is regulated by the G1/S-phase cyclins (Clns) 1-3 and B-type cyclins (Clbs) 1-6 [22-25]. Cyclin expression and CDK activity is tightly regulated in all eukaryotic organisms by pathways that integrate nutrient and mitogenic signals from within the cell and from receptors at the cell surface [25]. In budding yeast, the primary determinant for progression through the restriction point is cell size [26]. Completion of the preceding cell cycle results in mother and daughter cells of unequal size (Figure 1). As a result, the larger mother cells progress more rapidly through the next G1 phase compared to smaller daughter cells, which take longer to reach the critical size threshold. Integrating cell size with molecular preparation for S-phase entry, newborn mother and daughter cells must reach a critical size to express a sufficient quantity of Cln3 and bypass of C kinase 2 (Bck2) to activate Swi4/6 binding factor (SBF) and Mlu1-box binding factor (MBF), G1 transcription factors that stimulate transcription of Cln1, 2 and B-type cyclins [22, 25, 27-29]. Cdc28-Cln2 activity, which initiates bud emergence, precedes full activation of Cdc28-Clb5 as a result of persistent expression of the Cdc28-Clb inhibitors Sic1 [30-32]. Phosphorylation of CDK inhibitors by Cdc28-Cln triggers SCF (Skp, Cullin, F-box) binding and ubiquitylation, and results in full activation of the S-phase Cdc28-Clb kinases [33, 34]. Stabilization of Cdc28-Clb activity then reduces Cln expression in preparation for events later in the cell cycle [35]. Progression through START (G1 to S transition) is inhibited in the presence of mating factor, which activates a MAP kinase signaling-cascade and triggers activation of the CDK inhibitor factor arrest 1 (Far1) [36, 37].

Figure 1. The budding yeast cell cycle and checkpoints. Budding yeast enter the cell cycle in G1, following cytokinesis and the physical separation of mother and daughter cells. At this time, cells grow until they reach a critical size threshold after which they traverse START and begin DNA replication in S-phase. Coincident with DNA replication initiation is bud emergence and spindle pole body duplication. During G2 phase, cells monitor the genome for the presence of DNA damage and initiate nuclear migration towards the mother-daughter bud neck. Entry into mitosis is activated by increased catalytic activity of CDK (Cdc28-Clb) and is inhibited by morphological defects in the budding yeast. Formation of the intranuclear mitotic spindle drives nuclear division and the separation of sister chromatids into mother and daughter cells. Defects in bipolar spindle attachment and spindle orientation delay progression through mitosis until these checkpoints are satisfied. The cell cycle is complete following downregulation of CDK activity during mitotic exit and the separation of cells during abscission.

Budding Yeast Cell Cycle



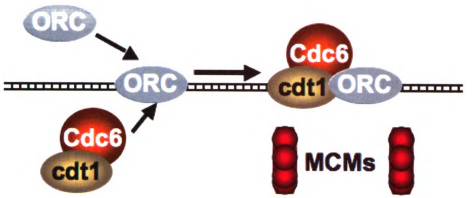
Events preceding DNA replication initiation, including origin binding and helicase loading, are best understood in *S. cerevisiae*. This is likely attributable to the well-defined replication origin binding sequence in this organism. The hexameric origin recognition complex (Orc1-6) interacts with the A and B1 elements (~30bp DNA) of origins in budding yeast [38, 39]. The sequences required for ORC binding in other organisms are not as well-defined and are thought to depend on mechanisms other than DNA sequence [40-42]. In budding yeast, fission yeast and *Drosophila*, ORC binding appears constitutive throughout the cell cycle; this is in contrast to human and *Xenopus* cells, which indicate at least a partial clearance of ORC from origins during metaphase [43-46]. ORC binding stimulates Cdc6 and Cdt1-dependent loading of minichromosome maintenance DNA helicase proteins (Mcm2-7), forming the pre-replicative complex (pre-RC) (Figure 2) [47]. In addition to DNA sequence and chromatin elements that regulate pre-RC formation, trans-acting proteins participate in coordinating cell cycle progression with assembly of the pre-RC to prevent unauthorized replication. Cdc28-Cln and Clb kinases negatively regulate pre-RC formation by phosphorylating Cdc6, which triggers its nuclear export (mammalian cells) or degradation (yeast) [48-54]. The pre-RC inhibitor Geminin also inhibits MCM loading in metazoans independent of CDK activity [55, 56].

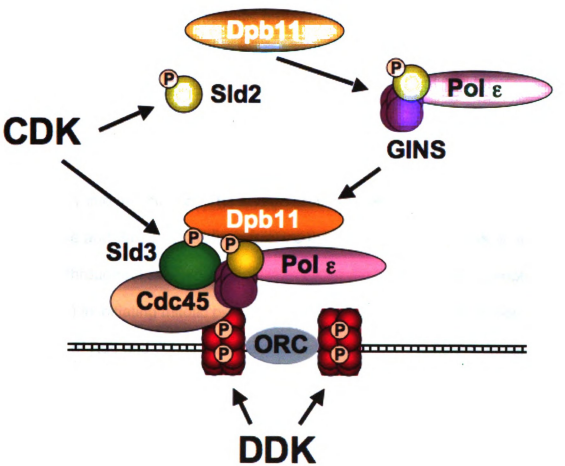
Two protein kinases Cdc28-Clb5 (S-phase CDK) and Cdc7-Dbf4 (Dbf4-dependent kinase; DDK) are required to trigger DNA replication initiation at origins in the budding yeast (Figure 2) [21, 57]. Phosphorylation events by CDK and DDK stimulate the assembly of additional proteins at the pre-RCs and

precipitate activation of the replicative helicase Mcm2-7 and origin unwinding [58-60]. Recent evidence suggests that the only essential proteins requiring Cdc28 phosphorylation at budding yeast origins are two initiation proteins: Sld2 (synthetic lethal with Dbp11-2) and Sld3 [61, 62]. Cdc28 phosphorylation of Sld2 and Sld3 facilitate their interaction with Dpb11, which is necessary to recruit Cdc45, GINS and replicative DNA polymerases to sites of origin unwinding (Figure 2) [61, 62].

S-phase is marked by the initiation of chromosome replication and duplication of the spindle-pole bodies (SPBs)/centrosomes. Eukaryotic DNA replication is bidirectional and semi-conservative with each strand serving as a template for a newly synthesized nascent strand [63-66]. The pace of S-phase progression is controlled by the regulation of origin firing throughout S-phase [67]. Early origins fire shortly after passage through the restriction point with late origins initiating later in S-phase [68-72]. DNA damage or low nucleotide levels initiate a delay in late origin firing to slow progression through S-phase and delay chromatid separation in mitosis [73, 74]. During every S-phase, replication forks encounter myriad genetic lesions that must be overcome for faithful duplication of the genome [75, 76]. Eukaryotes utilize a variety of polymerases for both leading and lagging-strand synthesis as well as translesion and mismatch–repair polymerases that repair and replace damaged or mismatched bases [77]. Polymerase α initiates replication at origins following helicase unwinding and prior to loading the highly processive polymerases (Pol ε and δ), which coordinate leading and lagging strand synthesis [77, 78]. A primary factor

Figure 2. Model of budding yeast DNA replication initiation. Initiation of DNA replication commences in late G2/M phase of the preceding cell cycle. During this period, the budding yeast initiator, ORC (origin recognition complex), binds origins of replication throughout the genome. Binding of ORC to origins allows the association of Cdt1 and Cdc6 with the initiator and facilitates MCM (minichromosome maintenance) loading on origins. The triggering mechanism for replication initiation at origins requires the catalytic activity of CDK (cyclin dependent kinase) and DDK (Dbf4-dependent kinase). Phosphorylation of Sld2 and Sld3 by CDK is thought to trigger association with the BRCT containing protein Dpb11 and facilitate polymerase association with origins of replication. Genetic and biochemical evidence suggests that MCM phosphorylation by DDK triggers DNA unwinding by helicases and DNA replication initiation.


ORC
ORC
Cdc6
cdt1
Cdc6
cdt1
ORC
MCMs


Dpb11
P
Sld2
CDK
P
Pol ε
GINS
Dpb11
Sld3
P
P
Pol ε
Cdc45
P
P
ORC
P
P
DDK

contributing to replication processivity is the homotrimeric clamp proliferating cell nuclear antigen (PCNA), which localizes polymerases to DNA at the replication fork [79, 80]. Studies have placed replication proteins at nuclear foci within replicating cells, suggesting that replication forks are organized at "replication factories" throughout eukaryotic chromatin [81]. This is in contrast to previous models where replication forks moved along stationary DNA.

Following DNA replication, cells prepare for chromosome division by initiating mitosis. Timely entry into mitosis in eukaryotic organisms requires the activation of a critical mitotic CDK (Cdc28 in budding yeast; Cdc2 in fission yeast and humans) [82]. In most eukaryotic organisms, activation of mitotic CDK requires the cooperative action of two events. First, expression of mitotic cyclins must reach a critical threshold to activate the catalytic CDK subunit [83, 84]. Secondly, reversal of Wee1 inhibitory phosphorylation on a critical CDK tyrosine residue by the Cdc25 class of phosphatases elevates mitotic CDK activity and triggers entry into mitosis [85]. The antagonistic function of the Cdc25 phosphatase and Wee1 kinase in controlling the activity of mitotic CDK is highly conserved throughout evolution. First evidence for the CDK1/mitotic promoting factor (MPF) in initiating mitotic progression was provided by the cell-fusion experiment of Rao and Johnson, which demonstrated that a diffusible factor could initiate mitosis in cells arrested in non-mitotic stages of the cell cycle [9]. Key studies in the fission yeast *Schizosaccharomyces pombe* identified and demonstrated the epistatic relationship between genes that delayed (*cdc2* and

cdc25) or sped up (*wee1*) the cell cycle, resulting in elongated or shortened cells respectively [86, 87].

In eukaryotic organisms, high CDK levels trigger the duplication of the spindle pole body/centrosome in cells [88]. Metazoan centrosomes have dramatically different structures than yeast spindle pole bodies. Animal cell centrosomes usually comprise a pair of centrioles that serve as microtubule organizing centers (MTOCs) for the capture and segregation of chromosomes [89]. Yeast MTOCs are fundamentally different in structure and are organized as molecular plaques that remain attached to the nuclear envelope throughout a closed mitosis [90]. Animal MTOCs anchor themselves to opposite cellular poles with cortically attached microtubules and promote microtubule invasion of the cellular midzone early in mitosis in an attempt to capture and bind to sister-chromatid pairs [89]. Chromosome centromere and arm attachment is maintained throughout mitosis by a class of chromatid binding proteins called cohesins (yeast cohesin complexes contain sister chromatid cohesion 1 (Scc1), Scc3, structural maintenance of chromosomes 1 (Smc1) and Smc3) [91]. Degradation of cohesin subunit Scc1 by the protease Separase (extra spindle pole bodies 1 (Esp1)) is required for cohesin removal at the metaphase-to-anaphase transition. Inhibition of Esp1-dependent separation during mitosis is regulated by the APC-Cdc20 substrate securin (precocious dissociation of sisters 1 (Pds1) in budding yeast) [92-94]. Activation of the APC by chromatid binding to the mitotic spindle triggers Pds1 degradation and activation of Separase [92]. In contrast to budding yeast, vertebrates remove chromosome cohesins in two

successive steps. Early in pro-metaphase, cohesins are removed from chromosome arms by a protease-independent mechanism requiring phosphorylation by Polo-like kinase 1 (Plk1) [95]. Cohesins located at the kinetechore are maintained until APC activation in early anaphase [92]. Sister chromatid separation in both metazoans and yeast occurs only after all kinetechores have achieved bipolar attachment to the mitotic spindle [96]. Failure of a single chromatid pair to properly align along the metaphase plate triggers stabilization of the spindle assembly checkpoint (SAC) and metaphase arrest. Sister chromatids migrate to opposite cellular poles following Cdc20 stabilization and activation of the APC [97, 98]. In contrast to metazoan chromosomes, budding yeast chromatids must traverse the mother-daughter bud neck prior to exit from mitosis to prevent stabilization of the spindle position checkpoint (described in more detail below) [96].

In budding yeast, activation of the nucleolar phosphatase Cdc14 is an important event in bringing about the resolution of mitosis following sister chromatid segregation. The first demonstration of the critical function of Cdc14 was in temperature sensitive (ts) mutants that arrested in late telophase with high CDK levels [99]. Cdc14 is a dual-specificity phosphatase and triggers mitotic exit and reentry into G1 phase [100-102]. The first well-characterized roles of Cdc14 were therefore its dephosphorylation and activation of the Cdk1 inhibitor Sic1 and its transcriptional activator, Swi5, and stabilization of the APC subunit Cdh1 [100]. Activation of this essential function of Cdc14 in mitotic exit requires the coordinated regulation of a group of signaling proteins within the mitotic exit

network (MEN) (Figure 3) [102, 103]. Critical to MEN regulation is the spatial and temporal regulation of the daughter bound spindle pole body relative to the bud neck [104]. Activation of the MEN is achieved by triggering a shift in the SPB GTPase Tem1 towards an active GTP-bound state [105, 106]. Prior to MEN activation, the two-component GTPase activating protein (GAP) Bfa1-Bub2 antagonizes MEN signaling by enhancing Tem1-GTPase activity at SPBs [106]. Coordination of MEN activation with spindle elongation is regulated by bud neck localization of Lte1 during mitosis [104]. During anaphase the daughter-bound SPB passes toward a region of high Lte1 concentration (budneck), effectively switching Tem1 towards the GTP bound state. Tem1-GTP then activates the SPB kinases Cdc15 and Dbf2-Mob1 upstream of Cdc14 localization [107, 108]. Phosphorylation of Cdc14 and its inhibitor Cfi/Net1 within the nucleolus releases Cdc14 and initiates mitotic exit [109-111].

In addition to the role SPB localization plays in determining mitotic exit onset, CDK activity and activation of the Polo-like kinase Cdc5 is absolutely required for MEN activation (Figure 3) [112]. Cdc14 dephosphorylation of CDK sites on Cdc15 is required for full activation of the mitotic exit network [113]. In contrast, CDK phosphorylation is required for Cdc14 nucleolar release by directly phosphorylating nucleolar Net1/Cfi1 [110]. Cdc5 localizes to both SPBs during mitosis and phosphorylates the Bfa1 subunit of the Bfa1-Bub2 GAP [114, 115]. This triggers Bfa1-Bub2 subunit dissociation and enhances MEN signaling [116]. Deletion of *BUB2* in budding yeast allows rebudding similar to mitotic arrest deficient (MAD) mutants in the presence of spindle poisons [117]. Consistent with

the model for Cdc5 MEN regulation, overexpression of wild type Cdc5 causes premature Cdc14 release in cells arrested in S-phase (hydroxyurea) and rebudding in cells treated with spindle poisons (nocodazole) [112, 118]. In addition, some experiments suggest an additional function for Cdc5 in regulating full Cdc14 release outside of the MEN, although these findings await further investigation [119].

The discovery of a parallel pathway that regulates Cdc14 localization led to the characterization of a group of factors involved in regulating events in early anaphase [120]. Transient release of Cdc14 by the Cdc14 early anaphase release (FEAR) network results in nuclear release, in contrast to full activation and cytoplasmic localization of Cdc14 by the MEN (Figure 3) [106, 121]. Cdc14 release in early anaphase is important for stabilization of elongated spindle microtubules, assembly of a spindle midzone and resolution of late segregating regions of the genome including the rDNA locus [122-125]. CDK substrates that are dephosphorylated at this time include Ask1, Ase1, Fin1 and Sli15 [126]. Chromosome XII in budding yeast contains ~150 tandem copies of the DNA encoding ribosomal subunits. Activation of the FEAR network is important for resolution of this locus during anaphase [124, 127, 128]. Key to uncovering this pathway was the observation that Cdc14 transient release is triggered by sister-chromatid resolution and phosphorylation of six sites on Cfi1/Net1 by CDK [110, 111]. CDK activity is antagonized throughout mitosis by protein phosphatase 2A (PP2A) (Figure 3) [129, 130]. PP2A is regulated by the targeting subunit Cdc55 and inhibits Cdc14 release through dephosphorylation of Cdc28 Net1 phospho-

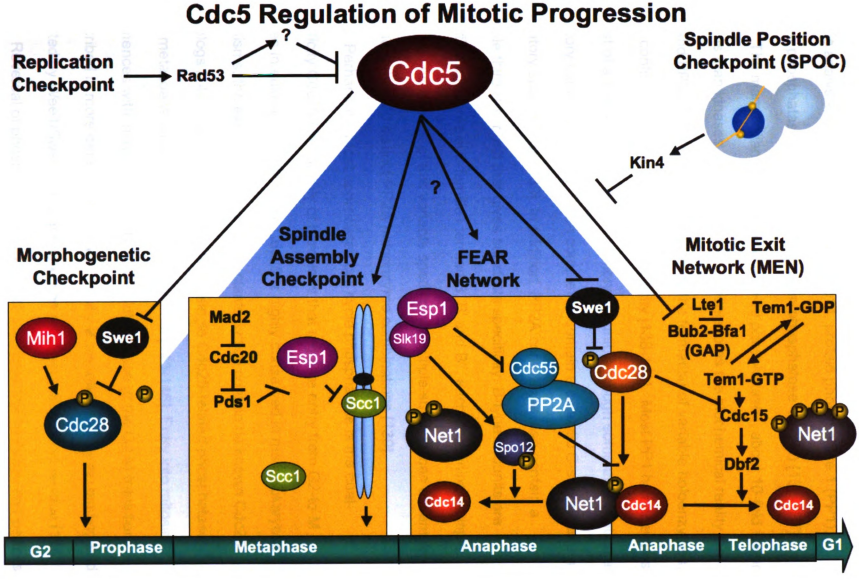
sites [129, 131]. Deletion of *CDC55* results in cellular rebudding in the presence of spindle poisons, although to a lesser extent than deletion of *BUB2* [117, 130]. Interestingly the FEAR components Esp1 and Slk19 are important for Cdc55 inactivation and Spo12 CDK phosphorylation, although the mechanism by which Spo12 and its binding partner Fob1 contribute to Cdc14 activation is not well understood [126].

The role for Cdc5 in regulating Cdc14 activity in the FEAR is even less clear (Figure 3). Epistasis analysis places Cdc5 downstream or in parallel of Esp1 and Slk19 [112, 120, 132]. Data suggesting that Cdc5 interacts with and phosphorylates Cdc14 suggests that Cdc5 coordinates Cdc14 release with Esp1, Slk19 and CDK [133]. A recent report suggests that Cdc5 activation of the FEAR occurs indirectly through inactivation of the CDK inhibitor Swe1, thereby increasing Cdk activity on Net1 [134]. Although this model is attractive, it awaits additional experimentation.

Phosphatase Regulation of the Cell Cycle

The critical role protein kinases play in controlling entry and exit from the cell cycle is well established, although the essential contribution that protein phosphatases play in this process has only recently been fully appreciated. It is now understood that phosphatases are critical players in regulating progression through many of the cell cycle phases. Of all the protein phosphatases identified, only a handful have been linked to mitotic regulation by biochemical or

Figure 3. Cdc5 is a master regulator of mitotic progression. The budding yeast Polo kinase Cdc5 positively regulates multiple stages of mitosis. Entry into mitosis is regulated by activation of the yeast CDK protein Cdc28. Inhibitory phosphorylation of Cdc28 by Swe1 is reversed by the phosphatase Mih1 (human Cdc25 homolog) during progression into mitosis. Cdc5 antagonizes Swe1 function during mitotic initiation through its association with proteins at the mother-daughter bud neck. Progression through the metaphase-anaphase transition requires bipolar spindle attachment at sister-chromatid kinetechores which triggers stabilization of the Cdc20-APC, ubiquitylation of Securin (Pds1) and proteolytic cleavage of chromatid cohesins by Separase (Esp1). Cdc5 phosphorylation of the cohesin subunit Scc1 is thought to enhance Separase activity on cohesins, but is not required for entry into anaphase in the budding yeast. The essential role for Cdc5 is in the activation of mitotic exit through release of Cdc14 from its inhibitory state within the nucleolus. This occurs by positive regulation of two pathways (MEN, mitotic exit network; FEAR, cdc14 early anaphase release) that affect Cdc14 activity. Cdc14 is inhibited during much of the cell cycle by association with the inhibitor Net1 within the nucleolus. Cdc5 activity is required at the beginning of anaphase for the transient release of Cdc14 into the nucleus. The precise requirement for Cdc5 is presently unknown, but recent evidence raises the possibility it may regulate FEAR by enhancing CDK phosphorylation of Net1 or direct phosphorylation of Cdc14. In addition to Cdc5, the Esp1-Slk19 complex and protein phosphatase PP2A-Cdc55 are also required for proper Cdc14 function in the FEAR network. MEN activation of Cdc14 requires Cdc5-dependent inhibition of the MEN antagonist Bfa1-Bub2, presumably at yeast spindle pole bodies. Disruption of the Bfa1-Bub2 interaction enhances Tem1-GTP activation of Cdc15, initiating a kinase cascade that results in hyperphosphorylation of Net1 and full and sustained release of Cdc14 resulting in mitotic exit.


Cdc5 Regulation of Mitotic Progression
Replication Checkpoint
Rad53
?
Cdc5
Spindle Position Checkpoint (SPOC)
Kin4
?
Morphogenetic Checkpoint
Spindle Assembly Checkpoint
FEAR Network
Mitotic Exit Network (MEN)
Mih1
Swe1
P
Cdc28
Mad2
Cdc20
Pds1
Esp1
Scc1
Scc1
Esp1
Slk19
Swe1
Cdc55
PP2A
Net1
Spo12
Cdc14
Net1
Cdc14
Cdc28
Lte1
Bub2-Bfa1 (GAP)
Tem1-GDP
Tem1-GTP
Cdc15
Net1
Dbf2
Cdc14
G2
Prophase
Metaphase
Anaphase
Anaphase
Telophase
G1

genetic analysis, most notably Cdc25, Cdc14, protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 4 (PP4) [135-137].

The mammalian genome encodes ~40 phosphatases, about 10-fold fewer proteins than kinases. This observation underscores the biochemical reality that PP1 and PP2A regulation is controlled by the formation of variable holoenzymes, which confer most of the substrate specificity [138-140]. Most PP1 holoenzymes consist of a single catalytic and regulatory subunit, although some also contain a inhibitory component [141]. PP2A complexes contain a catalytic C-subunit and a regulatory subunit [138, 139]. In addition, PP2A complexes also contain a variable third B-subunit that gives substrate specificity. B-type subunits are classified into three sub-families termed B, B' and B", each containing multiple isoforms. As a result of this variable specificity, multiple subunits can assemble into more than 70 distinct PP2A holoenzyme combinations [139].

Perhaps the most appreciated cell cycle phosphatase is the dual-specificity Cdc25/Mih1 family of homologs that regulate entry from G2-to-M phases in eukaryotic cells [142]. Cdc25 is highly conserved in most eukaryotic organisms; the exception being higher plants, which contain no known Cdc25 homologs [143]. The budding yeast has a single Cdc25-related phosphatase, while metazoans have multiple protein isoforms [143-146]. Entry into mitosis commences with increased activity of the Cdk1 kinase at the G2/M transition (described in more detail above). CDK activity is stimulated by cyclin binding and inhibited by Wee1/Swe1 phosphorylation on a conserved tyrosine residue [146, 147]. Reversal of phosphorylation by Cdc25 alleviates this inhibition and permits

Cdk activity on its substrates [148]. As a direct consequence of the pivotal role Cdc25 plays in regulating mitotic entry, several pathways that monitor cell cycle progression, most notably the G2/M DNA damage checkpoint, regulate Cdc25 activity [149]. In addition, activated Cdk1 phosphorylates Cdc25, inducing binding by PP1, which serves to activate Cdc25 and ensure an abrupt transition from G2 into mitosis [150].

The human Polo-like kinase 1 (Plk1) is also regulated by protein phosphatases during the cell cycle. Plk1 contains the protein targeting polo-box domain (PBD), which binds to the PP1 interacting partner, myosin phosphatase targeting subunit 1 (MYPT1) [151]. Decreased expression of MYPT1 increases phosphorylation on T210 of Plk1 by Aurora kinase and suggests that MYPT-PP1 may negatively regulate Plk1 activity [151]. In addition, the human homolog Cdc14B negatively regulates Plk1 prior to mitotic entry. In cells exposed to DNA damage, Cdc14B is released from the nucleolus into the cytoplasm, where it promotes APC-Cdh1-dependent degradation of Plk1[152].

Following chromosome segregation, cells exit mitosis during which CDK activity and reversal of mitotic CDK phosphorylation events decreases. In both yeast and higher organisms, protein phosphatases play a critically important role in promoting this process. In human cells more than 1000 proteins demonstrate increased protein phosphorylation during mitosis, most of them being Cdk1 substrates [153]. In budding yeast, activation of the mitotic exit network (MEN) results in the activation of the dual-specificity protein phosphatase Cdc14 [101, 102]. Following activation of the MEN, Cdc14 is released from within the

nucleolus and translocates through the nuclear membrane into the cytoplasm, where it dephosphorylates many Cdk1 substrates [100, 154]. Interestingly, humans contain two Cdc14 homologs (Cdc14A and Cdc14B) that functionally complement yeast Cdc14, but are not required for mitosis in human cells, indicating that additional phosphatases are sufficient for mitotic exit in higher organisms [137, 155]. Recent evidence suggests that human PP1 and PP2A holoenzymes effectively substitute for Cdc14 in this process [156-158].

Although budding yeast Cdc14 plays an essential role in regulating mitotic exit, additional phosphatases including PP2A make important contributions to regulating this process. PP2A is a heterotrimeric holoenzyme complex composed of a scaffolding unit (tRNA processing deficient (Tpd3)); a catalytic subunit (protein phosphatase 21 (Pph21) or Pph22); and a regulatory subunit (B-type, Cdc55 or B'-type, Rts1) [159]. Evidence suggests that both Cdc55 and Rts1 are involved in regulating MEN activation of Cdc14 in late mitosis [130, 160]. During mitosis, PP2A-Cdc55 antagonizes Cdc28 phosphorylation of the Cdc14 inhibitor Net1. Deletion of Cdc55 relieves this regulatory mechanism and results in premature mitotic exit and rebudding in the presence of spindle poisons [129, 130]. Recently, PP2A-Rts1 was shown to regulate the spindle position checkpoint kinase 4 (Kin4) [160]. Kin4 inhibits Cdc5/Plk1 activation of the MEN at spindle pole bodies. PP2A-Rts1 regulates Kin4 phosphorylation and localization at the SPB in the presence of mispositioned spindles.

REGULATION OF THE CELL CYCLE AND GROWTH BY CELLULAR CHECKPOINTS

Replication and DNA Damage Checkpoints

Organisms "sense" DNA damage during DNA replication and activate effector-signaling pathways to prolong S-phase and repair DNA lesions [161]. Activation of the replication checkpoint occurs when replication fork stalling is detected by "sensor kinases" within the cell. Activation of the replication checkpoint by hydroxyurea treatment depletes nucleotide pools and prolongs S-phase by delaying late origin firing [162-165]. Similarly DNA alkylating agents (methyl methane sulfanate) generate lesions that are detected at replication forks and delay S-phase progression until lesions can be efficiently repaired [166, 167].

Ataxia-telangiectasia mutated (ATM/scTel1) and ATM and Rad3-related (ATR/scMec1) are the sensor kinases involved in the S-phase checkpoint signaling cascade. Activation of these kinases is required for signaling to the downstream effector kinases checkpoint kinase 2 (Chk2)/yeast radiation sensitive 53 (Rad53) and Chk1, which is required for mitotic arrest in response to processed DNA double-strand breaks (DSBs) [168-170]. S-phase checkpoint activation is the result of extensive single-stranded DNA generated at perturbed replication forks. Single-stranded DNA is coated with the single-strand binding protein RPA and recruits the ATR/mitosis entry checkpoint 1 (Mec1)-ATR interacting protein (ATRIP)/scDdc2 heterodimeric protein complex to the fork [171-173]. Once at the fork, scMec1 phosphorylates the adaptor protein mediator

of replication checkpoint 1 (scMrc1), recruiting the scRad53 checkpoint kinase to phosphorylate and stabilize proteins at the replication fork [174-176].

In addition to replication fork damage, double-stranded DNA breaks pose a serious threat to genome stability and can result in aberrant genome rearrangements or chromatid loss during anaphase [177]. As a result, cells have evolved complex signaling networks to identify and correct such lesions. The exact nature of DNA structures that give rise to checkpoint responses remains obscure, however the prevailing view in the budding yeast is that dsDNA lesions are converted into ssDNA that gives rise to checkpoint responses. In yeast cells with single dsDNA breaks, generated by expression of the HO endonuclease, long tracts of RPA coated ssDNA triggers binding by scDdc2/ATRIP and scMec1/ATR [171, 178]. In the budding yeast, checkpoint activation by Mec1 activates two parallel pathways for cell cycle arrest. 1) Activation of Chk1 triggers phosphorylation and stabilization of the anaphase inhibitor Pds1 and 2) activation of the Chk2 homolog Rad53 inhibits Cdc5 activity in mitotic exit [73].

Spindle Assembly Checkpoint

During mitosis the spindle assembly checkpoint (SAC) delays the separation of sister chromatid pairs until every kinetechore has achieved bipolar attachment to the mitotic spindle [96]. Dynamic instability of microtubules allows the mitotic spindle to probe the intranuclear (in budding yeast) space for vacant kinetechores by a process termed 'search and capture' [179, 180]. A single binding site is present on smaller yeast kinetechores whereas vertebrate cells

strengthen chromatid attachment with multiple microtubule binding sites [181]. Once a kinetechore is attached to the mitotic spindle, a probing microtubule from the opposite pole attaches to the opposite kinetechore face. Bipolar attachment facilitates chromosome congression along the metaphase plate where the two sister chromatids come under tension as a result of antagonistic polar forces.

Proper attachment of all chromosomes triggers the activation of the Cdc20 version of the APC [96, 182, 183]. APC ubiquitylation and degradation of Pds1 (Securin) relieves Esp1 inhibition and permits degradation of chromosome cohesins [93, 184]. This exquisite and highly reliable mechanism of restraining APC activation prior to proper attachment of all sister chromatid pairs is essential for maintaining genome stability in cells [184]. Vertebrate cells with an inactive SAC have massive aneuploidy and quickly lose viability [185]. Interestingly, in budding yeast the SAC is not essential, but its proper function is required in the presence of mitotic mutants or spindle poisons. This peculiarity made the budding yeast a fruitful organism for the identification and elucidation of proteins involved in the SAC mechanism. Genetic screens in *S. cerevisiae* first identified the mitotic arrest deficient (Mad) 1-3 and the budding uninhibited by benzimidizole 1 (Bub1) proteins [117, 186]. These proteins are conserved in all model eukaryotic species and contribute to the regulation of precocious sister chromatid separation [187].

A critical feature of the SAC is that cells must differentiate between sister chromatids that are bound to opposite poles (amphitelic attachment) and those that are bound to a single pole (merotelic & syntelic). Therefore cells must

simultaneously monitor chromosome attachment and tension across the kinetechore. Two conserved kinetechore-binding proteins, Mad2 and Mad3/BubR1, are essential for proper regulation of the SAC. These proteins form a mitotic checkpoint complex (MCC) with Bub3 and Cdc20 thereby sequestering Cdc20 away from the APC and preventing Separase activation in response to unattached kinetechores [188].

In addition to the MCC, other critical components include Mad1, Aurora B/ increase in ploidy 1 (Ipl1), Bub1 and multipolar spindle 1 (Mps1) [189-193]. Aurora B/Ipl1 is important for correcting merotelic attachments that are not detected by the Mad2-dependent kinetechore attachment [194]. A "closed" isoform of the Mad2-Mad1 complex is recruited by the rough deal (Rod) - zeste white-10 (ZW10)–Rzz complex to kinetechore proteins and its activity regulated by Mps1 [195]. Closed-Mad2–Mad1 activates what is hypothesized to be the rate-limiting step of MCC formation, open-Mad2 binding to Cdc20. Additionally, in a separate SAC response pathway, centromere protein (CENP)-E interacts with and contributes to the activation of BubR1 on unattached kinetechores [196]. The substrates of BubR1 are currently unknown. Following bipolar microtubule attachment at kinetechores, a poleward force motor mechanism involving microtubules of the dynein–dynactin complex begins removing spindle assembly checkpoint proteins, including Mps1, Rzz, and closed-Mad2 complexes from kinetechores. It has been hypothesized that activation of the p31 comet protein may also occur at this moment in human cells to prevent the interaction of open-Mad2 with closed-Mad2 [197]. The affinity of Cenp-E to activate BubR1 also

decreases following formation of stable kinetochore microtubule attachment. This results in increased ubiquitylation of Cyclin B and Securin by the APC and subsequent proteolysis; this process activates Separase and ensures execution of anaphase. Ubiquitylation of Mps1 by the Cdc20-APC appears essential to permanently reverse the spindle assembly checkpoint at the commencement of anaphase [198].

Budding Yeast Spindle Position Checkpoint

Asymmetric cell division requires correct positioning of the mitotic spindle and cellular components to ensure the partitioning of the genetic material to each daughter cell. Budding in *S. cerevisiae* is an inherently asymmetric process; new cell wall is deposited (daughter bud) by the transport and accumulation of vesicles along actin cables to a growing bud tip [199]. This process ensures the unequal size of the mother and daughter cell (mother-larger and daughter-smaller) and pre-ordains the site of cytokinesis and abscission in yeast. Therefore, successful cell division requires the equal segregation of sister chromatids across a predetermined division plane (Figure 4). The budding yeast mitotic spindle is assembled during S-phase and is composed of intranuclear microtubules that remain within the intact nucleus and cytoplasmic/astral microtubules that interact with components of the cellular cortex [200]. Proper attachment of cortical microtubules to opposite spindle poles is coordinated by two redundant mechanisms involving karyogamy 9 (Kar9) and dynein 1 (Dyn1)

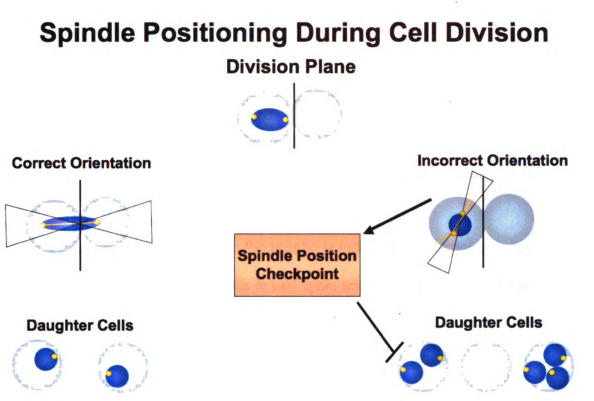


Figure 4. Budding yeast spindle orientation during mitosis. In the budding yeast, bud emergence during S-phase predetermines the cytokinetic division plane. This necessitates proper spindle orientation during mitosis to ensure all daughter-bound chromosomes traverse the bud neck during anaphase. Improper orientation of the mitotic spindle triggers stabilization of the SPOC (spindle position checkpoint) and prevents mitotic exit. Mutation of critical SPOC genes or hyperactivation of the MEN (mitotic exit network) induces premature exit from mitosis and ploidy abnormalities in cells.

[201-203]. Kar9 is attached to the plus end of cytoplasmic microtubules through association with Bim1 (binding to microtubules 1) and kinesin related protein 2 (Kip2) [204-206]. This complex attaches to myosin 2 (Myo2) on cortical actin cables where it is translocated through the bud neck and into the daughter cell ensuring bipolar division of sister chromatids [207, 208]. The plus-end microtubule motor protein Dyn1 contributes to proper spindle orientation by anchoring cytoplasmic microtubules within the cortex and contributing forces that result in correct spindle orientation [209].

Failure to properly position the mitotic spindle under normal or induced (*kar9Δ* or *dyn1Δ*) conditions results in cell cycle arrest and inhibition of the mitotic exit network [96, 201, 202]. The primary target of the spindle position checkpoint (SPOC) is the MEN GTPase Tem1 [210]. Bub2-Bfa1 and Lte1 regulate the antagonistic cycling of Tem1 between the active (Tem1-GTP) and inactive (Tem1-GDP) forms [210]. Activation of the SPOC prevents Cdc5-dependent phosphorylation of Bfa1 at the spindle pole body [116, 211, 212]. Inhibition of Cdc5 function requires proper localization of Kin4 to the spindle pole body and stable dephosphorylation of Kin4 by PP2A-Rts1 [160, 213]. Deletion of Rts1 or Kin4 induces premature mitotic exit in cells with misoriented spindles [160, 211, 212].

CDC7-DBF4 AND CDC5/POLO LITERATURE REVIEW

Cdc7-Dbf4: Structure and Function

The essential function of DDK in eukaryotic cell cycle progression was first identified by Lee Hartwell and colleagues who discovered that Cdc7 inactivation prevented cells from initiating S-phase in *Saccharomyces cerevisiae* [214]. Cdc7 was unique in this screen of mutants in that protein synthesis was not required after Cdc7 activity for the completion of S-phase [215]. Identification of a temperature-sensitive allele of *DBF4* defective in S-phase initiation eventually led to the hypothesis that Dbf4 and Cdc7 may function in a common pathway to control the start of replication [216]. Interestingly, Dbf4 was also identified in a screen for high-copy suppressors of *cdc7* (ts) [217]. Cdc7 was subsequently found to encode a serine/threonine protein kinase whose activity is controlled by cyclical expression of the kinase regulatory subunit Dbf4 [218-220]. It was shown that activity of Cdc7 depends on the direct binding and activation of the Dbf4 regulatory subunit [221, 222]. Together, these studies strongly suggested that Cdc7 and Dbf4 function in concert to regulate passage through the G1/S transition. Deletion of *DBF4* or *CDC7* is lethal, but *cdc7Δ* and *dbf4Δ* cells can be rescued by the *bob1-1* mutation in *MCM5*, a component of the eukaryotic replicative helicase [223].

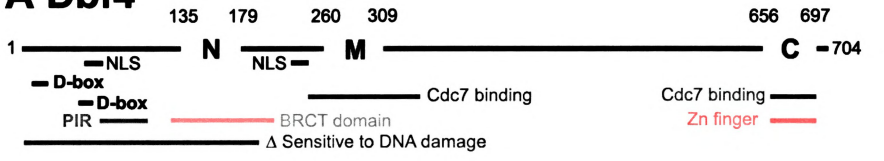
Homologs of Cdc7 and Dbf4 were not identified in other species until the discovery of *hsk1+*, an essential gene required for the initiation of S phase in fission yeast [224]. Like *CDC7*, *hsk1+* encodes a 507 amino acid protein comprising the catalytic subunit of DDK. Following the identification of *hsk1+*, putative Cdc7 homologs from additional species including humans were identified [225, 226]. Budding yeast *DBF4* encodes a 704 amino acid protein with

three motifs (N-terminal , Middle & C-terminal) of interspecies homology (Figure 5) [227]. All homologs share highest sequence similarity within the M-motif while the N and C-terminal regions show the highest degree of sequence divergence [227]. Activator of S-phase kinase (ASK), the human homolog of Dbf4, was identified in a 2-hybrid screen and encodes the 695 amino acid regulatory subunit of Cdc7 [228]. Although ASK shares the three common regions (N, M & C) of sequence similarity, the C-terminal half of the protein is less well conserved.

Detailed mutational analyses of budding and fission yeast *DBF4/dfp1+* identified regions essential for Cdc7 activity and binding to the origin recognition complex (ORC) [229]. In Dfp1, the individual M and C-motifs were sufficient to bind and partially activate fission yeast Hsk1, while intervening sequences were dispensable for kinase binding and activation [230]. These results suggested that activation of Cdc7/Hsk1 might occur through bipartite binding to the Dbf4/Dfp1 regulatory subunit. Similarly, the budding yeast Dbf4 C-terminal zinc-finger motif stimulated Cdc7 binding and catalytic activity, but was not essential for cell growth (mutants exhibit a slow S-phase) in the presence of an intact M-motif [231]. Mutations within region N in *DBF4/dfp1+* were found to confer general sensitivity to DNA damaging agents, but had little to no effect on growth under normal conditions [229, 230]. These results suggest a possible role for the Dbf4 N-terminus in regulating cellular responses to stress.

The Dbf4 N-terminus was originally described as having similarity to BRCT repeat sequences (Figure 5) [227]. Identified in the human gene *BRCA1*,

Figure 5. Schematic diagrams of functional Dbf4 and Cdc5 sequences. (A) Dbf4 contains three regions of high interspecies sequence similarity (N, N-terminal; M, Middle; C, C-terminal). Regions M and C are important for binding to the DDK catalytic subunit Cdc7 and for activation of the kinase. Two putative D-boxes (destruction boxes) and NLS (nuclear localization signal) sequences are present within the N-terminal half of the protein. Domain N is not required for viability and is contained within the Dbf4 BRCT domain. Deletion of this region causes sensitivity to DNA damaging agents. (B) *CDC5* encodes an N-terminal Ser/Thr kinase domain and a C-terminal polo box targeting domain (PBD). Both domains are essential for Cdc5 activity and viability in budding yeast. The polo box domain contains three regions (Pc, polo-cap; PB1, polo box 1; PB2, polo box 2) that are required for proper folding and phosphopeptide binding. Conserved residues (Blue font: W517, H641 and 643) are critical for substrate interaction. PBD phosphorylation by Cdc7-Dbf4 is decreased by alanine mutation of PBD residues T532, S573 and S630, but unaffected by the S647A mutation.


A Dbf4
135
179
260
309
656
697
1
N
M
C
704
NLS
NLS
D-box
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Cdc7 binding
Cdc7 binding
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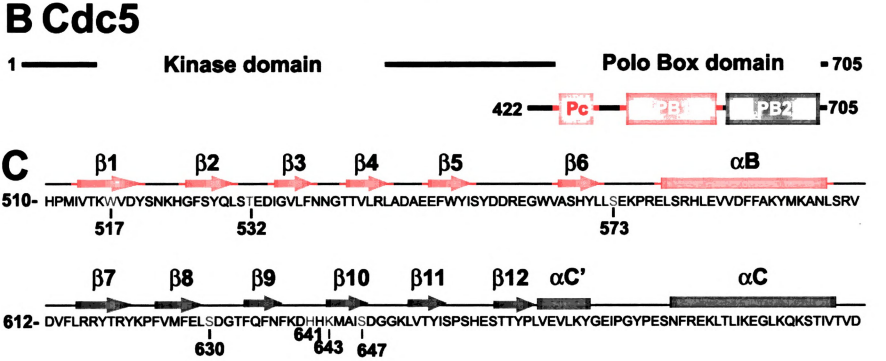
Zn finger
Δ Sensitive to DNA damage


B Cdc5
1
Kinase domain
Polo Box domain
705
422
Pc
PB1
PB2
705
C
β1
β2
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β5
β6
αB
510- HPMIVTKWVDYSNKHGFSYQLSTEDIGVLFNNGTTVLRLADAEEFWYISYDDREGWVASHYLLSEKPRELSRHLEVVDFFAKYMKANLSRV
517
532
573
β7
β8
β9
β10
β11
β12
αC'
αC
612- DVFLRRYTRYKPFVMFELSDGTFQFNFKDHHKMAISDGGKLVTYISPSHESTTYPLVEVLKYGEIPGYPESNFREKLTLIKEGLKQKSTIVTVD
630
641
643
647

BRCT domains are primarily found in DNA damage-response proteins [232, 233]. Crystallization of the Brca1 tandem BRCT repeat identified the domain as a phosphopeptide-binding module [234]. Recent work in our lab identified a c-terminal region of the BRCT sequence that is conserved in Dbf4 homologs prompting us to name it the BRDF (BRCT and Dbf4 similar) motif [229]. However, crystallization of this domain by Guarne' and colleagues suggests that it nonetheless adopts a canonical BRCT-fold [229, 235]. The function of the Dbf4 BRCT domain is unknown and proteins that interact exclusively with the BRCT domain have not been identified. Dbf4 is a target of Rad53-dependent checkpoint phosphorylation in response to replication stress and this regulation requires an intact Dbf4 N-terminus [229, 236]. In addition, Cds1/Rad53 kinase activation is severely perturbed in *hsk1-89* cells and the *hsk1-89* mutation is synthetically lethal with the checkpoint protein Rad3/Mec1 (ATR homolog in yeast) suggesting functional overlap between Cdc7-Dbf4 and the DNA damage response pathway [237]. A possible function of the Dbf4 BRCT domain is to interact with phosphorylated Rad53 FHA domain. Molecular modeling of the crystallized BRCT domain raises the possibility, but biochemical evidence for this is lacking [235]. Masai and others proposed that human Cdc7-Dbf4 interacts with components of stalled replication forks and may be required for replication restart in response to replication stress [238].

Expression and Regulation of DDK subunits during the Cell Cycle

Cdc7 catalytic activity is regulated by cyclical expression of Dbf4 throughout the cell cycle [239]. In contrast to its regulatory subunit, Cdc7 protein levels remain relatively constant during the yeast cell cycle [239]. Mammalian Cdc7 activity is positively regulated by serum stimulation and E2F transcription factor activity [226]. Dbf4 protein levels remain low throughout G1 and increase at the transition into S-phase and decrease at the end of mitosis [239]. Dbf4 promoter elements suggest that its transcription is under control of the MluI cell-cycle box (MCB) transcriptional element, however the observed protein oscillation within cells appears to be regulated at the level of protein stability [240]. Dbf4 contains two putative "RXXL" destruction boxes within the protein N-terminus (Figure 5) suggesting a role for APC-Cdc20 in regulating Dbf4 destruction. Mutation of the N-terminal destruction box can stabilize Dbf4 [241]. In addition, inactivation or mutation of genes encoding APC subunits stabilizes Dbf4 protein, however the precise regulation of Dbf4 destruction remains enigmatic [236, 242]. APC activity is regulated by the specificity components, Cdc20 and Cdh1, which become active at the metaphase-to-anaphase transition and during mitotic exit respectively [243]. The contribution each subunit plays in targeting Dbf4 for destruction is currently unclear, however, persistent expression of Dbf4 in mitotic exit mutants (*cdc5-1*, *dbf2-1* and *cdc14-1*) at non-permissive temperature argues against exclusive regulation by Cdc20 and suggests that Dbf4 levels persist late into mitosis, long after completion of S-phase [236, 242]. So how is Dbf4 stability regulated? Cdk phosphorylates Dbf4 during the normal cell cycle [244]. A serine within the N-terminal Dbf4 D-box ("RSPL") matches a

consensus CDK phosphorylation site and is phosphorylated *in vivo* after treatment with MMS (Jaime Lopez; personal communication). It is possible that CDK actively inhibits Dbf4 destruction prior to mitotic exit when the Cdc20 APC is active. Cdc14 dephosphorylation of CDK sites raises the possibility that Cdc14 may antagonize CDK phosphorylation of Dbf4 and allow the APC access to the Dbf4 destruction box late in mitosis.

Cdc7-Dbf4 Function In S-phase Initiation

In contrast to our understanding of the role for CDK in replication initiation, the precise biochemical role for DDK at origins is less clear. What is apparent is that localization of DDK to origins is critical for the timely and accurate firing of replication origins throughout S-phase. Diffley and colleagues demonstrated by a one-hybrid assay that Dbf4 interacts with origins, and that this genetic interaction is dependent on the origin recognition complex ORC [39]. Additionally, studies in Xenopus indicate that DDK associates with the pre-RC at origins and this association is dependent on Dbf4 [245]. The pre-RC proteins Orc2, Orc3, Mcm2 and Mcm4 all interact with Dbf4 suggesting a model whereby Dbf4 targets Cdc7 to sites of pre-RC binding [246]. Cdc7-Dbf4 can phosphorylate multiple proteins at the origin including several Mcm subunits, Cdc45 and polymerase alpha [58, 236, 247]. Mapping of Cdc7-Dbf4 phosphorylation sites on Mcm proteins and the identification of the *bob1-1* (*mcm5*, P83L) bypass suppressor of *cdc7Δ* and *dbf4Δ* cells led to the hypothesis that Cdc7-Dbf4 activates the Mcm replicative helicase [223, 248, 249]. It is thought that phosphorylation of Mcm

proteins by Cdc7 likely leads to a conformational shift in the helicase complex, initiating origin unwinding and DNA replication [250]. Recent evidence suggests a model whereby Cdc7 phosphorylation of MCM N-termini may trigger MCM double hexamer dissociation and DNA unwinding [251]. The mechanism whereby cells regulate the timing of origin firing throughout S-phase and delay the activity of Cdc7-Dbf4 on late origins in response to cellular stress remains to be established. Recent evidence suggests that an initiating phosphorylation event by a DDK-independent kinase primes the pre-RC Mcm2-7 complex for phosphorylation by Cdc7-Dbf4, suggesting a model in which the spatial and temporal regulation of Cdc7-Dbf4 activity on origins might be controlled [59].

Cdc5/Polo: Structure and Function

Molecular understanding of the important role of Polo kinases in regulating cell cycle progression and cellular homeostasis is accelerating. Two decades after the initial discovery in *Drosophila* that mutations in *polo* give rise to cells with aberrant mitotic spindles, we have achieved a relatively mature understanding of the biochemical role of polo kinases within cells [252]. This evidence is buttressed by the accelerated pursuit of molecular inhibitors of polo kinases for the treatment of human malignancy. Much of the knowledge about the polo family of kinases is the result of extensive investigation of homologs in model organisms. *Drosophila polo* is the homolog of *Xenopus* Plx1, human Polo-like kinase 1, fission yeast Plo1 and budding yeast Cdc5 [253-255]. Budding and

fission yeast have a single polo kinase, which most closely resembles *Drosophila* polo and human Plk1 in form and function; for this reason they are known collectively as polo kinases. In addition, metazoans have at minimum one additional PLK paralog (Plk4/SAK); human cells have two other family members Plk2 and Plk3 [256].

The polo kinase sequence and function is highly conserved throughout evolution [256]. Common among all polo kinases is the presence of an amino-terminal serine/threonine kinase domain and two distal polo boxes that fold into a functional polo-box domain (PBD) (Figure 5) [257, 258]. All of the known *polo* homologs and paralogs with the exception of Plk4/SAK, which has only a single polo-box, share this common structural fold [256]. The Plk1 PBD binds phosphorylated serine and threonine peptide sequences and demonstrates an extraordinary preference for serine and proline at the pThr -1 and +1 positions respectively [257-259]. Critical for phosphopeptide binding are conserved histidine and lysine residues (H538, K540 in Plk1) within the second polo-box that coordinate and neutralize the phosphate moiety. Additional residues including a conserved tryptophan (W414 in Plk1) within the first polo-box make significant contributions to phospho-peptide binding in Plk1 [257, 258].

In most organisms, including *S. cerevisiae*, increased protein expression of Polo kinase/Cdc5 precedes catalytic activation of the kinase domain [260]. In the budding yeast Cdc5 levels are regulated predominately by activity of the Cdh1-version of the APC [261]. Cdc5 protein levels are low throughout G1 when APC-Cdh1 activity is high, but rise with increasing Cdc28-Cln activity and peak in

late S-phase [260]. Protein levels remain stable throughout mitosis until activation of APC activity is fully restored following Cdc14 phosphatase release and mitotic exit [261]. In human cells regulation of Plk1 expression appears to be regulated by transcriptional repression in G1 and S-phase as opposed to transcriptional activation in late G2 [262]. The cell cycle-dependent element/cell cycle genes homology region (CDE/CHR) is immediately adjacent the Plk1 transcriptional start site and expression correlates negatively with CDE/CHR function [263]. In addition, the Rb family of pocket proteins (p107/p130) transcriptionally represses Plk1 by binding E2F4 directly on the promoter [264, 265].

Correct localization of Polo kinases within the cell is essential for proper regulation of cell cycle progression and growth. Experiments *in vivo* identified that Plk1 localizes to the mammalian centrosomes and kinetechores until the metaphase-to-anaphase transition [266-269]. At this stage of mitosis the affinity for these sites is diminished and Plk1 relocalizes to the spindle midzone in preparation for cytokinesis [266-269]. Similar to Plk1, Cdc5 localizes to the budding yeast spindle pole bodies and septin ring at the bud neck [94, 270]. In contrast to mammalian cells, Cdc5 targeting to SPBs occurs very early in S-phase and relocalizes to the bud neck prior to mitotic entry [271]. A functional Cdc5 PBD interacts with phosphorylated residues matching CDK consensus sites and is required for targeting Cdc5 by binding to SPBs and the bud neck [257, 272]. Interestingly, bud neck localization appears dispensable for viability in budding yeast with only SPB localization being required for viability [114].

Essential substrates involved in Cdc5 regulation of mitotic exit (discussed in more detail below) are located at the SPB and most likely explain this observation. Although the PBD is essential for targeting Cdc5 throughout the cell, the kinases that prime substrates for PBD are still largely unknown. Proteomic and structural analysis of optimal PBD binding sequences initially suggested that CDKs may prime proteins for Polo binding, however a recent study suggests that Plk1 binding can occur independent of substrate phosphorylation [244, 259, 273]. These studies suggest that our understanding of Polo targeting and regulation may be more complex than originally believed.

G2/M Transition

The key Cdk1 regulatory node for mitotic entry involves regulation by Plk1, however activation of Polo kinases and their relative importance in promoting mitotic entry varies considerably among organisms. This observed discrepancy might result from an inherent difference in the reliance of the G2 phase in monitoring cell cycle progression in divergent organisms. Vertebrates and fission yeast have a prolonged G2 phase and the G2/M checkpoint is a robust cell cycle arrest mechanism in the presence of DNA damage. Budding yeast by contrast have an extremely short G2 phase and rely more heavily on the spindle assembly checkpoint for delaying cell cycle progression. It may come as no surprise then that experiments in fission yeast, *Xenopus* and humans support a more prominent role for Polo kinases in acting upstream of Cdk1 [255, 274]. Plk1 phosphorylation and activation of the Cdc25 phosphatase at the G2/M transition

is necessary for Cdk1 dephosphorylation and mitotic entry [255, 274]. Catalytic activation of Plk1 is regulated by cooperative action of the Plk1 interacting protein Bora and the Aurora A kinase [275]. Bora accumulation and binding to Plk1 in G2 phase relieves PBD autoinhibition of the catalytic domain and increases the accessibility of Aurora A to the Plk1 T-loop [257, 275]. This mechanism for Plk1 and subsequent Cdk1 activation is a critical target for cell cycle arrest and recovery in response to DNA damage [276].

Budding yeast experiments suggest a relatively minor role for Cdc5 in regulating the G2/M transition. Published reports suggest that Cdc5 kinase activation is downstream of mitotic CDK, as catalytic activation of Cdc5 requires direct phosphorylation by Cdc28, although the cyclin specificity for this interaction awaits elucidation [277]. In addition, *cdc5-1* ts mutants progress through the G2/M transition normally and arrest in late mitosis with elongated spindles and partially divided nuclei [254]. In contrast to other organisms, Swe1 deletion has no measurable affect on growth or cell cycle progression in normal yeast [278]. It appears, however, that Swe1 plays a primary role in arresting cell cycle progression in response to activation of the morphogenetic checkpoint [279]. Chemicals that inhibit normal actin polymerization and bud formation prevent Hsl1-dependent localization of Swe1 to the bud neck [280, 281]. Once at the bud neck, Cdc5 and Cla4/PAK hyperphosphorylate Swe1, targeting it for ubiquitin-dependent proteosome degradation[282]. This degradation alleviates the antagonistic function of Swe1 on Cdk1 permitting mitotic entry.

Metaphase-to-Anaphase Transition

Sister chromatid segregation occurs after activation of the Cdc20-version of the APC at the metaphase-to-anaphase transition [98]. Prior to chromosome separation, sister chromatids are held together by a family of proteins called cohesins (described above). Degradation of the Scc1 cohesin subunit is essential for chromatid resolution during anaphase and Polo kinases facilitate this loss in both mammals and yeast cells [283]. In vertebrates, phosphorylation of Scc1 and SA1/SA2 by Polo kinase reduces the affinity of cohesins towards chromatin resulting in the loss of cohesion along chromosome arms in prometaphase [284]. In budding yeast, the entire chromosome arms segregate simultaneously (with the exception of the rDNA locus and telomeric regions) following cleavage of Scc1 by Esp1/separase[92]. It is thought that phosphorylation of Scc1 by Cdc5 increases the affinity of Esp1 for Scc1, however this function of Cdc5 is dispensable and cells initiate anaphase without Cdc5 phosphorylation [275].

Regulation of Cytokinesis

In addition to their role in mitotic exit, Cdc5, Cdc15 and Dbf2-Mob1 have additional roles in promoting cytokinesis in budding yeast. All three factors are required for proper actinomyosin ring formation at the budneck [285-288]. Following MEN activation and Cdc14 release, Cdc15 and Dbf2 relocalize from the SPB to the mother-daughter-budneck [119, 289, 290]. This is in contrast to Cdc5 localization, which targets to the budneck prior to mitosis where it participates in Swe1/Wee1 downregulation at the G2/M transition [114, 271, 282,

291]. *cdc5* (ts) mutants demonstrate defects in septum closure and membrane fusion resulting in chained cell morphologies [291]. These experiments suggest that Cdc5 has cytokinetic functions separate from MEN activation, as some mutants remain competent for mitotic exit. In fission yeast, some members of the MEN, including Plo1, participate in the septation initiation network (SIN), which is required for cytokinesis [292, 293]. As in fission yeast, human Plk1 localizes to the spindle midzone and regulates contraction of the actin ring prior to abscission [267, 286]. Although the exact nature of cytokinetic regulation by Polo-like kinases awaits further elucidation, the degree of conservation between highly divergent organisms is suggestive of a critical role in regulating this process.

CDC7-DBF4 AND PLK1 INVOLVEMENT IN CANCER ETIOLOGY

The development and progression of human cancers requires genetic modification of multiple pathways including apoptotic regulation, cellular proliferation, migration and invasion. Pathways regulated by Cdc7-Ask and polo kinases put these proteins in a unique position to control and regulate cell growth in cancer cells. Deregulation of the cell cycle is a hallmark of human cancer and the discovery that normal Cdc7 and polo kinase activity is perturbed in some tumors has important implications for the biology and treatment of these diseases.

Polo-like Kinases

Elevated Plk1 activity contributes to many of the phenotypes (cellular proliferation and genome instability) that are frequently associated with malignant disease. Indeed, overexpression of Plk1 in NIH 3T3 cells induces colony formation in soft agar and tumors in nude mice, suggesting a causative role in driving malignant progression [294]. It is not surprising, therefore, that deregulation of polo kinases is found in many human cancers.

Transcriptional expression of Plk1 has been extensively studied in myriad cancer types and tissues. Plk1 expression is elevated in breast, head and neck, squamous esophageal, ovarian, gastric, colon, endometrial carcinoma and glioblastomas [295]. In addition, increased expression is positively correlated with poor prognosis in many cancer types. In non-small cell lung adenocarcinomas, patients expressing moderate Plk1 levels demonstrated a significantly better prognosis (5-year survival mean; 52%) than patients expressing high levels of Plk1 (5-year survival mean; 24%) [296]. Similar results were observed in squamous cell esophageal carcinomas with low level expressers demonstrating a significant increase in 3-year survival (3-year survival mean; 54%) over high expressers (3-year survival mean; 25%) [297].

In large part, immunohistochemical analysis of Plk1 protein expression in solid tumors has supported these findings. In ovarian carcinoma patients, the percentage of Plk1 cells was positively associated with tumor grade, with more aggressive grades demonstrating more robust Plk1 expression (percent positive cells: grade 1, 13.5%; grade 2, 21.9%; grade 3, 35%) [298]. Similar findings were reported in glioma patients; tumors demonstrating more advanced anaplasia had

significantly higher levels of Plk1 expression compared to low-grade and "normal" tissues [299]. In addition, Plk was positively correlated ($r = 0.519$) with MIB-1 expression in these cells. Interestingly, Plk1 was also found to positively correlate ($r = 0.677$) with estrogen receptor (ER) expression in human breast carcinoma cells [296]. Although the biochemical significance of this observation awaits further elucidation, the relationship between these two proteins is interesting considering the strong association with ER status and patient prognosis.

Among polo kinase family members, Plk3 has also received significant attention with regard to its involvement in regulating cancer progression. In contrast to Plk1, however, a significant percentage of studies suggest that Plk3 expression is positively associated with patient survival. Like Plk1, the expression of Plk3 is regulated throughout the cell cycle and peaks in late S and M phase [300]. Plk3 mRNA and protein are undetectable in lung cancers and is similarly reduced in human head and neck carcinomas and rat colon tumors [300-302]. In support of these findings, induction of Plk3 reduces fibroblast proliferation and induces cell cycle arrest and apoptosis [303, 304].

Therapeutics Targeting Mitotic Kinases (Polo and Aurora kinases)

Cells undergoing mitosis are particularly susceptible to apoptotic-induced cell death [305]. For this reason, many of the most historically successful anti-neoplastic compounds target this stage of the cell cycle. In particular the vinca alkaloids and taxanes, which disrupt normal microtubule dynamics, perturb mitotic spindle mechanics resulting in activation of the mitotic checkpoint, cell

cycle arrest and apoptosis [306]. Absence of cancer cell specificity, however, results in deleterious off-target side effects and significant peripheral neuropathies in most patients.

As a result, pharmaceutical companies have invested significantly in the development of compounds targeting specific mitotic kinases: most notably the Aurora kinases and Plk1. Aurora kinases in particular have received increasing interest over the last several years because of their requirement for chromosome alignment and partitioning during mitosis [307]. Inhibition of Aurora A kinase activity generates cells with monopolar spindles that arrest at the SAC and eventually undergo apoptosis [308]. Aurora B inhibition by contrast results in SAC bypass and mitotic progression despite a failure to segregate chromosomes. This results in aneuploidy and tetraploidization of cells and ultimately apoptosis [309]. Specific (AZD1152, GSK1070916 and MLN8237) and pan-aurora kinase (CYC-116) inhibitors have been developed and are in Phase I and II clinical trials [310-312]. In preclinical studies, compounds showing marked anti-tumor activity, regardless of the pharmacokinetic profile, suggested that Aurora A and Aurora B are both effective targets for therapeutic intervention in cancer cells.

Currently, two strategies exist for targeting Plk1 in cancer cells. Firstly, inhibition of the Plk1 kinase domain has resulted in the development of numerous small ATP-binding pocket inhibitors (BI 2536, GSK461364, HMN-214 and ON 01910) [313-318]. These compounds are currently being tested in Phase I and II clinical trials and their effectiveness awaits publication. Secondly, peptides that

target the Plk1 polobox domain were shown to decrease the kinase activity. Development of the first non-peptide PBD inhibitor Poloxin was shown to induce mitotic arrest and apoptosis in HeLa cells [319, 320]. It might be expected that as our ability to more accurately design inhibitors targeting cell cycle kinases improves more promising results may emerge from clinical trials regarding cell cycle targeted therapies.

Cdc7-Dbf4 Cancer Involvement

Compared to Plk1, our current knowledge of the role Cdc7-Dbf4 plays in cancer development and progression is relatively modest, although recent studies indicate a possible role for DDK in driving malignant progression. Overexpression of *CDC7* mRNA in human tumors and cell lines first suggested a role for DDK in regulating cancer cell proliferation [321]. This observation is supported by further studies demonstrating increased Cdc7 and Dbf4 protein levels in a wide variety of human tumors and cell lines [322]. In support of this finding, Dbf4 expression levels negatively correlate with patient survival and tumor relapse in cutaneous melanoma cells. Consistent with this finding, knockdown of Cdc7 expression in cells induces apoptosis [322-324]. Interestingly, overexpression of Cdc7 and Dbf4 arrests normal chinese hamster ovary cells at the G2/M transition, suggesting that inactivation of specific checkpoints may be a precondition for increased Cdc7-Dbf4 activity [325].

Another hallmark of tumor development is that most malignant cells are genetically unstable and contain a high degree of DNA damage. As a

consequence, tumor cells develop mechanisms to bypass cell cycle checkpoints in response to damaged DNA. The tumor suppressor p53 is a key target of the DNA damage checkpoint in mammalian cells and transcriptionally activates key proteins involved in cell cycle arrest and apoptosis [326]. Depletion of Cdc7 also suggests a p53-dependent replication checkpoint in cancer cells. Some cells that are depleted of Cdc7 are susceptible to DNA damage during replication and require p53 to maintain cell viability and avoid apoptosis [324, 327]. A link between p53 and Cdc7 expression in maintaining cell viability in stressed cells is further supported by work in our lab demonstrating a correlation between p53 loss and increased Cdc7-Dbf4 expression [322]. These studies suggest functional interplay between Cdc7-Dbf4 and p53 in maintaining cancer cell viability in the presence of genome stress. As a result, these reports have important implications for the development of Cdc7 kinase inhibitors as a possible therapeutic drug target in p53 mutant tumors. Recent reports of novel Cdc7 kinase inhibitors report inhibition of cellular malignant growth *in vitro* and in xenograft mouse models [328-330]. Clearly, our understanding of the possible role Cdc7 plays in tumor development and/or progression is still in its infancy and further experimentation is required to investigate whether any serious effort at targeting Cdc7 in human tumors would be efficacious.

Significance and Concluding Remarks

Elucidating the mechanism of DNA replication and initiation during S-phase is a fundamental problem in eukaryotic cell biology. A well-established

causal link between DNA replication damage and cancer initiation suggests a thorough understanding of this process has the potential to improve human health. Critical to a more mature understanding of replication in eukaryotes is further elucidation of the role DDK (Cdc7-Dbf4) plays in regulating this process. In addition to the general involvement of replication and DNA damage repair defects in driving malignant progression, emerging evidence suggests direct involvement of human Cdc7-Dbf4 in cancer progression and/or maintenance and highlights the importance of additional investigation into DDK function.

Dbf4 structure/function studies identified and characterized three regions important for Cdc7-Dbf4 function [227, 229, 231]. As discussed, budding yeast *DBF4* encodes three regions (N, M & C) of high interspecies sequence similarity [227]. Although characterization of the M and C regions in Cdc7 binding and activation has improved our understanding of Dbf4, the biochemical function of the Dbf4 N-terminus remains enigmatic. The discovery that mutations within Dbf4 region N confer sensitivity to DNA damaging agents and eliminate Rad53-dependent checkpoint phosphorylation on Dbf4 raise the possibility that the Dbf4 N-terminus may regulate cellular responses to DNA damage. The well-established role for Chk2 (yeast Rad53 homolog) involvement in human cancers and identification of somatic mutations of Chk2 in human breast cancer patients suggests this research could have important implications for our understanding of cell biology and human disease.

Critical to understanding this process is the identification of additional proteins that interact with the N-terminal region of Dbf4. Masai and colleagues

reported that fission yeast DDK (Hsk1-Dfp1) interacts with components of stalled replication forks, but it was not clear whether this interaction was dependent on the Dbf4 N-terminus or conserved in the budding yeast [238]. To identify the function of the Dbf4 BRCT domain in these processes, our lab performed a yeast two-hybrid screen and identified an interaction between the N-terminus of Dbf4 and Cdc5. An interaction between Dbf4 and Cdc5 was initially reported in 1996, however this study did not determine the biochemical mechanism of the interaction and proposed a role for Cdc5 function in replication [331]. Subsequent studies failed to support this hypothesis on the primary basis that Cdc5 expression and kinase activity are not activated until late S or early G2 phase [260]. Additionally, evidence of a role for Cdc5 in the regulation of Dbf4 during DNA replication is unproven.

As a result of emerging evidence implicating members of the Cdc5 family of Polo-like kinases in positively regulating the G_2/M transition, events in anaphase, mitotic exit and cytokinesis, we hypothesized that Dbf4 may regulate Cdc5 to prevent premature activation of events in mitosis before completion of DNA replication [112, 115, 232, 233, 270, 271, 332]. We reasoned that this finding would represent a significant advance in our understanding of Cdc7-Dbf4 biology as it would be the first report of a function for Dbf4 outside S-phase. In addition, the central role for Dbf4 and Cdc5 in regulating replication and mitosis, respectively, their involvement in promoting cancer progression and recent interest in targeting both kinases therapeutically in human cancers underscored the importance for additional investigation into the functional regulation of these

proteins. As a result, we anticipated that successful completion of this project would significantly extend our understanding of the role for Cdc7-Dbf4 within the cell cycle and possibly uncover novel functions for this key regulatory protein outside of S-phase.

REFERENCES

1. Honigberg, S.M., and Purnapatre, K. (2003). Signal pathway integration in the switch from the mitotic cell cycle to meiosis in yeast. J Cell Sci *116*, 2137-2147.

2. Koch, A.L. (2002). Control of the bacterial cell cycle by cytoplasmic growth. Crit Rev Microbiol *28*, 61-77.

3. Sears, R.C., and Nevins, J.R. (2002). Signaling networks that link cell proliferation and cell fate. J Biol Chem *277*, 11617-11620.

4. Wansink, D.G., Manders, E.E., van der Kraan, I., Aten, J.A., van Driel, R., and de Jong, L. (1994). RNA polymerase II transcription is concentrated outside replication domains throughout S-phase. J Cell Sci *107 (Pt 6)*, 1449-1456.

5. Nurse, P., and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. Genetics *96*, 627-637.

6. Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell *33*, 389-396.

7. Masui, Y., and Markert, C.L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool *177*, 129-145.

8. Hunt, T. (1991). Cyclins and their partners: from a simple idea to complicated reality. Semin Cell Biol *2*, 213-222.

9. Johnson, R.T., and Rao, P.N. (1970). Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. Nature *226*, 717-722.

10. Newport, J.W., and Kirschner, M.W. (1984). Regulation of the cell cycle during early Xenopus development. Cell *37*, 731-742.

11. Sherr, C.J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

12. Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev *9*, 1149-1163.

13. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

14. Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M., and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev *8*, 9-22.

15. Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

16. Giacinti, C., and Giordano, A. (2006). RB and cell cycle progression. Oncogene *25*, 5220-5227.

17. Nevins, J.R., Chellappan, S.P., Mudryj, M., Hiebert, S., Devoto, S., Horowitz, J., Hunter, T., and Pines, J. (1991). E2F transcription factor is a target for the RB protein and the cyclin A protein. Cold Spring Harb Symp Quant Biol *56*, 157-162.

18. Zachariae, W., Schwab, M., Nasmyth, K., and Seufert, W. (1998). Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. Science *282*, 1721-1724.

19. Burton, J.L., Tsakraklides, V., and Solomon, M.J. (2005). Assembly of an APC-Cdh1-substrate complex is stimulated by engagement of a destruction box. Mol Cell *18*, 533-542.

20. Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K., and Toyoshima, K. (1992). Phosphorylation of the retinoblastoma protein by cdk2. Proc Natl Acad Sci U S A *89*, 7900-7904.

21. Hartwell, L.H., Culotti, J., and Reid, B. (1970). Genetic control of the cell-division cycle in yeast. I. Detection of mutants. Proc Natl Acad Sci U S A *66*, 352-359.

22. Cross, F.R. (1988). DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of Saccharomyces cerevisiae. Mol Cell Biol *8*, 4675-4684.

23. Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. (1989). An essential G1 function for cyclin-like proteins in yeast. Cell *59*, 1127-1133.

24. Hadwiger, J.A., Wittenberg, C., Richardson, H.E., de Barros Lopes, M., and Reed, S.I. (1989). A family of cyclin homologs that control the G1 phase in yeast. Proc Natl Acad Sci U S A *86*, 6255-6259.

25. Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, A.B. (1988). The WHI1+ gene of Saccharomyces cerevisiae tethers cell division to cell size and is a cyclin homolog. Embo J *7*, 4335-4346.

26. Hartwell, L.H., and Unger, M.W. (1977). Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division. J Cell Biol *75*, 422-435.

27. Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae. Embo J *14*, 4803-4813.

28. Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. Embo J *12*, 1955-1968.

29. Di Como, C.J., Chang, H., and Arndt, K.T. (1995). Activation of CLN1 and CLN2 G1 cyclin gene expression by BCK2. Mol Cell Biol *15*, 1835-1846.

30. Stuart, D., and Wittenberg, C. (1995). CLN3, not positive feedback, determines the timing of CLN2 transcription in cycling cells. Genes Dev *9*, 2780-2794.

31. Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell *79*, 233-244.

32. Calzada, A., Sacristan, M., Sanchez, E., and Bueno, A. (2001). Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases. Nature *412*, 355-358.

33. Verma, R., Annan, R.S., Huddleston, M.J., Carr, S.A., Reynard, G., and Deshaies, R.J. (1997). Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455-460.

34. Verma, R., Feldman, R.M., and Deshaies, R.J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. Mol Biol Cell *8*, 1427-1437.

35. Nasmyth, K., Adolf, G., Lydall, D., and Seddon, A. (1990). The identification of a second cell cycle control on the HO promoter in yeast: cell cycle regulation of SW15 nuclear entry. Cell *62*, 631-647.

36. Peter, M., Gartner, A., Horecka, J., Ammerer, G., and Herskowitz, I. (1993). FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell *73*, 747-760.

37. McKinney, J.D., Chang, F., Heintz, N., and Cross, F.R. (1993). Negative regulation of FAR1 at the Start of the yeast cell cycle. Genes Dev *7*, 833-843.

38. Bell, S.P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature *357*, 128-134.

39. Diffley, J.F., Cocker, J.H., Dowell, S.J., Harwood, J., and Rowley, A. (1995). Stepwise assembly of initiation complexes at budding yeast replication origins during the cell cycle. J Cell Sci Suppl *19*, 67-72.

40. Chesnokov, I., Remus, D., and Botchan, M. (2001). Functional analysis of mutant and wild-type Drosophila origin recognition complex. Proc Natl Acad Sci U S A *98*, 11997-12002.

41. Bielinsky, A.K., Blitzblau, H., Beall, E.L., Ezrokhi, M., Smith, H.S., Botchan, M.R., and Gerbi, S.A. (2001). Origin recognition complex binding to a metazoan replication origin. Curr Biol *11*, 1427-1431.

42. Ogawa, Y., Takahashi, T., and Masukata, H. (1999). Association of fission yeast Orp1 and Mcm6 proteins with chromosomal replication origins. Mol Cell Biol *19*, 7228-7236.

43. Romanowski, P., Madine, M.A., Rowles, A., Blow, J.J., and Laskey, R.A. (1996). The Xenopus origin recognition complex is essential for DNA replication and MCM binding to chromatin. Curr Biol *6*, 1416-1425.

44. Carpenter, P.B., Mueller, P.R., and Dunphy, W.G. (1996). Role for a Xenopus Orc2-related protein in controlling DNA replication. Nature *379*, 357-360.

45. Aparicio, O.M., Weinstein, D.M., and Bell, S.P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell *91*, 59-69.

46. Santocanale, C., and Diffley, J.F. (1996). ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. Embo J *15*, 6671-6679.

47. Tanaka, T., Knapp, D., and Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell *90*, 649-660.

48. Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol Cell Biol *18*, 2758-2767.

49. Drury, L.S., Perkins, G., and Diffley, J.F. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. Embo J *16*, 5966-5976.

50. Elsasser, S., Chi, Y., Yang, P., and Campbell, J.L. (1999). Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis. Mol Biol Cell *10*, 3263-3277.

51. Perkins, G., Drury, L.S., and Diffley, J.F. (2001). Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. Embo J *20*, 4836-4845.

52. Fujita, M., Yamada, C., Goto, H., Yokoyama, N., Kuzushima, K., Inagaki, M., and Tsurumi, T. (1999). Cell cycle regulation of human CDC6 protein. Intracellular localization, interaction with the human mcm complex, and CDC2 kinase-mediated hyperphosphorylation. J Biol Chem *274*, 25927-25932.

53. Jiang, W., Wells, N.J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A *96*, 6193-6198.

54. Petersen, B.O., Wagener, C., Marinoni, F., Kramer, E.R., Melixetian, M., Lazzerini Denchi, E., Gieffers, C., Matteucci, C., Peters, J.M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev *14*, 2330-2343.

55. Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science *290*, 2309-2312.

56. Stukenberg, P.T., Lustig, K.D., McGarry, T.J., King, R.W., Kuang, J., and Kirschner, M.W. (1997). Systematic identification of mitotic phosphoproteins. Curr Biol *7*, 338-348.

57. Schwob, E., and Nasmyth, K. (1993). CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genes Dev *7*, 1160-1175.

58. Nougarede, R., Della Seta, F., Zarzov, P., and Schwob, E. (2000). Hierarchy of S-phase-promoting factors: yeast Dbf4-Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. Mol Cell Biol *20*, 3795-3806.

59. Francis, L.I., Randell, J.C., Takara, T.J., Uchima, L., and Bell, S.P. (2009). Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. Genes Dev *23*, 643-654.

60. Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

61. Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature *445*, 328-332.

62. Zegerman, P., and Diffley, J.F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature *445*, 281-285.

63. Meselson, M., and Stahl, F.W. (1958). The Replication of DNA in Escherichia Coli. Proc Natl Acad Sci U S A *44*, 671-682.

64. Prescott, D.M., and Kuempel, P.L. (1972). Bidirectional replication of the chromosome in Escherichia coli. Proc Natl Acad Sci U S A *69*, 2842-2845.

65. Danna, K.J., and Nathans, D. (1972). Bidirectional replication of Simian Virus 40 DNA. Proc Natl Acad Sci U S A *69*, 3097-3100.

66. Masters, M., and Broda, P. (1971). Evidence for the bidirectional replications of the Escherichia coli chromosome. Nat New Biol *232*, 137-140.

67. Maya-Mendoza, A., Tang, C.W., Pombo, A., and Jackson, D.A. (2009). Mechanisms regulating S phase progression in mammalian cells. Front Biosci *14*, 4199-4213.

68. Taylor, J.H. (1958). The mode of chromosome duplication in Crepis capillaris. Exp Cell Res *15*, 350-357.

69. Crossen, P.E., Pathak, S., and Arrighi, F.E. (1975). A high resolution study of the DNA replication patterns of chinese hamster chromosomes using sister chromatid differential staining technique. Chromosoma *52*, 339-347.

70. Fangman, W.L., Hice, R.H., and Chlebowicz-Sledziewska, E. (1983). ARS replication during the yeast S phase. Cell *32*, 831-838.

71. Reynolds, A.E., McCarroll, R.M., Newlon, C.S., and Fangman, W.L. (1989). Time of replication of ARS elements along yeast chromosome III. Mol Cell Biol *9*, 4488-4494.

72. McCarroll, R.M., and Fangman, W.L. (1988). Time of replication of yeast centromeres and telomeres. Cell *54*, 505-513.

73. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166-1171.

74. Larner, J.M., Lee, H., Little, R.D., Dijkwel, P.A., Schildkraut, C.L., and Hamlin, J.L. (1999). Radiation down-regulates replication origin activity throughout the S phase in mammalian cells. Nucleic Acids Res *27*, 803-809.

75. Wang, Y., Vujcic, M., and Kowalski, D. (2001). DNA replication forks pause at silent origins near the HML locus in budding yeast. Mol Cell Biol *21*, 4938-4948.

76. Deshpande, A.M., and Newlon, C.S. (1996). DNA replication fork pause sites dependent on transcription. Science *272*, 1030-1033.

77. Hubscher, U., Maga, G., and Spadari, S. (2002). Eukaryotic DNA polymerases. Annu Rev Biochem *71*, 133-163.

78. Waga, S., Bauer, G., and Stillman, B. (1994). Reconstitution of complete SV40 DNA replication with purified replication factors. J Biol Chem *269*, 10923-10934.

79. Krishna, T.S., Kong, X.P., Gary, S., Burgers, P.M., and Kuriyan, J. (1994). Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. Cell *79*, 1233-1243.

80. Krishna, T.S., Fenyo, D., Kong, X.P., Gary, S., Chait, B.T., Burgers, P., and Kuriyan, J. (1994). Crystallization of proliferating cell nuclear antigen (PCNA) from Saccharomyces cerevisiae. J Mol Biol *241*, 265-268.

81. Spector, D.L. (1993). Macromolecular domains within the cell nucleus. Annu Rev Cell Biol *9*, 265-315.

82. Beach, D., Durkacz, B., and Nurse, P. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. Nature *300*, 706-709.

83. Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275-280.

84. Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339*, 280-286.

85. Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature *342*, 39-45.

86. Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49*, 559-567.

87. Nurse, P. (1975). Genetic control of cell size at cell division in yeast. Nature *256*, 547-551.

88. Haase, S.B., Winey, M., and Reed, S.I. (2001). Multi-step control of spindle pole body duplication by cyclin-dependent kinase. Nat Cell Biol *3*, 38-42.

89. Azimzadeh, J., and Bornens, M. (2007). Structure and duplication of the centrosome. J Cell Sci *120*, 2139-2142.

90. Jaspersen, S.L., and Winey, M. (2004). The budding yeast spindle pole body: structure, duplication, and function. Annu Rev Cell Dev Biol *20*, 1-28.

91. Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35-45.

92. Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature *400*, 37-42.

93. Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067-1076.

94. Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. Embo J *17*, 1336-1349.

95. Sumara, I., Vorlaufer, E., Stukenberg, P.T., Kelm, O., Redemann, N., Nigg, E.A., and Peters, J.M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. Mol Cell *9*, 515-525.

96. Lew, D.J., and Burke, D.J. (2003). The spindle assembly and spindle position checkpoints. Annu Rev Genet *37*, 251-282.

97. Schott, E.J., and Hoyt, M.A. (1998). Dominant alleles of Saccharomyces cerevisiae CDC20 reveal its role in promoting anaphase. Genetics *148*, 599-610.

98. Fang, G., Yu, H., and Kirschner, M.W. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell *2*, 163-171.

99. Hartwell, L.H., Culotti, J., Pringle, J.R., and Reid, B.J. (1974). Genetic control of the cell division cycle in yeast. Science *183*, 46-51.

100. Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol Cell *2*, 709-718.

101. Wan, J., Xu, H., and Grunstein, M. (1992). CDC14 of Saccharomyces cerevisiae. Cloning, sequence analysis, and transcription during the cell cycle. J Biol Chem *267*, 11274-11280.

102. Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. Mol Biol Cell *9*, 2803-2817.

103. Bardin, A.J., and Amon, A. (2001). Men and sin: what's the difference? Nat Rev Mol Cell Biol *2*, 815-826.

104. Bardin, A.J., Visintin, R., and Amon, A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell *102*, 21-31.

105. Shirayama, M., Matsui, Y., and Toh, E.A. (1994). The yeast TEM1 gene, which encodes a GTP-binding protein, is involved in termination of M phase. Mol Cell Biol *14*, 7476-7482.

106. Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol Cell *6*, 1-10.

107. Asakawa, K., Yoshida, S., Otake, F., and Toh-e, A. (2001). A novel functional domain of Cdc15 kinase is required for its interaction with Tem1 GTPase in Saccharomyces cerevisiae. Genetics *157*, 1437-1450.

108. Mah, A.S., Jang, J., and Deshaies, R.J. (2001). Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. Proc Natl Acad Sci U S A *98*, 7325-7330.

109. Yoshida, S., and Toh-e, A. (2002). Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. Biochem Biophys Res Commun *294*, 687-691.

110. Azzam, R., Chen, S.L., Shou, W., Mah, A.S., Alexandru, G., Nasmyth, K., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2004). Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science *305*, 516-519.

111. Shou, W., Azzam, R., Chen, S.L., Huddleston, M.J., Baskerville, C., Charbonneau, H., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2002). Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. BMC Mol Biol *3*, 3.

112. Visintin, R., Stegmeier, F., and Amon, A. (2003). The role of the polo kinase Cdc5 in controlling Cdc14 localization. Mol Biol Cell *14*, 4486-4498.

113. Menssen, R., Neutzner, A., and Seufert, W. (2001). Asymmetric spindle pole localization of yeast Cdc15 kinase links mitotic exit and cytokinesis. Curr Biol *11*, 345-350.

114. Park, J.E., Park, C.J., Sakchaisri, K., Karpova, T., Asano, S., McNally, J., Sunwoo, Y., Leem, S.H., and Lee, K.S. (2004). Novel functional dissection of the localization-specific roles of budding yeast polo kinase Cdc5p. Mol Cell Biol *24*, 9873-9886.

115. Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. Cell *107*, 655-665.

116. Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H., and Sedgwick, S.G. (2003). In vitro regulation of budding yeast Bfa1/Bub2·GAP activity by Cdc5. J Biol Chem *278*, 14591-14594.

117. Hoyt, M.A., Totis, L., and Roberts, B.T. (1991). S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507-517.

118. Hu, F., and Elledge, S.J. (2002). Bub2 is a cell cycle regulated phospho-protein controlled by multiple checkpoints. Cell Cycle *1*, 351-355.

119. Lee, S.E., Frenz, L.M., Wells, N.J., Johnson, A.L., and Johnston, L.H. (2001). Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. Curr Biol *11*, 784-788.

120. Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell *108*, 207-220.

121. Yoshida, S., Asakawa, K., and Toh-e, A. (2002). Mitotic exit network controls the localization of Cdc14 to the spindle pole body in Saccharomyces cerevisiae. Curr Biol *12*, 944-950.

122. Pereira, G., and Schiebel, E. (2004). Cdc14 phosphatase resolves the rDNA segregation delay. Nat Cell Biol *6*, 473-475.

123. Pereira, G., and Schiebel, E. (2003). Separase regulates INCENP-Aurora B anaphase spindle function through Cdc14. Science *302*, 2120-2124.

124. Sullivan, M., Higuchi, T., Katis, V.L., and Uhlmann, F. (2004). Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. Cell *117*, 471-482.

125. Zeng, X., Kahana, J.A., Silver, P.A., Morphew, M.K., McIntosh, J.R., Fitch, I.T., Carbon, J., and Saunders, W.S. (1999). Slk19p is a centromere protein that functions to stabilize mitotic spindles. J Cell Biol *146*, 415-425.

126. D'Amours, D., and Amon, A. (2004). At the interface between signaling and executing anaphase--Cdc14 and the FEAR network. Genes Dev *18*, 2581-2595.

127. Torres-Rosell, J., Machin, F., Jarmuz, A., and Aragon, L. (2004). Nucleolar segregation lags behind the rest of the genome and requires Cdc14p activation by the FEAR network. Cell Cycle *3*, 496-502.

128. D'Amours, D., Stegmeier, F., and Amon, A. (2004). Cdc14 and condensin control the dissolution of cohesin-independent chromosome linkages at repeated DNA. Cell *117*, 455-469.

129. Queralt, E., Lehane, C., Novak, B., and Uhlmann, F. (2006). Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. Cell *125*, 719-732.

130. Wang, Y., and Ng, T.Y. (2006). Phosphatase 2A negatively regulates mitotic exit in Saccharomyces cerevisiae. Mol Biol Cell *17*, 80-89.

131. Healy, A.M., Zolnierowicz, S., Stapleton, A.E., Goebl, M., DePaoli-Roach, A.A., and Pringle, J.R. (1991). CDC55, a Saccharomyces cerevisiae gene involved in cellular morphogenesis: identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. Mol Cell Biol *11*, 5767-5780.

132. Sullivan, M., and Uhlmann, F. (2003). A non-proteolytic function of separase links the onset of anaphase to mitotic exit. Nat Cell Biol *5*, 249-254.

133. Rahal, R., and Amon, A. (2008). The Polo-like kinase Cdc5 interacts with FEAR network components and Cdc14. Cell Cycle *7*, 3262-3272.

134. Liang, F., Jin, F., Liu, H., and Wang, Y. (2009). The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. Mol Biol Cell *20*, 3671-3679.

135. Sullivan, M., and Morgan, D.O. (2007). Finishing mitosis, one step at a time. Nat Rev Mol Cell Biol *8*, 894-903.

136. Trinkle-Mulcahy, L., and Lamond, A.I. (2006). Mitotic phosphatases: no longer silent partners. Curr Opin Cell Biol *18*, 623-631.

137. Queralt, E., and Uhlmann, F. (2008). Cdk-counteracting phosphatases unlock mitotic exit. Curr Opin Cell Biol *20*, 661-668.

138. Virshup, D.M., and Shenolikar, S. (2009). From promiscuity to precision: protein phosphatases get a makeover. Mol Cell *33*, 537-545.

139. Janssens, V., Longin, S., and Goris, J. (2008). PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). Trends Biochem Sci *33*, 113-121.

140. Hendrickx, A., Beullens, M., Ceulemans, H., Den Abt, T., Van Eynde, A., Nicolaescu, E., Lesage, B., and Bollen, M. (2009). Docking motif-guided mapping of the interactome of protein phosphatase-1. Chem Biol *16*, 365-371.

141. Ceulemans, H., and Bollen, M. (2004). Functional diversity of protein phosphatase-1, a cellular economizer and reset button. Physiol Rev *84*, 1-39.

142. Russell, P., and Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell *45*, 145-153.

143. Kerk, D., Templeton, G., and Moorhead, G.B. (2008). Evolutionary radiation pattern of novel protein phosphatases revealed by analysis of protein data from the completely sequenced genomes of humans, green algae, and higher plants. Plant Physiol *146*, 351-367.

144. Russell, P., Moreno, S., and Reed, S.I. (1989). Conservation of mitotic controls in fission and budding yeasts. Cell *57*, 295-303.

145. Nagata, A., Igarashi, M., Jinno, S., Suto, K., and Okayama, H. (1991). An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. New Biol *3*, 959-968.

146. Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell *56*, 829-838.

147. Parker, L.L., Atherton-Fessler, S., and Piwnica-Worms, H. (1992). p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. Proc Natl Acad Sci U S A *89*, 2917-2921.

148. Parker, L.L., and Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science *257*, 1955-1957.

149. Zeng, Y., Forbes, K.C., Wu, Z., Moreno, S., Piwnica-Worms, H., and Enoch, T. (1998). Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature *395*, 507-510.

150. Margolis, S.S., Perry, J.A., Weitzel, D.H., Freel, C.D., Yoshida, M., Haystead, T.A., and Kornbluth, S. (2006). A role for PP1 in the Cdc2/Cyclin B-mediated positive feedback activation of Cdc25. Mol Biol Cell *17*, 1779-1789.

151. Yamashiro, S., Yamakita, Y., Totsukawa, G., Goto, H., Kaibuchi, K., Ito, M., Hartshorne, D.J., and Matsumura, F. (2008). Myosin phosphatase-targeting subunit 1 regulates mitosis by antagonizing polo-like kinase 1. Dev Cell *14*, 787-797.

152. Bassermann, F., Frescas, D., Guardavaccaro, D., Busino, L., Peschiaroli, A., and Pagano, M. (2008). The Cdc14B-Cdh1-Plk1 axis controls the G2 DNA-damage-response checkpoint. Cell *134*, 256-267.

153. Dephoure, N., Zhou, C., Villen, J., Beausoleil, S.A., Bakalarski, C.E., Elledge, S.J., and Gygi, S.P. (2008). A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A *105*, 10762-10767.

154. Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Shevchenko, A., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell *97*, 233-244.

155. Berdougo, E., Nachury, M.V., Jackson, P.K., and Jallepalli, P.V. (2008). The nucleolar phosphatase Cdc14B is dispensable for chromosome segregation and mitotic exit in human cells. Cell Cycle *7*, 1184-1190.

156. Wu, J.Q., Guo, J.Y., Tang, W., Yang, C.S., Freel, C.D., Chen, C., Nairn, A.C., and Kornbluth, S. (2009). PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation. Nat Cell Biol *11*, 644-651.

157. Mochida, S., and Hunt, T. (2007). Calcineurin is required to release Xenopus egg extracts from meiotic M phase. Nature *449*, 336-340.

158. Skoufias, D.A., Indorato, R.L., Lacroix, F., Panopoulos, A., and Margolis, R.L. (2007). Mitosis persists in the absence of Cdk1 activity when proteolysis or protein phosphatase activity is suppressed. J Cell Biol *179*, 671-685.

159. Jiang, Y. (2006). Regulation of the cell cycle by protein phosphatase 2A in Saccharomyces cerevisiae. Microbiol Mol Biol Rev *70*, 440-449.

160. Chan, L.Y., and Amon, A. (2009). The protein phosphatase 2A functions in the spindle position checkpoint by regulating the checkpoint kinase Kin4. Genes Dev *23*, 1639-1649.

161. Nyberg, K.A., Michelson, R.J., Putnam, C.W., and Weinert, T.A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet *36*, 617-656.

162. Bousset, K., and Diffley, J.F. (1998). The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev *12*, 480-490.

163. Diffley, J.F., Bousset, K., Labib, K., Noton, E.A., Santocanale, C., and Tercero, J.A. (2000). Coping with and recovering from hydroxyurea-induced replication fork arrest in budding yeast. Cold Spring Harb Symp Quant Biol *65*, 333-342.

164. Young, C.W., and Hodas, S. (1965). Acute Effects of Cytotoxic Compounds on Incorporation of Precursors into DNA, Rna, and Protein of Hela Monolayers. Biochem Pharmacol *14*, 205-214.

165. Young, C.W., and Hodas, S. (1964). Hydroxyurea: Inhibitory Effect on DNA Metabolism. Science *146*, 1172-1174.

166. Tercero, J.A., and Diffley, J.F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature *412*, 553-557.

167. Loppes, R. (1966). Damage induced by methyl methanesulfonate (MMS) in Chlamydomonas reinhardi. Z Vererbungsl *98*, 193-202.

168. Gilbert, C.S., Green, C.M., and Lowndes, N.F. (2001). Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol Cell *8*, 129-136.

169. Walworth, N.C., and Bernards, R. (1996). rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint. Science *271*, 353-356.

170. Matsuoka, S., Huang, M., and Elledge, S.J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

171. Zou, L., and Elledge, S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

172. Osborn, A.J., Elledge, S.J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol *12*, 509-516.

173. Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.

174. Alcasabas, A.A., Osborn, A.J., Bachant, J., Hu, F., Werler, P.J., Bousset, K., Furuya, K., Diffley, J.F., Carr, A.M., and Elledge, S.J. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat Cell Biol *3*, 958-965.

175. Osborn, A.J., and Elledge, S.J. (2003). Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. Genes Dev *17*, 1755-1767.

176. Desany, B.A., Alcasabas, A.A., Bachant, J.B., and Elledge, S.J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev *12*, 2956-2970.

177. Khanna, K.K., and Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet *27*, 247-254.

178. Sugawara, N., and Haber, J.E. (1992). Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. Mol Cell Biol *12*, 563-575.

179. Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. Nature *312*, 237-242.

180. Belmont, L.D., Hyman, A.A., Sawin, K.E., and Mitchison, T.J. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. Cell *62*, 579-589.

181. Fitzgerald-Hayes, M., Clarke, L., and Carbon, J. (1982). Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. Cell *29*, 235-244.

182. Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A., and Murray, A.W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. Science *279*, 1041-1044.

183. Kim, S.H., Lin, D.P., Matsumoto, S., Kitazono, A., and Matsumoto, T. (1998). Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint. Science *279*, 1045-1047.

184. Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev *13*, 1936-1949.

185. Rajagopalan, H., and Lengauer, C. (2004). Aneuploidy and cancer. Nature *432*, 338-341.

186. Li, R., and Murray, A.W. (1991). Feedback control of mitosis in budding yeast. Cell *66*, 519-531.

187. Musacchio, A., and Salmon, E.D. (2007). The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol *8*, 379-393.

188. Sudakin, V., Chan, G.K., and Yen, T.J. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J Cell Biol *154*, 925-936.

189. Morrow, C.J., Tighe, A., Johnson, V.L., Scott, M.I., Ditchfield, C., and Taylor, S.S. (2005). Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. J Cell Sci *118*, 3639-3652.

190. Weiss, E., and Winey, M. (1996). The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. J Cell Biol *132*, 111-123.

191. Hardwick, K.G., Weiss, E., Luca, F.C., Winey, M., and Murray, A.W. (1996). Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. Science *273*, 953-956.

192. Chung, E., and Chen, R.H. (2002). Spindle checkpoint requires Mad1-bound and Mad1-free Mad2. Mol Biol Cell *13*, 1501-1511.

193. Tang, Z., Shu, H., Oncel, D., Chen, S., and Yu, H. (2004). Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol Cell *16*, 387-397.

194. Pinsky, B.A., Kung, C., Shokat, K.M., and Biggins, S. (2006). The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. Nat Cell Biol *8*, 78-83.

195. Buffin, A., Lefebvre, C., Huang, J., Gagou, M.E., and Karess, R.E. (2005). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. Curr Biol *10*, 856-861.

196. Regnier, V., Vagnarelli, P., Fukagawa, T., Zerjal, T., Burns, E., Trouche, D., Earnshaw, W., and W., B. (2005). CENP-A is required for accurate chromosome segregation and sustained kinetechore association of BubR1. Mol Biol Cell *10*, 3967-3981.

197. Xia, G., Luo, X., Habu, T., Rizo, J., Matsumoto, T., and Yu, H. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. Embo J *15*, 3133-3143.

198. Palframan, W.J., Meehl, J.B., Jaspersen, S.L., Winey, M., and Murray, A.W. (2006). Anaphase inactivation of the spindle checkpoint. Science *313*, 680-684.

199. Madden, K., and Snyder, M. (1998). Cell polarity and morphogenesis in budding yeast. Annu Rev Microbiol *52*, 687-744.

200. Byers, B., and Goetsch, L. (1975). Behavior of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J Bacteriol *124*, 511-523.

201. Eshel, D., Urrestarazu, L.A., Vissers, S., Jauniaux, J.C., van Vliet-Reedijk, J.C., Planta, R.J., and Gibbons, I.R. (1993). Cytoplasmic dynein is required for normal nuclear segregation in yeast. Proc Natl Acad Sci U S A *90*, 11172-11176.

202. Saunders, W.S., Koshland, D., Eshel, D., Gibbons, I.R., and Hoyt, M.A. (1995). Saccharomyces cerevisiae kinesin- and dynein-related proteins required for anaphase chromosome segregation. J Cell Biol *128*, 617-624.

203. Miller, R.K., and Rose, M.D. (1998). Kar9p is a novel cortical protein required for cytoplasmic microtubule orientation in yeast. J Cell Biol *140*, 377-390.

204. Korinek, W.S., Copeland, M.J., Chaudhuri, A., and Chant, J. (2000). Molecular linkage underlying microtubule orientation toward cortical sites in yeast. Science *287*, 2257-2259.

205. Lee, L., Tirnauer, J.S., Li, J., Schuyler, S.C., Liu, J.Y., and Pellman, D. (2000). Positioning of the mitotic spindle by a cortical-microtubule capture mechanism. Science *287*, 2260-2262.

206. Schwartz, K., Richards, K., and Botstein, D. (1997). BIM1 encodes a microtubule-binding protein in yeast. Mol Biol Cell *8*, 2677-2691.

207. Beach, D.L., Thibodeaux, J., Maddox, P., Yeh, E., and Bloom, K. (2000). The role of the proteins Kar9 and Myo2 in orienting the mitotic spindle of budding yeast. Curr Biol *10*, 1497-1506.

208. Yin, H., Pruyne, D., Huffaker, T.C., and Bretscher, A. (2000). Myosin V orientates the mitotic spindle in yeast. Nature *406*, 1013-1015.

209. Markus, S.M., Punch, J.J., and Lee, W.L. (2009). Motor- and tail-dependent targeting of dynein to microtubule plus ends and the cell cortex. Curr Biol *19*, 196-205.

210. Geymonat, M., Spanos, A., Smith, S.J., Wheatley, E., Rittinger, K., Johnston, L.H., and Sedgwick, S.G. (2002). Control of mitotic exit in budding yeast. In vitro regulation of Tem1 GTPase by Bub2 and Bfa1. J Biol Chem *277*, 28439-28445.

211. D'Aquino, K.E., Monje-Casas, F., Paulson, J., Reiser, V., Charles, G.M., Lai, L., Shokat, K.M., and Amon, A. (2005). The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. Mol Cell *19*, 223-234.

212. Pereira, G., and Schiebel, E. (2005). Kin4 kinase delays mitotic exit in response to spindle alignment defects. Mol Cell *19*, 209-221.

213. Maekawa, H., Priest, C., Lechner, J., Pereira, G., and Schiebel, E. (2007). The yeast centrosome translates the positional information of the anaphase spindle into a cell cycle signal. J Cell Biol *179*, 423-436.

214. Culotti, J., and Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division. Exp Cell Res *67*, 389-401.

215. Hartwell, L.H., Mortimer, R.K., Culotti, J., and Culotti, M. (1973). Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. Genetics *74*, 267-286.

216. Johnston, L.H., and Thomas, A.P. (1982). A further two mutants defective in initiation of the S phase in the yeast Saccharomyces cerevisiae. Mol Gen Genet *186*, 445-448.

217. Kitada, K., Johnston, L.H., Sugino, T., and Sugino, A. (1992). Temperature-sensitive cdc7 mutations of Saccharomyces cerevisiae are suppressed by the DBF4 gene, which is required for the G1/S cell cycle transition. Genetics *131*, 21-29.

218. Hollingsworth, R.E., Jr., and Sclafani, R.A. (1990). DNA metabolism gene CDC7 from yeast encodes a serine (threonine) protein kinase. Proc Natl Acad Sci U S A *87*, 6272-6276.

219. Oshiro, G., Owens, J.C., Shellman, Y., Sclafani, R.A., and Li, J.J. (1999). Cell cycle control of Cdc7p kinase activity through regulation of Dbf4p stability. Mol Cell Biol *19*, 4888-4896.

220. Patterson, M., Sclafani, R.A., Fangman, W.L., and Rosamond, J. (1986). Molecular characterization of cell cycle gene CDC7 from Saccharomyces cerevisiae. Mol Cell Biol *6*, 1590-1598.

221. Yoon, H.J., and Campbell, J.L. (1991). The CDC7 protein of Saccharomyces cerevisiae is a phosphoprotein that contains protein kinase activity. Proc Natl Acad Sci U S A *88*, 3574-3578.

222. Yoon, H.J., Loo, S., and Campbell, J.L. (1993). Regulation of Saccharomyces cerevisiae CDC7 function during the cell cycle. Mol Biol Cell *4*, 195-208.

223. Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M., and Sclafani, R.A. (1997). mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A *94*, 3151-3155.

224. Masai, H., Miyake, T., and Arai, K. (1995). hsk1+, a Schizosaccharomyces pombe gene related to Saccharomyces cerevisiae CDC7, is required for chromosomal replication. Embo J *14*, 3094-3104.

225. Jiang, W., and Hunter, T. (1997). Identification and characterization of a human protein kinase related to budding yeast Cdc7p. Proc Natl Acad Sci U S A *94*, 14320-14325.

226. Kim, J.M., Sato, N., Yamada, M., Arai, K., and Masai, H. (1998). Growth regulation of the expression of mouse cDNA and gene encoding a serine/threonine kinase related to Saccharomyces cerevisiae CDC7 essential for G1/S transition. Structure, chromosomal localization, and expression of mouse gene for s. cerevisiae Cdc7-related kinase. J Biol Chem *273*, 23248-23257.

227. Masai, H., and Arai, K. (2000). Dbf4 motifs: conserved motifs in activation subunits for Cdc7 kinases essential for S-phase. Biochem Biophys Res Commun *275*, 228-232.

228. Kumagai, H., Sato, N., Yamada, M., Mahony, D., Seghezzi, W., Lees, E., Arai, K., and Masai, H. (1999). A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol Cell Biol *19*, 5083-5095.

229. Gabrielse, C., Miller, C.T., McConnell, K.H., Deward, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCT-like domain required for the response to replication fork arrest in budding yeast. Genetics.

230. Ogino, K., Takeda, T., Matsui, E., Iiyama, H., Taniyama, C., Arai, K., and Masai, H. (2001). Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem *276*, 31376-31387.

231. Harkins, V., Gabrielse, C., Haste, L., and Weinreich, M. (2009). Budding Yeast Dbf4 Sequences Required for Cdc7 Kinase Activation and Identification of a Functional Relationship Between the Dbf4 and Rev1 BRCT Domains. Genetics.

232. Bork, P., Hofmann, K., Bucher, P., Neuwald, A.F., Altschul, S.F., and Koonin, E.V. (1997). A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. Faseb J *11*, 68-76.

233. Koonin, E.V., Altschul, S.F., and Bork, P. (1996). BRCA1 protein products ... Functional motifs. Nat Genet *13*, 266-268.

234. Clapperton, J.A., Manke, I.A., Lowery, D.M., Ho, T., Haire, L.F., Yaffe, M.B., and Smerdon, S.J. (2004). Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer. Nat Struct Mol Biol *11*, 512-518.

235. Matthews, L.A., Duong, A., Prasad, A.A., Duncker, B.P., and Guarne, A. (2009). Crystallization and preliminary X-ray diffraction analysis of motif N from Saccharomyces cerevisiae Dbf4. Acta Crystallogr Sect F Struct Biol Cryst Commun *65*, 890-894.

236. Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. Embo J *18*, 5334-5346.

237. Takeda, T., Ogino, K., Tatebayashi, K., Ikeda, H., Arai, K., and Masai, H. (2001). Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. Mol Biol Cell *12*, 1257-1274.

238. Matsumoto, S., Ogino, K., Noguchi, E., Russell, P., and Masai, H. (2005). Hsk1-Dfp1/Him1, the Cdc7-Dbf4 kinase in Schizosaccharomyces pombe, associates with Swi1, a component of the replication fork protection complex. J Biol Chem *280*, 42536-42542.

239. Jackson, A.L., Pahl, P.M., Harrison, K., Rosamond, J., and Sclafani, R.A. (1993). Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol *13*, 2899-2908.

240. Wu, X., and Lee, H. (2002). Human Dbf4/ASK promoter is activated through the Sp1 and MluI cell-cycle box (MCB) transcription elements. Oncogene *21*, 7786-7796.

241. Ferreira, M.F., Santocanale, C., Drury, L.S., and Diffley, J.F. (2000). Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. Mol Cell Biol *20*, 242-248.

242. Cheng, L., Collyer, T., and Hardy, C.F. (1999). Cell cycle regulation of DNA replication initiator factor Dbf4p. Mol Cell Biol *19*, 4270-4278.

243. Zachariae, W., and Nasmyth, K. (1999). Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev *13*, 2039-2058.

244. Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859-864.

245. Jares, P., Luciani, M.G., and Blow, J.J. (2004). A Xenopus Dbf4 homolog is required for Cdc7 chromatin binding and DNA replication. BMC Mol Biol *5*, 5.

246. Kneissl, M., Putter, V., Szalay, A.A., and Grummt, F. (2003). Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. J Mol Biol *327*, 111-128.

247. Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Johnston, L.H., and Sugino, A. (2000). Characterization of the yeast Cdc7p/Dbf4p complex purified from insect cells. Its protein kinase activity is regulated by Rad53p. J Biol Chem *275*, 35051-35062.

248. Sato, N., Arai, K., and Masai, H. (1997). Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. Embo J *16*, 4340-4351.

249. Masai, H., Matsui, E., You, Z., Ishimi, Y., Tamai, K., and Arai, K. (2000). Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. J Biol Chem *275*, 29042-29052.

250. Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol *20*, 3086-3096.

251. Remus, D., Beuron, F., Tolun, G., Griffith, J.D., Morris, E.P., and Diffley, J.F. (2009). Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell *139*, 719-730.

252. Sunkel, C.E., and Glover, D.M. (1988). polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. J Cell Sci *89 (Pt 1)*, 25-38.

253. Ohkura, H., Hagan, I.M., and Glover, D.M. (1995). The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev *9*, 1059-1073.

254. Kitada, K., Johnson, A.L., Johnston, L.H., and Sugino, A. (1993). A multicopy suppressor gene of the Saccharomyces cerevisiae G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. Mol Cell Biol *13*, 4445-4457.

255. Kumagai, A., and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. Science *273*, 1377-1380.

256. Lowery, D.M., Lim, D., and Yaffe, M.B. (2005). Structure and function of Polo-like kinases. Oncogene *24*, 248-259.

257. Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

258. Cheng, K.Y., Lowe, E.D., Sinclair, J., Nigg, E.A., and Johnson, L.N. (2003). The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. Embo J *22*, 5757-5768.

259. Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

260. Cheng, L., Hunke, L., and Hardy, C.F. (1998). Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. Mol Cell Biol *18*, 7360-7370.

261. Visintin, C., Tomson, B.N., Rahal, R., Paulson, J., Cohen, M., Taunton, J., Amon, A., and Visintin, R. (2008). APC/C-Cdh1-mediated degradation of the Polo kinase Cdc5 promotes the return of Cdc14 into the nucleolus. Genes Dev *22*, 79-90.

262. Uchiumi, T., Longo, D.L., and Ferris, D.K. (1997). Cell cycle regulation of the human polo-like kinase (PLK) promoter. J Biol Chem *272*, 9166-9174.

263. Martin, B.T., and Strebhardt, K. (2006). Polo-like kinase 1: target and regulator of transcriptional control. Cell Cycle *5*, 2881-2885.

264. Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R.A., and Dynlacht, B.D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev *16*, 245-256.

265. Gunawardena, R.W., Siddiqui, H., Solomon, D.A., Mayhew, C.N., Held, J., Angus, S.P., and Knudsen, E.S. (2004). Hierarchical requirement of SWI/SNF in retinoblastoma tumor suppressor-mediated repression of Plk1. J Biol Chem *279*, 29278-29285.

266. Arnaud, L., Pines, J., and Nigg, E.A. (1998). GFP tagging reveals human Polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. Chromosoma *107*, 424-429.

267. Golsteyn, R.M., Mundt, K.E., Fry, A.M., and Nigg, E.A. (1995). Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. J Cell Biol *129*, 1617-1628.

268. Lee, K.S., Yuan, Y.L., Kuriyama, R., and Erikson, R.L. (1995). Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. Mol Cell Biol *15*, 7143-7151.

269. Seong, Y.S., Kamijo, K., Lee, J.S., Fernandez, E., Kuriyama, R., Miki, T., and Lee, K.S. (2002). A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. J Biol Chem *277*, 32282-32293.

270. Song, S., Grenfell, T.Z., Garfield, S., Erikson, R.L., and Lee, K.S. (2000). Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol Cell Biol *20*, 286-298.

271. Sakchaisri, K., Asano, S., Yu, L.R., Shulewitz, M.J., Park, C.J., Park, J.E., Cho, Y.W., Veenstra, T.D., Thorner, J., and Lee, K.S. (2004). Coupling morphogenesis to mitotic entry. Proc Natl Acad Sci U S A *101*, 4124-4129.

272. Lee, K.S., Grenfell, T.Z., Yarm, F.R., and Erikson, R.L. (1998). Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. Proc Natl Acad Sci U S A *95*, 9301-9306.

273. Bishop, A.C., Shah, K., Liu, Y., Witucki, L., Kung, C., and Shokat, K.M. (1998). Design of allele-specific inhibitors to probe protein kinase signaling. Curr Biol *8*, 257-266.

274. Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J.C., and Doree, M. (1998). The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in Xenopus eggs. J Cell Sci *111 (Pt 12)*, 1751-1757.

275. Seki, A., Coppinger, J.A., Jang, C.Y., Yates, J.R., and Fang, G. (2008). Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. Science *320*, 1655-1658.

276. Macurek, L., Lindqvist, A., Lim, D., Lampson, M.A., Klompmaker, R., Freire, R., Clouin, C., Taylor, S.S., Yaffe, M.B., and Medema, R.H. (2008). Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature *455*, 119-123.

277. Mortensen, E.M., Haas, W., Gygi, M., Gygi, S.P., and Kellogg, D.R. (2005). Cdc28-dependent regulation of the Cdc5/Polo kinase. Curr Biol *15*, 2033-2037.

278. Booher, R.N., Deshaies, R.J., and Kirschner, M.W. (1993). Properties of Saccharomyces cerevisiae wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. Embo J *12*, 3417-3426.

279. Lew, D.J. (2003). The morphogenesis checkpoint: how yeast cells watch their figures. Curr Opin Cell Biol *15*, 648-653.

280. Edgington, N.P., Blacketer, M.J., Bierwagen, T.A., and Myers, A.M. (1999). Control of Saccharomyces cerevisiae filamentous growth by cyclin-dependent kinase Cdc28. Mol Cell Biol *19*, 1369-1380.

281. Shulewitz, M.J., Inouye, C.J., and Thorner, J. (1999). Hsl7 localizes to a septin ring and serves as an adapter in a regulatory pathway that relieves tyrosine phosphorylation of Cdc28 protein kinase in Saccharomyces cerevisiae. Mol Cell Biol *19*, 7123-7137.

282. Asano, S., Park, J.E., Sakchaisri, K., Yu, L.R., Song, S., Supavilai, P., Veenstra, T.D., and Lee, K.S. (2005). Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast. Embo J *24*, 2194-2204.

283. Nasmyth, K., Peters, J.M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. Science *288*, 1379-1385.

284. Hauf, S., Roitinger, E., Koch, B., Dittrich, C.M., Mechtler, K., and Peters, J.M. (2005). Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol *3*, e69.

285. Luca, F.C., Mody, M., Kurischko, C., Roof, D.M., Giddings, T.H., and Winey, M. (2001). Saccharomyces cerevisiae Mob1p is required for cytokinesis and mitotic exit. Mol Cell Biol *21*, 6972-6983.

286. Yoshida, S., Kono, K., Lowery, D.M., Bartolini, S., Yaffe, M.B., Ohya, Y., and Pellman, D. (2006). Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. Science *313*, 108-111.

287. Lippincott, J., and Li, R. (1998). Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. J Cell Biol *140*, 355-366.

288. Lippincott, J., Shannon, K.B., Shou, W., Deshaies, R.J., and Li, R. (2001). The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. J Cell Sci *114*, 1379-1386.

289. Frenz, L.M., Lee, S.E., Fesquet, D., and Johnston, L.H. (2000). The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis. J Cell Sci *113 Pt 19*, 3399-3408.

290. Yoshida, S., and Toh-e, A. (2001). Regulation of the localization of Dbf2 and mob1 during cell division of saccharomyces cerevisiae. Genes Genet Syst *76*, 141-147.

291. Park, C.J., Song, S., Lee, P.R., Shou, W., Deshaies, R.J., and Lee, K.S. (2003). Loss of CDC5 function in Saccharomyces cerevisiae leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. Genetics *163*, 21-33.

292. Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D.P., Glover, D.M., and Hagan, I.M. (2001). The role of Plo1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe. Embo J *20*, 1259-1270.

293. Mulvihill, D.P., and Hyams, J.S. (2002). Cytokinetic actomyosin ring formation and septation in fission yeast are dependent on the full recruitment of the polo-like kinase Plo1 to the spindle pole body and a functional spindle assembly checkpoint. J Cell Sci *115*, 3575-3586.

294. Smith, M.R., Wilson, M.L., Hamanaka, R., Chase, D., Kung, H., Longo, D.L., and Ferris, D.K. (1997). Malignant transformation of mammalian cells initiated by constitutive expression of the polo-like kinase. Biochem Biophys Res Commun *234*, 397-405.

295. Takai, N., Hamanaka, R., Yoshimatsu, J., and Miyakawa, I. (2005). Polo-like kinases (Plks) and cancer. Oncogene *24*, 287-291.

296. Wolf, G., Hildenbrand, R., Schwar, C., Grobholz, R., Kaufmann, M., Stutte, H.J., Strebhardt, K., and Bleyl, U. (2000). Polo-like kinase: a novel marker of proliferation: correlation with estrogen-receptor expression in human breast cancer. Pathol Res Pract *196*, 753-759.

297. Tokumitsu, Y., Mori, M., Tanaka, S., Akazawa, K., Nakano, S., and Niho, Y. (1999). Prognostic significance of polo-like kinase expression in esophageal carcinoma. Int J Oncol *15*, 687-692.

298. Takai, N., Miyazaki, T., Fujisawa, K., Nasu, K., Hamanaka, R., and Miyakawa, I. (2001). Expression of polo-like kinase in ovarian cancer is associated with histological grade and clinical stage. Cancer Lett *164*, 41-49.

299. Dietzmann, K., Kirches, E., von, B., Jachau, K., and Mawrin, C. (2001). Increased human polo-like kinase-1 expression in gliomas. J Neurooncol *53*, 1-11.

300. Dai, W., Huang, X., and Ruan, Q. (2003). Polo-like kinases in cell cycle checkpoint control. Front Biosci *8*, d1128-1133.

301. Dai, W., Liu, T., Wang, Q., Rao, C.V., and Reddy, B.S. (2002). Down-regulation of PLK3 gene expression by types and amount of dietary fat in rat colon tumors. Int J Oncol *20*, 121-126.

302. Li, B., Ouyang, B., Pan, H., Reissmann, P.T., Slamon, D.J., Arceci, R., Lu, L., and Dai, W. (1996). Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. J Biol Chem *271*, 19402-19408.

303. Conn, C.W., Hennigan, R.F., Dai, W., Sanchez, Y., and Stambrook, P.J. (2000). Incomplete cytokinesis and induction of apoptosis by overexpression of the mammalian polo-like kinase, Plk3. Cancer Res *60*, 6826-6831.

304. Wang, Q., Xie, S., Chen, J., Fukasawa, K., Naik, U., Traganos, F., Darzynkiewicz, Z., Jhanwar-Uniyal, M., and Dai, W. (2002). Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. Mol Cell Biol *22*, 3450-3459.

305. Wang, L., Liu, X., Kreis, W., and Budman, D. (2000). The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemotherapy Pharmacology *89*, 983-994.

306. Jordan, M.A., and Wilson, L. (2004). Microtubules as a target for anticancer drugs. Nat Rev Cancer *4*, 253-265.

307. Kops, G.J., Weaver, B.A., and Cleveland, D.W. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer *5*, 773-785.

308. Mortlock, A.A., Foote, K.M., Heron, N.M., Jung, F.H., Pasquet, G., Lohmann, J.J., Warin, N., Renaud, F., De Savi, C., Roberts, N.J., Johnson, T., Dousson, C.B., Hill, G.B., Perkins, D., Hatter, G., Wilkinson, R.W., Wedge, S.R., Heaton, S.P., Odedra, R., Keen, N.J., Crafter, C., Brown, E., Thompson, K., Brightwell, S., Khatri, L., Brady, M.C., Kearney, S., McKillop, D., Rhead, S., Parry, T., and Green, S. (2007). Discovery, synthesis, and in vivo activity of a new class of pyrazoloquinazolines as selective inhibitors of aurora B kinase. J Med Chem *50*, 2213-2224.

309. Taylor, S., and Peters, J.M. (2008). Polo and Aurora kinases: lessons derived from chemical biology. Curr Opin Cell Biol *20*, 77-84.

310. Yang, J., Ikezoe, T., Nishioka, C., Tasaka, T., Taniguchi, A., Kuwayama, Y., Komatsu, N., Bandobashi, K., Togitani, K., Koeffler, H.P., Taguchi, H., and Yokoyama, A. (2007). AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells in vitro and in vivo. Blood *110*, 2034-2040.

311. Manfredi, M.G., Ecsedy, J.A., Meetze, K.A., Balani, S.K., Burenkova, O., Chen, W., Galvin, K.M., Hoar, K.M., Huck, J.J., LeRoy, P.J., Ray, E.T., Sells, T.B., Stringer, B., Stroud, S.G., Vos, T.J., Weatherhead, G.S., Wysong, D.R., Zhang, M., Bolen, J.B., and Claiborne, C.F. (2007). Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. Proc Natl Acad Sci U S A *104*, 4106-4111.

312. Anderson, K., Lai, Z., McDonald, O.B., Stuart, J.D., Nartey, E.N., Hardwicke, M.A., Newlander, K., Dhanak, D., Adams, J., Patrick, D., Copeland, R.A., Tummino, P.J., and Yang, J. (2009). Biochemical characterization of GSK1070916, a potent and selective inhibitor of Aurora B and Aurora C kinases with an extremely long residence time1. Biochem J *420*, 259-265.

313. Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J.J., Hoffmann, M., Rettig, W.J., Kraut, N., and Peters, J.M. (2007). The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Curr Biol *17*, 304-315.

314. Steegmaier, M., Hoffmann, M., Baum, A., Lenart, P., Petronczki, M., Krssak, M., Gurtler, U., Garin-Chesa, P., Lieb, S., Quant, J., Grauert, M., Adolf, G.R., Kraut, N., Peters, J.M., and Rettig, W.J. (2007). BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Curr Biol *17*, 316-322.

315. Lansing, T.J., McConnell, R.T., Duckett, D.R., Spehar, G.M., Knick, V.B., Hassler, D.F., Noro, N., Furuta, M., Emmitte, K.A., Gilmer, T.M., Mook, R.A., Jr., and Cheung, M. (2007). In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1. Mol Cancer Ther *6*, 450-459.

316. Garland, L.L., Taylor, C., Pilkington, D.L., Cohen, J.L., and Von Hoff, D.D. (2006). A phase I pharmacokinetic study of HMN-214, a novel oral stilbene derivative with polo-like kinase-1-interacting properties, in patients with advanced solid tumors. Clin Cancer Res *12*, 5182-5189.

317. Takagi, M., Honmura, T., Watanabe, S., Yamaguchi, R., Nogawa, M., Nishimura, I., Katoh, F., Matsuda, M., and Hidaka, H. (2003). In vivo antitumor activity of a novel sulfonamide, HMN-214, against human tumor xenografts in mice and the spectrum of cytotoxicity of its active metabolite, HMN-176. Invest New Drugs *21*, 387-399.

318. Tanaka, H., Ohshima, N., Ikenoya, M., Komori, K., Katoh, F., and Hidaka, H. (2003). HMN-176, an active metabolite of the synthetic antitumor agent HMN-214, restores chemosensitivity to multidrug-resistant cells by targeting the transcription factor NF-Y. Cancer Res *63*, 6942-6947.

319. Reindl, W., Yuan, J., Kramer, A., Strebhardt, K., and Berg, T. (2009). A pan-specific inhibitor of the polo-box domains of polo-like kinases arrests cancer cells in mitosis. Chembiochem *10*, 1145-1148.

320. Reindl, W., Yuan, J., Kramer, A., Strebhardt, K., and Berg, T. (2008). Inhibition of polo-like kinase 1 by blocking polo-box domain-dependent protein-protein interactions. Chem Biol *15*, 459-466.

321. Hess, G.F., Drong, R.F., Weiland, K.L., Slightom, J.L., Sclafani, R.A., and Hollingsworth, R.E. (1998). A human homolog of the yeast CDC7 gene is overexpressed in some tumors and transformed cell lines. Gene *211*, 133-140.

322. Bonte, D., Lindvall, C., Liu, H., Dykema, K., Furge, K., and Weinreich, M. (2008). Cdc7-Dbf4 kinase overexpression in multiple cancers and tumor cell lines is correlated with p53 inactivation. Neoplasia *10*, 920-931.

323. Nambiar, S., Mirmohammadsadegh, A., Hassan, M., Mota, R., Marini, A., Alaoui, A., Tannapfel, A., Hegemann, J.H., and Hengge, U.R. (2007). Identification and functional characterization of ASK/Dbf4, a novel cell survival gene in cutaneous melanoma with prognostic relevance. Carcinogenesis *28*, 2501-2510.

324. Montagnoli, A., Tenca, P., Sola, F., Carpani, D., Brotherton, D., Albanese, C., and Santocanale, C. (2004). Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. Cancer Res *64*, 7110-7116.

325. Guo, B., Romero, J., Kim, B.J., and Lee, H. (2005). High levels of Cdc7 and Dbf4 proteins can arrest cell-cycle progression. Eur J Cell Biol *84*, 927-938.

326. Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

327. Tenca, P., Brotherton, D., Montagnoli, A., Rainoldi, S., Albanese, C., and Santocanale, C. (2007). Cdc7 is an active kinase in human cancer cells undergoing replication stress. J Biol Chem *282*, 208-215.

328. Ermoli, A., Bargiotti, A., Brasca, M.G., Ciavolella, A., Colombo, N., Fachin, G., Isacchi, A., Menichincheri, M., Molinari, A., Montagnoli, A., Pillan, A., Rainoldi, S., Sirtori, F.R., Sola, F., Thieffine, S., Tibolla, M., Valsasina, B., Volpi, D., Santocanale, C., and Vanotti, E. (2009). Cell division cycle 7 kinase inhibitors: 1H-pyrrolo[2,3-b]pyridines, synthesis and structure-activity relationships. J Med Chem *52*, 4380-4390.

329. Menichincheri, M., Bargiotti, A., Berthelsen, J., Bertrand, J.A., Bossi, R., Ciavolella, A., Cirla, A., Cristiani, C., Croci, V., D'Alessio, R., Fasolini, M.,

Fiorentini, F., Forte, B., Isacchi, A., Martina, K., Molinari, A., Montagnoli, A., Orsini, P., Orzi, F., Pesenti, E., Pezzetta, D., Pillan, A., Poggesi, I., Roletto, F., Scolaro, A., Tato, M., Tibolla, M., Valsasina, B., Varasi, M., Volpi, D., Santocanale, C., and Vanotti, E. (2009). First Cdc7 kinase inhibitors: pyrrolopyridinones as potent and orally active antitumor agents. 2. Lead discovery. J Med Chem *52*, 293-307.

330. Montagnoli, A., Valsasina, B., Croci, V., Menichincheri, M., Rainoldi, S., Marchesi, V., Tibolla, M., Tenca, P., Brotherton, D., Albanese, C., Patton, V., Alzani, R., Ciavolella, A., Sola, F., Molinari, A., Volpi, D., Avanzi, N., Fiorentini, F., Cattoni, M., Healy, S., Ballinari, D., Pesenti, E., Isacchi, A., Moll, J., Bensimon, A., Vanotti, E., and Santocanale, C. (2008). A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. Nat Chem Biol *4*, 357-365.

331. Hardy, C.F., and Pautz, A. (1996). A novel role for Cdc5p in DNA replication. Mol Cell Biol *16*, 6775-6782.

332. Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M.A., and Nasmyth, K. (2001). Phosphorylation of the cohesin subunit Scc1 by Polo/Cdc5 kinase regulates sister chromatid separation in yeast. Cell *105*, 459-472.

CHAPTER ONE

Cdc7p-Dbf4p Regulates Mitotic Exit by Inhibiting Polo Kinase

Miller, C.T., Gabrielse, C., Chen, Y.C., and Weinreich, M. (2009). Cdc7p-dbf4p regulates mitotic exit by inhibiting Polo kinase. PLoS Genet *5*, e1000498.

ABSTRACT:

Cdc7p-Dbf4p is a conserved protein kinase required for the initiation of DNA replication. The Dbf4p regulatory subunit binds Cdc7p and is essential for Cdc7p kinase activation, however the N-terminal third of Dbf4p is dispensable for its essential replication activities. Here we define a short N-terminal Dbf4p region that targets Cdc7p-Dbf4p kinase to Cdc5p, the single Polo kinase in budding yeast that regulates mitotic progression and cytokinesis. Dbf4p mediates an interaction with the Polo substrate-binding domain to inhibit its essential role during mitosis. Although Dbf4p does not inhibit Polo kinase activity it nonetheless inhibits Polo-mediated activation of the mitotic exit network (MEN), presumably by altering Polo substrate targeting. In addition, although *dbf4* mutants defective for interaction with Polo transit S-phase normally, they aberrantly segregate chromosomes following nuclear misorientation. Therefore, Cdc7p-Dbf4p prevents inappropriate exit from mitosis by inhibiting Polo kinase and functions in the spindle position checkpoint.

INTRODUCTION:

Accurate ordering of cell cycle events is an important requirement for the viability of all eukaryotic organisms. Once cells commit to duplicate their genome they must restrain mitosis until replication is complete and then accurately coordinate mitosis with cytokinesis to ensure the faithful transmission of chromosomes to daughter cells [1]. Importantly, errors in cell cycle checkpoints that enforce this ordering can be deleterious for accurate chromosome transmission. For

instance, DNA damage or replication fork arrest during S-phase elicits a reversible block to mitotic progression by the budding yeast Mec1p (HsATR) and Rad53p (HsChk2) checkpoint kinases [2, 3]. In the absence of Mec1p or Rad53p, replication fork arrest during S-phase is not sensed leading to premature mitotic events and cell death (reviewed by [4]). Additionally, since daughter cell growth is highly polarized in the budding yeast, exit from mitosis is prevented until sister chromatids segregate through the bud neck and into the daughter cell [5-7]. This ensures that spindle disassembly and mitotic exit are not initiated until accurate chromosome partitioning between mother and daughter cells has occurred. Failure to block mitotic exit when nuclear division takes place within the mother cell results in polyploid and anucleate progeny [8, 9]. It is not surprising therefore, that both entry into and exit from mitosis are delayed by cellular checkpoints that respond to replication stress, chromosome damage, or spindle disruption [1]. Errors in these mitotic checkpoints are catastrophic and result in ploidy defects and genetic alterations, which are frequently observed in human cancers (reviewed by [10]).

The Cdc7p-Dbf4p kinase is required to catalyze the initiation of DNA synthesis at the beginning of S-phase (reviewed by [11]). Cdc7p kinase activity is tightly regulated during the cell cycle by binding the Dbf4p regulatory subunit, which is cyclically expressed. Dbf4p accumulates in late G1, is present throughout S-phase and then is destroyed during mitosis and early G1 by anaphase promoting complex (APC)-dependent degradation [12-17]. Therefore, Cdc7p-Dbf4p kinase activity is low following exit from mitosis and entry into G1-

phase until it is needed to initiate a new round of DNA synthesis in late G1-phase of the following cell cycle. Multiple lines of evidence suggest that Cdc7p-Dbf4p activates the MCM DNA helicase [18-20] that is assembled at origins of replication in early G1 in an inactive form (reviewed in [21, 22]).

In addition to its essential role in replication initiation, several studies suggest that the Cdc7p-Dbf4p kinase responds to DNA damage or replication fork stalling but its precise role in these activities is unknown [17, 23-25]. Dbf4p encodes a dispensable BRCT-like domain in the N-terminus that might target the kinase to stalled replication forks [26, 27]. In fission yeast, the Cdc7p-Dbf4p ortholog Hsk1p-Dfp1p interacts with Swi1p (budding yeast Tof1p), a component of replication forks required for fork stability and also promotes centromeric cohesion in early mitosis [28, 29]. Rad53p also phosphorylates Dbf4p in response to replication stress and this regulation requires N-terminal Dbf4p sequences through which Rad53p physically interacts [17, 25, 30]. Interestingly, the absence of the BRCT-like domain results in a defect in late origin activation suggesting that this domain might alter Cdc7p-Dbf4p binding at early versus late replication origins [26]. Together, these data suggest that the Dbf4p N-terminus encodes non-essential regulatory functions that target the kinase to particular substrates.

To identify proteins that interact with the Dbf4p N-terminus, we performed a yeast two-hybrid screen with an N-terminal region of Dbf4p and identified an interaction with the Cdc5p kinase, the only Polo ortholog in yeast. Budding yeast Polo, like *Drosophila* Polo and human Polo-like kinase 1 (Plk1), functions as a

master regulator of mitotic progression and is also required for cytokinesis (reviewed by [31, 32]). Polo activity is regulated by several independent cellular mechanisms. Polo protein levels are controlled by APC-dependent degradation in mitosis/G1-phase and activation of Polo catalytic activity requires phosphorylation by Cdk1 kinase early in G2 [33-35]. In addition, Polo function is inhibited by cell cycle checkpoints that are induced following DNA or spindle damage [36-38]. A genetic and physical interaction between Dbf4 and Polo was described previously [39, 40], however the biological significance of this interaction was not known.

Polo controls multiple mitotic events to ensure accurate chromosome segregation. After anaphase initiation, Polo is required to activate the FEAR (Cdc14 early anaphase release) and MEN (mitotic exit network) pathways that promote nucleolar release of Cdc14p phosphatase [37, 41-43]. Limited Cdc14p release by the FEAR pathway promotes accurate rDNA and telomere segregation [44-46]. Subsequent full nucleolar release of Cdc14p by the MEN reverses Cdk substrate phosphorylation that leads to APC-Cdh1p activation, cyclin destruction and mitotic spindle disassembly (reviewed by [47]). Activation of the MEN is promoted by Tem1p-GTP and antagonized by Bfa1p-Bub2p, a two-component GTPase activating protein (GAP) [48-50]. To promote mitotic exit, Polo phosphorylates Bfa1p-Bub2p to inhibit its GAP activity and is also required for activation of Dbf2p kinase activity, independently of Bfa1p-Bub2p [37, 49, 51, 52]. The Polo requirement for Dbf2p kinase activation may reflect that Polo also promotes Cdc14p release in the FEAR pathway, which primes the

MEN [43]. Therefore, Polo promotes accumulation of Tem1p-GTP and activation of the downstream MEN kinases Cdc15p and Dbf2p, which ultimately cause full release of Cdc14p from the nucleolus. In response to replication fork arrest, Rad53 inhibits MEN activation, which may or may not impact Polo activity since the molecular basis of this regulation is not understood [53, 54]. Spindle position defects also counteract Polo activity by targeting Kin4p kinase to the spindle poles where it inhibits Polo-dependent Bfa1p phosphorylation [8, 9, 55]. Failure to execute the spindle position checkpoint (SPOC) results in premature exit from mitosis and nuclear partitioning defects.

Here we define an N-terminal Dbf4p polo-box interaction region (that we refer to as the "PIR") that binds directly to Polo and show that Dbf4p inhibits Polo and Dbf2p activity. Deletion of the PIR allows Cdc14p nucleolar release in a *cdc5-1* mutant at the non-permissive temperature. In response to nuclear mispositioning, a *dbf4* mutant lacking the PIR fails to arrest in mitosis and prematurely exits the cell cycle. Thus, Dbf4 protein is required for proper functioning of the spindle position checkpoint most likely by antagonizing the ability of Polo to promote Cdc14p release in either the FEAR or MEN pathways. Our work therefore reveals a previously unrecognized function for Dbf4p in the regulation of mitotic progression through a direct interaction with Polo.

RESULTS:

The Cdc5p polo-box domain interacts with the Dbf4p N-terminus. We conducted a yeast two-hybrid screen to identify proteins that interact with the

Dbf4p N-terminus (residues 67-227) and recovered multiple clones encoding the polo-box domain (PBD) of Cdc5p. Polo kinase has two conserved domains; an N-terminal kinase domain and a C-terminal region called the polo-box domain (PBD) (reviewed by [56]), which is a phospho-Ser/Thr binding module that targets the kinase to its mitotic substrates [57, 58]. The crystallographic structure of the Plk1 PBD bound to a phospho-threonine peptide has been solved [59]. Since the Dbf4p BRCT-like region alone (residues 110-227) failed to interact with the Polo PBD (Figure 6A), this suggested that the PBD interaction was occurring through Dbf4p N-terminal sequences from 67-109. Residues 67-109 were similarly required for the Polo interaction within the context of full length Dbf4p (Figure 6B) and were sufficient to interact with the Polo PBD (Figure 6A). Dbf4p residues 67-109 with all serines/threonines changed to alanine still interacted with the PBD (Figure 6A) suggesting that the PBD can bind to this Dbf4p region independently of phosphorylation. Further deletion and point mutant analysis (Y-C.C. and M.W., unpublished data) revealed that residues 82-88 are essential for the Dbf4p-Polo interaction (Figure 6A, B).

The PBD is composed of three conserved regions called the Polo-cap (Pc), Polo-box 1 (PB1) and Polo-box 2 (PB2) that fold together to form a functional phosphopeptide-binding domain [59]. We deleted conserved residues within the PBD to test their requirement for interaction with Dbf4p (Figure 6C). Deletion of residues preceding the PBD (GAD-Polo 454-705) had little effect on the Dbf4p-Polo interaction. However, elimination of the Pc (GAD-Polo 510-705)

Figure 6. The N-terminus of Dbf4p interacts with the Polo PBD. Cells co-transformed with various bait *GAL4* DNA binding domain fusions (GBD-*DBF4*) and prey *GAL4* activation domain fusions (GAD-*CDC5*) constructs were spotted at 10-fold serial dilutions on SCM–Trp –Leu and SCM–Trp–Leu–His plates containing 2mM 3AT (A, C) or 0.5mM 3AT (B) and incubated at 30 degrees Celsius for 2 to 3 days. The GBD-Dbf4 protein levels are shown in Figure 7.

A

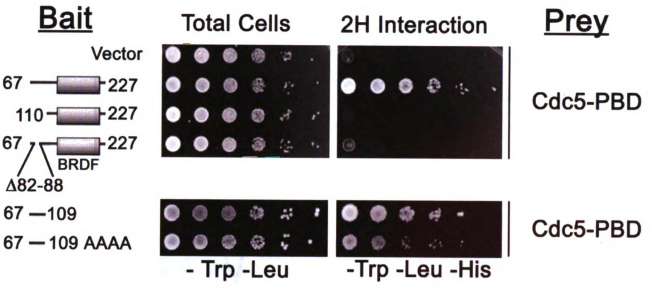
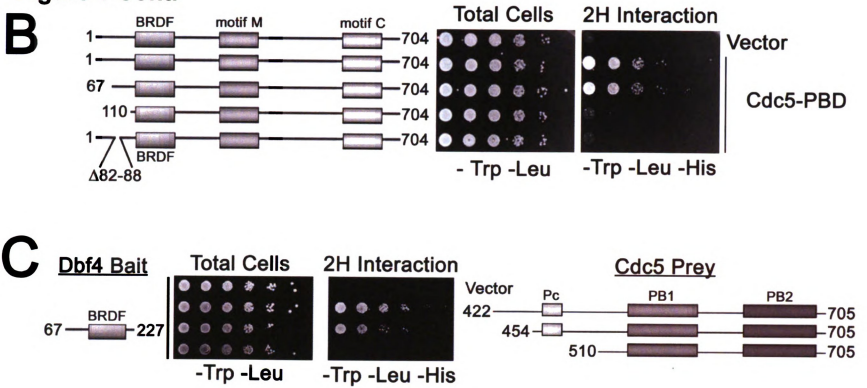

Bait
Total Cells
2H Interaction
Prey
Vector
67 — 227
110 — 227
67 — 227
BRDF
Δ82-88
67 — 109
67 — 109 AAAA
Cdc5-PBD
Cdc5-PBD
- Trp -Leu
-Trp -Leu -His

Figure 1 Cont.



completely disrupted the interaction with Dbf4p. These data suggest that the structural integrity of the Polo PBD is required for Dbf4p binding.

Dbf4p directly interacts with Polo. Dbf4p binds and activates the Cdc7p kinase subunit in yeast and has no known role apart from its interaction with Cdc7p [14, 60]. To determine whether the interaction between Dbf4p and Polo occurred in the context of the full-length Cdc7p-Dbf4p kinase, Sf9 cells were co-infected with baculoviruses expressing Polo, wild type HA-Cdc7p-Dbf4p or wild type HA-Cdc7p with various Dbf4p deletion derivatives. HA-Cdc7p-Dbf4p kinase was immunoprecipitated using an antibody against the HA tag and examined for the presence of Polo. All the Dbf4p deletion derivatives we examined interact with Cdc7p and activate normal Cdc7p kinase activity ([26] and data not shown). Whereas Polo interacted with full-length Cdc7p-Dbf4p and Cdc7p-Dbf4-NΔ65p, Polo did not interact with Cdc7p-Dbf4p complexes that lacked the Dbf4p N-terminal 109 residues required for the Polo two-hybrid interaction (Figure 8A). These data indicate that full length Cdc7p-Dbf4p kinase interacts with Polo but that Dbf4p residues 65-109 are required for this interaction. Importantly, HA-Cdc7p-Dbf4p interacts with Cdc5p in yeast when the proteins are expressed at endogenous levels and this interaction also depends on the Dbf4p N-terminus (Figure 8B).

We next tested whether Polo bound directly to Dbf4p using purified proteins. GST-PBD and Sumo-Dbf4 67-109 fusion proteins purified from *E. coli* were mixed, pulled down using glutathione-Sepharose and analyzed by

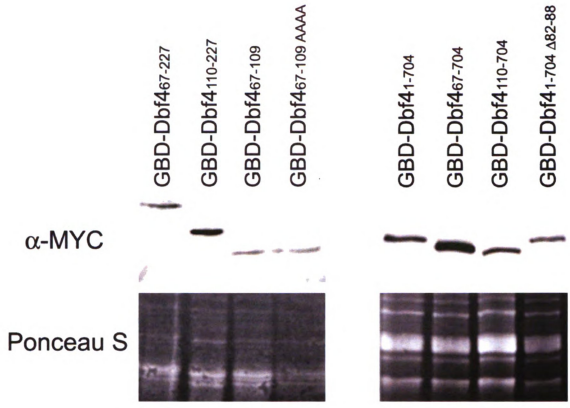


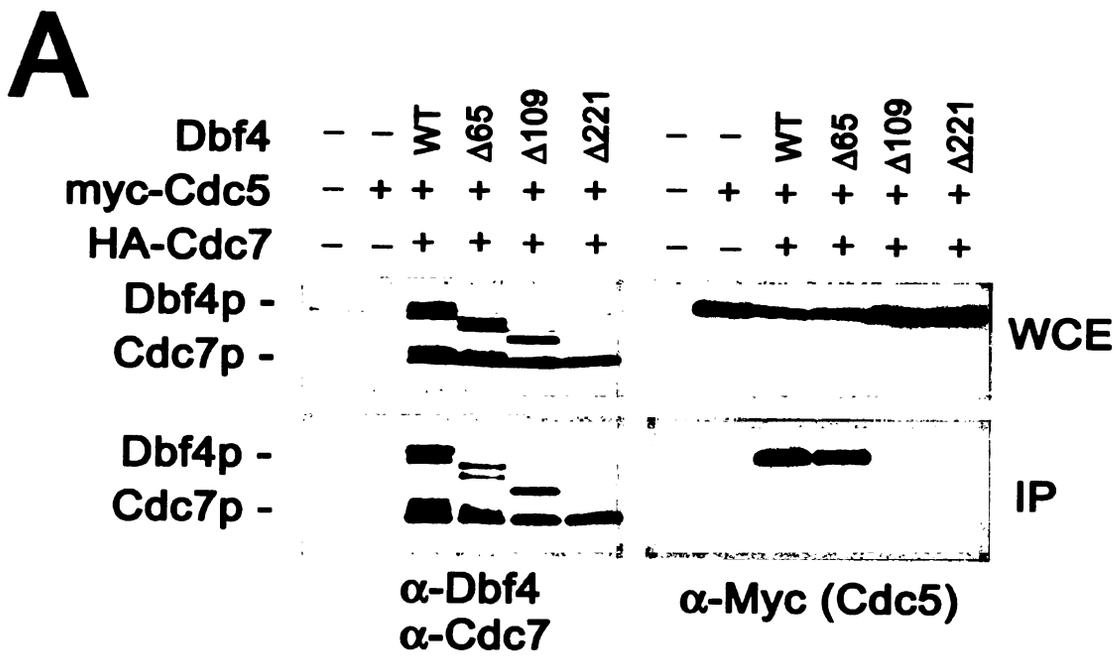
Figure 7. Western blot of Gal4-DNA binding domain fusions to various Dbf4 N-terminal fragments for the 2-hybrid assays shown in Figure 6.

immunoblotting. Although Sumo alone did not interact with GST-PBD, Sumo-Dbf4p 67-109 interacted with GST-PBD but not with GST alone (Figure 8C). These data indicate that Dbf4p residues 67-109 (that we refer to as the Dbf4p PIR) are sufficient for a direct interaction with the Polo PBD.

Dbf4p inhibits Polo activity. Cdc7p-Dbf4p is required to initiate DNA replication, but is present throughout S-phase and during the metaphase to anaphase transition. Dbf4p is subject to APC-Cdc20p dependent degradation [16] but some protein is still present in late mitotic mutants that have activated the Cdc20p but not the Cdh1p form of the APC [17]. We examined Dbf4 protein abundance relative to Pds1 protein in cells moving synchronously through the cell cycle, since Pds1p is degraded at the onset of anaphase by APC-Cdc20 [61]. We found that although the abundance of both proteins declines at the same time, Pds1p is absent during mitosis while some fraction of Dbf4p persists (Figure 9). In contrast, Dbf4p has very low abundance or is absent in cells arrested in G1-phase by mating-pheromone when the APC-Cdh1p is active [12, 13, 15, 17] and Dbf4p is stabilized by inactivation of the APC in G1 or by removal of its N-terminal D-box [13, 15-17]. Together these data suggest that Dbf4p degradation can occur via both APC-Cdc20p and APC-Cdh1p mediated ubiquitylation.

Since budding yeast Polo is not required for DNA replication but promotes multiple mitotic activities, we reasoned that Cdc7p-Dbf4p might influence Polo activity during mitosis. The *dbf4-NΔ109* mutant progresses normally through the

Figure 8. Dbf4p residues 67-109 interact directly with the Polo PBD. (A) HA-Cdc7p Dbf4p kinase or HA-Cdc7p plus Dbf4p truncation mutants were expressed in Sf9 cells together with Polo and immunoprecipitated using 12CA5 antibody. Blots were probed with anti-Myc (Cdc5p), anti-Cdc7p and anti-Dbf4p polyclonal antibodies. (B) Endogenous HA3-Cdc7p-Dbf4p complexes were immunoprecipitated using 12CA5 antibody from *HA3-CDC7 DBF4 CDC5-Myc15* (M2741) and *HA3-CDC7 dbf4-NΔ109 CDC5-Myc15* (M2743) yeast strains following nocodazole arrest and probed for Cdc7, Dbf4, and Cdc5-Myc. (C) Purified Sumo and Sumo-Dbf4 67-109 proteins were co-incubated with purified GST-PBD or GST alone; proteins were pulled down using glutathione-Sepharose beads and blotted with antibodies against GST or Sumo. (D) *In vitro* phosphorylation of GST-PBD using purified Cdc7p-Dbf4p kinase.


A
Dbf4
myc-Cdc5
HA-Cdc7
WT
Δ65
Δ109
Δ221
Dbf4p -
Cdc7p -
WCE
IP
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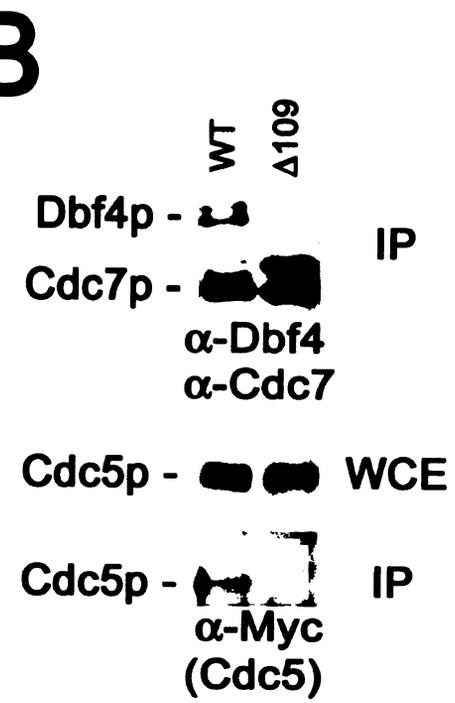
α-Cdc7
α-Myc (Cdc5)


B
WT
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Cdc7p -
IP
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Cdc5p -
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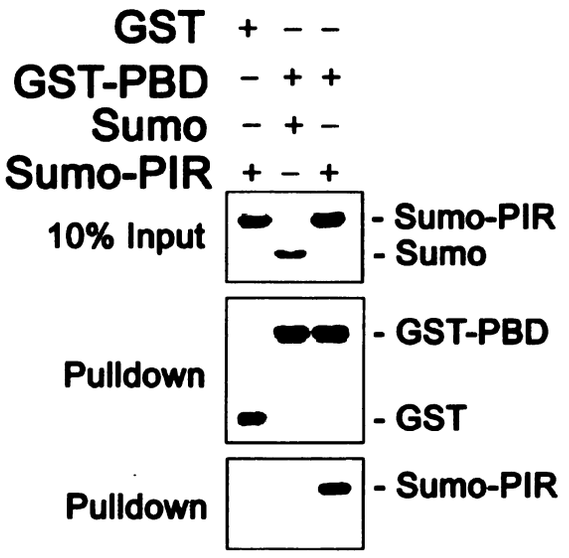
IP
α-Myc
(Cdc5)

Figure 8 Cont.

C



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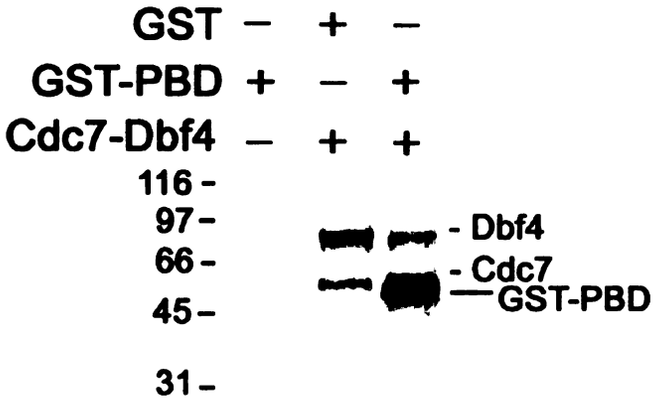


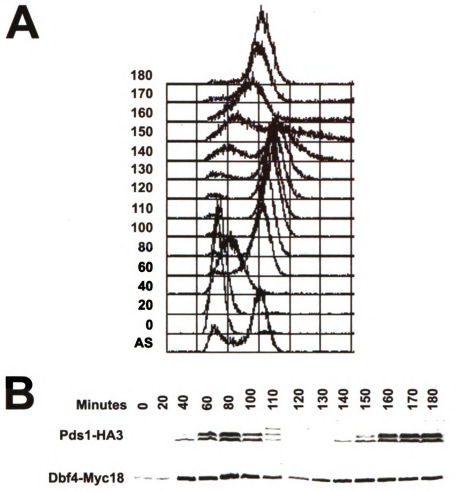


Figure 9. Some Dbf4p persists after Cdc20 activation during an unperturbed cell cycle. *PDS1-HA3 DBF4-Myc18* (M3161) was arrested in G1 with mating pheromone and released into the cell cycle at 20 degrees Celsius. Samples were taken at the indicated time points. Protein extracts were made for Western blotting and cells processed for DNA content analysis by flow cytometry. Western blots were probed with 9E10 α-Myc (Dbf4-Myc18) and 12CA5 α-HA (Pds1-HA) antibodies.

cell cycle and does not exhibit any obvious growth defects or temperature sensitivity (Figure 10A, B) [26]. We therefore tested for genetic interactions between *dbf4-NΔ109* and the *cdc5-1* temperature sensitive (ts) mutant. At the restrictive temperature *cdc5-1* cells arrest in late telophase with divided nuclei, elongated spindles and high Cdk1p-Clb2p levels indicating a failure to exit mitosis [34, 35, 62]. The *cdc5-1* mutant is ts at 30 degrees Celsius on rich media, but we found that *dbf4-NΔ109* suppressed the *cdc5-1* temperature sensitivity up to 35 degrees Celsius indicating a strong suppression of its growth defect (Figure 10A). The *dbf4-Δ82-88* mutant defective for interaction with Polo also suppressed the *cdc5-1* ts (Figure 10A). Since the *cdc5-1* ts suppression was reduced in heterozygous *dbf4-NΔ109/DBF4* diploids compared to *dbf4-NΔ109/dbf4-NΔ109* diploids, i.e. *dbf4-NΔ109* was haploinsufficient (Figure 10C), our data strongly suggests that Dbf4p is a Polo inhibitor and that loss of the Dbf4-Polo interaction leads to increased *cdc5-1* activity.

We confirmed that Dbf4p inhibited Polo activity using several independent genetic tests. An extra plasmid copy of wild type *DBF4* but not *dbf4-NΔ109* inhibited the growth of *cdc5-1* cells (Figure 10D). A *dbf4-NΔ65* mutant that disrupts the D-box (residues 62-70) resulting in elevated protein levels was synthetically sick or lethal in combination with *cdc5-1* (Figure 10E). Since the *dbf4-NΔ65* mutant exhibits a wild type growth rate and normal S-phase entry and progression (data not shown; [26]) but binds to Polo, this suggests that elevated Dbf4p levels are deleterious to *cdc5-1* activity. Finally, elevated expression of the Dbf4p N-terminus from the *GAL1* promoter completely inhibited the growth of

cdc5-1 cells but had no effect on the growth of wild type (not shown) or a *mcm2-1* ts mutant. Mcm2 is a component of the MCM helicase, which is thought to be the physiological target of Cdc7p-Dbf4p during the initiation of DNA replication [11]. The inhibition of *cdc5-1* growth depended on the Dbf4p-Polo interaction, since deletion of the PIR (residues 66-109) or Dbf4p residues 82-88 abrogated the growth inhibition (Figure 10A, B). Since the Dbf4p N-terminus interacts with the PBD, this data suggest that overexpression of Dbf4 N-terminal peptides interferes with essential Polo-substrate interactions by competitive inhibition. Together, these data indicate that the Dbf4p N-terminus inhibits Polo activity and that this inhibition requires residues 66-109, which are also required for the Dbf4p-Polo physical interaction.

We wanted to determine whether the Cdc7p kinase subunit is required to inhibit Polo in the FEAR or MEN pathways. This is not straightforward since Cdc7p is an essential protein kinase. Importantly, inhibiting Cdc7p activity would not only inhibit replication origin firing but would also likely induce the replication checkpoint that inhibits the metaphase to anaphase transition and MEN activation [4]. Inhibiting Cdc7p activity would thus interfere with the mitotic pathways we would like to measure. Therefore, we addressed this question indirectly by taking advantage of our observation that high copy *dbf4-NΔ109* suppressed the *cdc5-1* ts phenotype (Figure 11C). Since Dbf4p residues required for interaction with Cdc7p map between residues 312-704 (C.G. and M.W. unpublished data), Dbf4-NΔ109 protein (expressed in high copy) will compete with full length Dbf4p (in single copy) for Cdc7p binding. Therefore, our

Figure 10. The Dbf4p N-terminus inhibits Polo activity. (A) *dbf4-NΔ109* rescues the *cdc5-1* temperature sensitivity. Indicated strains were spotted at ten-fold serial dilution on YPD and grown at increasing temperatures. (B) Cell cycle progression of wild type and *dbf4-NΔ109* at 30 degrees Celsius by flow cytometry of alpha-factor arrested (t=0) and released cells (t=10 to 180 mins). (C) The indicated diploid strains were spotted at increasing temperatures (D) *DBF4 cdc5-1* was transformed with the indicated *ARS CEN* plasmids and spotted at 25 degrees Celsius and 32 degrees Celsius (E) Representative tetrads from a *dbf4-NΔ65* (M2007) cross to *cdc5-1* (M1614).

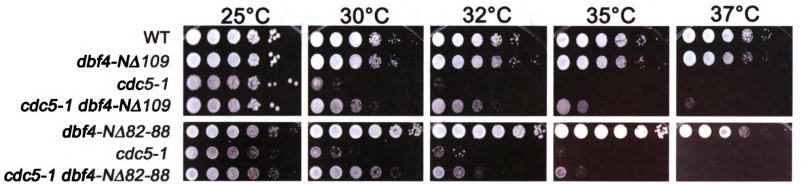

A
25°C
30°C
32°C
35°C
37°C
WT
dbf4-NΔ109
cdc5-1
cdc5-1 dbf4-NΔ109
dbf4-NΔ82-88
cdc5-1
cdc5-1 dbf4-NΔ82-88

Figure 10 Cont.

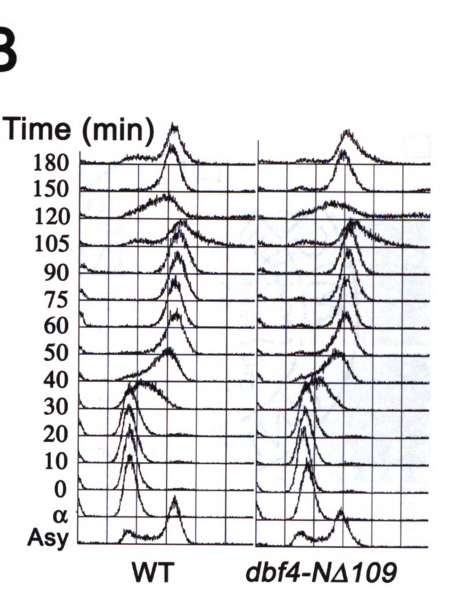
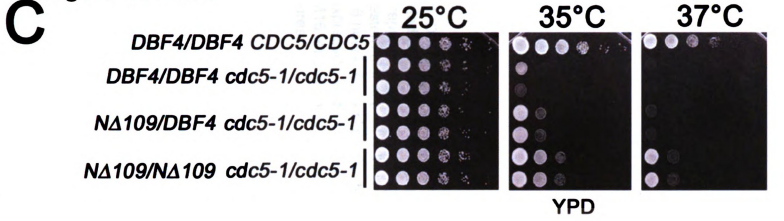
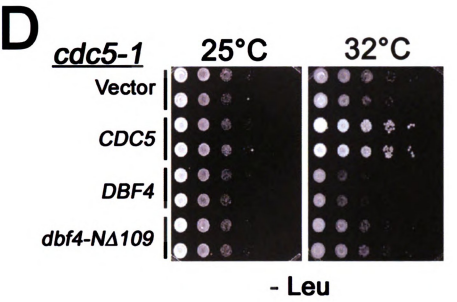


Figure 10 Cont.





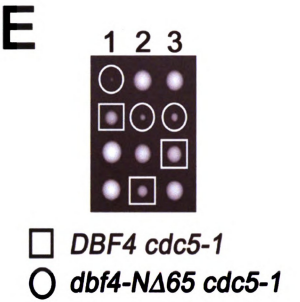
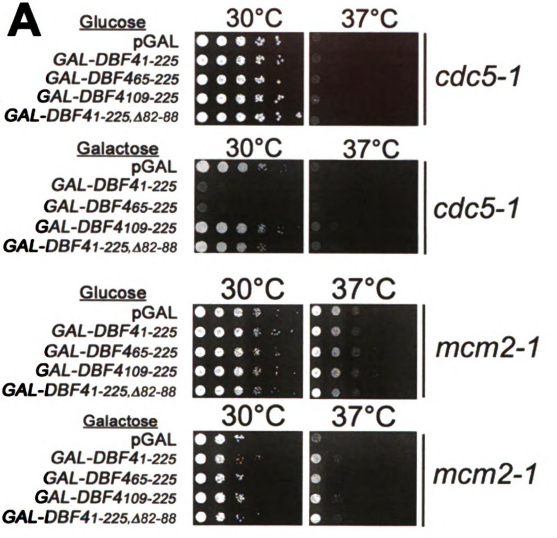
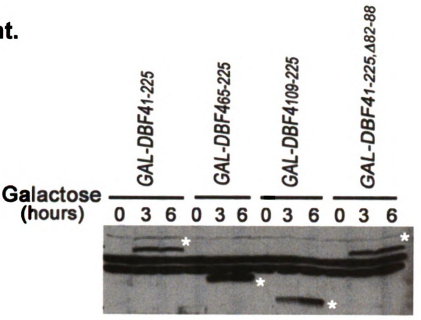


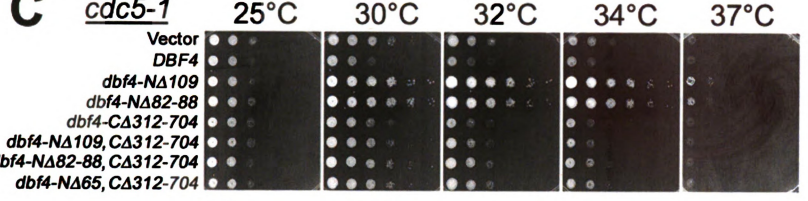
Figure 11. Dbf4p is a Polo inhibitor. (A) *cdc5-1* and *mcm2-1* strains expressing the indicated Dbf4 N-terminal regions from the *GAL1* promoter were plated onto glucose (repressing conditions) or galactose to induce Dbf4p expression. (B) Dbf4p Western blot of total cell extracts induced for *GAL1-DBF4* expression at 0, 3, and 6 hours. Asterisks indicate N-terminal Dbf4 peptides. (C) High copy vectors expressing wild type *DBF4* and mutant genes were transformed into *cdc5-1* (M1614) and spotted at 10-fold serial dilutions at the indicated temperatures.


A
Glucose
30°C
37°C
pGAL
GAL-DBF41-225
GAL-DBF465-225
GAL-DBF4109-225
GAL-DBF41-225,Δ82-88
cdc5-1
Galactose
30°C
37°C
pGAL
GAL-DBF41-225
GAL-DBF465-225
GAL-DBF4109-225
GAL-DBF41-225,Δ82-88
cdc5-1
Glucose
30°C
37°C
pGAL
GAL-DBF41-225
GAL-DBF465-225
GAL-DBF4109-225
GAL-DBF41-225,Δ82-88
mcm2-1
Galactose
30°C
37°C
pGAL
GAL-DBF41-225
GAL-DBF465-225
GAL-DBF4109-225
GAL-DBF41-225,Δ82-88
mcm2-1

B **Figure 11 Cont.**



C *cdc5-1*



finding suggested that high copy expression of Dbf4-NΔ109p reduced the cellular concentration of wild type Cdc7p-Dbf4p, the likely Cdc5p inhibitor, which suppressed the *cdc5-1* ts allele (Fig 11C). High copy expression of Dbf4-Δ82-88p that does not interact with Polo also suppressed the *cdc5-1* ts (Fig 11C). Importantly, deleting Dbf4p C-terminal residues required for interaction with Cdc7p (CΔ312-704) eliminated the ts suppression by high copy *dbf4-NΔ109* and *dbf4-Δ82-88*. These data are consistent with full-length Cdc7p-Dbf4p kinase acting as the physiological Polo inhibitor.

Cdc7p-Dbf4p phosphorylates the PBD. Wild type Cdc7p-Dbf4p might inhibit Polo abundance or kinase activity during the cell cycle and thus explain our genetic data. However, we saw little difference in Polo protein levels, cell cycle expression or Polo kinase activity comparing wild type yeast with the *dbf4-NΔ109* mutant (Figure 12). This suggests that Dbf4p inhibits Polo independently of altering its expression or kinase activity. This is consistent with our genetic data since loss of the Dbf4p PIR suppresses the *cdc5-1* allele yet the Cdc5-1 protein retains considerable protein abundance and kinase activity at the non-permissive temperature [63]. The mitotic exit defect associated with *cdc5-1* is due to a single P511L amino acid substitution preceding polo-box 1 of the PBD [64], strongly suggesting that the *cdc5-1* growth defect is caused by a defect in substrate recognition. Since genetically *DBF4* is a negative *CDC5* regulator we hypothesized that Cdc7p-Dbf4p phosphorylates Polo to prevent its access to key substrates in the MEN. Consistent with this possibility, we found that purified

Figure 12. Cdc7p-Dbf4p does not alter Polo kinase abundance or activity. (A) WT (M1585) and *dbf4-NΔ109* (M1874) containing *CDC5-HA3* were arrested in G1, released into the cell cycle and blotted for Cdc5-HA3 protein. Budding index is shown. (B) *DBF4 CDC5-4XGFP SPC42-eqRFP* (M2750) and *dbf4-NΔ109 CDC5-4XGFP SPC42-eqRFP* (M2748) were arrested in G1 phase with mating pheromone and released into nocodazole. These were scored for Cdc5-4XGFP localization to the spindle pole body. (C) Strains expressing Cdc5-3HA protein were arrested at 30°C with alpha-factor (a) and released into YPD containing 0.2M hydroxyurea (HU) or 15μg/ml nocodazole (Noc) for 2 hours. Extracts were blotted for Cdc5 protein and Cdc5p kinase activity was measured following IP (D) Quantitation of kinase activity from three independent experiments.

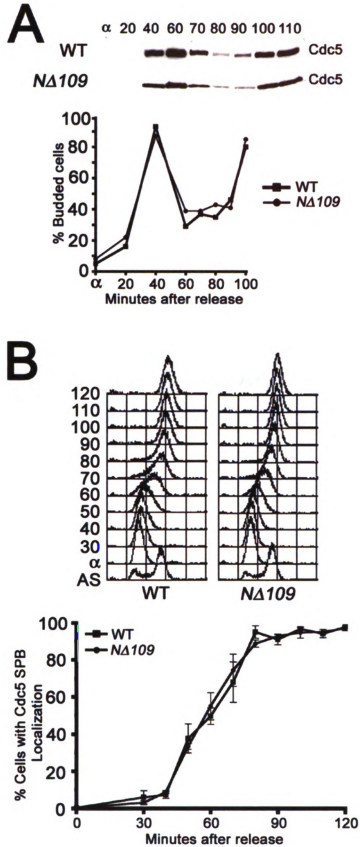
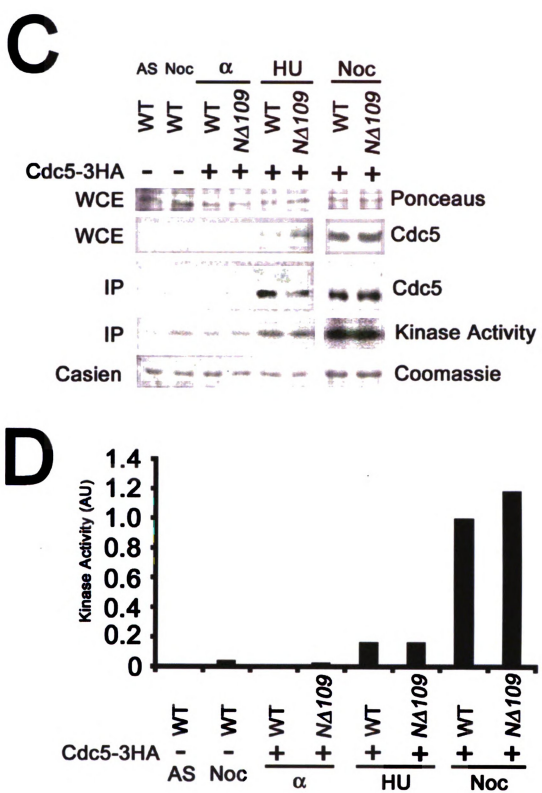

A
α 20 40 60 70 80 90 100 110
WT
Cdc5
NΔ109
Cdc5
% Budded cells
WT
NΔ109
α 20 40 60 80 100
Minutes after release
B
120
110
100
90
80
70
60
50
40
30
α
AS
WT
NΔ109
% Cells with Cdc5 SPB Localization
WT
NΔ109
0 30 60 90 120
Minutes after release

Figure 12 Cont.



Cdc7p-Dbf4p phosphorylated recombinant GST-PBD but not GST alone (Figure 8D). As a result of these findings we wanted to determine whether loss of the Dbf4-PIR interaction resulted in alterations in Cdc5 phosphorylation during the cell cycle. We examined Cdc5 for altered migration by PAGE in WT and *dbf4-NΔ109* cells during the cell cycle. We were able to observe two distinct Cdc5 isoforms, however no difference was observed between the two strains (Figure 13).

Dbf4p inhibits Cdc14p nucleolar release. The Cdc14p phosphatase is sequestered within the nucleolus during the cell cycle prior to FEAR and MEN pathway activation [50, 65]. Activation of the FEAR pathway allows limited Cdc14p nucleolar release, which promotes rDNA and telomere segregation during early anaphase [42, 44-46]. Cdc14p is then fully released by MEN activation and antagonizes Cdk activity to trigger exit from mitosis [66]. Since the *cdc5-1* mutant fails to release Cdc14p at the restrictive temperature [66], suppression of the *cdc5-1* ts by deletion of the Dbf4p PIR (Figure 14A) suggested that Cdc14p release is likely restored in these cells at higher temperatures. We quantitated the nucleolar release of Cdc14-EGFP in wild type, *dbf4-NΔ109*, *cdc5-1* and *cdc5-1 dbf4-NΔ109* cells at a restrictive temperature for *cdc5-1*. Cells were arrested in G1-phase at the permissive temperature and then released into the cell cycle at 34 degrees Celsius. The *dbf4-NΔ109* cells progressed through the cell cycle and released Cdc14p similarly to wild type cells (Figure 14A). Consistent with previous reports, the *cdc5-1* mutant failed to

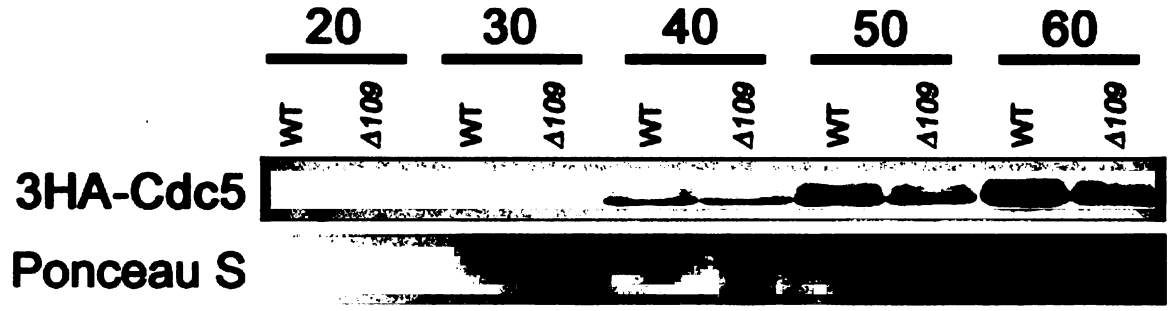


Figure 13. Dbf4 PIR deletion does not affect Cdc5 mobility or abundance. WT (M1585) and *dbf4-NΔ109* (M1874) containing *CDC5-HA3* were arrested in G1, released into the cell cycle and blotted for Cdc5-HA3 protein at the indicated time points (min after release).

Figure 14. *DBF4* inhibits Cdc14p nucleolar release and the MEN pathway. (A) Deletion of the Dbf4p PIR rescues Cdc14p nucleolar release at high temperature. M1992, M2005, M2139 and M2287 were arrested with alpha-factor at 25°C and released into YPD at 34°C followed by alpha-factor re-addition at 60 minutes. Entry and exit from the cell cycle was analyzed as a percentage of budded cells. Cdc14-EGFP release was analyzed in at least 100 cells at each time point and is reported as a percentage of cells with diffuse Cdc14p-EGFP nuclear staining (absence of nucleolar Cdc14p). (B) *dbf4-NΔ109* rescues the growth defect of the *cdc5-1* and *dbf2-1* strains at 35°C. Single and double mutant strains were spotted at 10-fold dilutions on YPD and incubated at increasing temperatures for 2 to 3 days.

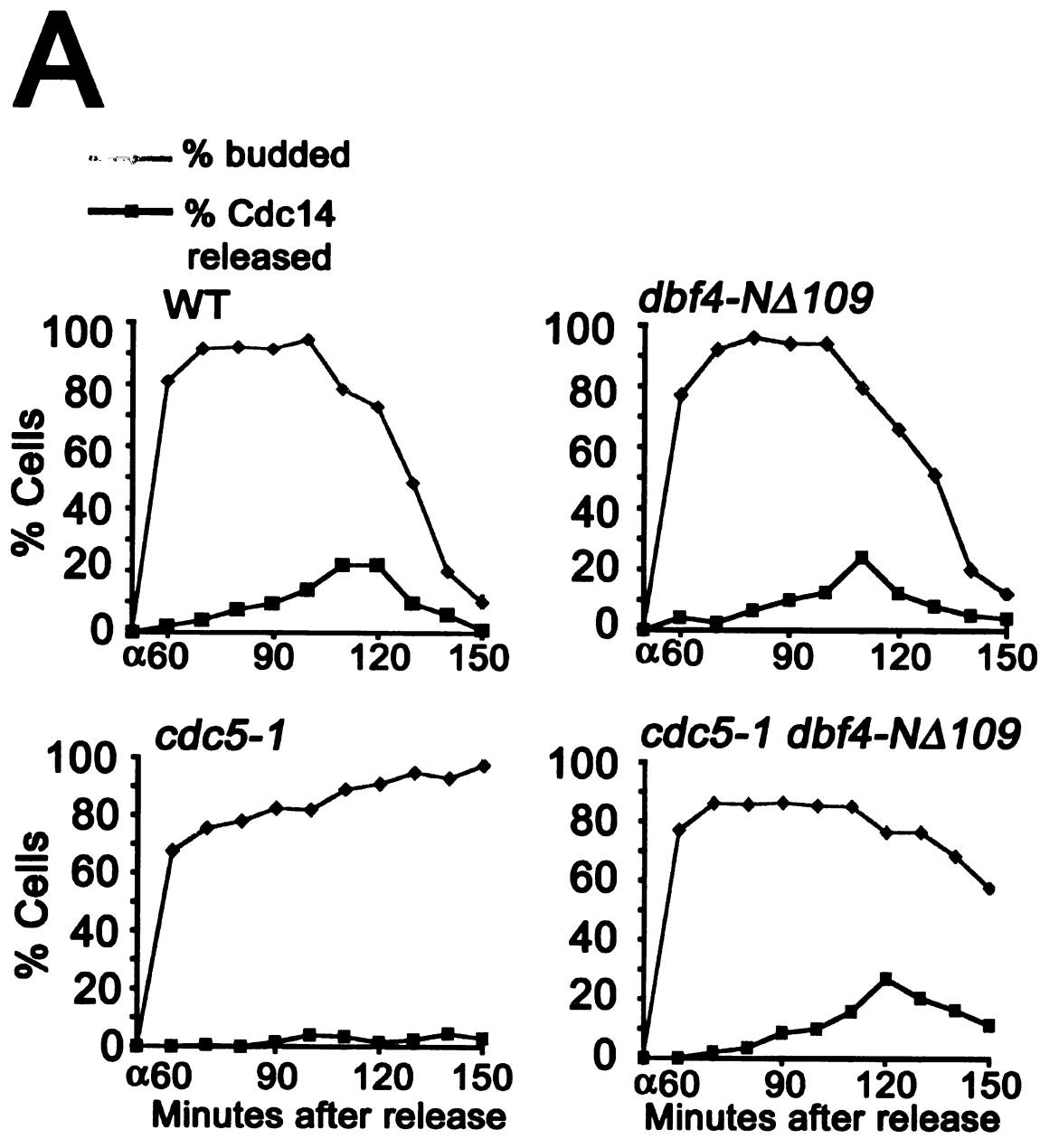
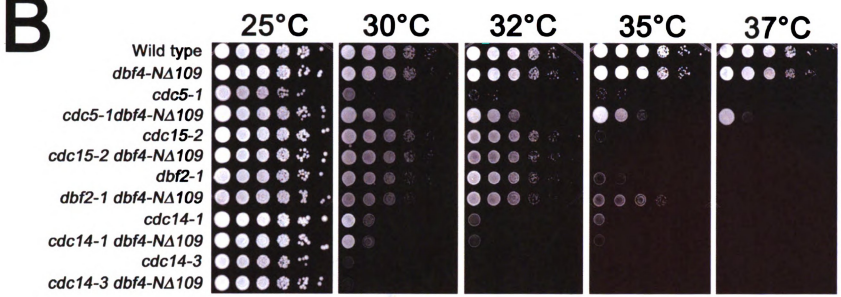

A
% budded
% Cdc14 released
WT
dbf4-NΔ109
cdc5-1
cdc5-1 dbf4-NΔ109
% Cells
100
80
60
40
20
0
α60
90
120
150
Minutes after release

Figure 14 Cont.



release Cdc14p from the nucleolus but a significant amount of Cdc14p was released from the nucleolus in the *cdc5-1 dbf4-NΔ109* mutant (Figure 14A). We noticed a delay in mitotic progression at 34 degrees Celsius in the *cdc5-1 dbf4-NΔ109* cells evidenced by a somewhat longer duration of Cdc14p release compared to the wild type and delayed cytokinesis (indicated by the delayed appearance of unbudded (G1) cells). Cdc14 release represents the final step in activating mitotic exit - as a result its activity is tightly controlled throughout the cell cycle and uncoupling this control adversely affects cell viability. Collectively, these data indicate that Dbf4p inhibits Polo activity to prevent Cdc14p release, which might be significant during a slowed S-phase or during periods of replication stress.

Dbf4p also inhibits the MEN kinase Dbf2p. Inactivation of Bfa1p-Bub2p is required to activate the MEN [37]. This signaling cascade is partially activated as a result of Bfa1p phosphorylation by Polo, which leads to activation of the Cdc15p and Dbf2p-Mob1p kinases (reviewed by [47]). Dbf2p kinase activation requires a Bub2p-Bfa1p independent function of Polo as well [49], indicating that Polo either directly promotes Dbf2p kinase activity or promotes a MEN-independent pathway that activates Dbf2p. We tested whether Dbf4p functions as a negative regulator of MEN activation by examining whether deletion of the Dbf4p PIR could suppress growth defects associated with additional ts mutants in the MEN (Figure 14B). We examined the growth of double mutants of *cdc5-1*, *cdc15-2*, *dbf2-1*, *cdc14-1*, or *cdc14-3* with *dbf4-Δ109*. As with *cdc5-1*, loss of the

DBF4 PIR rescued the growth of *dbf2-1* cells at the non-permissive temperature. In contrast, the *dbf4-Δ109* mutant failed to suppress the ts phenotype of *cdc15-2*, *cdc15-4* (not shown), *cdc14-1*, or *cdc14-3* mutants (Figure 14B). This suggests that Dbf4p may specifically inhibit Polo activation of Dbf2p and not Polo inactivation of Bub2p-Bfa1p GAP activity. Taken together, our observations suggest that Dbf4p antagonizes Polo activation of some Bfa1p-Bub2p independent step in MEN activation. This interpretation is further supported by the following experiments.

Dbf4p regulates a Bfa1p-Bub2p independent Polo activity during mitotic exit. Deletion of *BUB2* (or *BFA1*) or *CDC55* is sufficient to cause premature mitotic exit when cells are arrested in metaphase with the spindle poison nocodazole [67, 68]. This causes large budded cells to exit mitosis and rebud in the absence of chromosome segregation or cytokinesis. Since Dbf4p is a negative regulator of Polo activity, we examined whether deletion of the Dbf4p PIR was sufficient to induce rebudding in the presence of spindle poisons. Cells were arrested in G1, released into media containing nocodazole and quantitated for rebudding (Figure 15A; Figure 16). In contrast to *bub2Δ and cdc55Δ*, *dbf4-NΔ109* did not allow rebudding in a wild type background nor did this mutation advance rebudding in either background. This indicated that loss of the Dbf4p Polo interaction is not sufficient alone, or in combination with other Cdc14 regulators, to cause mitotic exit during metaphase and suggested that Dbf4p Polo inhibition may act independently of Bub2p-Bfa1p.

Null alleles of *CDC5* fail to activate the MEN and arrest in telophase with unphosphorylated Bfa1 [37]. Although the *cdc5-1* mutant is also defective in MEN activation it is proficient for Bfa1p phosphorylation and retains substantial Polo kinase activity at the non-permissive temperature (NPT) [37, 63]. This suggests that the *cdc5-1* mutant is defective in activating a Bfa1p-independent function of the MEN, perhaps in FEAR pathway activation or activating a downstream MEN target. In contrast to *cdc5-1*, *cdc5-2* cells neither phosphorylate Bfa1p nor activate the MEN at the NPT [37]. The *cdc5-2* temperature sensitivity is partially rescued by deletion of either *BFA1* or *BUB2* [37] but not by *dbf4-NΔ109* (Figure 15B). However, in a *bub2Δ* the *cdc5-2* temperature sensitivity was further suppressed by deletion of the Dbf4p PIR. So, eliminating the requirement for Bfa1p-Bub2p inactivation in *cdc5-2* cells (i.e. *cdc5-2 bub2Δ*), allowed *dbf4-NΔ109* to further suppress the *cdc5-2* ts and promote mitotic exit at 34 degrees Celsius (Figure 15B). These data are consistent with the interpretation that Dbf4p primarily inhibits Polo activation of a Bub2p-Bfa1p *independent* step in MEN activation, e.g. Dbf2p activity, Cdc14p release in the FEAR pathway, or some unknown activity.

***DBF4* prevents mitotic exit when the nucleus is mispositioned.** In the budding yeast, *KAR9* and *DYN1* encode cytoplasmic microtubule-associated and motor proteins, respectively, that operate in two redundant pathways essential for the correct positioning of the nucleus during anaphase (reviewed by [7]). Deletion of either gene results in a small percentage of cells with nuclear orientation

Figur
Deet
W3C3
releas
Samp
cells
suppr
scatte
Two

Figure 15. *DBF4* regulates mitotic exit independently of *BFA1-BUB2*. (A) Deletion of the *DBF4* polo-box interacting region does not cause rebudding. W303-1A, M1652, M1656 and M1860 were synchronized with alpha-factor and released into YPD containing 15ug/ml of nocodazole at 30 degrees Celsius. Samples were quantitated for the percentage of rebudded cells (large budded cells with a new bud). (B) Deletion of *BUB2* and the *DBF4* PIR cooperate to suppress the growth defect of *cdc5-2*. Strains of the indicated genotypes were spotted at 5-fold serial dilutions on YPD and grown at increasing temperature. Two isolates are shown for the *cdc5-2* recombinants.

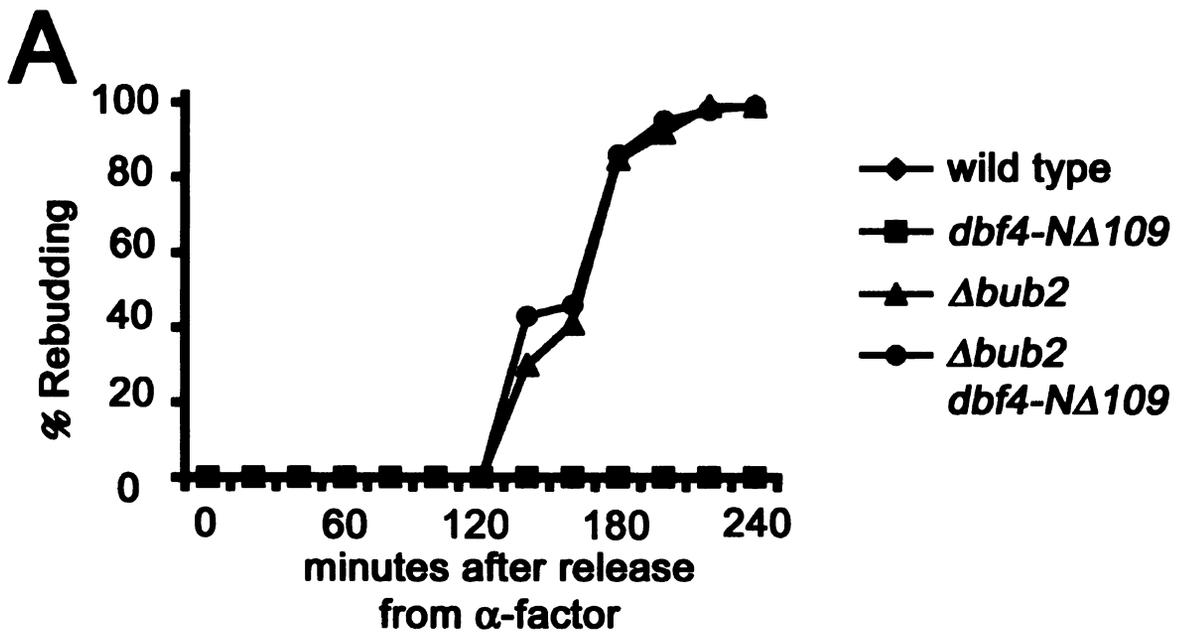
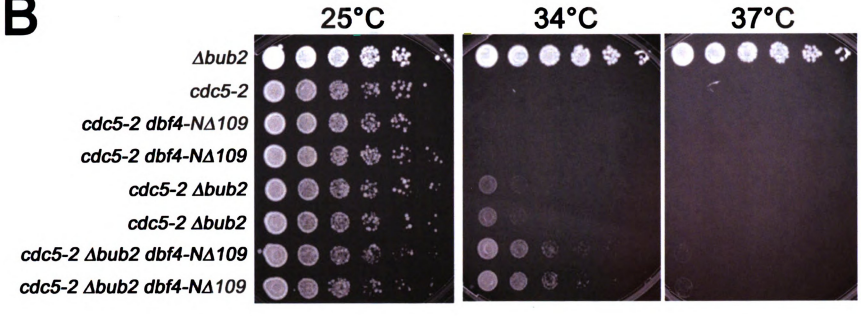

A
100
80
60
40
20
0
% Rebudding
0
60
120
180
240
minutes after release
from α-factor
wild type
dbf4-NΔ109
Δbub2
Δbub2
dbf4-NΔ109

Figure 15 Cont.



defects but deletion of both genes is lethal. In response to nuclear misorientation, *S. cerevisiae* inhibits premature activation of the MEN via the spindle position checkpoint (SPOC). When the SPOC is activated the Kin4p kinase localizes to spindle pole bodies (SPB) to counteract Polo Bfa1p phosphorylation [8, 9, 55]. Kin4p thus counteracts Polo inactivation of the Bfap1-Bub2p GAP and this inhibits MEN activation. Failure to adequately respond to nuclear mispositioning allows inappropriate nuclear division within the mother cell, leading to anueploidy and loss of viability. Given that Polo Bfa1p phosphorylation is prevented when the SPOC is activated, we hypothesized Dbf4p may also inhibit Polo to prevent mitotic exit in response to nuclear misorientation.

To examine whether Dbf4 regulated cell growth in dyn1 and kar9 mutants in the presence of spindle poisons, strains were spotted at serial dilutions on media containing the spindle poison benomyl. We analyzed wild type, *dbf4-NΔ109*, *kar9Δ*, *kar9Δ dbf4-NΔ109, dyn1Δ* and dyn1*Δ dbf4-NΔ109* for growth. Deletion of KAR9 or Dyn1 alone negatively affected growth on benomyl as compared to wild type; interestingly, PIR deletion further decreased cell viability in both genetic backgrounds suggesting a role for Dbf4 in regulating cell viability in the presence of a perturbed spindle (Figure 17).

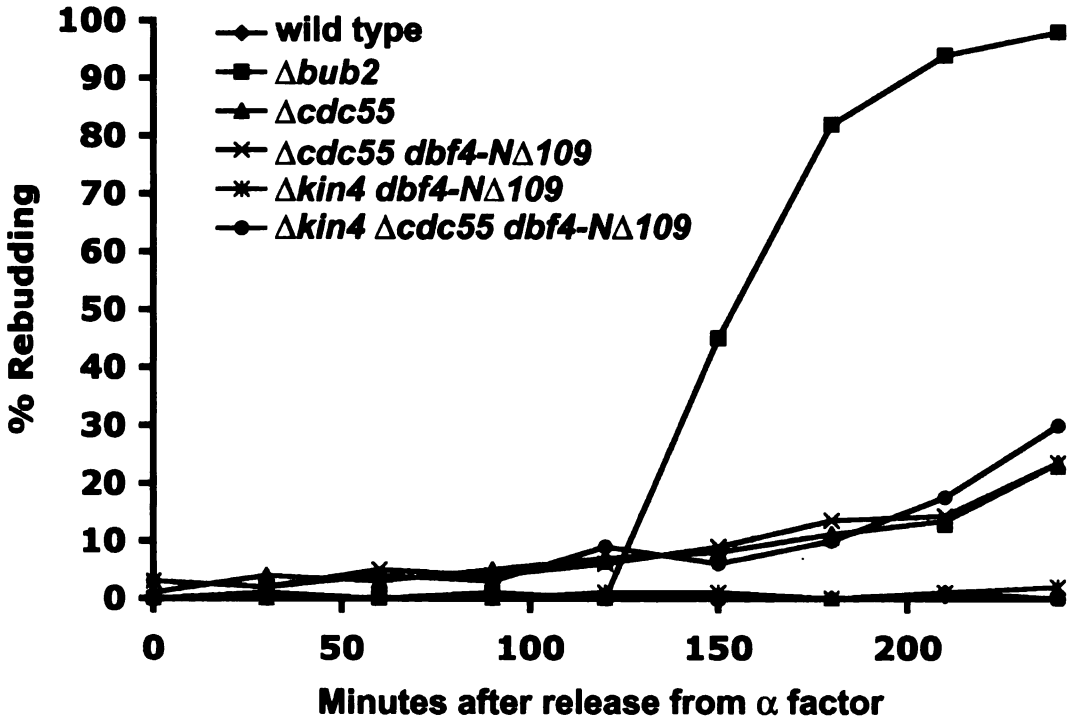


Figure 16. Deletion of the Dbf4 PIR does not affect rebudding in the presence of spindle poison. Wild type, *bub2Δ, cdc55Δ, cdc55Δ dbf4-NΔ109, kin4Δ dbf4-NΔ109 and kin4Δ cdc55Δ dbf4-NΔ109* cells were synchronized with alpha-factor and released into YPD containing 15ug/ml of nocodazole at 30 degrees Celsius. Samples were quantitated for the percentage of rebudded cells (large budded cells with a new bud).

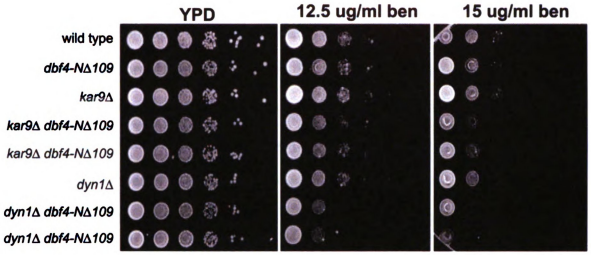


Figure 17. PIR deletion decreases cell viability in the presence of spindle poisons. Strains were spotted at 10-fold serial dilutions on YPD with and without the spindle poison benomyl. Cells were grown at 30 degrees Celsius for 3 days.

To test more specifically whether *DBF4* inhibited mitotic exit when nuclei were mispositioned, we examined wild type, *dbf4-NΔ109*, *kar9Δ* and *kar9Δ dbf4-NΔ109* strains for evidence of mitotic exit in the presence of mispositioned nuclei. Asynchronous cultures were grown at 25 degrees Celsius and shifted to 30 degrees Celsius for 4 or 24 hours prior to analysis to increase the penetrance of the nuclear mispositioning phenotype. Although wild type and *dbf4* mutant cells did not misorient their nuclei (Figure 18), both *kar9Δ* and *kar9Δ dbf4-NΔ109* strains had approximately equal number of cells with nuclear positioning defects. Importantly, deletion of the Dbf4p PIR resulted in a 2 to 3-fold increase (to 6%) in binucleate, anucleate and multinucleate cells at 4 hours (Figure 18A, C, D), which was *CDC5*-dependent, suggesting that premature mitotic exit occurred. At 24 hours the number of aberrant chromosome segregation events due to loss of the Dbf4p PIR increased six-fold (12%) relative to *kar9Δ* alone (2%). Since exit from mitosis causes spindle disassembly, we quantitated the spindle morphology in cells containing correctly segregated nuclei (between the mother and daughter cells) and in those cells where anaphase had initiated solely within the mother cell. Although *kar9Δ* and *kar9Δ dbf4–NΔ109* cells had similar frequencies of intact or disassembled spindles when nuclear division proceeded normally, *kar9Δ dbf4-NΔ109* cells showed a three-fold increase in spindle disassembly within the mother cell (leading to a bi-nucleate mother cell) compared to *kar9Δ* single mutants at 4 hours (Figure 18D). These data indicate that mitotic exit occurs in these cells in the absence of the Dbf4p PIR.

Figure 18. Deletion of the *DBF4* PIR allows exit from mitosis in cells with misoriented nuclei. (A, B) Representative cells showing nuclear positioning and mitotic arrest defects. Nuclear position was analyzed after DAPI staining. Cells with anaphase spindles located exclusively in the mother cell body are noted (white arrow). Anucleate (*), binucleate (**) and multinucleate (***) cells were frequently observed in *kar9Δ dbf4-NΔ109* (A) and *dyn1Δ dbf4-NΔ109* (B) cells. (C, E) Quantitation of nuclear segregation defects. >300 cells were counted for each strain and nuclear position was represented as a percentage of the total cells in culture. Black bars represent cells with nuclei segregated between mother and daughter cells; gray bars, divided nuclei in the mother cell; white bars, multinucleate and anucleate cells. (D, F) Spindle morphology of strains with misoriented nuclei and spindles. >50 cells were counted for each genotype. Black bars represent cells with mitotic spindles. Gray bars represent cells that have exited mitosis.

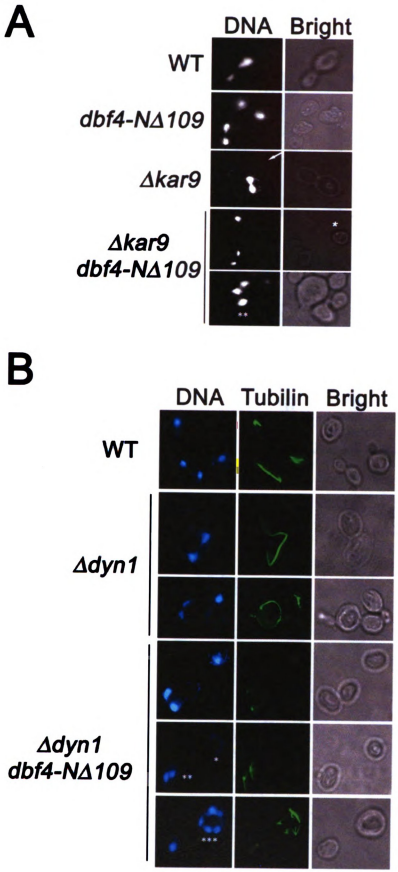
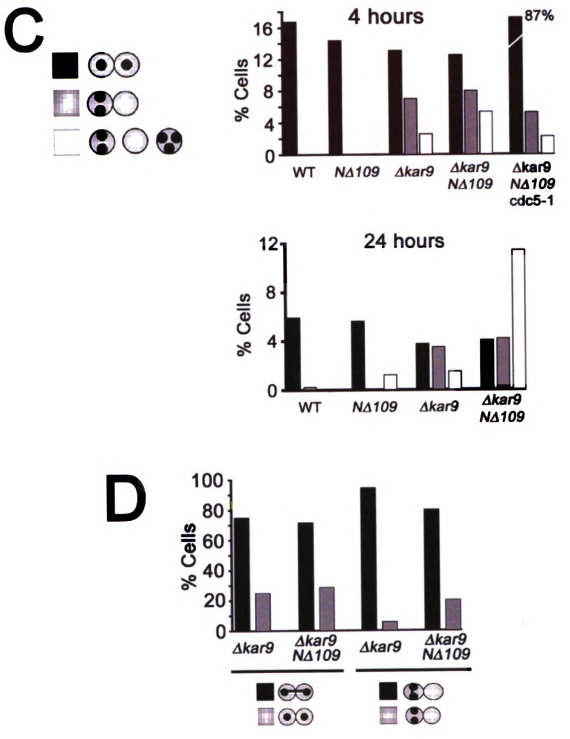
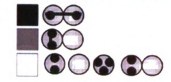
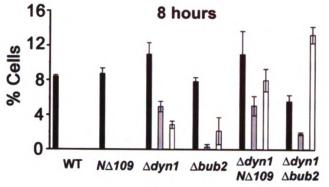

A
DNA
Bright
WT
dbf4-NΔ109
Δkar9
Δkar9
dbf4-NΔ109
B
DNA
Tubilin
Bright
WT
Δdyn1
Δdyn1
dbf4-NΔ109

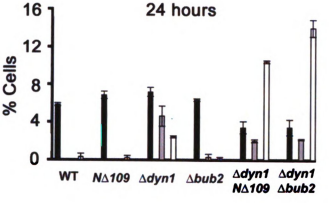
Figure 18 Cont.



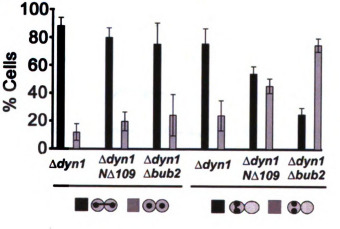
E **Figure 18 Cont.**







F



Similarly, we tested whether deletion of the Dbf4p PIR allowed premature mitotic exit in cells disrupted in the dynein pathway (Figure 18B, E, F). The spindle-positioning defect associated with deletion of *DYN1* is especially prominent at low temperatures. Although after 8 hours at 14 degrees Celsius only ~3% of *dyn1Δ* cells had exited mitosis inappropriately, deletion of the Dbf4p PIR resulted in a 3- to 4-fold increase in mitotic exit as evidence by the appearance of multinucleate and anucleate cells (Figure 18E) and this frequency was increased at 24 hours. For comparison, a *dyn1Δ bub2Δ* strain had a similar but higher frequency of segregation defects (Figure 18E, F). Therefore, the bypass of the SPOC following loss of the Dbf4p-Polo interaction is comparable to deletion of the MEN inhibitor *BUB2*. As with the *kar9Δ* mutant, we observed that a higher percentage of *dyn1Δ dbf4-NΔ109* cells compared to *dyn1Δ* single mutants that divided nuclei within the mother cell had disassembled their spindles (Figure 18F). These observations indicate that deletion of the Dbf4p N-terminus (including the PIR) overrides the mitotic arrest normally activated by the SPOC.

DISCUSSION:

We found that Dbf4p inhibited Polo kinase during mitosis very likely through a direct interaction with the polo-box domain. This interaction inhibited MEN pathway activation, nucleolar release of the Cdc14p phosphatase and was likely critical to maintain genome integrity during activation of the spindle position checkpoint. These results therefore have important implications for

understanding Dbf4p function and the regulation of mitotic progression in eukaryotic cells.

Defining the interaction between Dbf4p and Polo. The N-terminal third of Dbf4p encodes multiple functions: a destruction box (residues 62-70), two putative nuclear localization signals (residues 55-61, 251-257) and a BRCT-like domain (residues ~117-220). Nonetheless the 265 N-terminal amino acids of Dbf4p are not essential as long as a nuclear localization signal is present [26]. Deletion of the Dbf4p N-terminus through the PIR has no observable effect on growth, viability, or cell cycle progression either under normal growth conditions or in the presence of replication or spindle poisons ([26] and data not shown). Here, we discovered an interaction between Dbf4p and the Polo PBD that mapped to a short sequence of ~40 amino acids preceding the BRCT-like domain. Although a two-hybrid interaction between Polo and Dbf4p was reported before [39], the significance of this interaction was not determined.

The Polo PBD functions as a module for binding phosphorylated proteins and thereby targets Polo to its cellular substrates [57, 59]. The question naturally arises as to whether phosphorylation of the Dbf4p PIR is required for PBD binding. Currently, our observations suggest that phosphorylation is not required. A polo-box binding consensus sequence (S(pS/pT)P/X) is not present within this region of Dbf4p [59] and mutation of all putative serine and threonine residues within the Dbf4p PIR did not significantly diminish the interaction in the two-hybrid assay. These data suggest that phosphorylation of Dbf4p is not crucial for Polo

binding. Our observation that the Dbf4p PIR purified from *E. coli* directly interacted with the Polo PBD also supports this notion. Thus phosphorylation of Dbf4p was not critical for binding to Polo *in vitro*, but we cannot exclude the possibility that phosphorylation contributes to Dbf4p-Polo binding *in vivo* when the two proteins are present at physiological concentrations.

When does Dbf4p inhibit Polo and mitotic exit? The finding that deletion of the Dbf4p PIR significantly suppressed the ts phenotype of *cdc5-1* suggests that Cdc7p-Dbf4p inhibits Polo during the normal cell cycle and perhaps during periods of replication stress, when Cdc7p-Dbf4p is stabilized [17]. Our data clearly demonstrate a role for Dbf4p in inhibiting mitotic exit, since loss of the Dbf4p PIR suppressed both *cdc5* and *dbf2* ts mutants and allowed sustained Cdc14p phosphatase release and cytokinesis in the *cdc5-1* mutant at the NPT. However, during an unperturbed cell cycle the absence of this regulation had little impact. This is likely attributable to the fact that the cell cycle regulation of Polo activity is complex and modulated by multiple cell cycle checkpoints. Since *dbf4-NΔ109 bub2Δ* double mutants were more sensitive to growth on spindle poisons than either mutant alone (Figure 19), the Dbf4p-Polo and Bfa1p-Bub2p pathways may work together to suppress premature activation of the mitotic exit network.

It was shown very recently that increased expression of a non-destructible form of Dbf4p (Dbf4-NΔ65p) could delay rDNA segregation when Clb5p was also stabilized [16]. This raises the possibility that Dbf4p inhibits Cdc14p release via

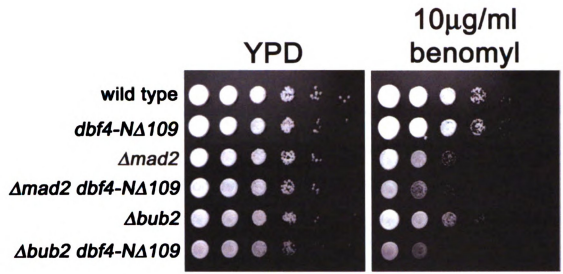


Figure 19. Deletion of the Dbf4p PIR causes decreased growth in the presence of spindle poisons. Indicated strains were spotted at 10-fold serial dilutions on YPD or YPD containing 10μg/ml benomyl.

the FEAR pathway under some circumstances and is consistent with our data showing that Dbf4p is a Polo inhibitor. However, we found no evidence that the *dbf4-NΔ109* allele promoted premature rDNA segregation (a FEAR pathway event) in the *cdc5-1* mutant (Figure 20A). Similarly, we found no evidence that *dbf4-NΔ109* caused premature Cdc14p release in a strain deleted for *FOB1*, which is thought help sequester Cdc14p in the nucleolus (Figure 20B). These data suggest that Dbf4p is not specifically inhibiting the FEAR pathway.

The budding yeast SPOC prevents premature exit from mitosis in part by inducing Kin4p phosphorylation of Bfa1p, which antagonizes the Polo-dependent inhibition of the Bfa1p-Bub2p GAP [8, 9, 55]. Premature exit from mitosis in *dbf4-NΔ109 dyn1* and *dbf4-NΔ109 kar9* double mutants suggests that Dbf4p regulation of Polo is critical for robust cell cycle control in response to nuclear mispositioning. What remains unclear however, is whether this Dbf4p activity is regulated following activation of the SPOC. For instance, Cdc7p-Dbf4p may inhibit Polo to buffer against premature release of Cdc14p during late S-phase or early M-phase whether or not the SPOC is activated. Therefore, in the absence of the Dbf4p-Polo regulation Polo may prematurely activate the MEN before a nuclear orientation defect is sensed by the SPOC. Following APC-Cdc20p and APC-Cdh1p activation during anaphase onset and exit, we suggest that degradation of Dbf4p provides a positive feedback loop for full Polo activation of the MEN and ultimately, cytokinesis.

Figure 20. (A) Deletion of the Dbf4p PIR does not enhance nucleolar segregation in *cdc5-1* cells. *DBF4*, *dbf4-NΔ109, cdc5-1* and *cdc5-1 dbf4-NΔ109* cells transformed with a centromeric EGFP-Nop1 plasmid were arrested in G1 with mating pheromone and released into the cell cycle in YPD at 34 degrees Celsius. Alpha-factor was added back after budding to permit a single cell cycle. Samples were taken at the indicated time points and scored for the presence of one or two GFP signals by florescence microscopy. (B) Deletion of the Dbf4p PIR does not enhance Cdc14 release when combined with deletion of *FOB1*. *fob1Δ CDC14-EGFP* (M3149) and *fob1Δ dbf4-NΔ109 CDC14-EGFP* (M3148) were arrested in G1 with mating pheromone and released into the cell cycle at 30 degrees Celsius. Alpha-factor was added back after budding to follow a single cell cycle. Samples were taken at the indicated time points and scored for release of Cdc14 from the nucleolus.

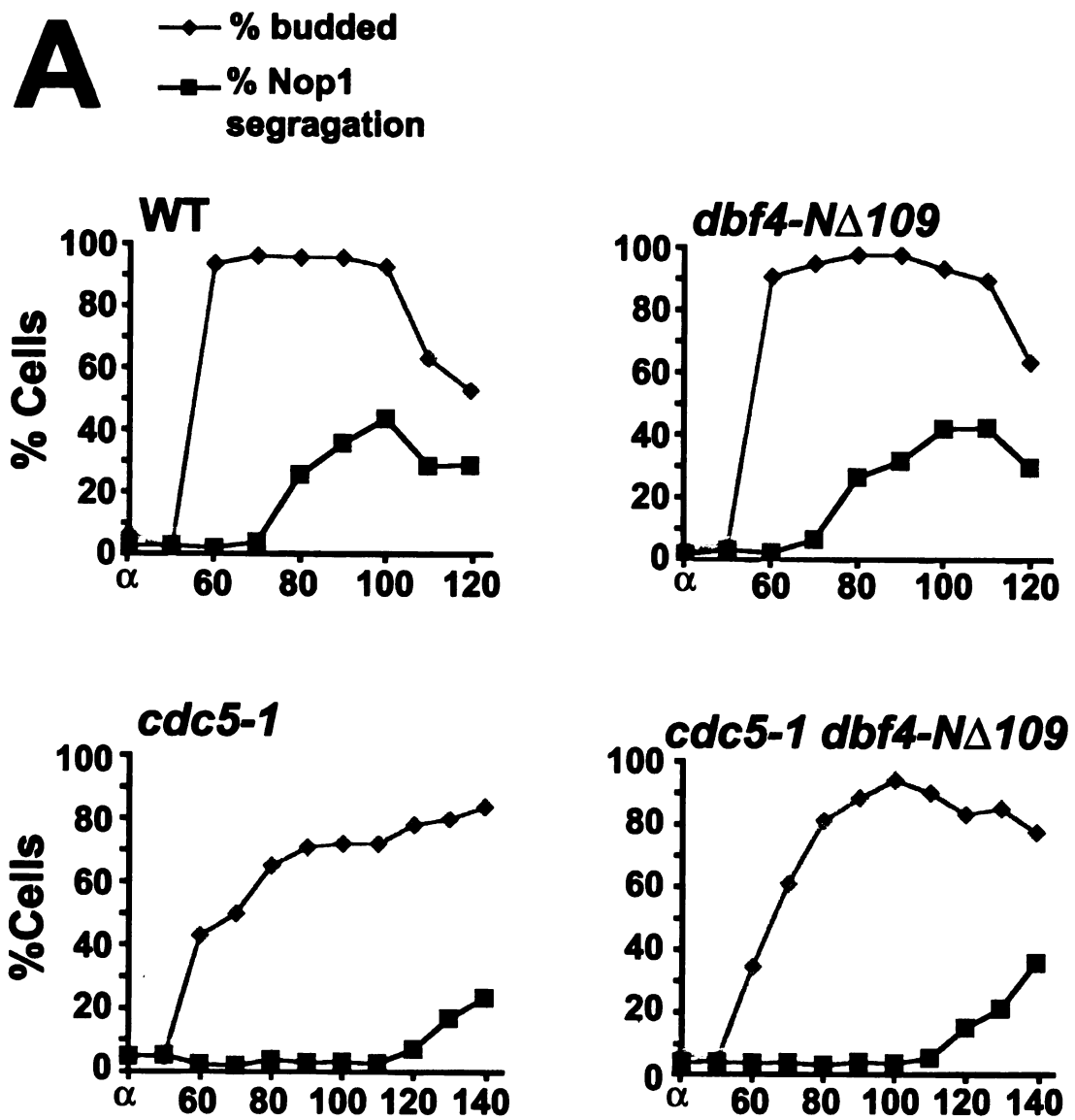
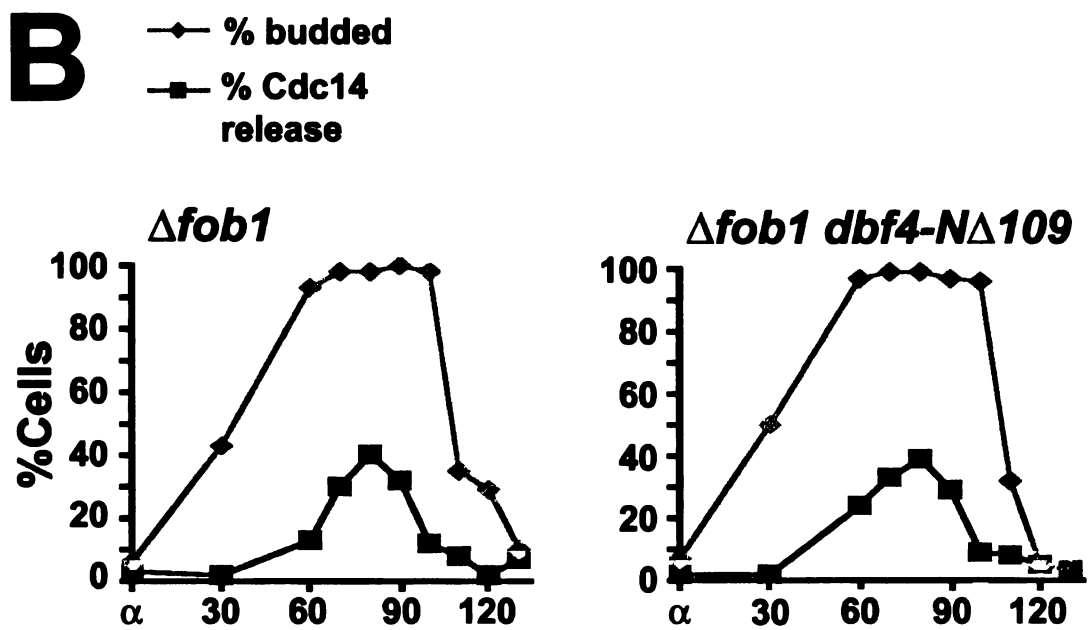

A
% budded
% Nop1 segragation
WT
dbf4-NΔ109
cdc5-1
cdc5-1 dbf4-NΔ109
% Cells
%Cells
100
80
60
40
20
0
α
60
80
100
120
140

Figure 20 Cont.



How does Dbf4p inhibit Polo and mitotic exit? Dbf4p did not influence Polo protein levels or overall kinase activity. Dbf4p nonetheless inhibited Polo activity since *dbf4* mutants unable to interact with Polo significantly suppressed the *cdc5-1* temperature sensitivity. Since the *cdc5-1* allele retains significant Polo protein expression, Bfa1p phosphorylation and overall Polo kinase activity at the non-permissive temperature [37, 63], the primary *cdc5-1* MEN defect is in a Bfa1p-independent requirement for MEN pathway activation, perhaps Dbf2p activation or some other MEN-dependent step. Our observation that the *dbf2-1* temperature sensitivity was also suppressed by loss of the Dbf4p PIR suggests that Dbf4p may specifically inhibit Polo activation of Dbf2p kinase independently of Bfa1p-Bub2p phosphorylation. Dbf2p was recently shown to promote cytoplasmic Cdc14p localization following MEN activation [69]. In addition, deletion of both *BUB2* and the *DBF4* PIR suppressed the *cdc5-2* ts better than either single mutant alone. In other words, since *dbf4-NΔ109* further suppressed the ts phenotype of a *cdc5-2 bub2Δ* strain, this supports the contention that Dbf4p regulates MEN activity independently of Bfa1p-Bub2p. Deletion of the Dbf4p PIR also did not allow rebudding in the presence of spindle poisons as seen in *bub2Δ* mutants, again suggesting that Dbf4p plays a minor or redundant role to inhibit Polo phosphorylation of Bfa1p-Bub2p.

Cdc7p-Dbf4p kinase phosphorylated the Polo PBD *in vitro* suggesting that Cdc7p-Dbf4p may antagonize Polo substrate binding. This possibility is consistent with the requirement for the PBD for targeting Polo to sites of MEN activity. Polo, Cdc15p, Dbf2p and Bfa1p-Bub2p are localized to SPB prior to

activation of the mitotic exit network [47]. Thus, we favor a model whereby Cdc7p-Dbf4p kinase inhibits precocious Polo binding to critical MEN substrate(s) by phosphorylating the Polo PBD. It will be interesting to investigate whether the Cdc7p-Dbf4p inhibition of Polo is regulated by cell cycle checkpoints and to determine the precise activity of Polo that is affected.

MATERIALS AND METHODS:

Images in this dissertation are presented in color.

Construction of Yeast Strains, Plasmids and Baculoviruses

Strains and plasmids used in this study are listed in Tables 1 and 2. PJ69-4a cells (*MAT***a** *trp1-901 leu2-3,-112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*) were used for two-hybrid experiments. All other strains were derivatives of W303 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3, -112 his3-11, -15 ura3-1*). Construction of Dbf4p N-terminal truncation mutants was previously described [26]. Cdc14-EGFP was constructed as described [70]. *bub2Δ* strains were created by replacement of the *BUB2* ORF via homologous recombination with *Sac*I-*Cla*I *bub2Δ::URA3* fragment from pTR24 (A. Hoyt). The *BAR1* ORF was deleted by homologous recombination with linearized pZV77 containing *bar1Δ::LEU2* (B. Futcher). Construction of *KAR9* and *DYN1* deletions were previously described [8].

For yeast two-hybrid analyses, a Gal4 DNA binding domain (GBD) fusion to Dbf4p 67-227 was constructed by PCR amplification of Dbf4p residues 67-227 (*Nco*I-*Pst*I) and cloned into pGBKT7 (Clonetech). Deletion of 71bp within the

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
W303-1A	*MATa ade2-1, ura3-1 his3-11, -15 trp1-1 leu2-3, -112 can1-100*	R. Rothstein
PJ69-4A	*MATa trp1-901 leu2-3, -112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	E.A. Craig
RHC15	W303 *MATa mad2Δ::URA3*	A. Murray
M1261	W303 *MATa dbf4-NΔ109*	C. Gabrielse
M319	W303 *MATa dbf2-1*	This study
M331	W303 *MATa cdc15-2*	"
M358	W303 *MATa mcm2-1*	"
M565	W303 *MATa cdc14-1*	"
M609	W303 *MATa cdc14-3*	"
M1585	W303 *MATa CDC5-HA-URA3*	
M1614	W303 *MATa cdc5-1*	"
M1652	W303 *MATa bub2Δ::URA3*	"
M1656	W303 *MATa dbf4-NΔ109-kanMX6*	"
M1804	W303 *MATa dbf4-NΔ109 cdc5-1*	"
M1860	W303 *MATa bub2Δ::URA3 dbf4-NΔ109-kanMX6*	"
M1864	W303 *MATa mad2Δ::URA3 dbf4-NΔ109-kanMX6*	"

Table 1 Cont.

M1866	W303 *MATa dbf4-NΔ109-kanMX6 cdc14-1*	"
M1868	W303 *MATa dbf4-NΔ109-kanMX6 cdc14-3*	"
M1870	W303 *MATa dbf4-NΔ109-kanMX6 dbf2-1*	"
M1872	W303 *MATa dbf4-NΔ109-kanMX6 cdc15-2*	"
M1918	W303 *MATa kar9Δ::klTrp1*	"
M1992	W303 *MATa CDC14-EGFP-kanMX6*	"
M2005	W303 *MATa CDC14-EGFP-kanMX6 dbf4-NΔ109-kanMX6*	"
M2007	W303 *MATα dbf4-NΔ65-kanMX6*	"
M2137	W303 *MATa CDC14-EGFP-kanMX6 cdc5-1*	"
M2139	W303 *MATa CDC14-EGFP-kanMX6 dbf4-NΔ109-kanMX6 cdc5-1*	"
M2179	W303 *MATa kar9Δ::klTrp1 dbf4-NΔ109-kanMX6*	"
M2180	W303 *MATα kar9Δ::klTrp1 dbf4-NΔ109-kanMX6*	"
M2269	W303 *MATa dyn1Δ::klTrp1*	"
M2280	W303 *MATa kin4Δ::his5 dbf4-NΔ109-kanMX6*	"
M2283	W303 *MATa bub2Δ::HIS3 dbf4-NΔ109-kanMX6 cdc5-2::URA3*	"
M2285	W303 *MATα bub2Δ::HIS3 dbf4-NΔ109-kanMX6 cdc5-2::URA3*	"

Table 1 Cont.

M2287	W303 *MATa cdc5-1 CDC14-EGFP-kanMX6*	"
M2289	W303 *MATa bub2Δ::HIS3 cdc5-2::URA3*	"
M2291	W303 *MATα bub2Δ::HIS3 cdc5-2::URA3*	"
M2293	W303 *MATa dyn1Δ::klTrp1 dbf4-NΔ109-kanMX6*	"
M2295	W303 *MATα dyn1Δ::klTrp1 dbf4-NΔ109-kanMX6*	"
M2357	W303 *MATa CDC5-3HA-URA3 bar1Δ::LEU2*	"
M2359	W303 *MATa CDC5-3HA-URA3 dbf4-NΔ109-kanMX6 bar1Δ::LEU2*	"
M2712	W303 *MATa kar9Δ::klTrp1 dbf4-NΔ109-kanMX6 cdc5-1*	"
M2728	W303 *MATa dyn1Δ::klTrp1 bub2Δ::HIS3*	"
M2741	W303 *MATa CDC5-15MYC 3HA-CDC7*	"
M2743	W303 *MATa CDC5-15MYC 3HA-CDC7 dbf4-NΔ109-kanMX6*	"
M2748	W303 *MATa CDC5-4xGFP-klTrp1 SPC42-eqFP-hphNT1*	"
M2750	W303 *MATa CDC5-4xGFP-klTrp1 SPC42-eqFP-hphNT1 dbf4-NΔ109-kanMX6*	"
M2804	W303 *MATa dbf4-NΔ82-88-kanMX6*	"

Table 1 Cont.

M2818	W303 *cdc5-1/cdc5-1*	"
M2822	W303 *cdc5-1/cdc5-1 DBF4/dbf4-NΔ109-kanMX6*	"
M2826	W303 *cdc5-1/cdc5-1 dbf4-NΔ109-kanMX6/dbf4-NΔ109-kanMX6*	"
M2908	W303 *MATα cdc5-1 dbf4-NΔ82-88-kanMX6*	"
M3027	W303 *MAT***a** *cdc55Δ::his5*	"
M3028	W303 *MAT***a** *cdc55Δ::his5 dbf4-NΔ109-kanMX6*	"
M3093	W303 *MAT***a** [pMHY193; pRS316-GFP-Nop1]	"
M3094	W303 *MAT***a** *dbf4-NΔ109-kanMX6* [pMHY193; pRS316-GFP-Nop1]	"
M3095	W303 *MAT***a** *cdc5-1 dbf4-NΔ109-kanMX6* [pMHY193; pRS316-GFP-Nop1]	"
M3096	W303 *MAT***a** *cdc5-1* [pMHY193; pRS316-GFP-Nop1]	"
M3148	W303 *MAT***a** *Δfob1 dbf4-NΔ109-kanMX6 CDC14-EGFP-kanMX6*	"
M3149	W303 *MAT***a** *Δfob1 CDC14-EGFP-kanMX6*	"
M3155	W303 *MAT***a** *kin4Δ::his5 cdc55Δ::his5 dbf4-NΔ109-kanMX6*	"
M3161	W303 *MAT***a** *PDS1-HA3 DBF4-Myc18*	"

Table 2. Plasmids used in this study.

Plasmid	Description	Source
pCG10	pRS415-*DBF4 110-704*	This study
pCG52	pGBKT7-*DBF4 67-227*	"
pCG53	pGBKT7-*DBF4 67-704*	"
pCG60	pGBKT7-*DBF4 67-227* Δ-732 to -802 ADH1 promoter	"
pCG61	pCG60-*DBF4 110-227*	"
pCG68	pGAD-*CDC5 422-705*	"
pCG74	pGBKT7-*DBF4 110-704*	"
pCG166	*pGAL-DBF4 1-225*	"
pCG163	*pGAL-DBF4 109-225*	"
pCG167	*pGAL-DBF4 65-225*	"
pCG213	*pGAL-DBF4 1-225 Δ 82-88*	"
pCM1	pGAD-*CDC5 422-705 H641A, K643M*	"
pCM3	pGAD-*CDC5 454-705*	"
pCM4	pGAD-*CDC5 510-705*	"
pCM16	pAcSG2-*3myc-CDC5 65-705*	"
pCM21	pCG60-*DBF4 67-109*	"
pCM24	pCM21 S84A, S92A, T95A, T105A	"
pGAD-CDC5.3	pGAD-*CDC5 421-705*	"
pHS4	pSUMO-*DBF4 66-109*	"
pMW489	pRS415-*DBF4*	"

Table 2 Cont.

pMW535	pRS415-*CDC5*	"
pMW537	pGEX-KG-*CDC5 357-705*	"
pYJ38	pCG60-*DBF4 67-227 Δ82-88*	"
pYJ150	pRS425-*DBF4 110-705*	"
pYJ152	pRS425-*DBF4 Δ82-88*	"
pYJ154	pRS425-*DBF4*	"
pYJ161	pRS425-*DBF4 ΔC312-704*	"
pYJ162	pRS425-*DBF4 Δ65 + ΔC312-704*	"
pYJ163	pRS425-*DBF4 Δ109 + ΔC312-704*	"
pYJ164	pRS425-*DBF4 Δ82-88 + ΔC312-704*	"
pYJ214	pGBKT7-*DBF4 Δ65 + Δ82-88*	"

ADH1 promoter sequence of pGBKT7 (-647 to -717 from the ATG) removed a Rap1p binding site and reduced the strength of GBD-Dbf4p 67-227 expression (which was otherwise lethal) to give pCG60. Point mutations and deletions were generated by site-directed mutagenesis using the QuikChange system (Stratagene). For all mutations, the entire coding sequence was verified by DNA sequencing. Construction of baculovirus plasmids encoding WT, NΔ65Dbf4p, NΔ109Dbf4p, NΔ221Dbf4p and HA-Cdc7p was previously described [26]. The baculovirus transfer plasmid containing 3Myc-NΔ65Polo was constructed in pAcSG2 (BD Biosciences). High-titer baculoviruses were generated by

transfection of Sf9 cells using the BaculoGold kit (BD Biosciences) followed by plaque purification and virus amplification.

For *in vitro* interaction assays, DNA encoding Polo amino acids 357-705 were PCR amplified with *Bam*HI-*Xma*I linkers and cloned into pGEX-KG for expression of GST-Polo PBD in *E. coli*. The region encoding Dbf4p amino acids 66-109 was PCR amplified from pMW489 with *Bsa*I-*Bam*HI linkers and cloned into pSUMO (LifeSensors Inc.) for expression of Sumo-Dbf4p 67-109.

Growth Media and Cell Cycle Experiments

Cells were cultured in YPD (1% yeast extract, 2% bacto peptone, 2% glucose). Synchronous G1 cultures were obtained after addition of 5μg/ml (0.1μg/ml in *bar1Δ* cells) alpha-factor to cells for 3 hours. DNA content was analyzed by flow cytometry as previously described [17]. Drugs were added directly to plates immediately before pouring.

Two-hybrid Experiments

PJ69-4a cells containing pCG60 were transformed with a *S. cerevisiae* two-hybrid library. Interacting clones were recovered on medium lacking tryptophan, leucine, and histidine but containing 2 mM 3-aminotriazole at 30 degrees Celsius. Positive interactors were streak-purified and also tested for *ADE2* reporter activity. Prey plasmids that activated both *HIS3* and *ADE2* expression were confirmed by retransformation in PJ69-4a and then sequenced. To quantify two-

hybrid interactions, co-transformed cells were spotted at ten-fold serial dilutions on selective media and grown for 2-3 days.

Yeast Whole-cell Extract Preparation, Immunoprecipitation and Blotting

Yeast protein extracts were prepared for Western blotting by trichloroacetic acid extraction [71] or for immunoprecipitation (IP) by bead-beating in NP-40 lysis buffer (20mM Tris-HCl, 150mM NaCl, 0.5% NP-40 and 1mM EGTA). HA-tagged proteins were immunoprecipitated using anti-HA monoclonal antibody (12CA5) conjugated to protein A-Sepharose. Blots were probed in phosphate-buffered saline containing 0.1% Tween and 1% dried milk. 12CA5 (1:1000) was used to detect HA-tagged proteins, 9E10 (1:1000) to detect Myc-tagged proteins, and polyclonal sera against Cdc7p (1:4000) and Dbf4p (1:1000) were used to detect those proteins.

Co-immunoprecipitation from Sf9 Insect Cells

Sf9 cells were co-infected with HA-Cdc7p, 3Myc-NΔ65Polo and Dbf4p derivatives and then immunoprecipitated as previously described [26]. Whole cell extracts and IPs were probed with polyclonal antibodies against Cdc7p (1:4000) and Dbf4p (1:1000) as described above. 3Myc-NΔ65Polo was probed with 9E10 monoclonal antibody against Myc (1:1000).

Protein Expression, Purification and GST-Pull down

Cdc7p-Dbf4p kinase was purified as described [17]. GST or GST-PBD was induced in BL21 cells for 3 hours at 37 degrees Celsius using 0.5mM IPTG. Cells were sonicated in PBS containing 1% Triton X-100 and GST proteins were purified from soluble extracts by binding to glutathione-agarose (Amersham), elution in (20mM Tris-HCl, 150mM NaCl, 1mM EDTA and 10% glycerol) containing 5mM glutathione followed by dialysis against the same buffer. 6His-tagged Sumo and Sumo-Dbf4p were expressed in BL21 cells and extracted in HEPES extraction buffer (50mM HEPES-KOH, pH 7.5, 150mM NaCl, 2M Urea and 10% glycerol). Proteins were loaded onto a Ni++ column and washed (20mM HEPES-KOH, pH 7.5, 200mM NaCl and 10% glycerol) before elution using an imidazole gradient. For GST pull-downs, Sumo, Sumo-Dbf4p, GST and GST-Polo were incubated with glutathione-agarose in the presence of buffer (20mM Tris-HCl pH 7.0, 300mM NaCl, 0.5% NP-40 and 1mM EGTA) for 1 hour at 4 degrees Celsius. The glutathione agarose beads were washed extensively and bound proteins separated on 12.5% SDS-PAGE gels. Blots were probed with polyclonal antisera raised against GST-Polo PBD (1:1000) and yeast Smt3p (Sumo) (1:1000).

Kinase Assays

For *in vitro* kinase assays, purified HACdc7p-Dbf4p (100ng) [17] was incubated with GST or GST-Polo PBD (300ng) at 30 degrees Celsius in kinase buffer (50mM Tris-HCl pH 7.5, 10mM MgCl2, 1mM DTT, 100μM ATP and 10μCi of [γ-

32 P] ATP) for 20 minutes. Proteins were separated on 10% SDS-PAGE and visualized by autoradiography.

Fluorescence Microscopy

For direct fluorescence analysis of Cdc14-EGFP, cells were fixed in 3.7% formaldehyde at room temperature for 1 hour. DNA was stained using DAPI (1mg/ml) for 10 minutes at room temperature. For the experiments in Figure 14A, the absence of a distinct Cdc14-EGFP fluorescent signal (but accompanied by diffuse nuclear and cytoplasmic fluorescence) was scored as "released." Any cell that had a distinct Cdc14-EGFP nucleolar fluorescence was counted as sequestered. Spindle morphology was detected after spheroplasting cells and incubation in methanol/acetone prior to incubation with antibodies: rat anti-tubulin (YOL1/34 Accurate Chemicals, 1:10) and goat anti-rat FITC (Jackson Immunoresearch, 1:50). Cells were imaged using a 60x objective.

Cdc5 Protein Abundance, Kinase Activity and SPB Localization

To analyze Cdc5 protein abundance during the cell cycle K6019 and M1874 were arrested with alpha-factor (5μg/ml) for 3h, released into the cell cycle at 30°C in YPD and TCA extracts were analyzed by Western blotting. Cdc5-HA3p kinase activity was analyzed in asynchronous, G1 (0.1μg/ml alpha-factor), HU (0.2M) and nocodazole (15μg/ml) arrested cultures. Bead-beated whole cell extracts from strains M2357 and M2359 were made in (20mM Tris-HCl, pH7.4, 150mM NaCl, 0.5% NP-40, 1mM EGTA), immunoprecipitated with 12CA5-protein

A Sepharose and washed 4x in the same buffer. Kinase activity was measured on half of the IP using casein as a substrate in (50mM Tris-HCl, pH 7.5, 10mM McCl2, 5mM DTT, 2mM EGTA, 100mM ATP, 10μCi [γ-32P] ATP). Bound proteins were separated on 10% SDS-PAGE and visualized by autoradiography or by Western blotting. To examine Cdc5p SPB localization, G1 arrested cells (M2748, M2750) were released into the cell cycle at 20 degrees Celsius in YPD media containing 15μg/ml nocodazole. Cells were fixed in 4% paraformaldeyde for 10 minutes, stained with DAPI (1μg/ml) and analyzed for Cdc5-4xGFP spindle pole body localization using fluorescence microscopy.

Nucleolar Segregation Assay

To assess nucleolar segregation *DBF4*, *dbf4-NΔ109, cdc5-1* and *cdc5-1 dbf4-NΔ109* cells transformed with a centromeric EGFP-Nop1 plasmid (pMHY193) were arrested in G1 with mating pheromone and released into the cell cycle in YPD at 34 degrees Celsius. Alpha-factor was added back after budding to permit a single cell cycle. Samples were taken at the indicated time points and scored for the presence of one or two GFP signals by florescence microscopy.

Dbf4 Cell Cycle Abundance

M3161 expressing Pds1-HA3p and Dbf4-Myc18p was arrested in G1 at 25 degrees Celsius with mating pheromone and released into the cell cycle at 20 degrees Celsius. Samples were taken at the indicated time points. Protein extracts were made for Western blotting by the TCA method [71] and cells were

processed for DNA content analysis by flow cytometry [17]. Western blots were probed using 9E10 (1:1000) and 12CA5 (1:1000) antibodies to detect Dbf4-Myc18p and Pds1-HA3p, respectively.

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REFERENCES:

1. Hartwell, L.H., and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629-634.

2. Allen, J.B., Zhou, Z., Siede, W., Friedberg, E.C., and Elledge, S.J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev *8*, 2401-2415.

3. Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev *8*, 652-665.

4. Osborn, A.J., Elledge, S.J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol *12*, 509-516.

5. Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol Cell *6*, 1-10.

6. Bardin, A.J., Visintin, R., and Amon, A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell *102*, 21-31.

7. Pearson, C.G., and Bloom, K. (2004). Dynamic microtubules lead the way for spindle positioning. Nat Rev Mol Cell Biol *5*, 481-492.

8. Pereira, G., and Schiebel, E. (2005). Kin4 kinase delays mitotic exit in response to spindle alignment defects. Mol Cell *19*, 209-221.

9. D'Aquino, K.E., Monje-Casas, F., Paulson, J., Reiser, V., Charles, G.M., Lai, L., Shokat, K.M., and Amon, A. (2005). The protein kinase Kin4 inhibits exit from mitosis in response to spindle position defects. Mol Cell *19*, 223-234.

10. Kops, G.J., Weaver, B.A., and Cleveland, D.W. (2005). On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer *5*, 773-785.

11. Sclafani, R.A. (2000). Cdc7p-Dbf4p becomes famous in the cell cycle. J Cell Sci *113 (Pt 12)*, 2111-2117.

12. Cheng, L., Collyer, T., and Hardy, C.F. (1999). Cell cycle regulation of DNA replication initiator factor Dbf4p. Mol Cell Biol *19*, 4270-4278.

13. Ferreira, M.F., Santocanale, C., Drury, L.S., and Diffley, J.F. (2000). Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. Mol Cell Biol *20*, 242-248.

14. Jackson, A.L., Pahl, P.M., Harrison, K., Rosamond, J., and Sclafani, R.A. (1993). Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol *13*, 2899-2908.

15. Oshiro, G., Owens, J.C., Shellman, Y., Sclafani, R.A., and Li, J.J. (1999). Cell cycle control of Cdc7p kinase activity through regulation of Dbf4p stability. Mol Cell Biol *19*, 4888-4896.

16. Sullivan, M., Holt, L., and Morgan, D.O. (2008). Cyclin-specific control of ribosomal DNA segregation. Mol Cell Biol *28*, 5328-5336.

17. Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J *18*, 5334-5346.

18. Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M., and Sclafani, R.A. (1997). mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A *94*, 3151-3155.

19. Lei, M., Kawasaki, Y., Young, M.R., Kihara, M., Sugino, A., and Tye, B.K. (1997). Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev *11*, 3365-3374.

20. Sato, N., Arai, K., and Masai, H. (1997). Human and Xenopus cDNAs encoding budding yeast Cdc7-related kinases: in vitro phosphorylation of MCM subunits by a putative human homologue of Cdc7. Embo J *16*, 4340-4351.

21. Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

22. Stillman, B. (2005). Origin recognition and the chromosome cycle. FEBS Lett *579*, 877-884.

23. Fung, A.D., Ou, J., Bueler, S., and Brown, G.W. (2002). A conserved domain of Schizosaccharomyces pombe dfp1(+) is uniquely required for chromosome stability following alkylation damage during S phase. Mol Cell Biol *22*, 4477-4490.

24. Pessoa-Brandao, L., and Sclafani, R.A. (2004). CDC7/DBF4 functions in the translesion synthesis branch of the RAD6 epistasis group in Saccharomyces cerevisiae. Genetics *167*, 1597-1610.

25. Takeda, T., Ogino, K., Tatebayashi, K., Ikeda, H., Arai, K., and Masai, H. (2001). Regulation of initiation of S phase, replication checkpoint signaling, and maintenance of mitotic chromosome structures during S phase by Hsk1 kinase in the fission yeast. Mol Biol Cell *12*, 1257-1274.

26. Gabrielse, C., Miller, C.T., McConnell, K.H., Deward, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCA1 C-Terminal-like domain required for the response to replication fork arrest in budding yeast. Genetics *173*, 541-555.

27. Masai, H., and Arai, K. (2000). Dbf4 motifs: conserved motifs in activation subunits for Cdc7 kinases essential for S-phase. Biochem Biophys Res Commun *275*, 228-232.

28. Matsumoto, S., Ogino, K., Noguchi, E., Russell, P., and Masai, H. (2005). Hsk1-Dfp1/Him1, the Cdc7-Dbf4 kinase in Schizosaccharomyces pombe, associates with Swi1, a component of the replication fork protection complex. J Biol Chem *280*, 42536-42542.

29. Bailis, J.M., Bernard, P., Antonelli, R., Allshire, R.C., and Forsburg, S.L. (2003). Hsk1-Dfp1 is required for heterochromatin-mediated cohesion at centromeres. Nat Cell Biol *5*, 1111-1116.

30. Duncker, B.P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S.M. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proc Natl Acad Sci U S A *99*, 16087-16092.

31. van Vugt, M.A., and Medema, R.H. (2005). Getting in and out of mitosis with Polo-like kinase-1. Oncogene *24*, 2844-2859.

32. Lee, K.S., Park, J.E., Asano, S., and Park, C.J. (2005). Yeast polo-like kinases: functionally conserved multitask mitotic regulators. Oncogene *24*, 217-229.

33. Mortensen, E.M., Haas, W., Gygi, M., Gygi, S.P., and Kellogg, D.R. (2005). Cdc28-dependent regulation of the Cdc5/Polo kinase. Curr Biol *15*, 2033-2037.

34. Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. Curr Biol *8*, 497-507.

35. Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are

regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. EMBO J *17*, 1336-1349.

36. Smits, V.A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E.A., and Medema, R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat Cell Biol *2*, 672-676.

37. Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. Cell *107*, 655-665.

38. Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev *13*, 1936-1949.

39. Hardy, C.F., and Pautz, A. (1996). A novel role for Cdc5p in DNA replication. Mol Cell Biol *16*, 6775-6782.

40. Kitada, K., Johnson, A.L., Johnston, L.H., and Sugino, A. (1993). A multicopy suppressor gene of the Saccharomyces cerevisiae G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. Mol Cell Biol *13*, 4445-4457.

41. Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. Mol Biol Cell *9*, 2803-2817.

42. Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell *108*, 207-220.

43. Visintin, R., Stegmeier, F., and Amon, A. (2003). The role of the polo kinase Cdc5 in controlling Cdc14 localization. Mol Biol Cell *14*, 4486-4498.

44. Pereira, G., and Schiebel, E. (2004). Cdc14 phosphatase resolves the rDNA segregation delay. Nat Cell Biol *6*, 473-475.

45. Sullivan, M., Higuchi, T., Katis, V.L., and Uhlmann, F. (2004). Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. Cell *117*, 471-482.

46. D'Amours, D., Stegmeier, F., and Amon, A. (2004). Cdc14 and condensin control the dissolution of cohesin-independent chromosome linkages at repeated DNA. Cell *117*, 455-469.

47. Stegmeier, F., and Amon, A. (2004). Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. Annu Rev Genet *38*, 203-232.

48. Fesquet, D., Fitzpatrick, P.J., Johnson, A.L., Kramer, K.M., Toyn, J.H., and Johnston, L.H. (1999). A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast. EMBO J *18*, 2424-2434.

49. Lee, S.E., Frenz, L.M., Wells, N.J., Johnson, A.L., and Johnston, L.H. (2001). Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. Curr Biol *11*, 784-788.

50. Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Shevchenko, A., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell *97*, 233-244.

51. Hu, F., and Elledge, S.J. (2002). Bub2 is a cell cycle regulated phospho-protein controlled by multiple checkpoints. Cell Cycle *1*, 351-355.

52. Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H., and Sedgwick, S.G. (2003). In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. J Biol Chem *278*, 14591-14594.

53. Liang, F., and Wang, Y. (2007). DNA damage checkpoints inhibit mitotic exit by two different mechanisms. Mol Cell Biol *27*, 5067-5078.

54. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166-1171.

55. Maekawa, H., Priest, C., Lechner, J., Pereira, G., and Schiebel, E. (2007). The yeast centrosome translates the positional information of the anaphase spindle into a cell cycle signal. J Cell Biol *179*, 423-436.

56. Lowery, D.M., Lim, D., and Yaffe, M.B. (2005). Structure and function of Polo-like kinases. Oncogene *24*, 248-259.

57. Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

58. Song, S., Grenfell, T.Z., Garfield, S., Erikson, R.L., and Lee, K.S. (2000). Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol Cell Biol *20*, 286-298.

59. Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

60. Kitada, K., Johnston, L.H., Sugino, T., and Sugino, A. (1992). Temperature-sensitive cdc7 mutations of Saccharomyces cerevisiae are suppressed by the DBF4 gene, which is required for the G1/S cell cycle transition. Genetics *131*, 21-29.

61. Cohen-Fix, O., Peters, J.M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev *10*, 3081-3093.

62. Hartwell, L.H., Mortimer, R.K., Culotti, J., and Culotti, M. (1973). Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. Genetics *74*, 267-286.

63. Park, C.J., Song, S., Lee, P.R., Shou, W., Deshaies, R.J., and Lee, K.S. (2003). Loss of CDC5 function in Saccharomyces cerevisiae leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. Genetics *163*, 21-33.

64. Lee, K.S., Grenfell, T.Z., Yarm, F.R., and Erikson, R.L. (1998). Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. Proc Natl Acad Sci U S A *95*, 9301-9306.

65. Visintin, R., Hwang, E.S., and Amon, A. (1999). Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. Nature *398*, 818-823.

66. Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol Cell *2*, 709-718.

67. Hoyt, M.A., Totis, L., and Roberts, B.T. (1991). S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507-517.

68. Li, R. (1999). Bifurcation of the mitotic checkpoint pathway in budding yeast. Proc Natl Acad Sci U S A *96*, 4989-4994.

69. Mohl, D.A., Huddleston, M.J., Collingwood, T.S., Annan, R.S., and Deshaies, R.J. (2009). Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. J Cell Biol *184*, 527-539.

70. Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

71. Foiani, M., Marini, F., Gamba, D., Lucchini, G., and Plevani, P. (1994). The B subunit of the DNA polymerase alpha-primase complex in Saccharomyces cerevisiae executes an essential function at the initial stage of DNA replication. Mol Cell Biol *14*, 923-933.

CHAPTER TWO

Cdc5 Phosphorylation Site Screen and Isolation of Phosphorylated Cdc5 Isoforms for Mass Spectrometry

ABSTRACT:

The Dbf4-dependent kinase (DDK) is essential for replication initiation in eukaryotic cells. In addition to a role in S-phase, DDK inhibits mitotic exit by interaction and inhibition of the polo kinase Cdc5; however, the mechanism of this regulation remains unknown. Disruption of the Dbf4-Cdc5 interaction has no affect on Cdc5 catalytic activity, protein abundance or SPB localization. *In vitro* kinase analyses suggest that the Cdc5 PBD is a substrate of Cdc7-Dbf4. Here I identify Cdc5 PBD residues required for *in vitro* phosphorylation by DDK. Substitution of residues T532, S573 and S630 within the PBD with alanine impairs phosphorylation on the Cdc5 PBD. In addition, substitution of nearby residues within a conserved PBD fold had no effect on Dbf4-Cdc5 binding, suggesting that the loss of phosphorylation did not result from a loss of protein-protein interaction. In addition, DDK phosphorylated PBD isoforms were successfully resolved by PAGE to facilitate the physical mapping of DDK Cdc5 phosphorylation sites by mass spectrometry.

INTRODUCTION:

Activation of the Cdc14 dual-specificity phosphatase triggers mitotic exit in *Saccharomyces cerevisiae* following sister chromatid separation and nuclear division [1-3]. The precise ordering of cell cycle events is crucial to this process and activation of the mitotic exit network (MEN) plays a key regulatory role in regulating Cdc14 activity [4, 5]. MEN activation is triggered by increased Tem1 GTP activity in telophase [6, 7]. The Bfa1-Bub2 complex antagonizes MEN

signaling prior to full activation of the mitotic exit network by enhancing Tem1-GTPase activity at SPBs and is inhibited by Lte1 [7-10]. Inactivation of Bfa1-Bub2 at SPBs increases the cellular Tem1-GTP concentration, activating the SPB-bound kinases Cdc15 and Dbf2-Mob1 [11, 12]. Phosphorylation of Cdc14 and Net1 within the nucleolus releases Cdc14 and initiates mitotic exit [13-15].

The polo kinase in budding yeast, Cdc5, is an essential regulator of mitotic exit; inactivation of Cdc5 arrests cells in telophase with partially divided nuclei and active Cdk [4, 16-18]. This defect is consistent with the role of Cdc5 in regulating Cdc14 by the Cdc14 early anaphase release (FEAR) and MEN [18-20]. Cdc5 phosphorylation of Bfa1-Bub2, presumably at SPBs, triggers Bfa1-Bub2 subunit dissociation and Tem1-GTP activation of Cdc14 regulators Cdc15 and Dbf2 [21, 22]. Interestingly, Cdc5 activity outside the MEN is required for robust Dbf2 activity in cells, although the precise mechanism underlying this requirement is unknown [5]. Cdc5 activity in the FEAR network may account for the additional requirement of Cdc5 activity outside the MEN. Cdc14 release from the nucleolus reverses Cdc15 inhibitory phosphorylation by CDK prior to MEN activation. Therefore, regulation of Cdc14 activity by Cdc5 in the FEAR may link the segregation of sister chromatids and spindle elongation with the faithful execution of mitotic exit. A recent report suggests that Cdc5 regulation of Cdc14 occurs indirectly by inactivation of Swe1 at the G2/M transition [23]. Swe1 is the budding yeast homolog of the human Wee1 kinase. Swe1 phosphorylation inhibits CDK activity prior to Mih1/Cdc25 dephosphorylation at the beginning of mitosis and prevents Cdc14 release by inhibiting Cdk activity on Net1 [23].

Although especially important in regulating mitotic entry in human and *S. pombe*, Swe1 plays only a minor role in budding yeast.

Limited understanding of the temporal and spatial mechanisms regulating Cdc5 activity complicates a complete interpretation of its function during mitosis. Ectopic Cdc5 activity causes untimely Cdc14 release in the presence of active checkpoints, suggesting that Cdc5 must be tightly regulated throughout the entire cell cycle [19, 24]. Cdc5 activity in G1 is primarily regulated by APC-dependent ubiquitination and degradation [25, 26]. Cdc5 protein levels rise in S-phase and catalytic activation by Cdc28 follows shortly thereafter [26]. Localization studies of Cdc5 have identified the bud neck and spindle pole bodies as sites of Cdc5 localization [27, 28]. Interestingly, Cdc5 appears to localize to SPB in S-phase, long before Bfa1 phosphorylation and MEN activation, implying a mechanism for inhibition of Cdc5 MEN activity until late mitosis [29]. Similarly, the mechanism of Cdc5 FEAR regulation prior to anaphase onset is currently unknown. An intact Cdc5 PBD is required for direct interaction with Cdc14 and proper SPB localization, suggesting that substrate priming and/or PBD inhibition may regulate Cdc5 activity at certain subcellular locations [30, 31].

Recent work in our lab demonstrated a novel role for Dbf4 in regulating Cdc5 activity during the cell cycle [29]. We reported a small PBD interacting region (PIR) within the Dbf4 N-terminus that when deleted suppressed the growth defects of MEN hypomorphs and rescued Cdc14 nucleolar release in *cdc5-1* cells. In addition, cells containing the Dbf4 PIR prematurely exit mitosis following spindle misorientation, resulting in ploidy abnormalities. As a result we

now appreciate a role for Dbf4 in regulating Cdc5 function during mitotic exit. Preliminary experiments eliminated altered SPB localization and decreased Cdc5 catalytic activity as mechanisms underlying Dbf4 regulation of Cdc5 [29]. Our discovery that Cdc7 is required for Dbf4-dependent inhibition of Cdc5 and identification of Cdc5 as a substrate for Cdc7-Dbf4 raises the possibility that DDK phosphorylation of the PBD is a mechanism for regulation of yeast polo kinase [29].

In an effort to identify the specific residues phosphorylated by DDK, I screened PBD mutants that were modified at putative Cdc7-Dbf4 phosphorylation sites by an *in vitro* phosphorylation assay. Purified Cdc7-Dbf4 was incubated with PBD phospho-ablation mutants matching previously published DDK consensus sites [29]. I identified T532, S573 and S630 as being required for efficient phosphorylation by DDK. The effect of the S630A, S573A and T532A mutations on the Cdc5-Dbf4 interaction was evaluated by two-hybrid analysis and found to have no effect on protein-protein interactions. In addition, *in vitro* phosphorylation of the Cdc5 PBD by purified Cdc7-Dbf4 identified multiple phosphorylated isoforms of Cdc5 for analysis by mass spectrometry. Identification of these phosphorylated residues should facilitate the development of reagents to examine the temporal and spatial regulation of Cdc5 by Cdc7-Dbf4.

RESULTS:

Mutation of Cdc5 PBD residues eliminates Cdc7-Dbf4 phosphorylation.

We previously reported that the Cdc5 PBD is a robust substrate of Cdc7-Dbf4 [29]. As a result, we hypothesize that DDK phosphorylation of Cdc5 inhibits its function in mitotic exit. Although the exact consensus sequence phosphorylated by DDK is not well defined, several recently published reports investigating Cdc7 phosphorylation of MCM helicase subunits identified residues important for *in vitro* and *in vivo* phosphorylation [32-36]. In these studies, Cdc7-Dbf4 demonstrated a strong preference for acidic residues at the pS +1 position, with a partial preference for hydrophobic residues immediately preceding the phosphorylation site [32-36]. Experiments in our lab support these findings [M. Weinreich unpublished data]. In an effort to identify PBD residues phosphorylated by Cdc7, I screened six putative Cdc7-Dbf4 phosphorylation sites matching published Cdc7-Dbf4 consensus sites within the Cdc5 PBD. Of all the Ser/Thr residues within the Cdc5 PBD, six (S-S383-D, A-S479-E, S-T532-E, L-S573-E, L-S630-D and I-S647-D) most closely matched the published consensus for Cdc7-Dbf4 phosphorylation. To investigate the role of these residues as sites of phosphorylation, Ser-to-Ala and Thr-to-Ala mutants were assessed for phosphorylation by DDK. Individual GST-PBD mutants were constructed via site-directed mutagenesis, expressed, purified and incubated in the presence of purified Cdc7-Dbf4 and radioactive ATP (Figure 21). Mutation of residues S383, S479 and S647 to alanine had no effect on substrate phosphorylation by kinase. In contrast, T532A, S630A and to a lesser extent S573A all significantly reduced Cdc7-Dbf4 phosphorylation.

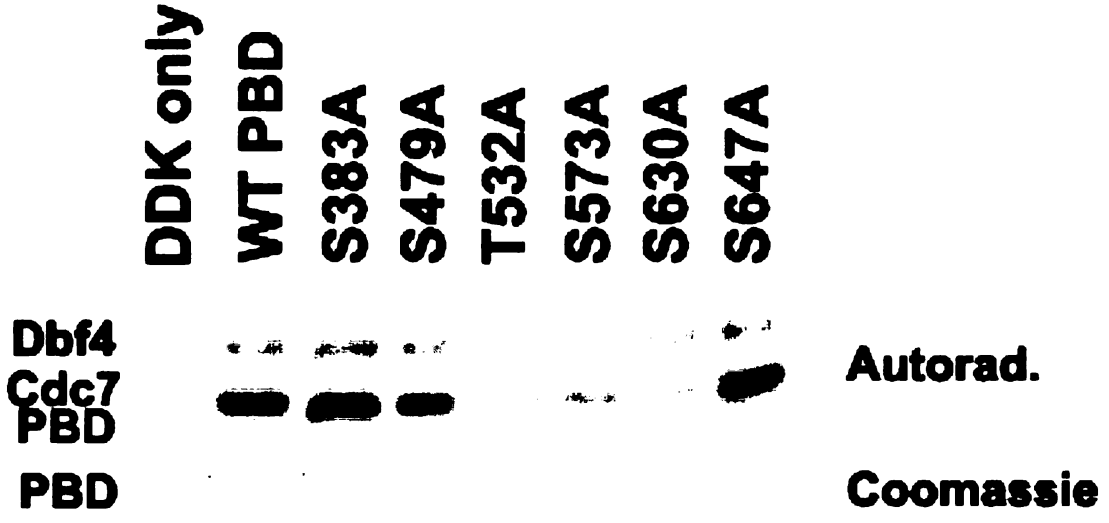

Figure 21. Substitution of PBD residues inhibits DDK phosphorylation of the Cdc5 PBD. Recombinant GST-PBD mutants were analyzed for Cdc7-Dbf4 substrate specificity by *in vitro* kinase assay. Mutant substrates were incubated with purified Cdc7-Dbf4 at 30 degrees Celsius for 30min. Proteins were resolved by PAGE (Cdc7 and GST-PBD proteins co-migrate) and phosphate incorporation measured by autoradiography.

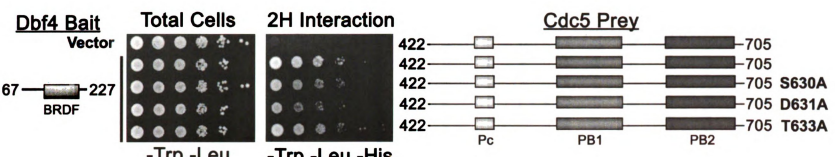

Figure 22. PBD substitutions near S630A do not influence the Dbf4-Cdc5 interaction. Cells were transformed with N-terminal Dbf4-Gal4 DNA binding domain and Gal4 activation domain-Cdc5 PBD mutants. Co-transformants were spotted at ten-fold serial dilutions on SCM –Trp –Leu and SC –Trp –Leu –His + 2mM 3AT for three days at 30 degrees Celsius. Gal4 activation domain mutant expression is shown in Figure 23.

Mutation of Cdc5 PBD residues does not affect the Dbf4-Cdc5 interaction. Identification of several residues that disrupt robust DDK phosphorylation on Cdc5 raised the possibility that some or all of these residues might regulate the interaction between Cdc5-Dbf4. The Cdc5 PBD is a highly conserved interaction domain composed of two sub-domains termed polo boxes and a single polo-cap. We hypothesized that mutation of residues within the PBD might significantly disrupt PBD folding and sufficiently disrupt DDK interaction with Cdc5. Serine 630 and Threonine 532 are located within PB2 and PB1 respectively. To investigate whether substitution of these amino acids might influence the Dbf4-Cdc5 interaction, putative phosphorylation sites (S630, T532 and S537) and adjacent amino acids within the Gal4 AD-Cdc5 422-705 (PBD) were substituted to alanine and the interaction with the Dbf4 polo box interacting region (PIR) analyzed by two-hybrid analysis. Cells were co-transformed with wild type or mutant GAD-PBD and DBD-Dbf4 67-227 constructs. Co-transformants were cultured and spotted at 10-fold serial dilutions on SC –trp -leu and SC –trp –leu -his with 2mM 3AT and grown for 3 days at 30 degrees Celsius. Mutant protein expression was assessed by western blot analysis (Figures 22 & 24). Non-expressed proteins were omitted from two-hybrid analysis (Figures 23 & 25). Interestingly, T532A, S537A and S630A substitutions all interacted with the Dbf4 PIR, suggesting that substitution of these sites was not sufficient to disrupt the interaction with Cdc5 in the two-hybrid system. In addition, all proteins with substitutions near T532 and S630 also interacted with Dbf4, suggesting that

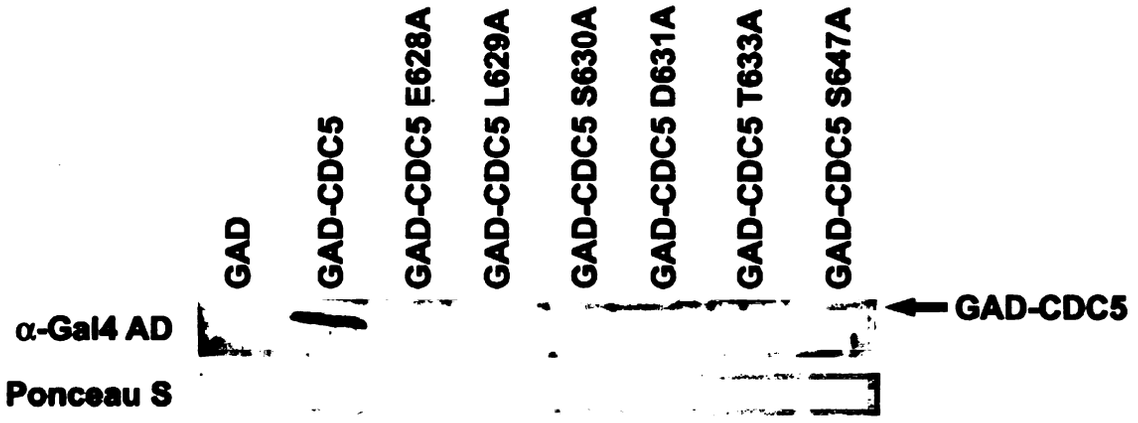

Figure 23. Substitution of PBD residues decreases GAD-Cdc5 expression. Western blot of various GAD-Cdc5 PBD mutants used in Figure 22. GAD-Cdc5 protein was probed using anti-Gal4 AD antibodies. Total protein expression was measured by staining with Ponceau S. Mutant Gal-PBD fusion proteins are indicated by arrow.

the Cdc5-Dbf4 interaction is not sensitive to sequence perturbations within these Cdc5 regions.

Prior to these studies, we observed that S630E mutants failed to complement the deletion of *CDC5* in cells; this is consistent with our model for Cdc5 regulation by Cdc7-Dbf4 and suggested that phosphorylation of this site might inhibit the essential function of Cdc5 during the cell cycle (unpublished data; M.W.). I analyzed the effect of phospho-mimetic substitutions of T532, S537 and S630 on the Dbf4-Cdc5 interaction using the two-hybrid system (Figure 22 & 24). Expression of S630E and T532E was not detectable, suggesting that substitution of these sites might destabilize domain folding (Figure 23 & 25). Together these results suggest that disruption of the Cdc5-Dbf4 protein interaction by substitution of putative phosphorylation sites is unlikely the cause of decreased DDK phosphorylation of Cdc5.

Isolation of phosphorylated isoforms of Cdc5 for mass spectrometry.

To isolate DDK-dependent phospho-specific isoforms of the Cdc5 PBD for use in mass spectrometry, recombinant full-length yeast HA-Cdc7 and Dbf4 were co-expressed in Sf9 insect cells and purified prior to *in vitro* kinase reactions with recombinant Cdc5 PBD protein. To maximize our chances for detecting a phosphorylation shifted isoform following kinase reactions, we wanted to use the lowest molecular weight form of recombinant PBD possible. I found that the original GST-PBD (~66 kDa) was highly resistant to proteolytic cleavage within

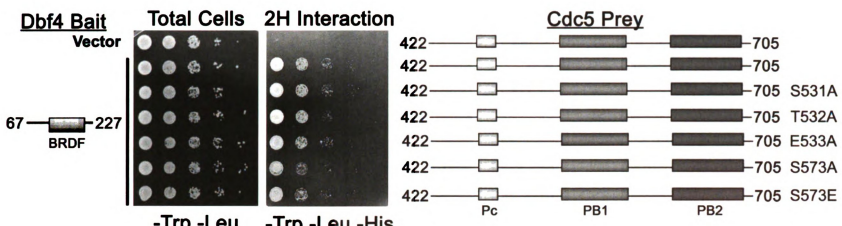

Figure 24. PBD substitutions do not affect the Dbf4-Cdc5 interaction. Cells were transformed with N-terminal Dbf4-Gal4 DNA Binding Domain and Gal4 activation domain-Cdc5 PBD mutants. Cotransformants were spotted at ten-fold serial dilutions on SCM –Trp –Leu and SC –Trp –Leu –His + 2mM 3AT for three days at 30 degrees Celsius. Gal4 activation domain mutant expression is shown in Figure 25.

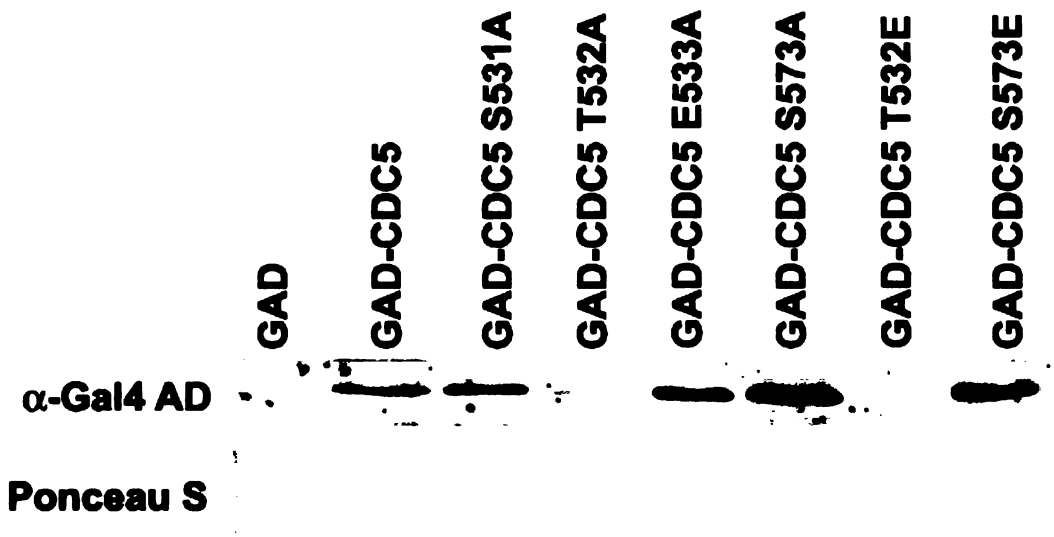

Figure 25. Substitution of PBD residues decreases GAD-Cdc5 Expression. Western blot of various GAD-Cdc5 PBD mutants used in Figure 24. GAD-Cdc5 protein was probed using anti-Gal4 AD antibodies. Total protein expression was measured by staining with Ponceau S.

the peptide linker, preventing me from using the PBD alone (~34kDa) as a substrate for DDK (data not shown). As an alternative, I utilized recombinant 6HIS-PBD (~45kDa) (Gift of Y.C. Chen). Following optimization of kinase reaction conditions (Magnesium and ATP concentration, incubation time, and kinase/substrate concentrations), active DDK was incubated with purified 6HIS-PBD in the presence of cold or radioactive ATP. Resolution of kinase reaction products by SDS-PAGE and silver staining identified a phosphorylation-dependent shift in Cdc5 mobility following incubation with active kinase (Figure 26). Three independent phosphorylated Cdc5 isoforms were visible following resolution by PAGE, suggesting that multiple DDK phosphorylation sites are present on Cdc5. A DDK consensus site following the HIS6 tag in our construct raises the possibility that this site is phosphorylated in our experiment, but the presence of multiple phosphorylated bands suggests that it is not the only residue being phosphorylated. Phosphorylated bands have been isolated and submitted for analysis by mass spectrometry.

DISCUSSION:

Consistent with the observation that the Cdc5 PBD is an effective substrate for *in vitro* DDK phosphorylation, we were able to identify specific residues within the PBD that when mutated decreased kinase phosphorylation. Of the six residues matching published Cdc7-Dbf4 consensus sites, we identified three sites (S630A, T532A and S573A) that resulted in a possible reduction in phosphorylation by DDK. Surprisingly, mutation of two of these sites individually (S630A or T532A)

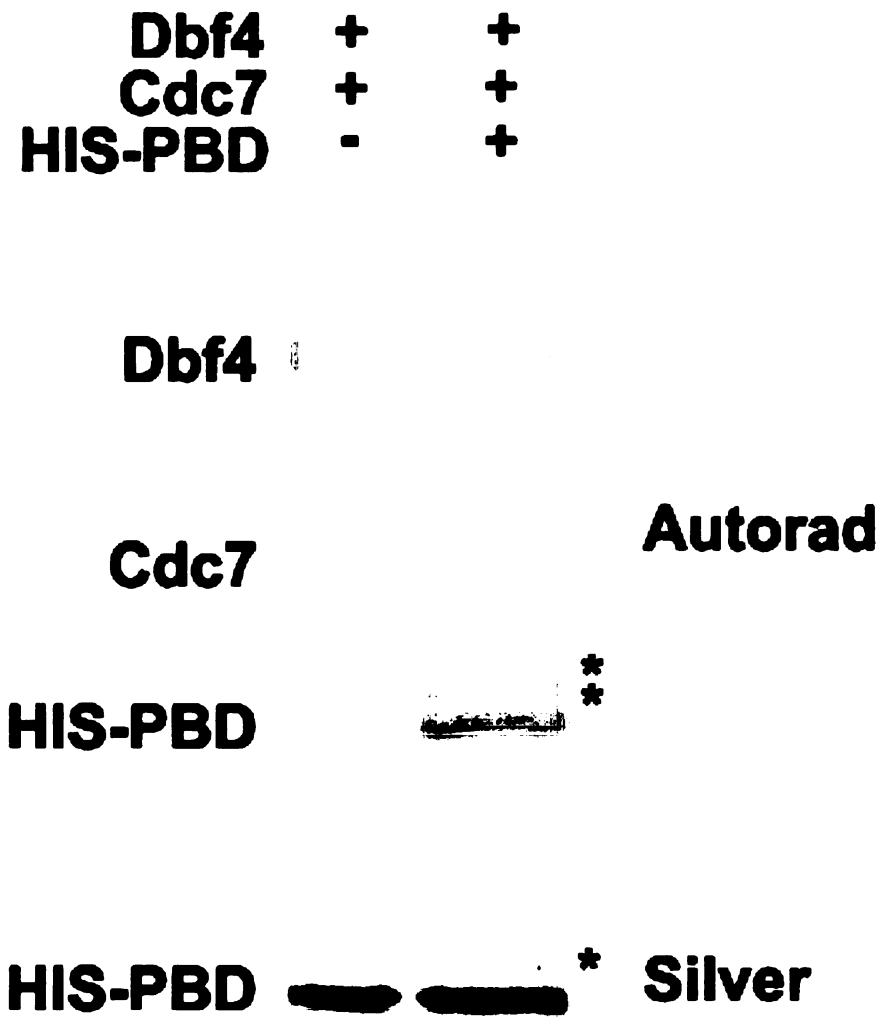

Figure 26. Resolution of phosphorylated Cdc5-PBD by SDS-PAGE. HIS-tagged Cdc5 PBD was phosphorylated by purified Cdc7-Dbf4 and separated from unphosphorylated substrate by PAGE. Phosphorylated Cdc5 was gel extracted and submitted for phosphopeptide mapping by mass spectrometry. (*) Shifted Cdc5 protein isoforms.

apparently caused complete loss of phosphorylation. This suggested that all three of these sites were not direct targets of DDK. To explain this finding, we proposed that one or more of these substitutions might disrupt the interaction with Dbf4, thereby indirectly affecting phosphorylation by DDK. Published reports indicate that the polo box domain is a phospho-dependent interaction domain that demonstrates strong preference for substrates with serine and proline at the pS -1 and +1 amino acid positions respectively [37-39]. Critical to this interaction are three highly conserved residues within polo box 1 (W517) and polo box 2 (H641 and K643) that contribute to phosphate binding. Histidine 641 and lysine 643 pincer residues coordinate direct binding with the negatively charged phosphate and tryptophan 517 is largely responsible for conferring specificity for serine at the pS -1 position [37, 39].

Interestingly, we have shown that the Dbf4-Cdc5 interaction occurs independent of phosphate binding and substitution of the conserved pincer residues (H641 and K643) has no affect on this interaction [29 and Y.C. and C. M. unpublished data]. Nonetheless, we hypothesized that disruption of these or flanking residues within conserved folding regions of the PBD might affect the interaction with Dbf4. We tested this by two-hybrid analysis using a Dbf4 N-terminal bait construct containing the polo box interaction region and Cdc5-PBD prey constructs containing mutations of interest. Surprisingly, the S630A, T532A and S573A mutant constructs all interacted with Dbf4, suggesting that a simple loss of Dbf4-Cdc5 interaction was not likely responsible for the observed loss of phosphorylation. Additionally surrounding residues D631A, T633A, S531A and

E533A also did not affect the Dbf4-Cdc5 interaction. Mutant residues preceding S630 (E628A and L629A) were not adequately expressed in the two-hybrid system, suggesting that these sites may contribute to protein stability. Although these results suggest that mutation of S630, T532 and S573 does not influence the interaction between Dbf4-Cdc5, it is possible that these mutations may affect protein conformation differently as a GST-PBD construct. Analysis of the interaction between mutant GST-PBD proteins with Dbf4 may be informative in this regard, nevertheless, these studies suggest that significant progress in the identification of Cdc5 phosphorylation sites will more likely come through the physical mapping of sites by mass spectrometry.

Phosphorylation of recombinant Cdc5 PBD protein for mass spectrometry. The results from our screen of putative DDK phosphorylation sites on Cdc5 strongly suggests that there are multiple DDK sites on the PBD. Initial attempts to map these sites on phosphorylated GST-PBD constructs were unsuccessful (C.M. and M.W. unpublished data). Identification of phosphorylated residues on proteins requires sufficient site occupancy of the target protein, which we may have now achieved by specifically isolating phosphorylated (shifted) Cdc5.

In an effort to resolve phospho-specific PBD protein I utilized a smaller molecular weight 6HIS-PBD construct and phosphorylated recombinant protein using purified Cdc7-Dbf4. Using this technique, two shifted Cdc5 bands were easily discernible in 6HIS-Cdc5 preps incubated in the presence of kinase (Figure 26). Radioactive *in vitro* kinase assays confirmed that shifted bands, in

addition to the primary fast-migrating band, all incorporated radioactive phosphate (Figure 26). This suggests that DDK phosphorylates Cdc5 on multiple sites and implies that mutation of a single residue (as in Figure 21) would not be sufficient to completely eliminate DDK phosphorylation.

Our current knowledge of the role Dbf4 plays in inhibiting Cdc5 during the cell cycle is complicated by our poor understanding of the specific timing and mechanism for Dbf4 inhibition of Cdc5. The absence of a biochemical marker to assess Dbf4 inhibition of Cdc5 represents a significant hurdle to our complete understanding of this interaction. This work provides important progress towards this goal. Identification of DDK phosphorylation sites on Cdc5 will facilitate the development of reagents to assess spatial and temporal Dbf4 regulation of Cdc5 and assist us in understanding the full significance Cdc5 regulation by Dbf4 during the cell cycle.

MATERIALS AND METHODS:

Construction of Yeast Strains, Plasmids and Baculoviruses

PJ69-4a cells (MATa *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*) were used for two-hybrid experiments. For yeast two-hybrid analyses, mutations were generated by site-directed mutagenesis of pGAD-Cdc5 422-705 using the Quikchange system (Stratagene). Quikchange deletion of 70bp within the *ADH1* promoter sequence of pCG60 (position -802 to -732) including the Rap1 binding site reduced the strength of Gal4 DBD-Dbf4 67-227 expression as previously described [29].

Construction of baculovirus plasmids encoding Dbf4 and HA-Cdc7 was previously described [40]. High-titer baculoviruses were generated by transfection of Sf9 cells using the BaculoGold kit (BD Biosciences) followed by plaque purification and virus amplification.

Two-hybrid Experiments

PJ69-4a cells were co-transformed with bait and prey plasmids. Double transformants were recovered on medium lacking tryptophan (bait) and leucine (prey). Co-transformants were streak-purified and tested for growth on SC –trp –leu –his plates containing 2 mM 3-aminotriazole. Interactions were confirmed by retransformation of plasmids. To quantify two-hybrid interactions, co-transformed cells were spotted at 10-fold serial dilutions on selective media and grown for 3 days at 30 degrees Celsius.

Protein Expression and Purification

Methods used for expression and purification of the Cdc7-Dbf4p complex were previously described [41]. BL21 cells were used for expression and purification of recombinant proteins out of *E. coli*. GST and GST-tagged Cdc5 PBD aa422-705 were expressed for 3 hours at 37°C and released from cells by sonication in PBS containing 1% Triton X-100. Binding to a glutathione-conjugated matrix purified GST-conjugated proteins. Protein was washed (20mM Tris-HCL, 150mM NaCl, 1mM EDTA and 10% glycerol), eluted with 5mM free glutathione and dialyzed.

Kinase Assays

For *in vitro* kinase assays, purified recombinant Dbf4-HACdc7 (50ng) was co-incubated with GST-Cdc5 PBD (500ng) in the presence of kinase buffer (50mM Tris-HCl, 10mM $McCl_2$, 5mM DTT and 2mM EGTA) with 100μM cold ATP and 10μCi of [γ-^{32}P] ATP for 30 min at 30 degrees Celsius. Proteins were separated on 10% SDS-PAGE and visualized by silver staining or autoradiography. Mutant screen experiments were repeated following repurification of independent mutant proteins.

Table 3. Plasmids used in this study.

Plasmid	Description	Source
pCG60	pGBKT7-*DBF4 67-227* Δ-732 to -802 ADH1 promoter	[29]
pGAD-CDC5.3	pGAD-*CDC5 PBD 422-705*	[29]
pMW537	pGEX-KG-*CDC5 357-705*	[29]
pCM37	pGEX-KG-*CDC5 357-705* S647A	This study
pCM50	pGEX-KG-*CDC5 357-705* S630A	"
pCM52	pGAD-*CDC5 422-705* S647A	"
pCM54	pGAD-*CDC5 422-705* E628A	"
pCM55	pGAD-*CDC5 422-705* L629A	"

	Table 3 Cont.	
pCM56	pGAD-*CDC5 422-705* S630A	"
pCM57	pGAD-*CDC5 422-705* D631A	"
pCM58	pGAD-*CDC5 422-705* T633A	"
pCM59	pGAD-*CDC5 422-705* S630E	"
pCM60	pGAD-*CDC5 422-705* S647A	"
pCM61	pGAD-*CDC5 422-705* S531A	"
pCM62	pGAD-*CDC5 422-705* T532A	"
pCM63	pGAD-*CDC5 422-705* T532E	"
pCM64	pGAD-*CDC5 422-705* E533A	"
pCM65	pGAD-*CDC5 422-705* S537A	"
pCM66	pGAD-*CDC5 422-705* S537E	"
pFJ240	pGEX-KG-*CDC5 357-705* S573A	"
pFJ244	pGEX-KG-*CDC5 357-705* S383A	"
pFJ245	pGEX-KG-*CDC5 357-705* T532A	"
pFJ252	pGEX-KG-*CDC5 357-705* S479A	"

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REFERENCES:

1. Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W., Jang, J., Shevchenko, A., Charbonneau, H., and Deshaies, R.J. (1999). Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell *97*, 233-244.

2. Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol Cell *2*, 709-718.

3. Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. Genes Dev *13*, 1936-1949.

4. Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A., and Morgan, D.O. (1998). The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae. Curr Biol *8*, 497-507.

5. Lee, S.E., Frenz, L.M., Wells, N.J., Johnson, A.L., and Johnston, L.H. (2001). Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. Curr Biol *11*, 784-788.

6. Shirayama, M., Matsui, Y., and Toh, E.A. (1994). The yeast TEM1 gene, which encodes a GTP-binding protein, is involved in termination of M phase. Mol Cell Biol *14*, 7476-7482.

7. Pereira, G., Hofken, T., Grindlay, J., Manson, C., and Schiebel, E. (2000). The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol Cell *6*, 1-10.

8. Bardin, A.J., Visintin, R., and Amon, A. (2000). A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell *102*, 21-31.

9. Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H., and Sedgwick, S.G. (2003). In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. J Biol Chem *278*, 14591-14594.

10. Geymonat, M., Spanos, A., Smith, S.J., Wheatley, E., Rittinger, K., Johnston, L.H., and Sedgwick, S.G. (2002). Control of mitotic exit in budding yeast. In vitro regulation of Tem1 GTPase by Bub2 and Bfa1. J Biol Chem *277*, 28439-28445.

11. Asakawa, K., Yoshida, S., Otake, F., and Toh-e, A. (2001). A novel functional domain of Cdc15 kinase is required for its interaction with Tem1 GTPase in Saccharomyces cerevisiae. Genetics *157*, 1437-1450.

12. Mah, A.S., Jang, J., and Deshaies, R.J. (2001). Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. Proc Natl Acad Sci U S A *98*, 7325-7330.

13. Yoshida, S., and Toh-e, A. (2002). Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. Biochem Biophys Res Commun *294*, 687-691.

14. Azzam, R., Chen, S.L., Shou, W., Mah, A.S., Alexandru, G., Nasmyth, K., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2004). Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science *305*, 516-519.

15. Shou, W., Azzam, R., Chen, S.L., Huddleston, M.J., Baskerville, C., Charbonneau, H., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2002). Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. BMC Mol Biol *3*, 3.

16. Hartwell, L.H., Culotti, J., and Reid, B. (1970). Genetic control of the cell-division cycle in yeast. I. Detection of mutants. Proc Natl Acad Sci U S A *66*, 352-359.

17. Kitada, K., Johnson, A.L., Johnston, L.H., and Sugino, A. (1993). A multicopy suppressor gene of the Saccharomyces cerevisiae G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. Mol Cell Biol *13*, 4445-4457.

18. Jaspersen, S.L., Charles, J.F., Tinker-Kulberg, R.L., and Morgan, D.O. (1998). A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. Mol Biol Cell *9*, 2803-2817.

19. Visintin, R., Stegmeier, F., and Amon, A. (2003). The role of the polo kinase Cdc5 in controlling Cdc14 localization. Mol Biol Cell *14*, 4486-4498.

20. Stegmeier, F., Visintin, R., and Amon, A. (2002). Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell *108*, 207-220.

21. Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J., and Elledge, S.J. (2001). Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. Cell *107*, 655-665.

22. Lee, S.E., Jensen, S., Frenz, L.M., Johnson, A.L., Fesquet, D., and Johnston, L.H. (2001). The Bub2-dependent mitotic pathway in yeast acts every cell cycle and regulates cytokinesis. J Cell Sci *114*, 2345-2354.

23. Liang, F., Jin, F., Liu, H., and Wang, Y. (2009). The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. Mol Biol Cell *20*, 3671-3679.

24. Hu, F., and Elledge, S.J. (2002). Bub2 is a cell cycle regulated phospho-protein controlled by multiple checkpoints. Cell Cycle *1*, 351-355.

25. Shirayama, M., Zachariae, W., Ciosk, R., and Nasmyth, K. (1998). The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae. Embo J *17*, 1336-1349.

26. Cheng, L., Hunke, L., and Hardy, C.F. (1998). Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. Mol Cell Biol *18*, 7360-7370.

27. Park, J.E., Park, C.J., Sakchaisri, K., Karpova, T., Asano, S., McNally, J., Sunwoo, Y., Leem, S.H., and Lee, K.S. (2004). Novel functional dissection of the localization-specific roles of budding yeast polo kinase Cdc5p. Mol Cell Biol *24*, 9873-9886.

28. Park, C.J., Song, S., Giddings, T.H., Jr., Ro, H.S., Sakchaisri, K., Park, J.E., Seong, Y.S., Winey, M., and Lee, K.S. (2004). Requirement for Bbp1p in the proper mitotic functions of Cdc5p in Saccharomyces cerevisiae. Mol Biol Cell *15*, 1711-1723.

29. Miller, C.T., Gabrielse, C., Chen, Y.C., and Weinreich, M. (2009). Cdc7p-dbf4p regulates mitotic exit by inhibiting Polo kinase. PLoS Genet *5*, e1000498.

30. Rahal, R., and Amon, A. (2008). The Polo-like kinase Cdc5 interacts with FEAR network components and Cdc14. Cell Cycle *7*, 3262-3272.

31. Song, S., Grenfell, T.Z., Garfield, S., Erikson, R.L., and Lee, K.S. (2000). Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. Mol Cell Biol *20*, 286-298.

32. Masai, H., Matsui, E., You, Z., Ishimi, Y., Tamai, K., and Arai, K. (2000). Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. J Biol Chem *275*, 29042-29052.

33. Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006). Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem *281*, 10281-10290.

34. Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

35. Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., Tamai, K., Ohtani, K., and Arai, K. (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem *281*, 39249-39261.

36. Charych, D.H., Coyne, M., Yabannavar, A., Narberes, J., Chow, S., Wallroth, M., Shafer, C., and Walter, A.O. (2008). Inhibition of Cdc7/Dbf4 kinase activity affects specific phosphorylation sites on MCM2 in cancer cells. J Cell Biochem *104*, 1075-1086.

37. Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

38. Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

39. Cheng, K.Y., Lowe, E.D., Sinclair, J., Nigg, E.A., and Johnson, L.N. (2003). The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. Embo J *22*, 5757-5768.

40. Gabrielse, C., Miller, C.T., McConnell, K.H., Deward, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCA1 C-Terminal-like domain required for the response to replication fork arrest in budding yeast. Genetics *173*, 541-555.

41. Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. Embo J *18*, 5334-5346.

CHAPTER THREE

Summary and Conclusions

SUMMARY AND CONCLUSIONS

The essential function of DDK in eukaryotic cells is in regulating DNA replication initiation in S-phase [1, 2]. Recent structure/function analyses of conserved Dbf4 regions implicate motif M and C in regulating Cdc7 binding and catalytic activity and suggest that the Dbf4 N-terminus may regulate additional functions in S-phase [3-6]. To gain a better understanding of the role of the Dbf4 N-terminus in regulating DDK function, we carried out a two-hybrid screen to identify unknown N-terminal interacting proteins and rediscovered an interaction with the yeast polo kinase Cdc5 (Figure 6) and identified a novel function for Dbf4 in regulating Cdc5-dependent cell cycle functions within mitosis [7]. Given the important role for Polo kinases in regulating mitosis and cancer progression, this discovery suggests a more sophisticated role for Dbf4 in eukaryotic biology and raises the possibility that increased understanding of Dbf4-Cdc5 regulation might have important implications for human health.

Initial discovery and analysis of the Cdc5-Dbf4 interaction by Hardy and colleagues identified a minimal 147 amino acid region within the Dbf4 N-terminus that was required for interaction with full length Cdc5 [8]. The current study improves substantially on this finding in several ways. We identified that Dbf4 interacts with the Cdc5 PBD and narrowed the Dbf4 PIR to 43 amino acids (67-109) adjacent the Dbf4 BRCT domain (Figure 6). Identification of this smaller sequence first raised the possibility that Dbf4 binds the Cdc5 PBD independent of protein phosphorylation. The polo box domain is a highly conserved protein-targeting domain within the C-terminal region of Cdc5 and demonstrates binding

specificity for S-pT/pS-P sites on substrates [9-11]. Mutation of potential Cdc5 phosphopeptide binding sites within the Dbf4 PIR did not affect the Dbf4-Cdc5 interaction (Figure 6) and pulldown experiments using recombinant Cdc5 and Dbf4 proteins purified from E. coli recapitulated the Dbf4-Cdc5 interaction (Figure 8). These observations support a phosphorylation-independent role for Cdc5-Dbf4 binding and have important implications for understanding polo kinase regulation. Presently, the Cdc5 residues required for Dbf4 binding have not been defined. Preliminary results suggest that Cdc5 binds Dbf4 using alternate residues than those used for phosphopeptide binding (Y.C. Chen, unpublished results). Future identification of the Dbf4 binding site on Cdc5 has the potential to provide additional insights into the regulation of polo kinases in cells.

Cdc5 has many roles in mitosis, including its essential function in regulating Cdc14 during mitotic exit (Figure 3). This work identifies a significant function for Dbf4 in regulating this pathway. Importantly, this is the first report of a role for DDK in mitosis and suggests a regulatory function for Cdc7-Dbf4 in controlling cell cycle exit. Our observations that Dbf4 inhibits Cdc14 release and that deletion of the Dbf4 PIR bypasses the spindle position checkpoint provide evidence that DDK activity persists well after DNA replication (Figures 14 & 18). What remains unclear is whether Dbf4 inhibition of Cdc5 is regulated directly or indirectly by spindle position checkpoint activation. The SPOC checkpoint protein Kin4 inhibits Cdc5 MEN activation at spindle pole bodies in response to mispositioned spindles. It is unclear whether Dbf4-dependent inhibition of Cdc5 is a target of this pathway or if Dbf4 indirectly affects mitotic exit during spindle

misorientation by global inhibition of Cdc5 function. Further experiments addressing this question will have important implications for our understanding of Dbf4-Cdc5 regulation during mitosis.

Future studies have the potential to focus on several critical issues, which should continue to enhance our understanding of Dbf4 and Cdc5 cell cycle regulation. In particular, the specific mechanism by which Dbf4 inhibits Cdc5 function is of considerable interest. Genetic analyses of *cdc5-1* suppression in *dbf4* mutants that do not interact with Cdc7 suggest that both DDK subunits are required for inhibition of Cdc5 function (Figure 11C). This raises the possibility that DDK phosphorylation of Cdc5 may inhibit its function in mitotic exit. My observation that DDK phosphorylates Cdc5 *in vitro* and determination of putative sites of phosphorylation suggest this may be the possible (Figure 8D & Figure 21). The identification of DDK phosphorylation sites using mass spectrometry will allow us to test the model that Cdc7-Dbf4 phosphorylates Cdc5 to inhibit PBD function and would provide a marker to assess the temporal and contextual regulation of Cdc5 by Dbf4. In addition, although my initial studies have focused on phosphorylation of the Cdc5 PBD, Cdc5 residues outside this region might also be targets of DDK phosphorylation.

Cdc5 influences Cdc14 function at multiple mitotic stages. In this work, we provide strong evidence that Dbf4 regulates Cdc5-dependent release of Cdc14, independent of Bfa1-Bub2 MEN signaling. I identified that Cdc5 localization to SPBs (Figure 12B), the site of Bfa1-Bub2, is unperturbed in *dbf4-NΔ109* cells and provided genetic evidence for additional regulation of Cdc5 in *bub2Δ*

(hyperactive MEN) mutants (Figure 15). This raises the possibility that Dbf4-dependent regulation of Cdc14 may act through an alternative pathway/s.

While this dissertation was in preparation, it was proposed that Cdc5-dependent regulation of the FEAR pathway occurs through inhibition of Swe1 (Wee1 homolog), resulting in enhanced CDK activity and promoting Cdc14 release through dissociation with its inhibitor Net1 [12]. CDK activity is essential to activate Cdc14 during mitotic exit as well, presumably by maintaining Net1 in an inhibitory state. This raises the possibility that Dbf4 may regulate Cdc5-dependent mitotic exit through inhibition of Net1. Interestingly, Cdc55-PP2A is thought to directly antagonize CDK in this function [13]. Deletion of *CDC55* results in rebudding in the presence of spindle poisons, although to a much lesser extent than deletion of *BUB2* (Figure 16 & [14]). Our observation that *dbf4-NΔ109* failed to advance rebudding in *cdc55Δ* cells is consistent with a model whereby Dbf4 could inhibit Cdc5-dependent Net1 phosphorylation (Figure 16), however studies that Cdc5 also interacts with additional members of the FEAR network including Seperase and Slk19 implies that our current understanding of Cdc5 function in the FEAR remains incomplete [15].

Another untested hypothesis is the possibility that Dbf4 directly inhibits the Cdc5 interaction with Cdc14. A recent report suggests that Cdc14 and Cdc5 interact directly via the polo-box domain, possibly to modulate Cdc14 activity directly [15]. This model would be consistent with our previous data suggesting that Dbf4 regulates Cdc5 independent of Bfa1-Bub2. Although the significance of the Cdc14-Cdc5 interaction remains to be established, it raises the possibility

that inhibition of the Cdc5 PBD by DDK phosphorylation might prevent association of Cdc5 with Cdc14 and direct activation of the phosphatase in mitosis.

Our lab's initial discovery that deletion of the Dbf4 1-109aa region leads to complete loss of both the Cdc5 interaction and Dbf4 phosphorylation in response to HU treatment, raised the possibility that Rad53 might regulate Dbf4-dependent inhibition of Cdc5, possibly in response to DNA damage [5]. This hypothesis was complicated by the observation that *dbf4-NΔ109* cells exhibit no growth phenotypes, compared to WT cells, in response to DNA damaging agents or spindle poisons [5]. These data combined with our *cdc5-1* suppression experiments (Figure 10) suggest that Dbf4 regulates Cdc5 activity during every cell cycle, not just in response to checkpoint activation. Still, several published reports suggesting a direct interaction between Rad53 and Dbf4 and Rad53-dependent regulation of Cdc5 in response to DNA damage leave open the possibility that Rad53 and/or other checkpoint proteins may enhance Dbf4 regulation of Cdc5 to coordinate events in S-phase with progression through mitosis [16, 17]. These studies will provide an important basis for future interrogation of the precise mechanisms of Cdc5 regulation and provide a broader framework for our understanding of Cdc7-Dbf4 in the cell cycle.

REFERENCES

1. Culotti, J., and Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division. Exp Cell Res *67*, 389-401.

2. Johnston, L.H., and Thomas, A.P. (1982). A further two mutants defective in initiation of the S phase in the yeast Saccharomyces cerevisiae. Mol Gen Genet *186*, 445-448.

3. Takeda, T., Ogino, K., Matsui, E., Cho, M.K., Kumagai, H., Miyake, T., Arai, K., and Masai, H. (1999). A fission yeast gene, him1(+)/dfp1(+), encoding a regulatory subunit for Hsk1 kinase, plays essential roles in S-phase initiation as well as in S-phase checkpoint control and recovery from DNA damage. Mol Cell Biol *19*, 5535-5547.

4. Ogino, K., Takeda, T., Matsui, E., Iiyama, H., Taniyama, C., Arai, K., and Masai, H. (2001). Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem *276*, 31376-31387.

5. Gabrielse, C., Miller, C.T., McConnell, K.H., Deward, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCT-like domain required for the response to replication fork arrest in budding yeast. Genetics.

6. Harkins, V., Gabrielse, C., Haste, L., and Weinreich, M. (2009). Budding Yeast Dbf4 Sequences Required for Cdc7 Kinase Activation and Identification of a Functional Relationship Between the Dbf4 and Rev1 BRCT Domains. Genetics.

7. Miller, C.T., Gabrielse, C., Chen, Y.C., and Weinreich, M. (2009). Cdc7p-dbf4p regulates mitotic exit by inhibiting Polo kinase. PLoS Genet *5*, e1000498.

8. Hardy, C.F., and Pautz, A. (1996). A novel role for Cdc5p in DNA replication. Mol Cell Biol *16*, 6775-6782.

9. Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

10. Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

11. Cheng, K.Y., Lowe, E.D., Sinclair, J., Nigg, E.A., and Johnson, L.N. (2003). The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. Embo J *22*, 5757-5768.
12. Liang, F., Jin, F., Liu, H., and Wang, Y. (2009). The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. Mol Biol Cell *20*, 3671-3679.

13. Queralt, E., Lehane, C., Novak, B., and Uhlmann, F. (2006). Downregulation of PP2A(Cdc55) phosphatase by separase initiates mitotic exit in budding yeast. Cell *125*, 719-732.

14. Wang, Y., and Ng, T.Y. (2006). Phosphatase 2A negatively regulates mitotic exit in Saccharomyces cerevisiae. Mol Biol Cell *17*, 80-89.

15. Rahal, R., and Amon, A. (2008). The Polo-like kinase Cdc5 interacts with FEAR network components and Cdc14. Cell Cycle *7*, 3262-3272.

16. Duncker, B.P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S.M. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proc Natl Acad Sci U S A *99*, 16087-16092.

17. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S.J. (1999). Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science *286*, 1166-1171.

APPENDIX

An ectopic NLS sequence on *dbf4-NΔ221* partially rescues cell growth.

Coincident with experiments investigating the functional role of Dbf4 in regulating Cdc5 function, members of the Weinreich lab were completing a detailed structure-function analysis of the Dbf4 N-terminus [1]. Recent evidence suggested a role for the BRCT-like region (encoding motif N) in fission yeast Dfp1/Dbf4 in maintaining cell viability during replication and DNA damage stress [2]. Building on this foundation, our lab identified additional sequences within Dbf4 proteins that differed significantly from other BRCT containing proteins and significantly affected Dbf4 function during replication stress [1].

To assist in bringing this project to completion, I conducted several experiments to help solidify our understanding of the role Dbf4 N-terminal sequences contribute to normal Dbf4-Cdc7 function. Deletion of the Dbf4 N-terminal 221 amino acids caused significant delays in S-phase entry and replication [1]. To analyze the growth of this and other N-terminal Dbf4 mutants, strains were grown overnight to saturation and released into fresh YPD at low density (Figure A1). I recorded culture densities every hour following growth at 25 degrees Celsius. Deletion of N-terminal Dbf4 amino acids had a significant affect on cell growth compared to WT, *dbf4-NΔ65* and *dbf4-NΔ109* mutants. Previous experiments demonstrated S-phase initiation and progression defects in *dbf4-NΔ221* mutants [1]. Addition of an ectopic NLS to *dbf4-NLS-NΔ221* mutants partially overcame these defects and showed significant improvement in growth

suggesting that the Dbf4 BRCT domain did not affect Dbf4-Cdc7 replication activity.

Initiation mutants show broad sensitivity to DNA damaging agents.

Decreased origin activity and prolonged S-phase was a possible explanation for *dbf4-NΔ221* sensitivity to a broad spectrum of DNA damaging agents. I compared the DNA damage phenotypes of several initiation mutants including *cdc7-1*, *dbf4-1*, *cdc6-1*, *cdc46-1* (mutant *mcm5*) and *cdc47-1* (mutant *mcm7*) in addition to the *dbf4-NΔ221* strain (Figure A2). Mutants were grown overnight and spotted at ten-fold serial dilutions on YPD with or without MMS, bleomycin or hydroxyurea and grown for 3 days at 25 degrees Celsius. All replication mutants were sensitive to MMS, suggesting that a decrease in origin efficiency may be responsible for this defect. The *dbf4-NΔ221* mutant was sensitive to bleomycin and HU treatment and other mutants demonstrated varying phenotypes when grown on these compounds (Figure A2). Appending an ectopic NLS to the *dbf4-NΔ221* mutant in an attempt to eliminate replication defects eliminated the sensitivity to MMS and bleomycin and suggested that the primary function of the BRCT region is response to replication stress (HU treatment) [1].

Dbf4 regulates Rad53 expression.

Deletion of the Dbf4 N-terminal 221 amino acids increased HU sensitivity in cells raising the possibility that Dbf4 was inhibiting checkpoint function during replication stress. Experiments by other lab members, however, found that

cellular checkpoints, including Rad53, were not affected by this deletion [1]. Rad53 activation is accompanied by a characteristic gel mobility shift in the presence of HU. To determine whether this shift was present in Dbf4 mutants, asynchronous cultures of WT, *dbf4-NΔ109 and dbf4-NΔ221* cells were incubated in the presence of HU for 0, 1.5 and 3 hours. Rad53-HA activation was examined by gel mobility shift (1.5 and 3 hours) after probing with anti-HA antibodies. As expected, incubation of HU still resulted in a Rad53 mobility shift in both mutants (Figure A3). More surprisingly, I observed decreased expression of Rad53 in the *dbf4-NΔ221* mutant. Additional experiments by other lab members demonstrated that decreased Rad53 expression was independent of BRCT deletion and likely resulted from decreased overall Dbf4 activity. The significance of this finding remains uncertain, but raises the possibility that Dbf4 may counteract Rad53 activity during checkpoint activation.

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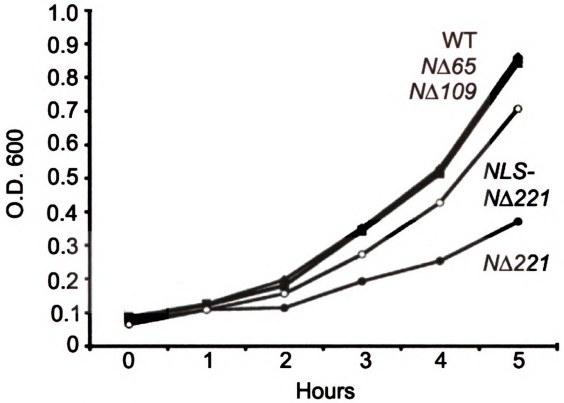
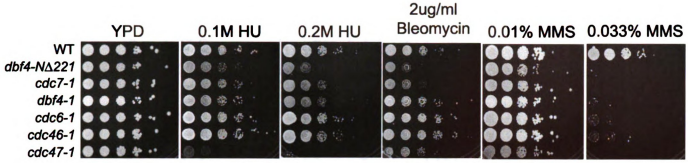


Figure A1. Growth curve of N-terminal Dbf4 mutants. WT (W303-1A), *dbf4-NΔ65* (M1642), *dbf4-NΔ109* (M1261), *dbf4-NΔ221* (M1356) *and dbf4-NLS-NΔ221* (M1830) yeast strains were grown to saturation overnight then diluted in fresh YPD at equivalent optical densities. Strains were grown at 25 degrees Celsius and OD measurements recorded every hour.

Figure A2. Sensitivity of initiation mutants to DNA damaging agents. WT (W303-1A), *dbf4-NΔ221* (M1356), *cdc7-1* (M199), *dbf4-1* (M361), *cdc6-1* (M378), *cdc46-1* (M323) and *cdc47-1* (M317) yeast strains were spotted at ten-fold serial dilution on YPD with or without DNA damaging agents and incubated for 3 days at 25 degrees Celsius.


YPD
0.1M HU
0.2M HU
2ug/ml Bleomycin
0.01% MMS
0.033% MMS
WT
dbf4-NΔ221
cdc7-1
dbf4-1
cdc6-1
cdc46-1
cdc47-1

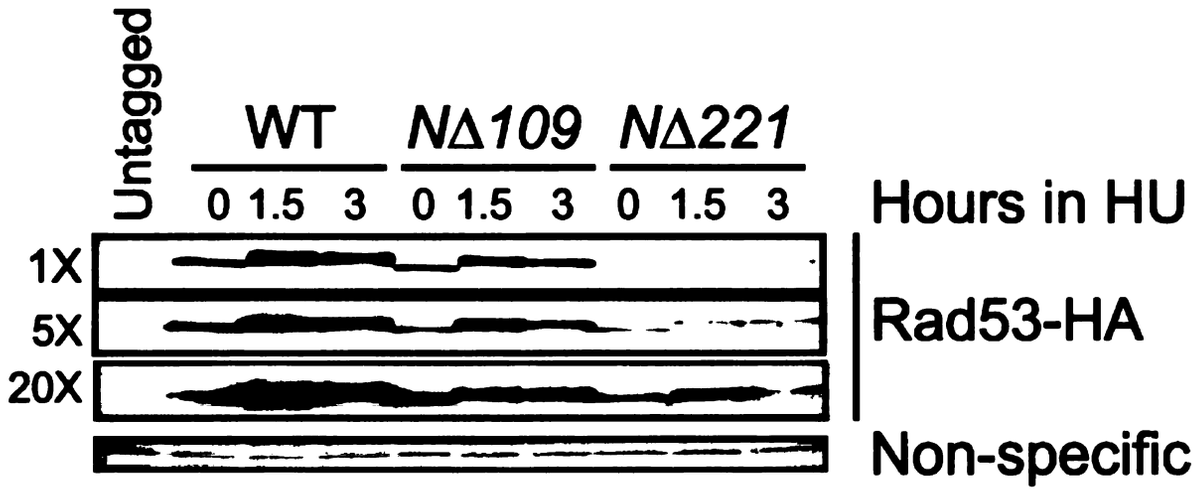


Figure A3. Dbf4 regulates Rad53 expression. Asynchronous cultures of WT Rad53-HA (M1763), *dbf4-NΔ109* Rad53-HA (M1792) and *dbf4-NΔ221* Rad53-HA (M1763) indicated strains were grown in the YPD + 0.2M HU for 0-3 hours at 30 degrees Celsius. Protein extracts were probed with anti-HA antibody and exposed for increasing times (1X, 5X, 20X) to assess Rad53 expression.

REFERENCES

1. Gabrielse, C., Miller, C.T., McConnell, K.H., Deward, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCT-like domain required for the response to replication fork arrest in budding yeast. Genetics.

2. Ogino, K., Takeda, T., Matsui, E., Iiyama, H., Taniyama, C., Arai, K., and Masai, H. (2001). Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem *276*, 31376-31387.

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