

Regulation of the DNA Damage Response and Spindle Checkpoint Signaling Pathways

by

Kristen Marie Foss

Department of Pharmacology and Cancer Biology
Duke University

Date: _____

Approved:

Sally Kornbluth, Supervisor

Gerard Blobel

Jeffrey Rathmell

Neil Spector

Xiao-Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
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ABSTRACT

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Abstract

The ultimate goal of any living cell is to pass on a complete, unaltered copy of its DNA to its daughter cell. The DNA damage response (DDR) and spindle checkpoint are two essential signaling pathways that make it possible for a cell to achieve this goal. The DDR protects genetic integrity by sensing errors in the DNA sequence and activating signaling pathways to arrest the cell cycle and repair the DNA. The spindle checkpoint protects chromosomal integrity by preventing the separation of chromosomes during mitosis until all chromosomes are correctly attached to the mitotic spindle. Proper regulation of both the DDR and the spindle checkpoint is critical for cell survival. In this dissertation I will describe our discovery of novel regulatory mechanisms involved in each of these signaling networks.

The first research chapter of this dissertation describes our findings concerning how the DDR regulates cyclin F levels. Cyclin F is an F-box protein that associates with the SCF E3 ubiquitin ligase complex to target proteins for degradation. In response to DNA damage, cyclin F levels are downregulated to facilitate increased dNTP production for efficient DNA repair, but the molecular mechanisms regulating this downregulation of cyclin F are largely unknown. We discovered that cyclin F downregulation by the DDR is the combined result of increased protein degradation and decreased mRNA expression. At the level of protein regulation, cyclin F is targeted for proteasomal

degradation by the SCF complex. Interestingly, we found that the half-life of cyclin F protein is significantly increased in cells treated with the phosphatase inhibitor calyculin A, which caused cyclin F to be hyper-phosphorylated. Calyculin A also partially prevented cyclin F downregulation following DNA damage. This result suggests that cyclin F phosphorylation stabilizes the protein, and dephosphorylation of cyclin F may be required for its degradation in both unperturbed and DNA damaged cells. We also found that cyclin F downregulation is dependent on the Chk1 kinase, which is predominately activated by the ATR kinase. In examining the mechanism by which Chk1 promotes cyclin F downregulation, we determined that Chk1 represses cyclin F transcription. Lastly, we investigated the role of cyclin F in cell cycle regulation and discovered that both increased and decreased cyclin F expression delay mitotic entry, indicating that an optimal level of cyclin F expression is critical for proper cell cycle progression.

The second research chapter of this dissertation details our discovery of the requirement for phosphatase activity to inhibit the APC/C E3 ubiquitin ligase during the spindle checkpoint. Early in mitosis, the mitotic checkpoint complex (MCC) inactivates the APC/C until the chromosomes are properly aligned and attached to the mitotic spindle at metaphase. Once all the chromosomes are properly attached to the spindle, the MCC dissociates, and the APC/C targets cyclin B and securin for degradation so that the cell progresses into anaphase. While phosphorylation is known to drive many of the

events during the checkpoint, the precise molecular mechanisms regulating spindle checkpoint maintenance and inactivation are still poorly understood. In our studies, we sought to determine the role of mitotic phosphatases during the spindle checkpoint. To address this question, we treated spindle checkpoint-arrested cells with various phosphatase inhibitors and examined their effect on the MCC and APC/C activation. Using this approach we found that two phosphatase inhibitors, calyculin A and okadaic acid (1 μ M), caused MCC dissociation and APC/C activation in spindle checkpoint-arrested cells. Although the cells were able to degrade cyclin B, they did not exit mitosis as evidenced by high levels of Cdk1 substrate phosphorylation and chromosome condensation. Our results provide the first evidence that phosphatases are essential for maintenance of the MCC during operation of the spindle checkpoint.

Dedication

This dissertation is lovingly dedicated to my family for all of their love and support. To my dad, who lost his battle with cancer far too early in life; he showed me how to live life to the fullest and never lose hope. To my mom, who taught me patience and humility. To my sister, Jackie, who reminds me what is truly important in life. To my sister, Sara, who was my exercise and baking partner through it all. Finally, to my husband, Kent, who always believes in me and makes every day more fun.

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List of Abbreviations

53BP1	p53 binding protein
APC/C	Anaphase promoting complex/cyclosome
ARPP-19	Cyclic AMP-regulated 19 kDa phosphoprotein
ATP	Adenosine triphosphate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
β -TrCP	β -Transducin repeat containing protein
BRCA1	Breast cancer 1, early onset
Bub1	Budding uninhibited by benzimidazole 1
Bub3	Budding uninhibited by benzimidazole 3
BubR1	Budding uninhibited by benzimidazole-related
CDC20	Cell division cycle 20
CDC25	Cell division cycle 25
CDH1	CDC20 homologue 1
CDK	Cyclin-dependent kinase
CE	Cell extract
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2

CHX	Cycloheximide
CIP/KIP	CDK interacting protein/Kinase inhibitory protein
CKI	Cyclin-dependent kinase inhibitor
CPT	Cisplatin
Cul1	Cullin-1
DDR	DNA damage response
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
dNTP	Deoxyribonucleotide
Dox	Doxycycline
DRB	Doxorubicin
DSB	DNA double strand break
Emi1	Early mitotic inhibitor 1
ENSA	α -endosulfine
Etop	Etoposide
FBXW7	F-box/WD repeat-containing protein 7
GFP	Green Fluorescent Protein
GST	Glutathione S-transferase
Gwl	Greatwall kinase
HR	Homologous recombination
HU	Hydroxyurea

INK4	Inhibitor of CDK4
IP	Immunoprecipitation
KNL1	Kinetochore null protein 1
Luc	Luciferase
Mad1	Mitotic arrest deficient 1
Mad2	Mitotic arrest deficient 2
MCC	Mitotic checkpoint complex
MDC1	Mediator of DNA damage checkpoint 1
MDM2	Murine double minute 2
MEFs	Mouse embryonic fibroblasts
MIS12	Missegregation 12
MPS1	Monopolar spindle 1 kinase
MRN	Mre11-Rad50-Nbs1
NAE	NEDD8-activating enzyme
NCS	Neocarzinostatin
NDC80	Nuclear division cycle 80
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
Noc	Nocodazole
OA	Okadaic acid

PCR	Polymerase chain reaction
Plk1	Polo-like kinase 1
PPM	Protein phosphatase Mg ²⁺ -dependent
PPP	Phosphoprotein phosphatase
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B (calcineurin)
PP4	Protein phosphatase 4
PP5	Protein phosphatase 5
PP6	Protein phosphatase 6
PP7	Protein phosphatase 7
pRB	Retinoblastoma protein
qPCR	Quantitative polymerase chain reaction
Rbx1	RING-box protein 1
RFC	Replication factor C
RNR	Ribonucleotide reductase
RPA	Replication protein A
RRM1	Ribonucleotide reductase M2
RRM2	Ribonucleotide reductase M1
SCF	Skp1/Cul1/F-box protein

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
ssDNA	Single-stranded DNA
TOPBP1	Topoisomerase binding protein 1
UBA	Ubiquitin-activating enzyme
UBC	Ubiquitin-conjugating enzyme
UV	Ultraviolet
WT	Wild-type

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provided the mass spectrometry analysis and helpful discussion in designing experiments.

1. Introduction

1.1 *The cell cycle*

The cell cycle is the process whereby one cell grows and divides into two daughter cells. The primary goal of the cell cycle is to ensure accurate replication and division of the DNA such that each daughter cell inherits an identical and complete copy. In eukaryotic cells, this process, which is also known as the cell division cycle, consists of a series of three highly ordered events: interphase, mitosis, and cytokinesis (Figure 1.1). Interphase can be further divided into G1 (Gap 1), S (DNA Synthesis), and G2 (Gap 2) phases.¹ During the G1 phase, organelles are replicated and the DNA is prepared for replication. G1 is a preparatory phase of the cell cycle during which crucial cell fate decisions are determined.² During this phase, the cell uses several molecular mechanisms to sense whether the external environment is favorable for cell division. The cell also receives internal signals that indicate if the cell has reached a large enough size and if all damaged DNA has been repaired before committing to cell division.¹ If these conditions are satisfied, the cell will progress from G1 into S phase and begin the process of DNA replication. Alternatively, if the cell does not receive signals indicating that it should undergo cell division, it will exit the cell cycle and enter G0 phase, which is a resting, quiescent state. Once all of the DNA has been replicated during S phase, the

cell enters G2 phase, which consists of additional cell growth, repairing any DNA damage that may have occurred during replication, and preparing for mitosis.¹

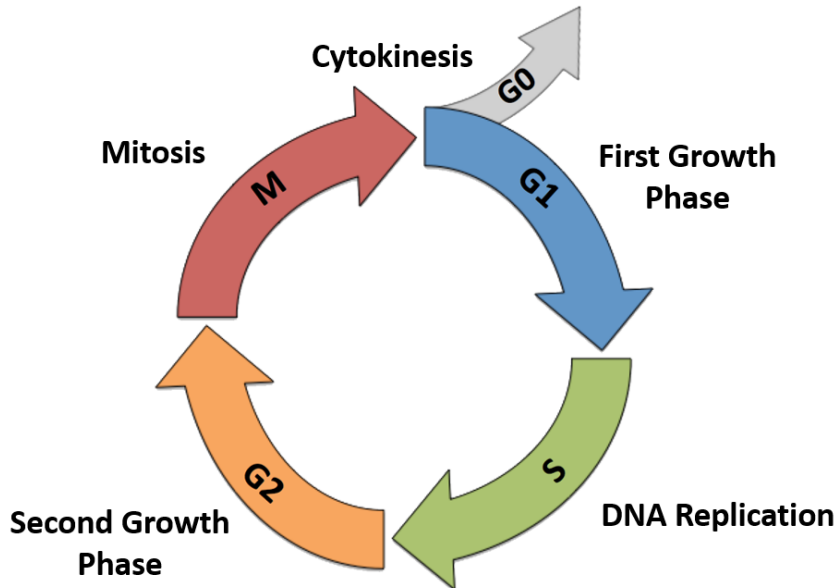


Figure 1.1: The Cell Cycle. The cell cycle is divided into interphase, mitosis, and cytokinesis. Interphase can be further subdivided into G1, S, and G2 phases. During G1 phase, the cell senses whether it should commit to cellular division or leave the cell cycle and enter G0 phase. In S phase, the cell replicates its DNA. During G2 phase, the cell continues to grow and prepare for mitosis, which is when the duplicated chromosomes and the nuclear contents of the cell are divided. Following mitosis, the two daughter cells separate during cytokinesis.

Following the successful completion of G2, the cell enters mitosis. During mitosis, the two copies of the DNA generated during S phase are separated in a series of five stages: prophase, prometaphase, metaphase, anaphase, and telophase (Figure 1.2). In **prophase**, the newly replicated DNA condenses into structures called chromosomes, which consist of two identical sister chromatids joined together at the centromere. The

initial steps of mitotic spindle formation also occur during prophase. Next, the cell progresses into **prometaphase**, during which the nuclear envelope breaks down, the kinetochore protein structures form on the centromeres, and the microtubules of the mitotic spindle interact with the kinetochores to begin to pull the chromosomes into alignment. During **metaphase**, the mitotic spindle pulls the chromosomes back and forth until they are in alignment at the metaphase plate (an imaginary line in the middle of the cell). Once the chromosomes have aligned at the metaphase plate, the cell enters **anaphase**. During anaphase, the sister chromatids are pulled to opposite poles of the cell by the mitotic spindle. The final stage of mitosis is **telophase** when the chromosomes begin to decondense, and nuclear envelopes reform around the two daughter nuclei. Lastly, during cytokinesis the cytoplasm is divided, and the two daughter cells separate from each other.³

Cells receive numerous signals both from within the cell and from the extracellular environment that regulate the process of cell division. Regulatory mechanisms orchestrate the transition from one stage of the cell cycle to the next and are essential to protect the genomic integrity of the cell. If the cell encounters a problem during the cell cycle, for example if the DNA is damaged, the cell cycle will be arrested until the damage has been repaired. Alternatively, if the problem cannot be corrected, the cell will either undergo programmed cell death (apoptosis) or exit the cell cycle and

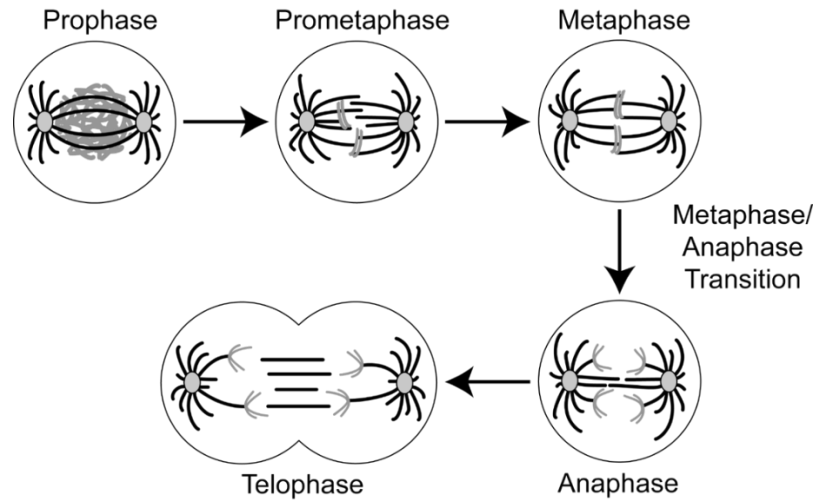


Figure 1.2: The Five Stages of Mitosis. Mitosis can be divided into five stages. (1) In **prophase**, the DNA condenses and the mitotic spindle begins to form. (2) During **prometaphase**, the microtubules of the mitotic spindle make their initial contact with the kinetochore proteins and begin to pull the chromosomes into alignment. (3) At **metaphase**, the chromosomes are aligned at the equator of the cell, the metaphase plate. (4) Once all of the chromosomes are properly attached to the mitotic spindle and aligned at the metaphase plate, the cell passes through the metaphase to anaphase transition and the sister chromatids are pulled apart to opposite poles during **anaphase**. (5) Lastly, during **telophase**, nuclear envelopes form around the two daughter nuclei marking the completion of mitosis.

enter G₀ phase (senescence). There are three designated points in the cell cycle when cell division may be arrested. These time points are called cell cycle checkpoints, and they will be described in section 1.1.3. The checkpoint regulating the metaphase to anaphase transition (the spindle checkpoint) will be described in detail in section 1.2. Defects in cell cycle regulatory mechanisms can impair the ability of the cell to arrest the cell cycle at these checkpoints, which may lead to unrestrained cell division and, ultimately, the development of cancer. The events of the cell cycle are largely coordinated by reversible

phosphorylation changes and irreversible protein degradation. The next two sections will take a closer look at these two modes of regulation.

1.1.1 Cell cycle regulation by phosphorylation

Protein phosphorylation is a type of post-translational modification that involves the addition of a phosphate group to a serine, threonine, or tyrosine residue. This modification can regulate protein stability, localization, function, or interaction with other proteins. Kinases are proteins that add phosphate groups to proteins (phosphorylation), and conversely, phosphatases remove phosphate groups (dephosphorylation). Many kinases and phosphatases are involved in regulating the cell cycle, but the cyclin-dependent kinases (CDKs), in partnership with cyclin proteins, are the primary drivers.¹ There are 21 CDKs encoded in the human genome, seven of which have been implicated directly in cell cycle regulation.⁴ CDKs become active only when bound to cyclins, which are the CDK regulatory subunits. Cyclin protein levels oscillate throughout the cell cycle to regulate CDK activation.^{5,6} Therefore, different cyclin/CDK complexes regulate cell cycle progression at different cell cycle phases (Figure 1.3).

Cyclins D, E, and A in partnership with CDK2, CDK4, or CDK6 are important regulators of interphase cell cycle transitions. Cyclin D levels begin to increase early in G1, which drives the activation of its partner CDKs, CDK4 and CDK6.⁴ As the cell transitions through G1, cyclin E expression increases, and cyclin E/CDK2 triggers entry

into S-phase.^{4,7} Early in S phase, cyclin A binds and activates CDK2 to promote DNA replication.^{7,8} In G2 phase, cyclin A interacts with CDK1 to promote the transition into mitosis.⁷ The primary mitotic cyclin is cyclin B, which regulates the G2/M transition and progression through mitosis by binding and activating CDK1. Cyclin degradation via the ubiquitin-proteasome degradation pathway inactivates their partner CDKs and is crucial for proper cell cycle progression. For example, cyclin B degradation, which occurs when the cell transitions from metaphase to anaphase, causes decreased CDK1

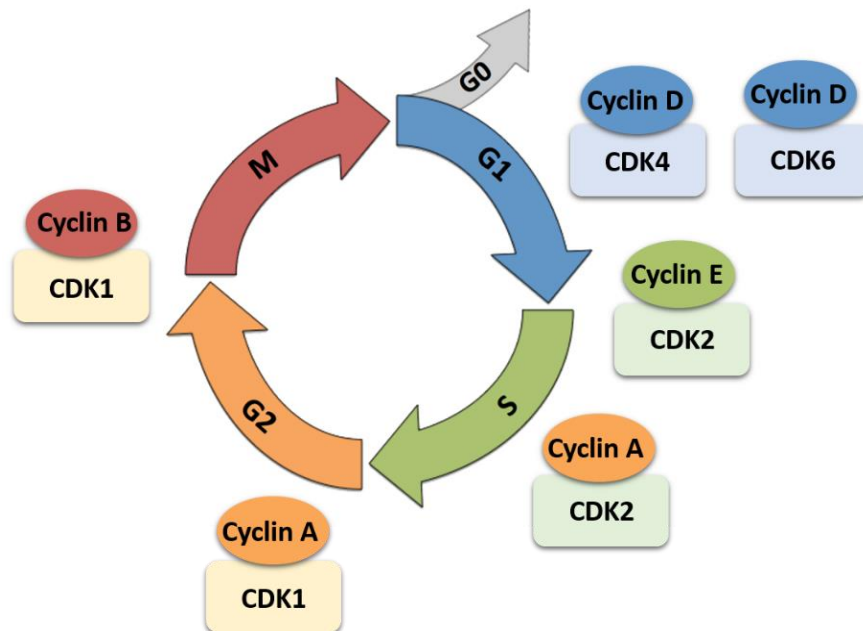


Figure 1.3: Cyclins and Cyclin-Dependent Kinases. Cyclin/CDK complexes drive the progression through the different phases of the cell cycle by phosphorylating important regulatory proteins. In G1 phase, cyclin D binds to CDK4 and CDK6, which promotes cyclin E expression. CDK2 is activated by increasing cyclin E levels, which drives entry into S phase. Cyclin E is replaced by cyclin A early in S phase to maintain CDK2 activity and initiate DNA replication. Beginning in late S-phase, cyclin A also binds to CDK1 to help promote mitotic entry along with cyclin B/CDK1.

activity, which is required for mitotic exit.

In addition to changes in cyclin B levels, mitotic progression is also regulated by inhibitory phosphorylation on CDK1 by the kinases Myt1 and Wee1, which keeps cyclin B/CDK1 inactive during interphase.^{7,9} The inhibitory CDK1 phosphorylation sites are dephosphorylated by the Cdc25 (cell division cycle 25) phosphatase to activate CDK1 and promote mitotic entry.⁷ Positive feedback loops involving cyclin B/CDK1 activating and inactivating phosphorylation of Cdc25 and Wee1, respectively, ensure that entry into mitosis happens rapidly and irreversibly.

CDKs are also regulated by a class of proteins known as cyclin dependent kinase inhibitors (CKIs). CKIs can be divided into two different families, the INK4 (Inhibitor of CDK4) family and the CIP/KIP (CDK interacting protein/Kinase inhibitory protein) family.^{7,10} The INK4 family contains four members (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19^{INK4d}), and they only inhibit the cyclin D-dependent CDKs, CDK4 and CDK6.¹¹ The CIP/KIP family consists of p21^{CIP1/WAF1}, p27^{KIP1}, p57^{KIP2}, and these proteins can bind to all of the CDKs.¹¹ Interestingly, while the CIP/KIP proteins negatively regulate cyclin E/CDK2, cyclin A/CDK2, and cyclin B/CDK1, they actually promote the assembly of cyclin D/CDK4 and cyclin D/CDK6 complexes in early G1.¹² When cyclin D levels rise during G1, CIP/KIP proteins are recruited to cyclin D/CDK4 and cyclin D/CDK6 and away from cyclin E/CDK2 and cyclin A/CDK2, which allows for the activation of the latter

complexes.¹² Conversely, INK4 family members bind and inhibit CDK4/6 by preventing its interaction with cyclin D.¹³ Cell cycle regulation by CKIs is imperative for preventing uncontrolled cell division. Accordingly, it is not surprising that the INK4 gene is often deleted in many types of cancers.¹¹⁻¹³

Two other essential families of kinases involved in cell cycle regulation are the Aurora kinases and the Polo-like kinases (Plks). These kinases are primarily involved in safeguarding fidelity during cell division, while the CDKs facilitate driving the cell cycle forward. Plks are involved in multiple stages of the cell cycle, including mitotic spindle formation and chromosome segregation.¹⁴ Aurora A localizes to the centrosomes during mitosis where it is essential for proper centrosome maturation, mitotic spindle formation, and cytokinesis.¹⁵ Aurora B localizes at the centromeres of chromosomes where it is required for proper binding of the mitotic spindle to the kinetochores. Similar to Aurora A, Aurora B is essential for cytokinesis.¹⁵

Phosphatases are also important regulators of the cell cycle. Most notably, protein phosphatase 1 (PP1) and protein phosphatase 2A in complex with its B55 δ regulatory subunit (PP2A-B55 δ) are essential for mitotic exit as they dephosphorylate numerous cyclin B/CDK1 mitotic substrates.¹⁶⁻¹⁸ Importantly, entry into mitosis relies not only on kinase activation but also on the inhibition of the counteracting phosphatases.¹⁹ This phosphatase inhibition originates from another kinase known as Greatwall (Gwl)

kinase, which indirectly inhibits PP2A-B55 δ to allow for mitotic entry.¹⁹⁻²¹ Gwl is activated by increased cyclin B/CDK1 activity at the G2/M transition. Active Gwl phosphorylates two important substrates, cyclic AMP-regulated 19 kDa phosphoprotein (ARPP-19) and α -endosulfine (ENSA), which converts these proteins into potent inhibitors of PP2A-B55 δ .^{19,21} The inhibition of PP2A-B55 δ allows for the rapid accumulation of phosphorylated cyclin B/CDK1 mitotic substrates, which drive the cell into mitosis.¹⁹ In addition to their essential function in mitotic exit, PP1 and PP2A also dephosphorylate key proteins to promote spindle checkpoint silencing and sister chromatid separation, which will be discussed in section 1.2.4.^{22,23}

1.1.2 Cell cycle regulation by the ubiquitin proteasome pathway

In addition to changes in phosphorylation, the cell cycle is also regulated by irreversible proteolysis of important regulatory proteins, most notably the cyclin proteins and the CKIs.²⁴ This system of protein degradation is mediated by ubiquitination, which is a second type of post-translational modification. Ubiquitin is a small protein that is covalently attached to lysine residues on target proteins through the coordinating actions of three classes of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Figure 1.4). The ubiquitin protein is first activated by binding to the E1 enzyme via an ATP dependent process. Next, the ubiquitin is transferred from the E1 to the E2 enzyme. Finally, the E3 ubiquitin

ligase catalyzes the transfer of the ubiquitin from the E2 enzyme to the target protein.²⁴ While there are very few known E1 and E2 enzymes, there are estimated to be between 500 and 1000 different human E3 ubiquitin ligases.²⁴ As the E3 ubiquitin ligase recognizes the substrate for ubiquitination, this large number of E3 ligases conveys specificity to the ubiquitin-conjugating system. Once attached to a protein, ubiquitin can affect a protein in several different ways, but the most well characterized type of

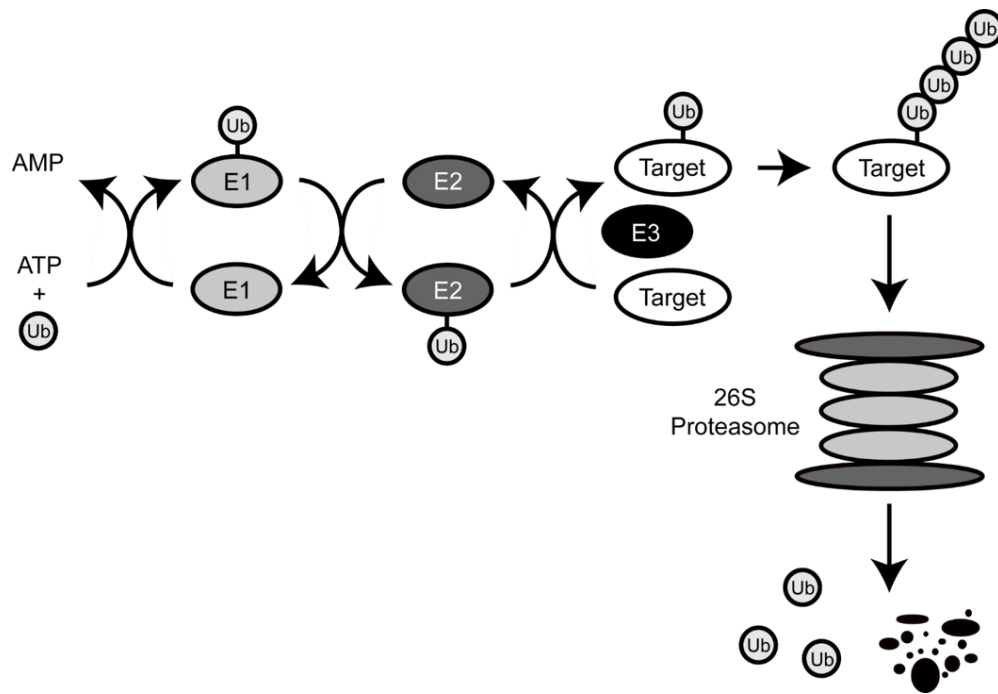


Figure 1.4: The Ubiquitin Proteasome Degradation Pathway. The process of targeting a protein for degradation via the ubiquitin proteasome degradation pathway begins with ubiquitin activation, which is an ATP dependent process mediated by an E1 ubiquitin-activating enzyme. The ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme, before it is ultimately transferred to the target protein by an E3 ubiquitin ligase. This process may be repeated several times to generate polyubiquitin chains, which target the protein for degradation by the 26S proteasome.

ubiquitin modification, Lys 48 polyubiquitination, targets the protein for degradation via the 26S proteasome.²⁵

The two most prominent E3 ligases involved in regulating the cell cycle are the Anaphase Promoting Complex/Cyclosome (APC/C) and the SCF complex (Skp1-Cullin-1-F-Box protein). The APC/C functions primarily during mitosis and G1, whereas the SCF E3 ubiquitin ligase complex targets proteins for degradation during G1, S, and early mitosis.^{25,26} The APC/C is a large multi-protein complex composed of at least 11 core subunits.²⁶ Similar to CDK activation, the APC/C requires the binding of an additional regulatory protein to become active. Cell division cycle 20 (CDC20) and CDC20 homologue 1 (CDH1) are the two coactivators of the APC/C, and they target specific substrates for degradation by recognizing KEN box and destruction box (D box) protein sequences, respectively.²⁷

CDC20 and CDH1 interaction with the APC/C varies throughout the cell cycle to regulate APC/C activity.²⁸ During mitosis, APC/C^{CDC20} is activated once all sister chromatids are properly attached to the mitotic spindle at the end of metaphase.²⁹ Prior to this occurrence, the APC/C is inhibited by the spindle checkpoint, which will be discussed in detail in section 1.2. Of note, the APC/C is able to recognize certain substrates, such as cyclin A, prior to the metaphase to anaphase transition.³⁰ This ability of the APC/C to target cyclin A for degradation while the spindle checkpoint is active is

not completely understood but is thought to be due in part to the high affinity between cyclin A and the APC/C.³⁰ Once the spindle checkpoint is satisfied, APC/C^{CDC20} triggers entry into anaphase by targeting cyclin B and securin for degradation, which leads to CDK1 inactivation and sister chromatid separation, respectively.²⁹ As CDC20 interaction with the APC/C is dependent on cyclin B/CDK1 activity, when cyclin B/CDK1 activity declines during anaphase due to cyclin B degradation, CDC20 is no longer able to bind the APC/C.³¹ At this point in late mitosis, CDH1 takes over for CDC20 and promotes mitotic exit by continuing to target cyclin B for degradation, along with other late mitotic substrates.³¹ APC/C^{CDH1} remains active during G1 and prevents premature entry into S phase by ubiquitinating substrates such as cyclins A and B and one of the SCF F-box proteins, S-phase kinase-associated protein 2 (Skp2).¹⁶ Prior to entry into S-phase, APC/C^{CDH1} must be inactivated. This inactivation is mediated by several different mechanisms including inhibitory phosphorylation on CDH1, SCF targeting of CDH1 for degradation, and APC/C^{CDH1} mediated ubiquitination of its E2 ubiquitin-conjugating enzyme.^{16,32} Also, in late G1, the E2F1 transcription factor upregulates the APC/C inhibitor Early mitotic inhibitor 1 (Emi1), which inactivates APC/C^{CDH1} activity by acting as a pseudo-substrate.^{16,26} Following the inactivation of APC/C^{CDH1} at S phase, the APC/C remains inactive until the next round of mitosis when cyclin B/CDK1 activity activates APC/C^{CDC20}.

The SCF complex is much smaller than the APC/C, consisting only of RING-box protein 1 (Rbx1), S-phase kinase-associated protein 1 (Skp1), Cullin-1 (Cul1), and one of a large family of substrate binding proteins known as F-box proteins (Figure 1.5).³³ Rbx1 contains a RING finger domain, which is a small zinc binding domain that recruits the E2 ubiquitin-conjugating enzyme. The structural protein Cul1 forms a scaffold that links Rbx1 and Skp1. An important requirement for SCF activation is the addition of the small ubiquitin-like protein NEDD8 to the Cul1 subunit.³⁴ This neddylation reaction is initiated by the NEDD8-activating enzyme (NAE). De-neddylation of Cul1 causes dissociation of the SCF complex.³⁴

The F-box protein conveys substrate specificity to the SCF complex; it binds to the substrate independently of the rest of the complex and is then recognized by Skp1 to bring the target protein into close proximity with the E2 ubiquitin-conjugating enzyme. Most F-box proteins recognize phosphorylation sites on their substrates, which presents an additional layer of regulation.³⁵ There are over 60 known human F-box proteins, each capable of recognizing and targeting different substrates for degradation.³⁶ The two most well-known F-box proteins are Skp2 and β -TrCP (β -Transducin Repeat Containing Protein), and both have important roles in cell cycle regulation. SCF^{Skp2} promotes CDK activation by recognizing and targeting CKIs, such as p27 and p21, for degradation thereby allowing cells to enter S-phase.³⁶⁻³⁹ SCF ^{β -TrCP} has both positive and negative

regulatory effects on the cell cycle. By inducing the degradation of phosphorylated Wee1, which is the kinase responsible for inhibiting cyclin B/CDK1, SCF^{β-TrCP} promotes CDK1 activity and mitotic progression at G2/M.^{16,36} Conversely, SCF^{β-TrCP} impedes CDK activity by targeting Cdc25A for degradation; Cdc25A is phosphorylated by the checkpoint kinase Chk1 to target it for degradation by SCF^{β-TrCP} both during S-phase and following DNA damage.^{16,40} SCF^{β-TrCP} also regulates APC/C^{CDC20} activation by targeting the APC/C inhibitor Emi1 for degradation following CDK1 and Plk1 phosphorylation of Emi1 during prometaphase.^{16,36,41} Another F-box protein that appears to play a role in

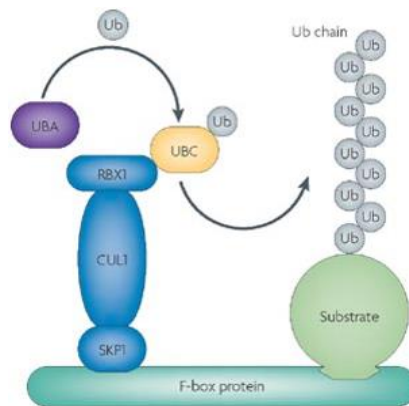


Figure 1.5: The SCF E3 Ubiquitin Ligase Complex. The SCF E3 ubiquitin ligase complex targets proteins for degradation via the ubiquitin proteasome pathway. The SCF complex is composed of four proteins: Rbx1, Cul1, Skp1, and an F-box protein. The F-box protein recruits the protein substrate that will be targeted for degradation. Skp1 interacts with the F-box protein to bring the substrate into close proximity with the rest of the complex. Cul1 is a large adaptor protein that links Skp1 and the F-box protein with Rbx1, which recognizes the E2 ubiquitin-conjugating enzyme (UBC) via its RING finger domain. The ubiquitin is transferred from the E1 ubiquitin-activating enzyme (UBA) to the UBC, and then the SCF E3 ubiquitin ligase mediates the transfer of the ubiquitin from the UBC to the substrate. (Adapted from Welcker and Clurman, 2008)

cell cycle regulation is cyclin F, which will be discussed in more detail in section 1.4.

Cyclin F is a member of the cyclin family of proteins, but as opposed to activating CDKs, cyclin F recruits substrates to the SCF complex to target them for proteasomal degradation.

Interestingly, the APC/C and SCF complexes also regulate each other by targeting different regulatory subunits and inhibitors for degradation. As mentioned above, APC/C^{CDH1} targets the SCF F-box protein Skp2 for degradation during G1 thereby stabilizing the CDK inhibitors p21 and p27.²⁵ The SCF complex can activate or inhibit the APC/C at different phases of the cell cycle. Early in mitosis, SCF ^{β -TrCP} targets the APC/C inhibitor Emi1 for degradation to promote APC/C activation. Then during S phase, the SCF complex degrades the APC/C co-activator CDH1 to inactivate the APC/C.³²

1.1.3 Cell cycle checkpoints

Each round of cell division occurs with the end goal of producing two cells, each containing an identical and complete copy of the parental DNA. In order to ensure that this goal is met, there are three primary checkpoints during the cell cycle that allow the cell to detect errors that might prevent the cell from achieving this goal. The checkpoints arrest the cell cycle to allow time for error correction before cell cycle progression resumes. These checkpoints are the G1/S checkpoint, G2/M checkpoint, and the spindle checkpoint (Figure 1.6).

The G1/S checkpoint controls entry into S-phase, and it halts the cell cycle until the environment is favorable for DNA replication and cell division. Once the cell passes this checkpoint, it is committed to undergo cell division. During G1, the transcription repressor pRb binds to the E2F transcription factor, which inhibits progression into S phase. If the environment is favorable for cell division, pRb is phosphorylated by cyclin D/CDK4/6 and cyclin E/CDK2, which causes it to dissociate from E2F. Once E2F is free from pRb, it can induce transcription of numerous S phase-promoting genes. If the

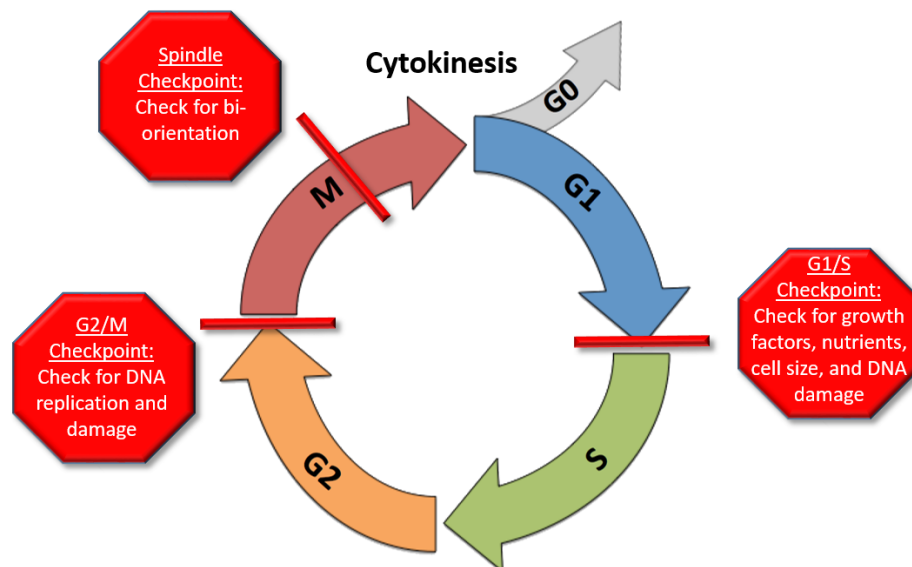


Figure 1.6: Cell Cycle Checkpoints. The cell cycle contains control mechanisms known as checkpoints that prevent the cell from progressing onto the next phase of the cycle until certain requirements are met. The cell may be arrested at the **G1/S checkpoint** if there are insufficient growth factors or nutrients available, the cell size is too small, or if there is any DNA damage. The **G2/M checkpoint** monitors for DNA damage, and it also checks to make sure DNA replication is complete. If the G2/M checkpoint is satisfied, the cell enters mitosis. In mitosis, the **spindle checkpoint** prevents cells from passing into anaphase until all of the chromosomes are properly attached to the mitotic spindle.

environment is unfavorable for cell division, transcription of CDK inhibitors such as INK4, prevent entry into S phase. The cell may then remain in G1 phase until the environment is favorable, or it may enter a quiescent state known as G0.

The G2/M checkpoint prevents entry into mitosis until all of the DNA has been accurately replicated and any DNA damage that may have occurred has been repaired. If any DNA damage is detected, the DNA damage response (DDR) is initiated, which will be discussed in detail in section 1.3. Once the G2/M checkpoint has been satisfied, Plk1 phosphorylates Wee1 to mark it for degradation by the SCF complex, thereby promoting cyclin B/CDK1 activation. Plk1 also phosphorylates and activates Cdc25 to further promote cyclin B/CDK1 activation and mitotic entry. Lastly, the spindle checkpoint prevents entry into anaphase until all of the chromosomes are properly attached to the mitotic spindle. The next section will detail the spindle checkpoint.

1.2 The spindle checkpoint

The spindle checkpoint, also known as the spindle assembly checkpoint or mitotic checkpoint, is activated in early mitosis to protect chromosomal integrity by preventing entry into anaphase until all chromosomes are properly attached to the mitotic spindle. The spindle checkpoint is essential for maintaining genomic stability, as defects in the checkpoint may lead to missegregated chromosomes and aneuploidy, which could contribute to tumorigenesis. Fortunately, the mechanism regulating the

checkpoint is very sensitive and even a single unattached kinetochore can generate the “wait anaphase” signal and arrest the cell cycle. Once the spindle checkpoint is satisfied, the “wait anaphase” signal is reversed, and the cell progresses into anaphase.

1.2.1 Spindle checkpoint activation

During cell division, it is imperative that all chromosomes align at the metaphase plate to ensure that each daughter cell receives only one sister chromatid from each pair when they are separated at anaphase. This alignment and ensuing separation is achieved by the mitotic spindle, a system of microtubules emanating from the centrosomes at the spindle poles. Microtubules generated at opposite spindle poles bind to the kinetochore proteins at the centromere of each sister chromatid and pull the chromosomes toward the poles. Kinetochores themselves also generate microtubules which aid in the initial kinetochore-microtubule interaction.⁴² Dozens of proteins make up the large kinetochore protein complex, which is important both for microtubule attachment and spindle checkpoint activation.⁴² One set of kinetochore proteins that is particularly important during the spindle checkpoint is the KMN network, which consists of kinetochore null protein 1 (KNL1), the missegregation 12 (MIS12) complex, and the nuclear division cycle 80 (NDC80) complex.^{22,42,43} NDC80 and KNL1 directly interact with microtubules.⁴² KNL1 also acts as a docking site for other essential proteins that mediate the spindle checkpoint.^{22,42}

In order for the spindle checkpoint to be satisfied, one sister kinetochore must be attached to microtubules from one pole while the other sister is attached to microtubules from the opposite pole. This attachment state is known as bi-orientation. However, early in prometaphase it is common for sister kinetochores to become erroneously attached to microtubules from the same spindle pole (syntelic attachment) or for one sister kinetochore to be attached to microtubules from both spindle poles (merotelic attachment).^{22,42} To allow for error correction in the event that kinetochores are bound to microtubules from the wrong pole, the initial interaction between the kinetochore and microtubule is quite unstable. This instability is caused by high Aurora B kinase activity at the centromere, which phosphorylates several kinetochore proteins, including NDC80 and KNL1, to weaken the kinetochore-microtubule interaction.⁴² Once bi-orientation is achieved, tension is generated at the kinetochore, which physically separates Aurora B (located at the inner centromere) from the outer kinetochore proteins and stabilizes the kinetochore-microtubule interaction.⁴² PP1 is also required for the stabilization of kinetochore-microtubule interactions as it dephosphorylates the Aurora B substrates at the kinetochore.⁴² PP1 is recruited to kinetochores through its interaction with KNL1. KNL1-PP1 interaction is decreased by Aurora B phosphorylation of KNL1, and thus when Aurora B activity decreases due to increased tension at kinetochores, PP1 recruitment increases. This positive feedback loop promotes rapid stabilization of

kinetochore-microtubule interactions once the proper tension is applied at the kinetochore.⁴²

There are still many unanswered questions regarding the molecular mechanisms regulating how the spindle checkpoint is generated, but it is clear that the “wait anaphase” signal originates from kinetochores that are unattached or weakly attached to the mitotic spindle. While the exact molecular details are still being deciphered, the primary function of the spindle checkpoint is known to be inactivation of the APC/C E3 ubiquitin ligase complex. As briefly described in section 1.1.2, the APC/C controls the degradation of cyclin B and securin, which leads to CDK1 inactivation, separation of the sister chromatids, and mitotic exit. Therefore, to prevent missegregation of the chromosomes, the APC/C must be inhibited from degrading these two proteins until all chromosomes are bi-oriented at the metaphase plate. The next section will discuss how the spindle checkpoint achieves this inactivation of the APC/C.

1.2.2 Inhibition of the APC/C by the Mitotic Checkpoint Complex

The primary inhibitor of the APC/C during spindle checkpoint operation is the mitotic checkpoint complex (MCC), which is composed of the APC/C coactivator CDC20 in complex with the proteins mitotic arrest deficient 2 (Mad2), budding uninhibited by benzimidazoles 3 (Bub3), and budding uninhibited by benzimidazoles-related (BubR1) (Figure 1.7).^{29,44} Kinetochores lacking tension or attachment to the mitotic spindle form a

platform for MCC formation, which occurs via sequential recruitment of the MCC components. While there are still many unknowns regarding the molecular mechanisms regulating MCC assembly, it is well established that phosphorylation is essential for MCC assembly and checkpoint activation. One of the key mediators of MCC formation is the monopolar spindle 1 (MPS1) kinase, which is required for kinetochore localization of all known spindle checkpoint components. Early in the checkpoint, MPS1 phosphorylation of the outer kinetochore protein KNL1 forms a docking site for the checkpoint proteins Bub1 and Bub3, which in turn recruit BubR1 and a heterodimer of Mad1 and Mad2. The Mad1-Mad2 complex recruits an additional Mad2 to the kinetochore, where it undergoes a conformational change and binds CDC20.⁴⁵ The Mad2-CDC20 complex then binds BubR1-Bub3 to form the functional MCC, which diffuses away from the kinetochore to inhibit the APC/C. Although the MCC complex can be formed in the cytosol, the rate of formation is much higher at the kinetochore, where there is a higher concentration of Mad2-CDC20 and BubR1-Bub3 complexes. Sub-complexes of Mad2-CDC20 and BubR1-Bub3-CDC20 also have some inhibitory effect on the APC/C, but the complete MCC is a much stronger inhibitor.^{46,47}

MCC-mediated inhibition of the APC/C occurs through a number of different mechanisms. First, the MCC component BubR1 contains a KEN box domain (KEN1) that allows it to act as a pseudo-substrate for the APC/C thereby blocking the KEN box

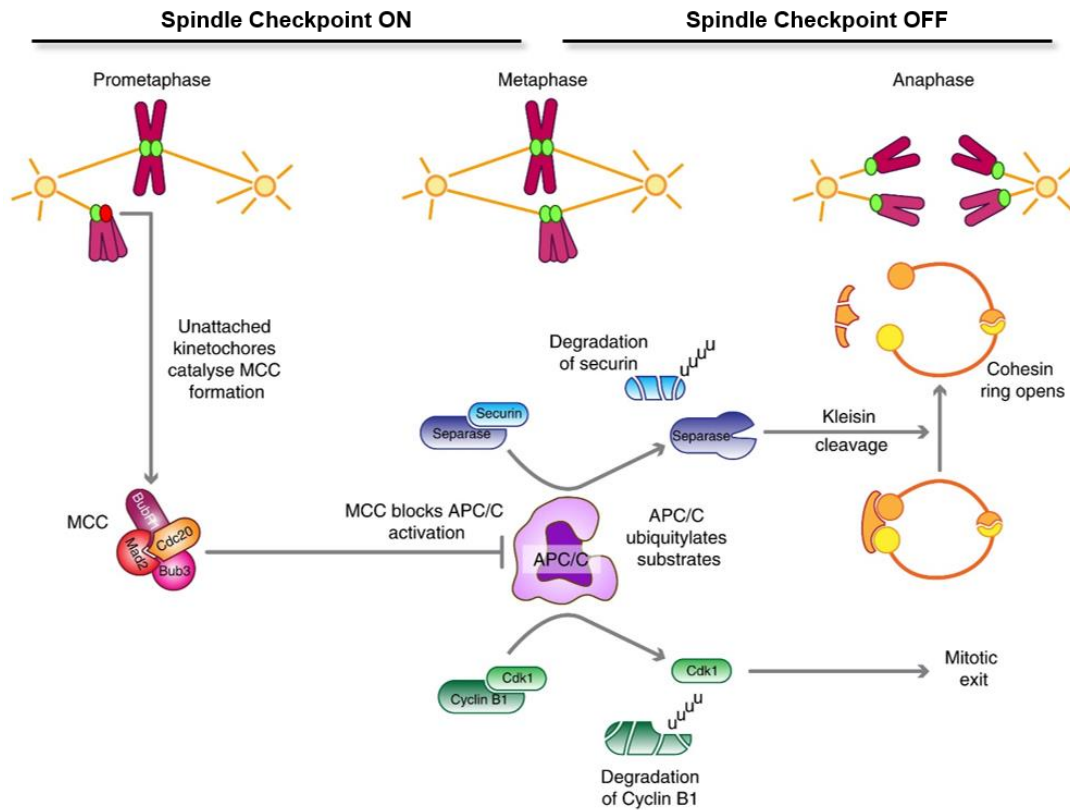


Figure 1.7: MCC Inhibition of the APC/C. The primary effector of the spindle checkpoint is the mitotic checkpoint complex (MCC), which is composed of four proteins: Mad2, Bub3, BubR1, and CDC20. Formation of the MCC is catalyzed by kinetochores that are not attached to the mitotic spindle. The MCC inhibits APC/C activation until all chromosomes are properly attached to the mitotic spindle, at which point the MCC dissolves and the APC/C targets cyclin B and securin for degradation. Cyclin B degradation leads to the inactivation of CDK1 and mitotic exit. Securin degradation releases separase to cleave the kleisin subunit of the cohesin ring that holds the sister chromatids together, resulting in the separation of the sister chromatids. (Adapted from Lara-Gonzalez et al., 2012)

recognition site for its other substrates, namely cyclin B and securin.⁴⁸⁻⁵⁰ BubR1 contains a second KEN box domain (KEN2) that is also required for preventing cyclin B from binding with the APC/C, although the requirement for the KEN2 domain is not well

understood.⁴⁸ Second, MCC-bound CDC20 binds to the APC/C in a different configuration than individual CDC20 proteins, which is thought to prevent the APC/C from functioning properly.^{48,49,51} Finally, it has recently been shown that the MCC is able to bind to a second CDC20 that is already bound to active APC/C to inhibit APC/C activity.⁴⁷

1.2.3 Regulation of the spindle checkpoint by kinases

As mentioned previously, the kinase MPS1 plays a critical role in recruiting MCC components to the kinetochore and activating the spindle checkpoint. The importance of MPS1 activity in the spindle checkpoint is evidenced by experiments in which MPS1 is inhibited. MPS1 inhibition leads to MCC disassembly, decreased cyclin B and securin levels, and mitotic exit.⁴⁸ Aurora B kinase is also required for the spindle checkpoint and has been shown to contribute to the kinetochore recruitment of several essential checkpoint proteins. Additionally, as mentioned above, Aurora B indirectly promotes spindle checkpoint maintenance by destabilizing improperly attached microtubules at the kinetochore. Unstable kinetochore-microtubule interactions lead to the production of additional MCCs which potentiates the spindle checkpoint. Another important kinase for checkpoint activation and maintenance is Plk1. Plk1 was recently shown to phosphorylate KNL1 and MPS1 thereby enhancing MPS1 activity and promoting localization of MCC components to kinetochores.⁵²

1.2.4 Regulation of the spindle checkpoint by phosphatases

As the MPS1, Aurora B, and Plk1 kinases all promote spindle checkpoint activation, it is unsurprising that protein phosphatases have been implicated in checkpoint silencing. The two main phosphatases known to be involved in checkpoint silencing are PP1 and PP2A-B56. These phosphatases exist in both positive and negative feedback loops with the kinases MPS1 and Aurora B to allow for robust checkpoint activation and also rapid inactivation and dissociation of the MCC upon proper microtubule attachment. MPS1 phosphorylation of KNL1 recruits PP2A to kinetochores through its interaction with BubR1. PP1 is also recruited to KNL1, but its binding is inhibited early in prometaphase by strong Aurora B phosphorylation of KNL1, which displaces PP1. Interestingly, BubR1-associated PP2A-B56 opposes Aurora B phosphorylation of KNL1 thereby promoting PP1 recruitment to the kinetochore. In addition to PP2A-B56, PP1 has also been shown to oppose Aurora B at the kinetochore thereby stabilizing kinetochore-microtubule attachments and promoting checkpoint silencing.

PP1 and PP2A-B56 have also both been implicated in dephosphorylating MPS1 phosphorylation sites on KNL1, which in turn dissociates PP2A-B56 and the MCC components from the kinetochore once bi-orientation has occurred. Additionally, preventing PP1 interaction with KNL1 results in constitutive spindle checkpoint

activation, demonstrating the requirement for PP1 in checkpoint silencing. Taken together, these findings indicate that PP1 and PP2A-B56 are both essential for MCC disassembly and spindle checkpoint silencing. Although phosphatases are known to be required for spindle checkpoint silencing, it remains to be seen if phosphatases contribute to spindle checkpoint maintenance. We investigated this question and found that phosphatases are required for maintenance of the MCC during the spindle checkpoint. Our results will be described in chapter 4.

1.2.5 Spindle checkpoint silencing

Spindle checkpoint silencing occurs once all the chromosomes are bi-oriented such that tension is applied on all kinetochores. Several different mechanisms are thought to contribute to spindle checkpoint silencing. Mad1 and Mad2 are stripped from kinetochores through the movement of dynein along microtubules, which impedes the formation of new MCC complexes.⁵³ Active MCC complexes are also disassembled by the protein p31^{comet}, which promotes MCC turnover by removing Mad2 from the MCC. Additionally, the APC/C can target both CDC20 and BubR1 for degradation, which may also promote MCC disassembly. Lastly, as mentioned above, the phosphatases PP1 and PP2A-B56 play critical roles in silencing the checkpoint by dephosphorylating MPS1 and Aurora B substrates.

Once the spindle checkpoint has been turned off, the MCC dissociates, releasing CDC20 to activate the APC/C, which can then target cyclin B and securin for degradation. Cyclin B degradation leads to decreased cyclin B/CDK1 activity, and the phosphatases mentioned previously rapidly dephosphorylate the many cyclin B/CDK1 mitotic substrates. Securin is an inhibitor of separase, an enzyme that cleaves the kleisin subunit of the cohesin ring complex that is responsible for holding the sister chromatids together. APC/C-mediated degradation of securin allows separase to cleave cohesin so the sister chromatids can be pulled to opposite poles by the mitotic spindle.^{54,55}

1.3 The DNA damage response

Our cells are constantly encountering DNA damage as a result of environmental agents, metabolic insults, replication errors, and spontaneous mutations.⁵⁶⁻⁵⁸ To maintain genetic integrity, it is imperative that our cells have the ability to repair this damage, especially before the cell divides and passes on the altered DNA to its daughter cells. As discussed above, movement through the cell cycle is highly regulated, in part to prevent the persistence of DNA damage. Cells use complementary and highly refined mechanisms to sense and repair damaged DNA before the cell cycle can proceed. When DNA damage is detected, our cells activate the DNA damage response (DDR), which halts the cell cycle and activates repair mechanisms so the DNA can be repaired before the cell divides.⁵⁹ The DDR can be initiated and affect the fate of the cell during any stage

of the cell cycle. If severe DNA damage occurs during interphase, the DDR may induce senescence. Alternatively, a prolonged DDR may induce cell death by activating apoptotic pathways.⁵⁸ Importantly, if the DDR is faulty and the cell continues to divide with damaged DNA, the resulting mutations may cause the cell to become cancerous.⁵⁹

1.3.1 The DNA damage checkpoint signaling network

DNA damage can be caused by a wide array of damaging insults, which can then lead to several different types of DNA lesions, including single base pair mismatches, abasic sites, insertions, deletions, bulky DNA adducts, intra and inter-strand crosslinks, single strand breaks, and double strand breaks (DSBs).⁶⁰ This damage initiates the DDR, which is a complex signaling network that consists of DNA damage sensors, mediators, transducers, and effectors (Figure 1.8).

The DNA damage sensors are the first proteins on the scene once DNA damage has occurred. The type of sensor proteins that are recruited to the damaged DNA is specific for the type of damage that has occurred. DNA damage that results in single stranded DNA (ssDNA), such as single strand breaks, stalled replication forks, nucleotide depletion, and ultraviolet (UV) light, are first recognized by the ssDNA binding protein RPA (Replication Protein A).^{59,61} This checkpoint sensor protein coats the ssDNA and recruits the apical kinase ATR via its binding partner, ATR-interacting protein (ATRIP).^{61,62} In order for ATR to fully activate the DDR, two Rad checkpoint

sensor complexes must also co-localize to the site of damage, the Rad9-Rad1-Hus1 (9-1-1) complex and Rad17-RFC.^{62,63} DSBs often result from ionizing radiation or radio-mimetic chemicals and are recognized by the MRE11-Rad50-NBS1 (MRN) sensor protein complex. The MRN complex binds to sites of DSBs and recruits the apical kinase ATM, which is activated through autophosphorylation.^{59,61} Activated ATM/ATR phosphorylate several downstream proteins including histone H2AX (known as γ H2AX), which is a critical step in the recruitment of key mediator proteins to the site of DNA damage.⁵⁹

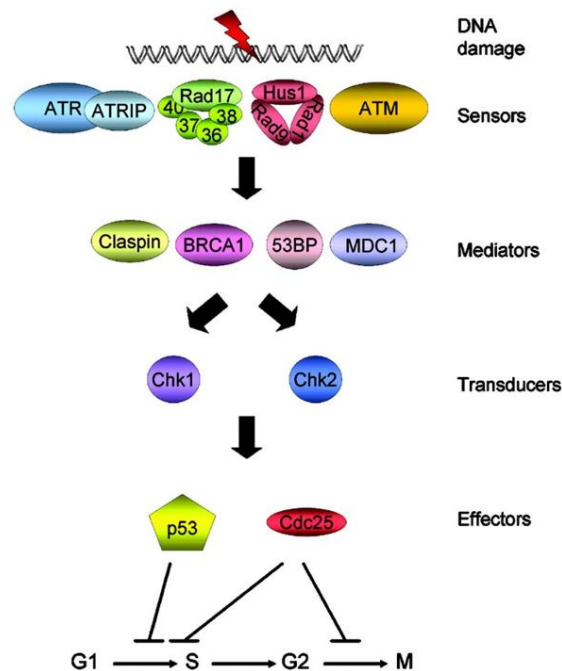


Figure 1.8: The DNA Damage Response. A hierarchy of sensors, mediators, transducers, and effectors respond to DNA damage to activate the cell cycle checkpoint and DNA repair processes. DNA damage is detected by sensor proteins, and together with the mediators, they activate the traducer checkpoint proteins Chk1 and Chk2. Chk1 and Chk2 then activate or inhibit several effector proteins, such as p53 and Cdc25, which directly mediate cell cycle arrest or DNA repair. (Adapted from Sancar et al., 2004)

Checkpoint mediator proteins relay the DNA damage signal from the checkpoint sensors to the downstream transducers and effectors that elicit the cell cycle checkpoint and DNA repair. These proteins are often adaptor proteins involved in localizing the downstream checkpoint and repair proteins to the sites of DNA damage and making them available for ATM/ATR phosphorylation. Most if not all of the checkpoint mediators are themselves substrates of ATM/ATR, such as Topoisomerase Binding Protein 1 (TopBP1), claspin, mediator of DNA damage checkpoint 1 (MDC1), p53-binding protein 1 (53BP1), and Breast Cancer 1, Early Onset (BRCA1). TopBP1 is recruited by the 9-1-1 complex to sites of damage, where it bolsters the kinase activity of ATR.^{61,63} Claspin is another mediator downstream of ATR that enhances ATR activity.⁶¹ Additionally, claspin is required for ATR mediated activation of the transducer protein kinase Chk1.^{63,64} MDC1 binds to γ H2Ax at sites of DSBs where it is phosphorylated by ATM and recruits additional MRN complexes, thereby creating a positive feedback loop that amplifies the ATM signal.⁶¹ 53BP1 also facilitates ATM recruitment to γ H2Ax and, along with BRCA1, plays a key role in DNA repair.^{61,64}

Downstream of the sensors and mediators are the checkpoint transducer kinases Chk1 and Chk2. Chk1 is predominantly activated by ATR phosphorylation while ATM primarily activates Chk2. However, there is a lot of cross-talk between these two pathways. For instance, ATR can phosphorylate Chk2 in response to certain genotoxic

agents such as cisplatin.⁶⁵ Additionally, ATR and Chk1 are activated downstream of ATM in response to DSBs when ssDNA is generated during the DSB resection.^{63,64,66,67} Conversely, other types of DNA damage induce ATM activation downstream of ATR, such as in DNA damage resulting from UV^{67,68} or elevated oxygen levels (hyperoxia).⁶⁹ Consequently, the ATR/Chk1 and ATM/Chk2 pathways are often activated simultaneously.

Although the ATR/Chk1 and ATM/Chk2 pathways activate many of the same downstream effectors, they are differentially required for survival. ATR and Chk1 individual knockouts are embryonic lethal in mice, while ATM and Chk2 individual knockout mice can survive with minor phenotypes, including an increased susceptibility to cancer.⁷⁰⁻⁷³ This difference is due in part to the role of the ATR/Chk1 pathway in protecting genomic stability during S-phase.⁷⁴ Chk1 has also been shown to have additional roles outside of the DDR, including regulating mitotic entry⁷⁵ and the spindle checkpoint.^{67,76,77} In humans, loss of ATM function results in a severe disease known as Ataxia Telangiectasia (AT) that is primarily characterized by neurodegeneration, immunodeficiency, and a predisposition to cancer.^{78,79}

As Chk1 and Chk2 are diffusible kinases, they propagate the DNA damage signal away from the site of damage to several downstream effector proteins, including transcription factors, cell cycle regulators, DNA repair proteins, and if necessary, the

apoptotic machinery.^{59,80} One of the principal effector proteins is p53, which can be phosphorylated by ATR, ATM, Chk1, or Chk2.⁶² p53 phosphorylation protects it from being targeted for proteasomal degradation by the E3 ubiquitin ligase MDM2 and prevents nuclear export.⁸¹ Additionally, MDM2 is destabilized following DNA damage leading to its inability to target p53 for degradation.^{81,82} Elevated nuclear p53 levels results in transcriptional upregulation of genes involved in cell cycle arrest, DNA repair, senescence, and apoptosis. One of the key p53 target genes is the CDK inhibitor p21, which promotes cell cycle arrest.

Another important effector protein targeted by Chk1 and Chk2 is Cdc25. Cdc25 has three family members, designated Cdc25A, Cdc25B, and Cdc25C. All three family members can both dephosphorylate CDK1 and induce cyclin B/CDK1 activation to promote mitotic entry, as described in section 1.1.1.⁸³ Cdc25A also has a role earlier in the cell cycle as it dephosphorylates CDK2 to activate cyclin E/CDK2 and cyclin A/CDK2 to promote S-phase entry.⁸³ Following DNA damage, Chk1/Chk2 phosphorylate Cdc25A, which causes it to be degraded via SCF ^{β -TrCP} mediated ubiquitination and thereby promotes cell cycle arrest at the G1/S or intra-S checkpoint.^{59,64,67,73} Chk1/Chk2 phosphorylation of Cdc25C creates a binding site for 14-3-3 proteins, which sequesters and inactivates Cdc25C, thereby preventing cyclin B/CDK1 activity and promoting G2/M arrest in response to DNA damage.^{64,67,73} Another Chk1 substrate that promotes

cell cycle arrest is the CDK1 inhibitor Wee 1, which becomes activated following Chk1 phosphorylation.^{64,67}

1.3.2 DNA damage repair and the regulation of deoxynucleotide production

DNA damage repair is an essential component of the DDR and is highly coordinated with the DNA damage checkpoint. In fact some of the DNA repair pathways directly contribute to DNA damage-induced cell cycle arrest and apoptosis.⁸⁴ As there are several types of DNA damage, there are consequently several different DNA repair mechanisms. When DNA damage occurs on only a single strand of DNA, three different excision mechanisms may be employed to repair the damage. (1) Base excision repair (BER) uses DNA glycosylase enzymes to repair damage to a single nitrogenous base.⁵⁸ (2) In the nucleotide excision repair (NER) pathway, the damaged region of DNA is excised as part of a 22-30 base oligonucleotide.⁵⁸ DNA polymerase and DNA ligase are then recruited to the resulting ssDNA to replace the missing DNA strand.⁵⁸ This pathway is used to repair damage resulting in bulky DNA lesions, such as pyrimidine dimers.³ (3) Mismatch repair (MMR) is used to repair insertions, deletions, and base pair mismatches generated during DNA replication.⁸⁴ A fourth mechanism of single strand DNA repair does not use excision mechanisms but rather involves the direct chemical reversal of damage (direct repair), but this mechanism is not very widely employed.

DSBs, which may be induced by ionizing radiation, oxidizing agents, and replication errors, pose the highest threat to genome integrity of any type of damage because there is no template strand available for the repair. DSBs are primarily repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR). In NHEJ, the Ku protein recognizes the DSB and then recruits and activates DNA-PKcs, a protein kinase that subsequently promotes the recruitment of several proteins involved in DSB repair. This repair pathway is not ideal as it often leads to a mutation at the site of the break. Another less well-understood Ku-independent NHEJ mechanism is the microhomology-mediated end-joining (MMEJ) pathway, and this pathway is even less ideal than NHEJ because it always leads to deletions in the DNA sequence.⁵⁸ HR is a more precise form of DSB repair because it uses the DNA sequence of a homologous chromosome, such as a sister chromatid, as a template for repairing the damage. While HR is more reliable and accurate than NHEJ and MMEJ, it is primarily used to repair DNA only in S and G2 when a sister chromatid is nearby, whereas NHEJ and MMEJ can repair DNA in any cell cycle phase.

The DDR regulates DNA damage repair in three different ways. Firstly, as described in the previous section, the DDR induces post-translational modifications on DNA repair proteins that can directly alter protein activity, stability, binding partners, and localization. Secondly, chromatin modifications resulting from DDR kinase activity

allow for improved access and recruitment of repair proteins to the site of damage. Lastly, the DDR induces global changes in the cell that result in an overall more favorable environment for DNA repair. One of these global responses of the DDR is increasing deoxynucleotide (dNTP) levels. Increased nucleotide production at the site of damaged DNA is important for efficient DNA repair, but excessively high levels or imbalanced pools of dNTPs increases the frequency of mutations.^{85,86} Therefore dNTP production is tightly regulated both during normal cell cycle progression and during the DDR.

The ribonucleotide reductase (RNR) enzyme is responsible for catalyzing the conversion of ribonucleotides to dNTPs, which can then be incorporated into DNA. This rate-limiting enzyme is a complex consisting of two identical large subunits (Ribonucleotide Reductase M1 or RRM1) and two identical small subunits (Ribonucleotide Reductase M2 or RRM2).⁸⁷ While RRM1 protein levels remain stable throughout the cell cycle, RRM2 levels fluctuate to regulate overall RNR activity.^{88,89} During G1 when dNTP levels should be kept low, APC/C^{CDH1} targets RRM2 for degradation.⁸⁹ Then as the cell is preparing for DNA replication at the G1/S transition, RRM2 levels are increased by E2F1-mediated transcriptional upregulation to elevate dNTP production.^{90,91} Additionally, APC/C^{CDH1} activity is decreased at the G1/S transition, which also likely contributes to increased RRM2 levels. Following DNA

replication, RRM2 is targeted for degradation by the SCF^{cyclin F} complex.⁹² CDK phosphorylation of RRM2 during G2 exposes the cyclin F binding motif.⁹² Cyclin F-mediated RRM2 degradation is important for regulating dNTP levels and promoting genome stability.⁹²

In response to DNA damage, RNR function is upregulated by several mechanisms to ensure adequate availability of dNTPs for the DNA repair process. One method of regulation following DNA damage is the translocation of the RRM1 and RRM2 subunits from the cytoplasm to the sites of DNA damage.⁹³ A second mode of regulation is the stabilization of RRM2 protein levels.^{88,89} This stabilization of RRM2 was recently shown to be the result of cyclin F downregulation in response to DNA damage.⁹² As mentioned above, cyclin F targets RRM2 for degradation, and thus when cyclin F levels decrease, RRM2 levels increase. Increased dNTP production promotes DNA repair, and if the damaged DNA is successfully repaired, cell cycle progression will continue. Alternatively, as mentioned previously, if the damage cannot be repaired, the cell will undergo apoptosis or become senescent.

1.4 Emerging roles for cyclin F and the SCF complex

As mentioned in section 1.1.2, cyclin F is an F-box protein that targets substrates for degradation via the SCF E3 ubiquitin ligase complex. Discovered in 1994, cyclin F was named after its sequence homology with the cyclin family of proteins, of which it

shares the most homology with cyclins A and B.^{94,95} Structurally, cyclin F contains three primary regions (Figure 1.9). At the N-terminus, it has an F-box domain that interacts with the Skp1 subunit of the SCF complex. This domain is referred to as the “pseudo-catalytic” domain because cyclin F itself lacks catalytic activity, but by recruiting proteins to the SCF complex, cyclin F is able to direct the catalytic activity of the SCF complex.⁹⁶ Near the middle of the protein, cyclin F contains a cyclin box domain, which is found in all cyclin proteins. However, while the other cyclins interact with CDKs via their cyclin box domain, this domain is used by cyclin F to interact with SCF substrates. Most F-box proteins recognize phosphorylation sites on their substrates, but cyclin F uses the hydrophobic patch within its cyclin box to recognize and bind CY motifs (RxL) on its substrates.⁹⁶ Lastly, near the C-terminus of cyclin F is a regulatory region that contains both a nuclear localization signal (NLS) and a PEST domain, which is a short

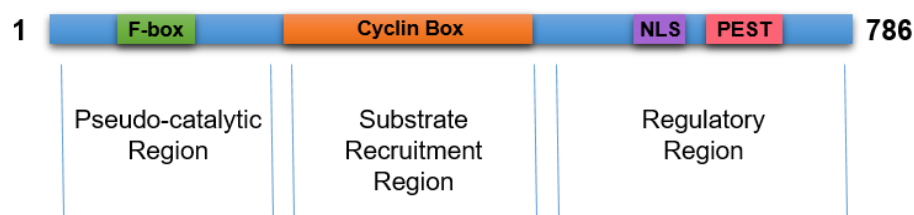


Figure 1.9: Cyclin F Protein Regions. This schematic representation shows the three primary regions of the cyclin F protein. The N-terminus of the protein contains the F-box domain common to all F-box proteins. The F-box domain mediates the interaction between cyclin F and the SCF subunit Skp1, and thus this segment of the protein is called a “pseudo-catalytic” region. The substrate recruitment region contains the cyclin box, which recognizes SCF substrates. The C-terminal region of cyclin F contains two regulatory domains: a nuclear localization sequence (NLS) and a PEST sequence. (Adapted and modified from D’Angiolella et al., 2013)

protein sequence with a high content of proline, glutamate, serine, and threonine.⁹⁶ PEST sequences are often found on proteins that have a short half-life and therefore are thought to signal proteolysis.

While there are still many unanswered questions about the function of cyclin F, recent studies have indicated that cyclin F has a role in cell cycle regulation, DNA damage repair, and maintaining genome integrity.⁹⁷ Additionally, cyclin F may have a tumor suppressor role as it was recently shown that cyclin F downregulation in hepatocellular carcinoma samples was correlated with increased tumor size, advanced clinical stage, and worse survival.⁹⁸

1.4.1 Cyclin F and the cell cycle

Cyclin F is similar to other cyclins in that both its mRNA and proteins levels oscillate throughout the cell cycle, but its role in the cell cycle is largely unknown. Cyclin F mRNA and protein levels follow a pattern similar to cyclin A as their expression is low during G1, accumulates during S phase, and peaks at G2/M before dropping off during mitosis prior to cyclin B degradation.⁹⁵ This expression pattern is suggestive of a role for cyclin F during the G2 phase and possibly at the G2/M transition. The observed low cyclin F levels during S phase are important for elevated RRM2 levels and the increased dNTP production that is necessary for DNA replication. Interestingly, overexpression of cyclin F in human cells causes a significant accumulation of cells in G2 phase as

compared with normal cells.⁹⁵ This altered cell cycle pattern could be the result of (1) a shortened G1 or S-phase or (2) a lengthened G2 phase in the presence of excess cyclin F protein.

Knockout studies in mice also indicate a role for cyclin F in cell cycle regulation. Interestingly, cyclin F is essential for development, as cyclin F knockout mice die at around embryonic day 10.5 due largely to a failure of proper placental development.⁹⁹ However, cyclin F is not required for the survival of mouse embryonic fibroblasts (MEFs). These cyclin F null MEFs do, however, have defects in cell cycle progression. The population doubling time is decreased in cyclin F null cells and they are also slower to reenter the cell cycle from a quiescent state.⁹⁹ Although the molecular mechanisms of cyclin F cell cycle regulation are still unknown, these findings demonstrate that cyclin F plays an important role in cell cycle progression. We have investigated the cell cycle role of cyclin F, and our findings will be described in section 3.2.3.

1.4.2 Cyclin F and the DNA damage response

As described in section 1.3.2, the DDR coordinates a number of changes in the cell to create a favorable environment for DNA repair, including dNTP production. It was recently demonstrated that an important step in increasing dNTP production during the DDR is the downregulation of cyclin F and subsequent accumulation of the RNR subunit RRM2.⁹² DNA damage-induced cyclin F downregulation was shown to be

dependent on the ATR signaling pathway and independent of ATM.⁹² The importance of DNA damage-induced downregulation of cyclin F is demonstrated by experiments in which cyclin F is overexpressed at high enough levels such that RRM2 cannot accumulate following DNA damage. These cells display a reduced ability to repair damaged DNA and reduced cell survival following DNA damage.⁹² Therefore, cyclin F is an important downstream effector of the DDR. However, the specific signaling pathways that promote cyclin F downregulation in response to DNA damage are largely unknown. In chapter 3, we will describe our efforts to define the molecular mechanisms that regulate cyclin F degradation both in unperturbed cells and in cells faced with DNA damage.

2. Materials and Methods

2.1 Cell culture

2.1.1 Cell lines and maintenance

A549, H1299, HCT116, 293T, HeLa, and HeLa S3 cells were purchased from the Duke Cell Culture Facility. HeLa Tet-Off Advanced cells were purchased from Clontech. HeLa Tet-Off cells were a kind gift from Donald McDonald (Duke University, Durham, NC). hTERT-RPE1 cells were a kind gift from Christopher Counter (Duke University, Durham, NC). HeLa Tet-On and HeLa S3 Tet-On cells were generated using the Retro-X Tet-On Advanced Inducible Expression System from Clontech according to manufacturer's instructions and as described in section 2.1.4. All cultures were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO₂.

2.1.2 Cell synchronization

HeLa Tet-Off cells were single or double thymidine blocked using 2.5 mM thymidine (Sigma-Aldrich), washed twice with PBS, and released into medium containing 100 ng/mL nocodazole (Calbiochem) or 100 nM taxol (LC Laboratories) for 16 hours to activate the spindle checkpoint and arrest cells at prometaphase. Phosphatase inhibitors were then added for two hours before collection. In certain experiments, MG132 or the APC/C inhibitor proTAME was added for one hour prior to phosphatase inhibitor treatment. For mitotic exit assays, nocodazole or taxol-arrested cells were pre-

treated for 30 minutes with inhibitor, washed twice with PBS, and re-plated in media containing an inhibitor or DMSO.

2.1.3 DNA transfections

DNA was transfected into cells using X-tremeGENE 9 DNA Transfection Reagent (Roche) per the manufacturer's instructions 48 hours before collection. The following DNA constructs were used: mCherry-FLAG-Cyclin F (Addgene plasmid #32975, from the laboratory of Michele Pagano), pBABE-FLAG-HA-puro-Cyclin F (Addgene plasmid #32976, from the laboratory of Michele Pagano), pLVX-Tight-mCherry-FLAG-Cyclin F-Tet-Off (Addgene plasmid #32977, from the laboratory of Michele Pagano), pcDNA3-FLAG-Cyclin F, pRetroX-Tet-On Advanced (Clontech), pRetroX-Tight-pur (Clontech), pEGFP-C1, pEGFP-C1-cyclin F 407-565, pEGFP-C1 cyclin F 407-660, pCS2-HA-CDC20, pcDNA3-Myc-BubR1, and pCS2-Myc-Mad2.

2.1.4 Virus generation and infections

To generate HeLa Tet-On and HeLa S3 Tet-On cells, retrovirus was first generated in 293T by transfecting the cells with VSV-G, gag/pol, and the pRetroX-Tet-On Advanced vector, which encodes the doxycycline-controlled transactivator rtTA-Advanced (Clontech). Virus-containing media was collected 48 and 72 hours post transfection and filtered through a 0.45 μ M filter. HeLa S3 or regular HeLa cells were then infected with the retrovirus encoding the transactivator in the presence of polybrene (10 μ g/mL), and infected cells were selected with 500 μ g/mL G418 (Hyclone).

To generate HeLa S3 Tet-On cells with doxycycline-inducible cyclin F (full length or fragments), the HeLa S3 Tet-On cells were infected with a second virus encoding either pLVX-tight-mCherry-FLAG-Cyclin F-Tet-Off or pRetroX-tight-pur-EGFP-cyclin F 407-565/407-660 and selected with 1 $\mu\text{g}/\text{mL}$ puromycin (Sigma-Aldrich). Expression was induced with 1 $\mu\text{g}/\text{mL}$ doxycycline (Sigma-Aldrich).

2.2 Inhibitors

The following inhibitors were used: okadaic acid (0.1 μM or 1 μM , Enzo), fostriecin (10 μM , Enzo), FK506 (10 μM , Enzo), cyclosporin A (10 μM , Enzo), calyculin A (20 nM, Calbiochem), MG132 (20 μM , Enzo), proTAME (12 μM , Boston Biochem), roscovitine (10 μM , Calbiochem), MLN4924 (1 μM , BostonBiochem), actinomycin D (5 $\mu\text{g}/\text{mL}$, Enzo), cycloheximide (100 $\mu\text{g}/\text{mL}$, Sigma-Aldrich), VE-821 (10 μM , Selleckchem), KU-55933 (10 μM , R&D Systems), Chk2 Inhibitor II (10 μM , Calbiochem), PF-477736 (0.4 μM , Sigma-Aldrich), etoposide (50 μM , Sigma-Aldrich), doxorubicin (1 μM , Calbiochem), neocarzinostatin (0.1 $\mu\text{g}/\text{mL}$, Sigma-Aldrich), cisplatin (20 μM , Sigma-Aldrich), and hydroxyurea (20 μM , Sigma-Aldrich).

2.3 Antibodies

The following antibodies were obtained from Santa Cruz: anti-cyclin A (sc-751), anti-cyclin B (sc-245), anti-actin (sc-1616-R), anti-CDC20 (sc-5296, used for immunoprecipitation), anti-Mad2 (sc-6329), anti-PP1 α (sc-6104), anti-PP1 γ (sc-6108), anti-myc (sc-789), anti-HA (sc-805), anti-cyclin F (sc-952), anti-Chk1 (sc-8408), and anti-

p53 (sc-126). The following antibodies were obtained from Abcam: anti-CDC20 (ab26483), anti-BubR1 (ab54894), anti-PP1 β (ab53315), anti-Chk1 (pS296) (ab79758), anti-actin (ab8224), anti-ATM (ab78), and anti-ATM (pS1981) (ab81292). The following antibodies were obtained from Cell Signaling: anti-phospho-histone H3 (Ser10) (9701), anti- β TrCP (4394), anti-ATR (2790), and anti-Chk1 (pS345) (2348). The following antibodies were obtained from BD Biosciences: anti-Bub3 (611730), anti-PP5 (611020), p21 (556431), and cyclin D1 (556470). We also used anti-vinculin (Sigma-Aldrich, V9131), anti-GFP (chromotek, 3H9), anti-MPM2 (Millipore, 05-368), and anti-PP2Ac (Millipore, 05-421). The following secondary antibodies were used: Alexa Fluor 680 goat anti-rabbit (Life Technologies, A21076), IRDye 800CW goat anti-mouse (LI-COR, 926-32210), and IRDye 800CW donkey anti-goat (LI-COR, 926-32214).

2.4 Immunoblotting and immunoprecipitations

For immunoblotting, cell pellets were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.5, 1% NP-40, 0.25% Sodium deoxycholate, 1mM EDTA, 0.1% SDS, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin) or Co-IP buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 0.5% Triton X-100, 1.5 mM MgCl₂, 2 mM EGTA, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin) by vortexing samples at 4°C for 30 minutes, followed by centrifugation at 16,000 g for 15 minutes. Supernatants were collected and protein concentration was measured by a Bradford protein assay (Bio-Rad). SDS sample buffer was added to the lysates before

samples were heated at 95°C for 5 minutes. Lysates were then separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using appropriate antibodies.

For immunoprecipitations, cell pellets were lysed in Co-IP buffer and protein concentration was measured as indicated above. Lysates containing equal amounts of protein were incubated with CDC20, Cyclin F, or IgG control antibody for 2-4 hours at 4°C. Proteins were collected by adding Protein G Sepharose (Sigma-Aldrich) or Protein A/G Dynabeads (Life Technologies) for 1 hour at 4°C. Alternatively, Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) or Anti-c-Myc Agarose Affinity Gel (Sigma-Aldrich) was used to immunoprecipitate tagged proteins. The beads were washed 3 times with Co-IP buffer + 150 mM NaCl, heated in SDS sample buffer for 5 minutes, and analyzed by SDS-PAGE and immunoblotting.

2.5 siRNA and shRNA

siRNAs were synthesized by Sigma-Aldrich or Invitrogen and transfected using Lipofectamine RNAiMax (Invitrogen) per the manufacturer's instructions immediately prior to synchronization. Luciferase, AllStars Negative Control (Qiagen, 1027281) siRNA, or control siRNA from Santa Cruz (sc-37007) were used as negative controls. esiRNAs (Sigma-Aldrich) were used to knockdown six phosphatases identified by mass spectrometry: DUSP6 (EHU123191), NUDT5 (EHU013191), PNKP (EHU132321), PPP1R12A (EHU072171), PPA1 (EHU109021), and DCTPP1 (EHU042641). Sigma-

Aldrich's pLKO.1 lentiviral shRNA constructs were used for PP4 and PP6, and the control pLKO.1 shRNA construct is from Addgene (Plasmid #1864).

The following siRNA sequences were used:

Luciferase: UCGAAGUAUCCGCGUACG

Cyclin F-1: UAGCCUACCUCUACAAUGA

Cyclin F-2: GCACCCGGUUUAUCAGUAA

ATM: L-003201-00-0005, Dharmacon SMARTpool ON-TARGETplus

ATR A: AACCUCCGUGAUGUUGCUUGA

ATR B: AAGCCAAGACAAAUUCUGUGU

ATR C: EHU040341, Sigma-Aldrich MISSION esiRNA

Chk1 A: AACUGAAGAAGCAGUCGCAGU

Chk1 B: UACAUUUUCAUGAUUUAGCAU

B-TrCP: GUGGAAUUUGUGGAACAUC

Cullin1: GGUUAUAUCAGUUGUCUAA

Skp1: AACAUCAAGCAAACCUUUG

CDC20: CGGAAGACCUGCCGUUACA

Pan-PP1: sc-43545, Santa Cruz

PP1 α : SIHP0511-250PMOL, Sigma-Aldrich

PP1 β -1: AAAUGCGAUUGAUGCUAGC

PP1 β -2: AUUCAGUCCACCAUACUGG

PP1 γ : SIHP0518-250PMOL, Sigma-Aldrich

PP2A: CAACGUGCAAGAGGUUCGAUGUCCA

PP5-1: AACAUAUUCGAGCUCAACGGU

PP5-2: CUCAACAUAUUCGAGCUCA

The following shRNA sequences were used:

PP4-1: TRCN0000002760

PP4-2: TRCN0000002761

PP4-3:TRCN0000002762

PP6-1: TRCN0000002764

PP6-2: TRCN0000002765

PP6-3: TRCN0000002766

2.6 Cloning and mutagenesis

pEGFP-C1 containing cyclin F fragments (amino acids 407-565 and 407-660) were generated by subcloning cyclin F fragment PCR products generated with primers encoding a N-terminal XhoI restriction site

(AGACTCGAGCTGTCCCCACTGTGGTGGAT) and a C-terminal EcoRI restriction site

(565: CTCGAATTCTCAGGGAGAGCTGAGGAA ; 660:

CTCGAATTCTCATGGACAAGCCTCCTC) into pEGFP-C1 digested with XhoI and

EcoRI. pRetroX-tight-pur-EGFP-cyclin F 407-565/660 was generated by subcloning

EGFP-cyclin F 407-565/660 PCR products generated with primers encoding a N-terminal

BamHI restriction site (CTCGGATCCATGGTGAGCAAGGGCGAGGA) and a C-terminal EcoRI site (same as above) into pRetroX-tight-pur digested with BamHI and EcoRI.

Stratagene's site-directed mutagenesis kit was used to mutate serine and threonine residues to alanine per the manufacturer's instructions. Primers were designed using Agilent Technologies QuikChange Primer Design Tool and are as follows:

Cyclin F S195A: Forward 5- CTCATCCTCGAACAGAGCCAGCACTCTGCCCAAG -3;

Reverse 5- CTTGGGCAGAGTGCTGGCTCTGTTCGAGGATGAG -3

Cyclin F S509A: Forward 5- ACTACAGGCAAGTCGCTCTGACCGCCGTG -3; Reverse

5- CACGGCGGTCAGAGCGACTTGCCTGTAGT -3

Cyclin F S577A: Forward 5- CTGTCTTCCTGGAGGGCGTTCTCCCGCTTCCG -3;

Reverse 5- CGGAAGCGGGAGAACGCCCTCCAGGAAGACAG -3

Cyclin F S754A: Forward 5- CTCCGGGGGAGCTGGGGGACGACACTGTAAA -3;

Reverse 5- TTTACAGTGTCGTCCCCCAGCTCCCCCGGAG -3

Cyclin F S774A: Forward 5-

GTTTCATGTCCTCCTCCTCAGCGTGTATGCATAGGTTTATC -3; Reverse 5-

GATAAACCTATGCATACACGCTGAGGAGGAGGACATGAAC -3

Cyclin F L35A/P36A (LP/AA): Forward 5-

GAAACCTGACCATCTTGAGTGCCGCCGAAGATGTGCTCTTTCAC -3; Reverse 5-

GTGAAAGAGCACATCTTCGGCGGCACTCAAGATGGTCAGGTTTC -3

CDC20 S84A: Forward 5- CTATATCCCCCATCGCGCTGCTGCCCAGATGGAG -3;

Reverse 5- CTCCATCTGGGCAGCAGCGCGATGGGGGATATAG -3

CDC20 S92A: Forward 5- CAGATGGAGGTGGCCGCCTTCCTCCTGAGCAA -3;

Reverse 5- TTGCTCAGGAGGAAGGCGGCCACCTCCATCTG -3

CDC20 S491A: Forward 5- AGGCCAGTGCAGCCAAAGCCAGCCTCATCCAC -3;

Reverse 5- GTGGATGAGGCTGGCTTTGGCTGCACTGGCCT -3

CDC20 S492A: Forward 5- CAGTGCAGCCAAAAGCGCCCTCATCCACCAAGGC -3;

Reverse 5- GCCTTGGTGGATGAGGGCGCTTTTGGCTGCACTG -3

BubR1 S12A/S16A: Forward 5- TGCTCTGGCTGAAGCCATGGCCCTGG -3; Reverse 5-

CCAGGGCCATGGCTTCAGCCAGAGCA -3

BubR1 S619A: Forward 5- CCAGAGCAGCTCGTTTTGTAGCCACTCCTTTTCA -3;

Reverse 5- TGAAAAGGAGTGGCTACAAAACGAGCTGCTCTGG -3

BubR1 T710A: Forward 5-

AATTCCTGAGAACTAGAACTTGCTAATGAGACTTCAGAAAACCC -3; Reverse 5-

GGGTTTTCTGAAGTCTCATTAGCAAGTTCTAGTTTTCTCAGGAATT -3

2.7 Mass spectrometry

To identify etoposide-induced phosphorylation sites on cyclin F, pcDNA3-FLAG-cyclin F was transfected into HeLa S3 Tet-On cells. Two days post transfection, the cells were treated with 3 different conditions: (1) DMSO, (2) MLN4924 (1 μ M) treatment for 5 hours total, or (3) MLN4924 (1 μ M) pre-treatment for 1 hour followed by etoposide (50 μ M) treatment for 4 hours. Cells were collected and lysed in Co-IP buffer. FLAG-tagged cyclin F was immunoprecipitated using Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) at 4°C for 2 hours and then washed 5 times with Co-IP buffer + 150 mM NaCl. The protein was then eluted in SDS sample buffer, separated by SDS-PAGE, and phosphorylation sites on cyclin F were detected by mass spectrometry.

To identify calyculin A-induced phosphorylation sites on CDC20 and BubR1, pCS2-HA-CDC20 and pcDNA3-Myc-BubR1 were transfected into 293T cells, which were then arrested with nocodazole for 16 hours followed by DMSO or Calyculin A (20 nM) treatment for 2 hours. Cells were harvested 48 hours post transfection and immunoprecipitated using HA Epitope Tag Antibody Agarose Conjugate (Pierce, 26181) or Anti-c-Myc Agarose Affinity Gel (Sigma-Aldrich, A7470). After washing beads 3 times with Co-IP Buffer + 150 mM NaCl, proteins were eluted in SDS sample buffer, separated by SDS-PAGE, and analyzed by mass spectrometry.

To identify MCC binding proteins, pCS2-Myc-Mad2 and pcDNA3-Myc-BubR1 were transfected into 293T cells, which were then arrested with nocodazole and

harvested 48 hours post transfection. DSP crosslinking was performed¹⁰⁰, and Myc-Mad2 and Myc-BubR1 were co-immunoprecipitated using Anti-c-Myc Agarose Affinity Gel (Sigma-Aldrich, A7470). Beads were washed 3 times with Co-IP Buffer + 150 mM NaCl, and proteins were eluted with 0.1 M Ammonium Hydroxide, which was neutralized with 1 N Acetic Acid before samples were analyzed by mass spectrometry.

Mass spectrometry was performed by the Duke University School of Medicine Proteomics and Metabolomics Shared Resource, and Scaffold Proteome Software was used to analyze the data.

2.8 mRNA quantification

cDNA was generated using BioRad's iScript cDNA Synthesis Kit (170-8891) per the manufacturer's instructions. qPCR was performed using BioRad's iQ SYBR Green Supermix (170-8882) per the manufacturer's instructions. qPCR cycling parameters were as follows: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 30 seconds. All samples were amplified in triplicate. mRNA levels were determined relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method. The following primers were used:

Cyclin F: Forward 5- ATGAAGGCCTGTCTGTGTCTG -3; Reverse 5-

TCAGCGAGACTGAAGAAGCG -3

PP4: Forward 5- ATCAAGGAGAGCGAAGTCAAG -3; Reverse 5-

CCTACTCTGAACAGCTCTTTGAG -3

PP6: Forward 5- CCTGAAGGTGAGCCCTATTTG -3; Reverse 5-

ACAAACGTAGTCACATAGCCG -3

PPP1R12A: Forward 5- GAGACGGACCTCGAGCCT -3; Reverse 5-

CATCAATGCAAGCCTGGTGC -3

DUSP9: Forward 5- TTCCGGTGGCGTTAGGCTG -3; Reverse 5-

GATCGGCTCCCTACACGCTG -3

PNKP: Forward 5- ACCGGTTTCGAGAGATGACG -3; Reverse 5-

TCGAACTGCTTCCTGTAGCC -3

PPA1: Forward: 5- TGGA ACTATGGTGCCATCCC -3; Reverse 5-

CACCTCTTGACATACCTTGC -3

DCTPP1: Forward 5- AGCTGGCAGAACTCTTTCAGTG -3; Reverse 5-

AGGACGTCACTAAGCTCCTCT -3

NUDT5: Forward 5- TCTCCAGCGGTCTGTATGGA -3; Reverse 5-

CTCTCCATCCCCTGGCTTTG -3

GAPDH: Forward 5- CTCCTGTTGACAGTCAGCC -3; Reverse 5-

ACCAAATCCGTTGACTCCGAC -3

GAPDH (#2): Forward 5- ACATCGCTCAGACACCATG -3; Reverse 5-

ATGACAAGCTTCCCGTTCTC -3

2.9 Chromosome spreads

Mitotic cells were swelled in a prewarmed hypotonic solution containing 75 mM KCl for 20 minutes at 37°C. Cells were adjusted to a density of 2×10^5 /mL and fixed with methanol:acetic acid (v/v=3:1) for 15 minutes at room temperature. Cells were dropped onto microscope slides, dried at room temperature and stained with Hoechst 33258 (Molecular Probes) for 5 minutes. The slides were washed, sealed, and viewed using a Leica SP5 confocal scanning microscope.

2.10 In vitro protein assays

2.10.1 In vitro lambda protein phosphatase assay

To assess the phosphorylation status of CDC20 and BubR1, cells were lysed in Co-IP buffer, and whole cell lysates or immunoprecipitated endogenous proteins were incubated with 1 μ L lambda protein phosphatase (New England Biolabs) for 1 hour at 30°C.

2.10.2 In vitro kinase assay with Chk1

293T cells were transfected with mCherry-FLAG-cyclin F, pEGFP-C1, pEGFP-C1-cyclin F 407-565, or pEGFP-C1 cyclin F 407-660 and collected 48 hours post transfection in Co-IP buffer. mCherry-FLAG-cyclin F was immunoprecipitated from whole cell extracts using Anti-FLAG M2 Affinity Gel (Sigma-Aldrich). EGFP and EGFP-cyclin F fragments were immunoprecipitated using GFP-Trap (Chromotek). The beads were washed 4 times in Co-IP buffer + 150 mM NaCl (or 850 mM NaCl for GFP-Trap)

followed by 2 washes with Chk1 buffer (10mM HEPES pH7.5, 10mM MgCl₂, 10mM MnCl₂, and 1mM DTT) and then incubated with 0.3 µg GST-Chk1 (Millipore) in the presence of ATP [γ -³²P] for 45 minutes at 30°C. The beads were then washed 3 times with Co-IP buffer + 150mM NaCl. Bound proteins were eluted off the affinity gel with SDS sample buffer at 95°C for 5 minutes. Lysates were separated by SDS-PAGE and transferred to a PVDF membrane. ³²P incorporation was detected by autoradiography, and protein loading was detected by Coomassie blue staining.

2.11 Cell cycle analysis by flow cytometry

Cells were washed twice in PBS and fixed overnight in 70% ethanol in PBS. The following day, cells were centrifuged at 500 g for 5 minutes at 4°C and washed once with 2 mL PBS. Fixed cells were re-suspended in 500 µL PBS containing RNase A (1 µL) and propidium iodide (PI, 15 µL) and incubated at 37°C for 30 minutes prior to flow cytometry analysis of PI staining.

3. Cyclin F and the DNA damage response

3.1 Introduction

The DNA damage response (DDR) is an essential signaling network that protects the genetic material of a cell by initiating DNA repair processes and arresting the cell cycle until the damage has been repaired. As described in section 1.4.2, an important step of the DDR is the ATR-dependent downregulation of cyclin F. This decrease in cyclin F levels leads to an increase in the protein levels of the machinery responsible for dNTP production.⁹² Cyclin F downregulation in response to DNA damage is, therefore, essential for efficient DNA repair; however, the molecular mechanisms controlling cyclin F downregulation following DNA damage are largely unknown. This chapter focuses on determining the signaling pathways responsible for decreased cyclin F levels in response to DNA damage. Herein, we also investigate the regulation of cyclin F in unperturbed cells and explore how cyclin F levels affect cell cycle progression.

To elucidate the molecular mechanisms controlling cyclin F downregulation, we investigated several modes of regulation. Interestingly, we found that cyclin F is downregulated at both the mRNA and protein level following DNA damage. The protein level downregulation is due to increased proteasomal degradation, while the mRNA downregulation appears to be due to transcriptional repression as we did not observe any changes in mRNA stability following DNA damage. We also determined that, contrary to a previous report, Chk1 is required for cyclin F downregulation

following DNA damage. Interestingly, Chk1 inhibition prevents DNA damage-induced downregulation of cyclin F mRNA levels, indicating that Chk1 represses cyclin F mRNA levels in response to DNA damage. Through our studies, we also increased the number of DNA damaging agents known to induce cyclin F downregulation. Previously, the DNA damage-inducing chemotherapeutics doxorubicin, camptothecin, and methyl methanesulfonate along with UV radiation were all shown to induce cyclin F downregulation.⁹² We tested several other DNA damaging agents and found that the chemotherapeutics etoposide, neocarzinostatin, hydroxyurea, and cisplatin all induced cyclin F downregulation. In addition to these chemotherapeutic DNA damaging agents, we found that X-irradiation also causes cyclin F levels to decline. The wide-range of DNA damaging agents that trigger cyclin F downregulation is indicative of the importance of this event in the DDR. In most of our studies, we used the DNA damaging agent etoposide, which is a topoisomerase II inhibitor that causes single and double strand DNA breaks.

In this chapter, we also examined whether the SCF (Skp1-Cul1-F-box protein) complex is involved in cyclin F degradation. We found that the SCF complex plays a role in targeting cyclin F for degradation both under basal conditions and in response to DNA damage. Inhibiting the SCF complex did not completely prevent cyclin F downregulation following DNA damage, which would be expected given our finding

that regulation at the mRNA level also contributes to cyclin F downregulation following DNA damage.

In studying cyclin F protein regulation, we noticed that cyclin F appeared to migrate at two different molecular weights when separated by SDS-PAGE, and preventing cyclin F degradation by inhibiting SCF activity made these two different versions of cyclin F even more apparent. We investigated these two forms of cyclin F and discovered that the higher molecular weight cyclin F is phosphorylated. Furthermore, cyclin F is rapidly phosphorylated in the presence of the phosphatase inhibitor calyculin A, which suggests that cyclin F is constantly being dephosphorylated under normal conditions. As described in chapter 1, protein phosphorylation often regulates protein stability, and F-box proteins often recognize phosphorylated residues on proteins, which are then recruited to the SCF complex and ubiquitinated. Therefore, the discovery of phosphorylated cyclin F lead us to investigate the role of phosphorylation in the regulation of cyclin F degradation. We hypothesized that activated Chk1 may directly phosphorylate cyclin F to target it for degradation in response to DNA damage. Indeed, we found that Chk1 can directly phosphorylate cyclin F *in vitro*, but we were unable to identify Chk1 phosphorylation sites required for promoting cyclin F degradation. Interestingly, we discovered that phosphorylation of cyclin F may actually stabilize the protein, rather than lead to degradation. Treating cells with the phosphatase inhibitor calyculin A significantly increased the half-life of cyclin F

in untreated cells and prevented cyclin F downregulation in DNA damaged cells. This finding suggests dephosphorylation of cyclin F may be necessary for its degradation following DNA damage. However, it is possible that while certain cyclin F phosphorylation sites may stabilize the protein, other phosphorylation sites may promote its degradation.

Lastly, we investigated the role of cyclin F in cell cycle regulation. As mentioned in section 1.4.1, cyclin F has been suggested to be important for proper cell cycle progression, but its exact function is still being elucidated. Using cyclin F siRNA in synchronized cells, we determined that decreased levels of cyclin F delay mitotic entry. This delayed mitotic entry in the absence of cyclin F may be important for preventing cells from progressing into mitosis during the DDR, when cyclin F levels are downregulated. Interestingly, cyclin F overexpression also delayed mitotic exit, which we hypothesize is due to decreased RRM2 levels during S phase. This finding signifies the importance of maintaining low cyclin F levels during S phase to allow for efficient dNTP production and DNA synthesis.

The data presented in this chapter increase our understanding of how cyclin F levels are regulated in both normal and DNA damaged cells and how changes in cyclin F regulation may affect cell cycle progression. Understanding the mechanisms regulating the DDR and cell cycle progression is important because failures of these control systems can lead to uncontrolled cell growth and tumorigenesis. Advancing our

understanding of these pathways may enable us to identify new targets for cancer treatments and to develop anti-cancer therapies that are more effective at killing cancer cells with fewer off-target effects on healthy cells.

3.2 Results

3.2.1 Regulation of cyclin F protein in unperturbed and DNA damaged cells

3.2.1.1 Cyclin F is targeted for proteasomal degradation by the SCF E3 ubiquitin ligase complex in response to DNA damage.

To begin our investigation into the molecular mechanisms mediating the downregulation of cyclin F in response to DNA damage, we examined the amount of time required for this downregulation to occur in response to etoposide treatment in our cell lines. Cyclin F was consistently downregulated following etoposide treatment in multiple cell lines (Figure 3.1A). Although the initial downregulation of cyclin F was detectable just one hour after etoposide treatment, the most robust downregulation occurred after four hours of etoposide treatment (Figures 3.1A and B). For this reason, most of our studies were carried out using a four hour etoposide treatment. To confirm that the DDR signaling pathway was activated, we immunoblotted for Chk1 S345 phosphorylation, which is phosphorylated by ATR. Once activated, Chk1 undergoes autophosphorylation at S296, which we measured as a readout of Chk1 activity. ATM S1981 phosphorylation was also measured as a positive control for the presence of DNA damage.

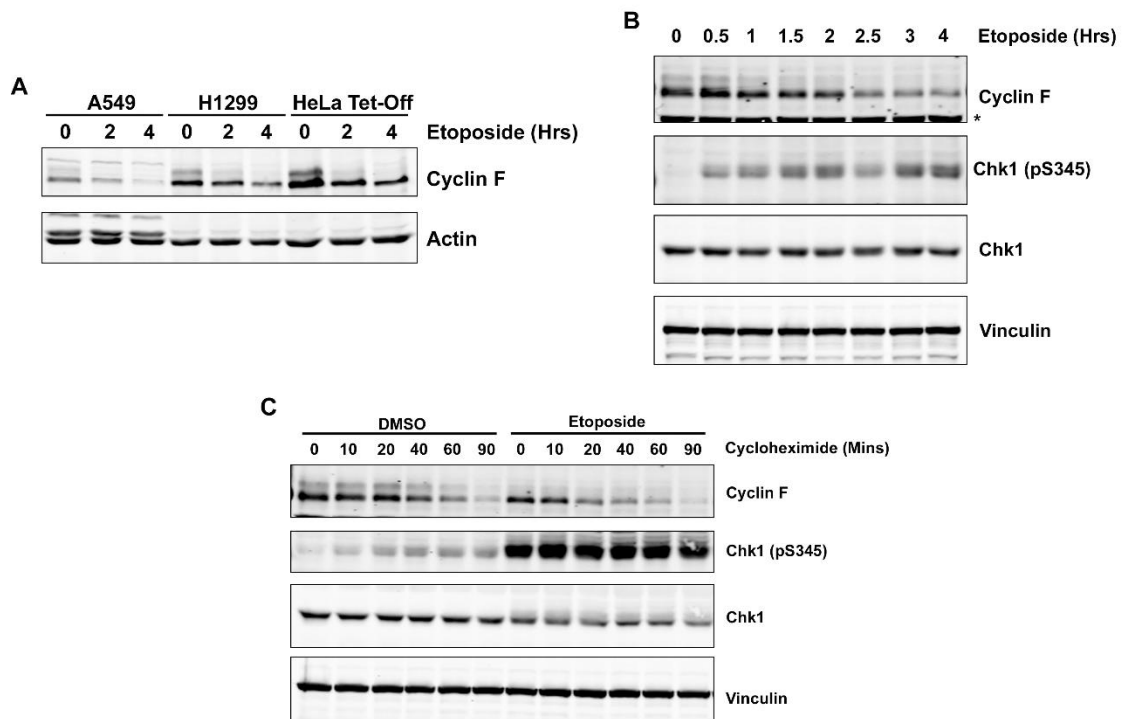


Figure 3.1: Cyclin F is degraded following etoposide-induced DNA damage. (A) A549, H1299, and HeLa Tet-Off cells were treated with etoposide (50 μ M) and collected at the indicated time points. (B) HeLa Tet-Off cells were treated with etoposide (50 μ M) and collected at the indicated time points. (C) HeLa Tet-Off cells were pre-treated with DMSO or etoposide (50 μ M) for 2 hours prior to treatment with cycloheximide (100 μ g/mL). Cells were collected at the indicated time points. (A-C) Protein levels were detected by immunoblotting with the indicated antibodies. * denotes a non-specific band.

Lowered cyclin F levels could be the result of downregulation of the protein, mRNA, or both. A previous report suggested that cyclin F downregulation following DNA damage was due primarily to protein degradation and not changes in mRNA expression.⁹² According to this report, cyclin F protein was degraded via the proteasome by an ATR-dependent, Chk1-independent mechanism following DNA damage, while mRNA levels were not significantly altered.⁹² Therefore, we began our study of DNA

damage-induced cyclin F downregulation by investigating regulation at the protein level. To investigate if cyclin F protein is degraded following DNA damage, we assessed changes in protein stability by measuring the half-life of cyclin F in the presence or absence of etoposide. Cycloheximide was used to inhibit new protein synthesis, and because cyclin F is a very short lived protein, we used a short time course to observe changes in cyclin F stability. We determined the half-life of cyclin F protein was considerably decreased in the presence of etoposide. The half-life of cyclin F in unperturbed cells was around 40-50 minutes, but in cells pre-treated with etoposide, the half-life of cyclin F was reduced by nearly half to around 20-30 minutes (Figure 3.1C). This finding indicates that in response to DNA damage, cyclin F is downregulated at the protein level by increased protein degradation. However, as will be discussed in section 3.2.2, cyclin F mRNA is also downregulated following DNA damage, and thus the total decrease in cyclin F protein following etoposide treatment is a result of both mRNA and protein downregulation.

Once we determined that cyclin F is degraded in response to etoposide, we examined whether the SCF complex is involved in targeting cyclin F for degradation. The SCF complex is known to mediate the degradation of some of its own F-box proteins, such as Skp2.¹⁰¹ To investigate whether this “auto-ubiquitination” plays a role in cyclin F degradation, we used the Nedd8 activating enzyme (NAE) inhibitor MLN4924. As mentioned earlier, SCF activity requires NAE-mediated neddylation of

the Cul1 subunit, so by inhibiting NAE, we prevent neddylation of Cul1, thus inhibiting SCF activity. Strikingly, cells treated with MLN4924 had dramatically increased cyclin F levels indicating that the SCF complex is responsible for the basal turnover of cyclin F (Figure 3.2A and B). We also assessed whether MLN4924 could prevent DNA damage-induced cyclin F degradation. DNA damaged cells that were pre-treated with MLN4924 had elevated cyclin F protein levels as compared with control treated DNA damaged cells (Figure 3.2A and B). However, DNA damage still induced a significant decrease in cyclin F levels in the presence of MLN4924 (Figure 3.2A and B). Quantification of the immunoblot data confirmed that SCF inhibition through MLN4924 treatment could partially rescue DNA damage-induced cyclin F downregulation. The remaining downregulation of cyclin F in the presence of MLN4924 could be due to cyclin F protein degradation via an SCF-independent pathway, or it could simply be due to downregulation of cyclin F mRNA levels, as will be discussed in the next section. The proteasome inhibitor MG132 nearly completely rescued cyclin F protein downregulation following etoposide treatment, although MG132 did not cause as much of an increase in basal cyclin F protein levels as MLN4924 (Figure 3.2A). One possible explanation for this result is that a small amount of cyclin F is degraded in the presence of MG132 via a proteasome-independent mechanism. Another possible explanation for the more dramatic increase in cyclin F levels following MLN4924 as compared with MG132 is that by inhibiting all proteasomal degradation, MG132 may be affecting other processes in

the cell that mediate cyclin F levels, such as mRNA transcription. Indeed, as will be described in section 3.2.2.2, MG132 treatment resulted in decreased cyclin F mRNA levels. Etoposide treatment induced Chk1 autophosphorylation on S296 confirming the activation of the DDR in all etoposide treated samples (Figure 3.2A).

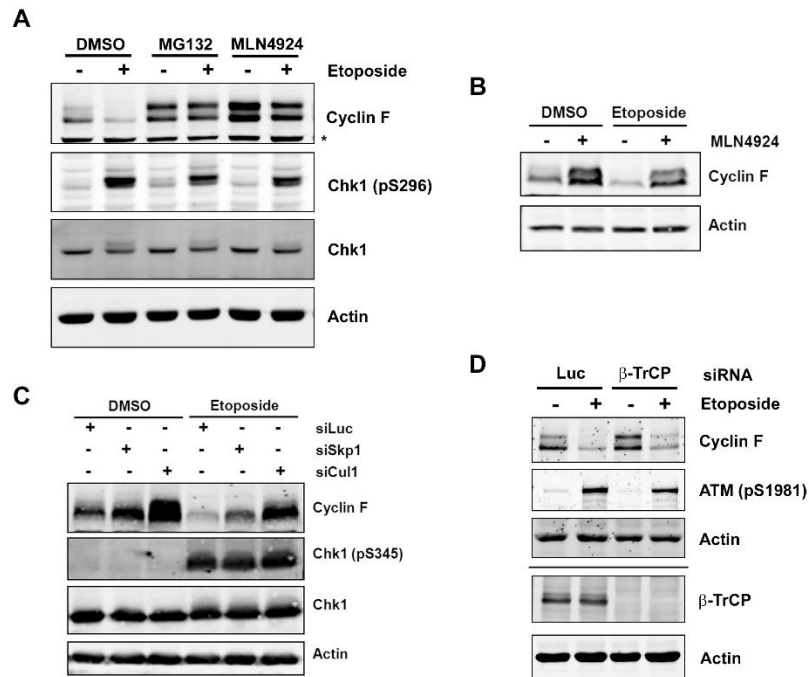


Figure 3.2: Cyclin F is targeted for proteasomal degradation via the SCF complex. (A) HeLa Tet-Off Advanced cells were pre-treated for 1 hour with DMSO, MG132 (20 μ M), or MLN4924 (1 μ M) prior to etoposide (50 μ M) treatment for 4 hours in the indicated samples. (B) H1299 cells were pre-treated for 1 hour with MLN4924 (1 μ M) prior to etoposide treatment (50 μ M) for 4 hours in the indicated samples. (C) HeLa Tet-Off cells were transfected with luciferase (Luc), Skp1, or Cul1 siRNA. 48 hours post transfection, cells were treated with etoposide (50 μ M) for 4 hours. (D) HeLa cells were transfected with luciferase (Luc) or β -TrCP siRNA. 48 hours post transfection, cells were treated with etoposide (50 μ M) for 4 hours before harvesting. (A-D) Protein levels were detected by immunoblotting with the indicated antibodies. * denotes a non-specific band.

We further investigated the role of the SCF complex in cyclin F degradation using siRNAs directed against the Skp1 and Cul1 subunits of the SCF complex. We observed that knocking down either subunit increased cyclin F levels in unperturbed cells; thereby validating the MLN4924 results by demonstrating that the SCF complex mediates cyclin F degradation under normal conditions (Figure 3.2C). Furthermore, as with MLN4924 inhibition of the SCF complex, Skp1 or Cul1 knockdown partially abrogated DNA damage-induced downregulation of cyclin F but did not fully rescue cyclin F levels (Figure 3.2C). Again, these findings indicate that while the SCF complex targets cyclin F protein for degradation, other factors contribute to cyclin F downregulation following DNA damage, as mentioned above.

We also attempted to identify the F-box protein that targets cyclin F for SCF-mediated degradation. As there are more than 60 human F-box proteins, we focused on β -TrCP because of its known role in the DDR pathway. As mentioned in section 1.3.1, SCF $^{\beta$ -TrCP is activated following DNA damage to target Cdc25A for degradation, which promotes cell cycle arrest. Using β -TrCP-targeted siRNA, we observed a modest increase in cyclin F levels under basal conditions and also a slight rescue of cyclin F levels following DNA damage as compared with control knockdown cells (Figure 3.2D). This result suggests that β -TrCP contributes to the targeting of cyclin F for degradation, but it is likely not the primary F-box protein mediating cyclin F degradation.

3.2.1.2 ATR and Chk1 mediate cyclin F downregulation in response to DNA damage

We next investigated the upstream signaling pathways that mediate DNA damage-induced cyclin F downregulation. As described in section 1.3, ATR and ATM are the two apical kinases in the DDR signaling pathways, and they canonically signal through Chk1 and Chk2, respectively. To elucidate which pathways are required for DNA damage-induced cyclin F downregulation, we first treated cells with specific ATR, ATM, Chk1, and Chk2 small molecule inhibitors and measured cyclin F protein levels following etoposide treatment. Neither the ATM inhibitor (KU-55933) nor the Chk2 inhibitor had any effect on cyclin F downregulation, indicating that etoposide-induced cyclin F downregulation is not dependent on the ATM/Chk2 pathway (Figure 3.3A). Conversely, the ATR inhibitor (VE-821) and the Chk1 inhibitor (PF-477736) both decreased the amount of cyclin F downregulation following etoposide treatment (Figure 3.3A). While ATR inhibition only partially restored cyclin F levels following DNA damage, Chk1 inhibition fully restored cyclin F levels (Figure 3.3A). We therefore concluded that Chk1 is the pivotal kinase responsible for cyclin F downregulation in response to DNA damage. Because ATR is the primary activator of Chk1, ATR inhibition dramatically repressed Chk1 activity as demonstrated by decreased Chk1 S296 autophosphorylation; however Chk1 S296 phosphorylation was still elevated in

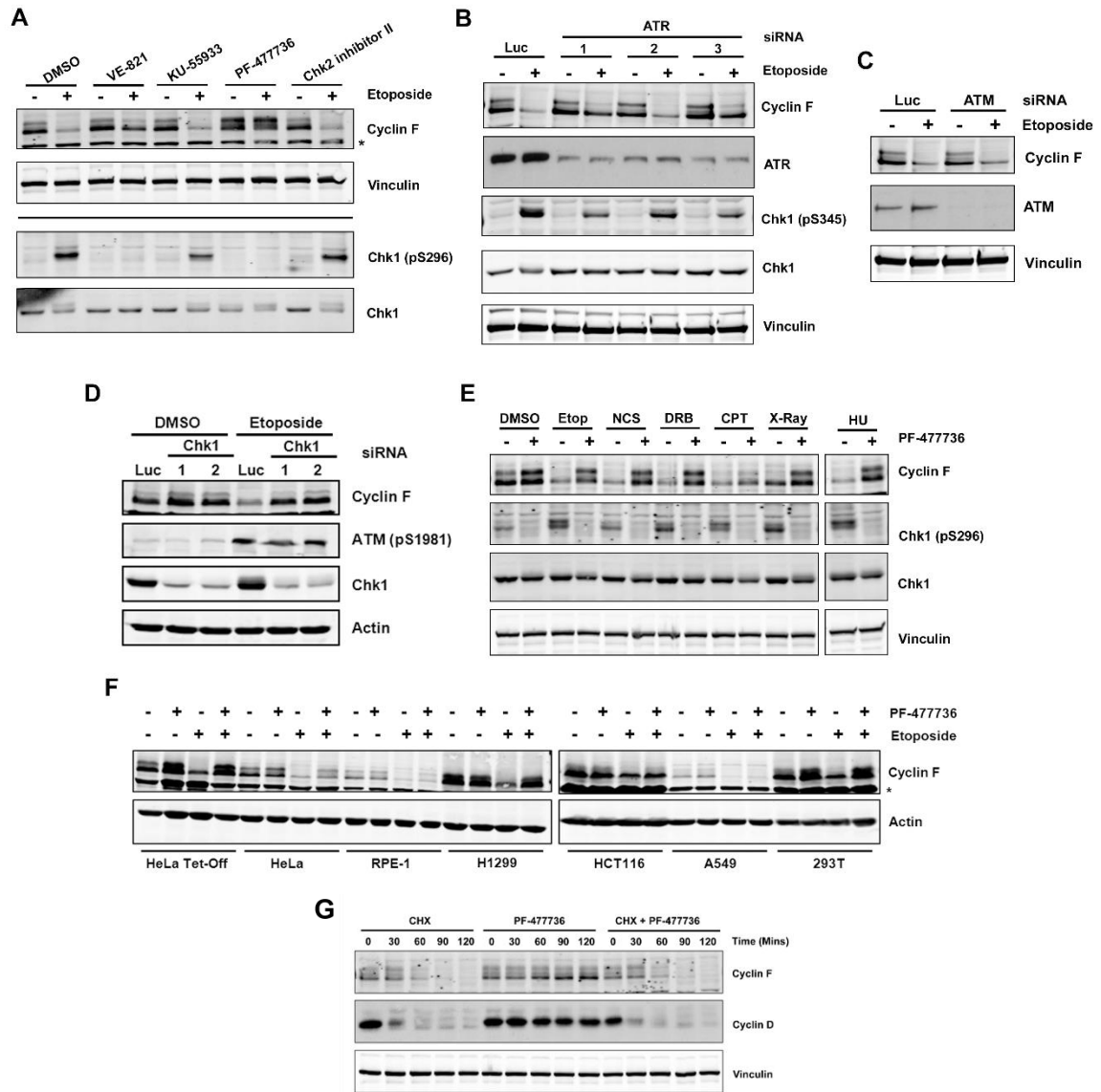


Figure 3: The ATR/Chk1 signaling pathway targets cyclin F for degradation in response to several DNA damaging agents. (A) HeLa Tet-Off Advanced cells were pre-treated for 1 hour with VE-821 (ATR inhibitor, 10 μ M), KU-55933 (ATM inhibitor, 10 μ M), PF-477736 (Chk1 inhibitor, 0.4 μ M), or Chk2 inhibitor II (10 μ M). Cells were treated with etoposide for 4 hours before harvesting. (B) HeLa Tet-Off Advanced cells were transfected with luciferase (Luc) or 1 of 3 different ATR siRNAs. 48 hours post transfection, cells were treated for 4 hours with etoposide (50 μ M) before harvesting. (C) HeLa Tet-Off Advanced cells were transfected with luciferase (Luc) or ATM siRNA. 48 hours post transfection, cells were treated for 4 hours with etoposide (50 μ M) before harvesting. (D) HeLa Tet-Off cells were transfected with luciferase (Luc) or 1 of 2

different Chk1 siRNAs. 48 hours post transfection, cells were treated with etoposide (50 μM) for 4 hours before harvesting. (E) HeLa Tet-Off cells were pre-treated with PF-477736 (0.4 μM) for 1 hour prior to treatment with the indicated DNA damaging agent for 4 hours. (F) The indicated cell lines were pre-treated for 1 hour with PF-477736 (0.4 μM) prior to etoposide (50 μM) treatment for 4 hours. (G) HeLa Tet-On cells were pre-treated for 1 hour with PF-477736 (0.4 μM) and then treated with cycloheximide (CHX, 100 $\mu\text{g}/\text{mL}$). Cells were harvested at the indicated time points. (A-G) Protein levels were detected by immunoblotting with the indicated antibodies. Etop: etoposide (50 μM); NCS: neocarzinostatin (0.1 $\mu\text{g}/\text{mL}$); DRB: doxorubicin (1 μM); CPT: cisplatin (20 μM); X-ray (5 Gy); HU: hydroxyurea (20 μM). * denotes a non-specific band.

ATR inhibitor treated cells as compared with cells treated with the Chk1 inhibitor (Figure 3.3A). This result suggests that Chk1 is still partially activated in the presence of the ATR inhibitor, which likely explains why ATR inhibition was not as effective as Chk1 inhibition at rescuing cyclin F levels following DNA damage. This activation of Chk1 in the presence of an ATR inhibitor could be due to residual ATR activity resulting from incomplete inhibition. Alternatively, ATM may also be contributing to Chk1 activation in response to etoposide. Indeed, Chk1 autophosphorylation of S296 was decreased in the presence of the ATM inhibitor indicating that ATM activity does promote Chk1 activation (Figure 3.3A). However, it is clear that ATR is the primary activator of Chk1 as ATR inhibition had a much stronger effect on Chk1 autophosphorylation and restoration of cyclin F levels in the presence of etoposide.

To validate our kinase inhibitor findings, we used siRNAs targeted toward ATR, ATM, or Chk1 and measured etoposide-induced cyclin F downregulation. For each kinase, the siRNA mediated knockdown had the same effect on cyclin F levels as when the kinase was inhibited by the small molecule inhibitors. Two of the three ATR siRNAs

(#1 and #3) partially restored cyclin F levels and significantly decreased Chk1 S296 autophosphorylation in response to etoposide (Figure 3.3B). ATR siRNA #2 was not as effective as siRNAs #1 and #3 at knocking down ATR levels or decreasing Chk1 autophosphorylation, which likely explains the lack of cyclin F rescue following DNA damage (Figure 3.3B). Consistent with the ATM inhibitor results, ATM knockdown by siRNA had no effect on etoposide-induced cyclin F downregulation (Figure 3.3C). While ATM siRNA had no effect on cyclin F downregulation and ATR siRNA could only partially restore cyclin F levels, Chk1 knockdown by two different siRNA sequences fully restored cyclin F levels following etoposide treatment, consistent with the effect of the Chk1 inhibitor (Figure 3.3D). Taken together, the small molecular inhibitor data and siRNA data both indicate that Chk1, activated primarily through ATR, is the key mediator of cyclin F downregulation following etoposide treatment.

Further examination of the role of Chk1 in DNA damage-induced cyclin F downregulation revealed that Chk1 inhibition could rescue cyclin F downregulation in response to multiple DNA damaging agents, including neocarzinostatin (NCS), doxorubicin (DRB), X-irradiation (X-rays), and hydroxyurea (HU) (Figure 3.3E). Interestingly, Chk1 inhibition did not appear to be as effective at rescuing cyclin F levels following cisplatin (CPT) treatment suggesting there are also Chk1-independent mechanisms that can mediate cyclin F downregulation in response to certain types of DNA damage. In addition to testing multiple DNA damaging agents, we also

investigated whether etoposide-induced cyclin F downregulation is dependent on Chk1 across multiple cell lines. We observed that certain cell lines were highly dependent on Chk1 for cyclin F downregulation following etoposide treatment, while other cell lines were not as dependent on Chk1. For example, Chk1 inhibition could completely restore cyclin F levels in HeLa Tet-Off and 293T cells, but in A549 and hTERT-RPE1 cells, the effect of Chk1 inhibition was barely detectable (Figure 3.3F). We also observed an intermediate effect of Chk1 inhibition in certain cell lines suggesting that while Chk1 plays a role in cyclin F downregulation, other factors are also involved in these cell lines (Figure 3.3F). Interestingly, in some cell lines the basal level of cyclin F protein was increased following Chk1 inhibition, suggesting a role for Chk1 in the regulation of cyclin F levels in unperturbed cells. We investigated the possibility that Chk1 may promote cyclin F degradation in unperturbed cells by measuring cyclin F half-life in the presence or absence of the Chk1 inhibitor. We found that Chk1 inhibition did not alter the half-life of cyclin F protein under basal conditions, indicating that Chk1 may repress cyclin F levels by another mechanism such as by repressing translation, transcription, or mRNA stability (Figure 3.3G). In section 3.2.2.2, we describe our finding that Chk1 represses cyclin F mRNA levels in response to DNA damage and that Chk1 may also affect cyclin F mRNA levels under basal conditions.

3.2.1.3 Cyclin F exists in both a hypo and hyperphosphorylated state

In studying cyclin F, we consistently detected a subset of cyclin F that migrated slightly slower than the majority of the cyclin F protein when separated by SDS-PAGE. Treating cell lysates with lambda protein phosphatase caused all of the cyclin F protein to migrate at the lower molecular weight, thereby demonstrating that the higher molecular weight band of cyclin F is a phosphorylated version of the protein (Figure 3.4A). The downshift of cyclin F following lambda phosphatase treatment was particularly apparent when we pretreated cells with MG132 or MLN4924 to increase overall cyclin F levels (Figure 3.4A). The phosphorylated form of cyclin F was generally less abundant than the non-phosphorylated form, and in certain experiments, phosphorylated cyclin F appeared to be enriched when cyclin F degradation was prevented with MG132 or MLN4924 (Figure 3.2A, 3.2B, and 3.4A). As phosphorylation is commonly affects the stability of proteins, we hypothesized that phosphorylation on cyclin F may target it for degradation. To test our hypothesis, we first sought to identify phosphatase inhibitors that would prevent dephosphorylation of cyclin F and enrich the phosphorylated form of cyclin F. We could then measure cyclin F levels in the presence of the phosphatase inhibitor to determine if phosphorylation is affecting its degradation. We tested the phosphoprotein phosphatase (PPP) family phosphatase inhibitors calyculin A and okadaic acid and found that calyculin A was a very potent inhibitor of the cyclin F-targeted phosphatase as it induced phosphorylation of all cyclin F protein

(Figure 3.4B). High concentrations (1 μ M) of okadaic acid also increased the phosphorylated form of cyclin F, but low concentrations (0.1 μ M) of okadaic acid had no effect (Figure 3.4B). The PPP family phosphatases PP1, PP2A, PP4, and PP5 are known to be inhibited by calyculin A, but low concentrations of okadaic acid are only known to inhibit PP2A and PP4.¹⁰² PP1 and PP5 are both inhibited at higher concentrations of okadaic acid, but since PP5 is less sensitive to calyculin A than PP1, we hypothesized that PP1, or a PP1-like phosphatase, is responsible for cyclin F dephosphorylation.¹⁰²

Having identified phosphatase inhibitors that could drive cyclin F phosphorylation, we sought to test our hypothesis that forcing cyclin F phosphorylation by phosphatase inhibition would lead to increased cyclin F degradation. We treated cells with the phosphatase inhibitors okadaic acid or calyculin A and then measured cyclin F levels. However, we found that neither okadaic acid nor calyculin A-induced cyclin F phosphorylation was sufficient to cause cyclin F degradation (Figure 3.4B). We tested the effect of calyculin A on multiple cell lines and determined that while calyculin A strongly induced cyclin F phosphorylation in each cell line, it did not induce cyclin F degradation (Figure 3.4C). Interestingly, a time course of calyculin A treatment showed that cyclin F becomes phosphorylated very rapidly in the presence of calyculin A, suggesting that kinases and phosphatases are continuously acting on cyclin F to alter its phosphorylation status (Figure 3.4D). These rapid phosphorylation changes would provide a very agile, adaptable mechanism to regulate cyclin F function and/or stability

both during unperturbed cell cycles and in response to DNA damage. We will further explore the effects of cyclin F phosphorylation in the following two sections.

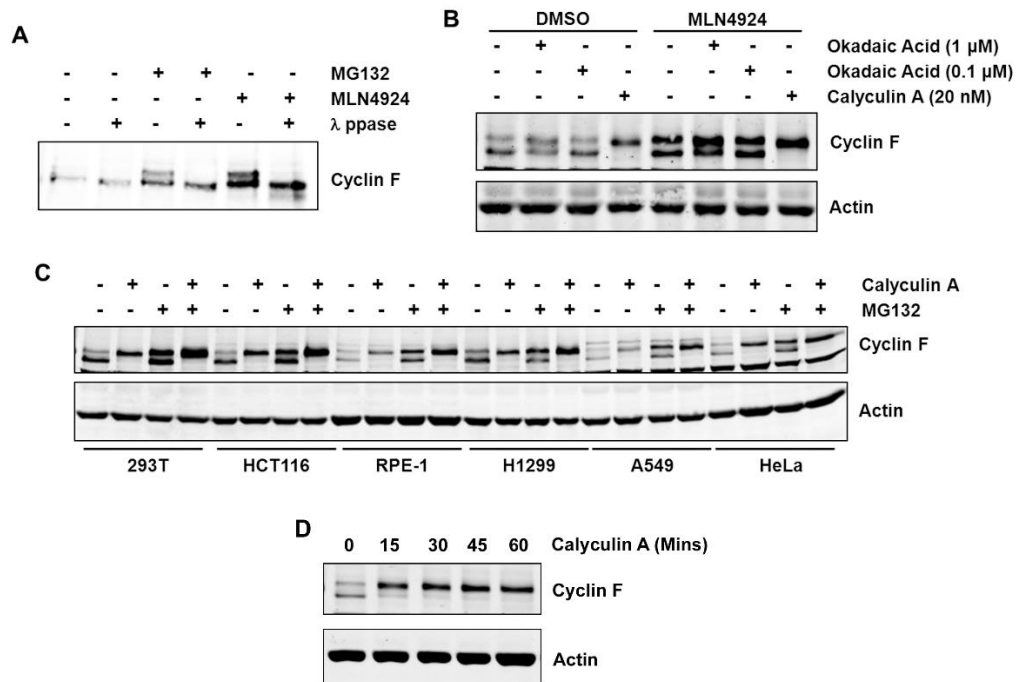


Figure 3.4: Cyclin F exists in both a hypo- and hyper-phosphorylated state. (A) HeLa Tet-off cells were treated with MG132 (20 μ M) or MLN4924 (1 μ M) for 3 hours prior to collection. Cyclin F was immunoprecipitated from cell lysates and treated with lambda protein phosphatase (λ ppase). The cyclin F mobility shift was detected by SDS-PAGE and immunoblotting. (B) HeLa cells were treated pre-treated for 1 hour with MLN4924 (1 μ M) prior to treatment with the indicated phosphatase inhibitors for 1 hour. Protein levels were detected by immunoblotting with the indicated antibodies. (C) The indicated cell lines were pre-treated with MG132 (20 μ M) for 1 hour prior to treatment with calyculin A (20 μ M) for 1 hour. Protein levels were detected by immunoblotting. (D) HeLa cells were treated with calyculin A (20 μ M) and collected at the indicated time points. Protein levels were detected by immunoblotting.

3.2.1.4 Chk1 can phosphorylate cyclin F *in vitro*

Although calyculin A-induced phosphorylation was not sufficient to induce cyclin F degradation, it remains possible that phosphorylation may still regulate cyclin F

stability in a more nuanced way. We hypothesized that specific phosphorylation sites on cyclin F may be inhibitory towards degradation while other sites may promote degradation. Given that Chk1 is required for DNA damage-induced cyclin F downregulation, we investigated the possibility that Chk1 may directly phosphorylate cyclin F to target cyclin F for degradation in response to DNA damage. We first sought to determine if Chk1 could phosphorylate cyclin F *in vitro*. To test this, we immunoprecipitated mcherry and FLAG-tagged cyclin F from overexpressing 293T cells and performed an *in vitro* kinase assay with recombinant GST-tagged Chk1 in the presence of ATP [γ - 32 P]. Interestingly, we found that mcherry-FLAG-cyclin F was phosphorylated even in the absence of GST-Chk1, indicating that a kinase was immunoprecipitated with cyclin F (Figure 3.5A). However, mcherry-FLAG-cyclin F phosphorylation was significantly enhanced when incubated in the presence of GST-Chk1, indicating that Chk1 can phosphorylate mcherry-FLAG-cyclin F *in vitro* (Figure 3.5A).

We next attempted to identify etoposide-induced phosphorylation sites on cyclin F using mass spectrometry. We overexpressed FLAG-tagged cyclin F in 293T cells, pre-treated cells with MLN4924 to prevent cyclin F degradation, and then treated cells with DMSO or etoposide to induce DNA damage. In addition to the MLN4924 treated samples, we also analyzed an untreated sample as a negative control. FLAG-cyclin F was immunoprecipitated from each of the three samples and separated by SDS-PAGE

prior to mass spectrometry analysis. Unfortunately, the protein coverage by mass spectrometry was rather poor with only 18% of the cyclin F protein identified. Nonetheless, four cyclin F phosphorylation sites were still able to be identified: S195, S577, S754, and S774 (Figure 3.5B). All four of the identified phosphorylation sites were present in each of the three samples, but there was a trend toward increased phosphorylation in the etoposide-treated sample. To investigate whether

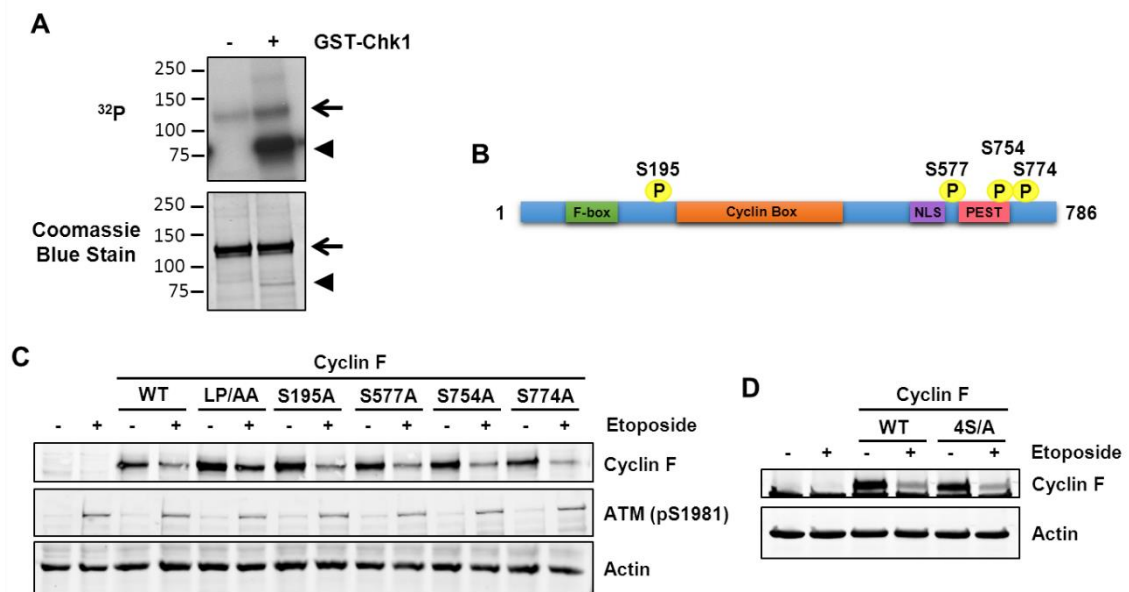


Figure 3.5: Chk1 can phosphorylate cyclin F *in vitro*. (A) 293T cells were transfected with mCherry-FLAG-cyclin F and collected 48 hours post transfection. mCherry-FLAG-cyclin F was immunoprecipitated from whole cell extracts using Anti-FLAG M2 beads and incubated with GST-Chk1 in the presence of ATP [γ - 32 P]. 32 P incorporation was detected by autoradiography, and protein loading was detected by Coomassie blue staining. Arrows: mCherry-FLAG-Cyclin F; Arrowheads: GST-Chk1. (B) Schematic representation of cyclin F protein indicating the location of the 4 phosphorylation sites identified by mass spectrometry. (C and D) HeLa cells were transfected with wild-type (WT) pBABE-FLAG-HA-puro-cyclin F or the indicated cyclin F mutants. 48 hour later, cells were treated for 4 hours with etoposide (50 μ M) before harvesting. Protein levels were detected by immunoblotting.

phosphorylation at any of these sites is required for cyclin F degradation in response to etoposide treatment, we first mutated the identified serine residues to alanine to prevent phosphorylation at each site. The mutated cyclin F constructs were transfected into 293T cells, and etoposide-induced cyclin F degradation was assessed by immunoblot. Each of the serine-to-alanine mutant cyclin F proteins was degraded by etoposide treatment demonstrating that phosphorylation on the mutated serine is not required for etoposide-induced cyclin F degradation (Figure 3.5C). We also mutated all four serine residues to alanine in the same construct, but this mutant version of cyclin F was also degraded following etoposide treatment (Figure 3.5D).

In addition to testing the degradation of the serine-to-alanine cyclin F mutant proteins, we also tested the degradation of a cyclin F F-box mutant (LP/AA) that was previously reported to not bind Skp1, the SCF subunit that interacts with F-box proteins. This mutant protein would allow us to determine if cyclin F mediates its own degradation via the SCF complex following DNA damage. The LP/AA cyclin F protein was degraded following etoposide treatment (Figure 3.5C). However, when we tested the interaction of the LP/AA mutant of cyclin F with Skp1, we found that this mutant could still bind to Skp1, and therefore we cannot make any conclusions about the possibility that cyclin F may target itself for degradation via the SCF complex based on this data.

Although the four cyclin F phosphorylation sites identified by mass spectrometry are not required for cyclin F degradation, given the low protein coverage in our mass spectrometry analysis, it is possible that other unidentified phosphorylation sites may still exist to target cyclin F for degradation. To increase the likelihood of identifying etoposide-induced phosphorylation sites, we attempted to narrow down the region of cyclin F that is required for its degradation in response to etoposide. We generated several cyclin F truncation mutants and fragments and tested whether they could be degraded following etoposide treatment. A previous study had demonstrated that a fragment of cyclin F containing amino acids 406-660 could be degraded following DNA damage; this was also sufficient to induce the degradation of Green Fluorescent Protein (GFP) when the sequence was added to the GFP protein.⁹² We confirmed this finding and also demonstrated that an even shorter fragment of cyclin F (406-565) could target EGFP (Enhanced GFP) for degradation following DNA damage (Figure 3.6A).

To determine if there were any Chk1 phosphorylation sites on these fragments, we performed an *in vitro* kinase assay using the EGFP-cyclin F fragment fusion proteins. The EGFP-cyclin F proteins were immunoprecipitated from overexpressing 293T cells using the GFP-Trap and then incubated with GST-Chk1 in the presence of ATP [γ -³²P]. We established that Chk1 could phosphorylate both the 407-565 and 407-660 EGFP-cyclin F fusion proteins but not EGFP alone, indicating that Chk1 phosphorylation occurred on the cyclin F portion of the fusion protein (Figure 3.6B). Chk1 has a known

preferential phosphorylation sequence of RXXS/T, and so we analyzed the 407-565 region of cyclin F for this sequence. We found only one sequence match in this region, which was RQVS at S509. However, when we mutated S509 to alanine to prevent

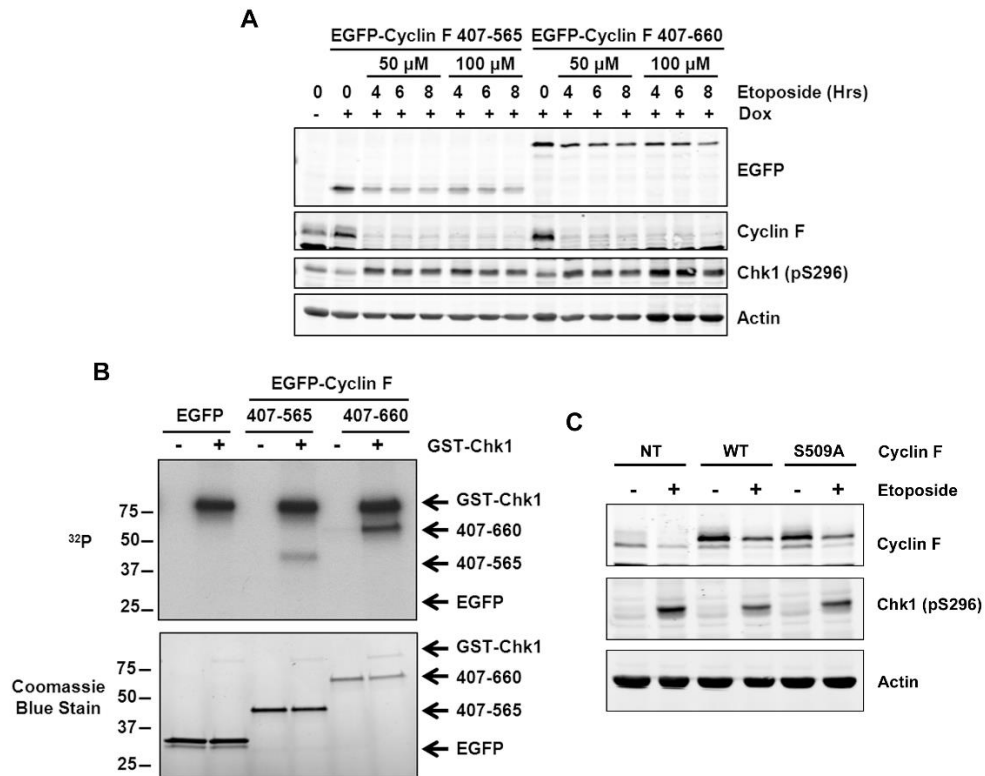


Figure 3.6: GFP-tagged cyclin F fragments are degraded by etoposide and can be phosphorylated by Chk1 *in vitro*. (A) HeLa S3 Tet-On cells containing doxycycline-inducible EGFP-cyclin F fragments (407-565 or 407-660) were treated overnight with doxycycline (1 μ g/mL) to induce expression. The next day, cells were treated with etoposide (50 μ M or 100 μ M) for 4, 6, or 8 hours. Protein levels were detected by immunoblotting. (B) 293T cells were transfected with pEGFP-C1, pEGFP-C1-cyclin F 407-565, or pEGFP-C1-cyclin F 407-660 and collected 48 hours post transfection. EGFP was immunoprecipitated from whole cell extracts using GFP-Trap and incubated with GST-Chk1 in the presence of ATP [γ -³²P]. ³²P incorporation was detected by autoradiography, and protein loading was detected by Coomassie blue staining. (C) HeLa cells were transfected with wild-type (WT) or S509A pBABE-FLAG-HA-puro-cyclin F. 48 hour later, cells were treated for 4 hours with etoposide (50 μ M) before harvesting. Protein levels were detected by immunoblotting.

phosphorylation at this site in the full length protein, it did not prevent etoposide-induced degradation (Figure 3.6C). Future studies investigating cyclin F phosphorylation sites will be important for determining whether etoposide-induced phosphorylation mediate cyclin F degradation.

3.2.1.5 Calyculin A prevents cyclin F protein downregulation in unperturbed and DNA damaged cells

As mentioned in section 3.2.1.2, inhibiting phosphatase activity with calyculin A caused rapid phosphorylation of cyclin F, but this phosphorylation did not induce an observable decrease in cyclin F protein levels as we hypothesized. To further investigate the effect of cyclin F phosphorylation on cyclin F stability, we measured changes in the half-life of cyclin F using cycloheximide to inhibit translation in cells treated with calyculin A. Unexpectedly, we found that calyculin A treatment dramatically increased the half-life of cyclin F (Figure 3.7A). Given the large accumulation of phosphorylated cyclin F following calyculin A treatment, we hypothesized that the calyculin A-induced phosphorylation sites on cyclin F actually stabilize the protein, as opposed to targeting it for degradation as we had initially proposed. Another possible explanation for the increased half-life of cyclin F is that calyculin A treatment phosphorylates and inhibits another component of the cyclin F degradation pathway. For example, calyculin A treatment may inhibit the SCF complex or the proteasome. To investigate this possibility, we measured the half-life of other short-lived proteins to see if calyculin A treatment was having a general effect on protein degradation or if it was specifically

inhibiting cyclin F degradation. Interestingly, we found that calyculin A actually had the opposite effect on the half-life of the other proteins we investigated; calyculin A treatment alone induced the degradation of p21, cyclin D, and p53 (Figure 3.7A). p21 and cyclin D are both known to be targeted for degradation by the SCF complex, which indicates that the SCF complex is still functional in the presence of calyculin A.¹⁰³ Given

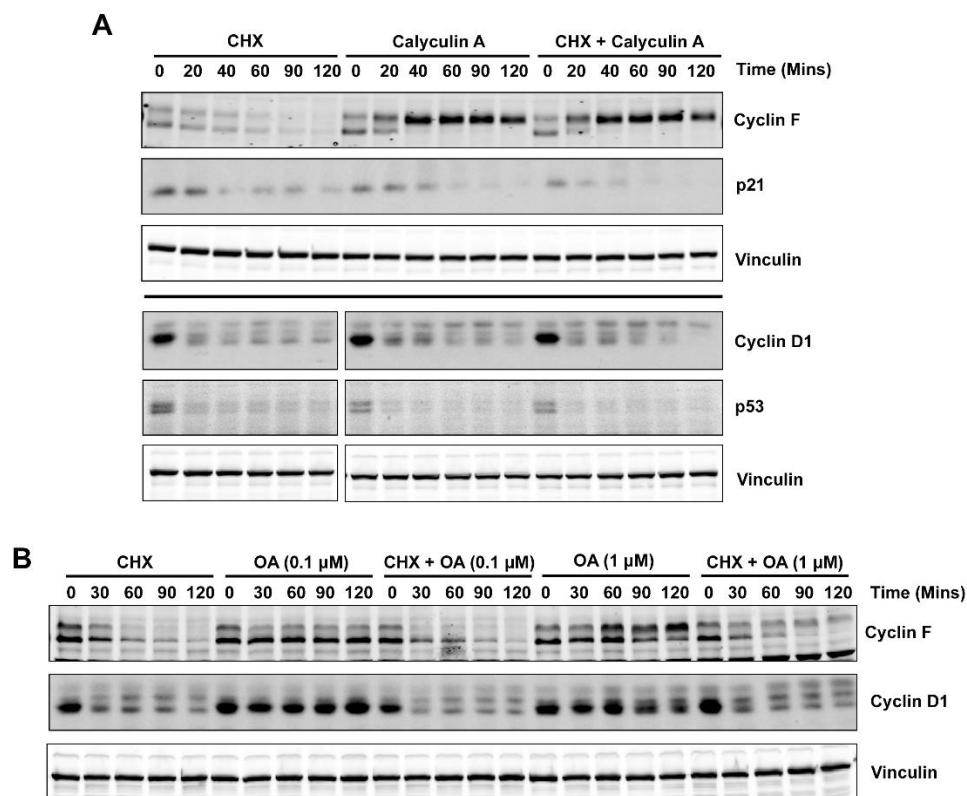


Figure 3.7: Phosphatase inhibition prevents cyclin F degradation. (A) HeLa Tet-On cells were treated with calyculin A (20 nM) and/or cycloheximide (CHX, 100 μg/mL) and collected at the indicated time points. Protein levels were detected by immunoblotting with the indicated antibodies. (B) HeLa Tet-On cells were pre-treated for 30 minutes with okadaic acid (0.1 μM or 1 μM) prior to treatment with cycloheximide (CHX, 100 μg/mL). Cells were harvested at the indicated time points. Protein levels were detected by immunoblotting with the indicated antibodies.

that we know the SCF complex also mediates cyclin F degradation, these results suggest that the cyclin F degradation pathway is still intact and that phosphorylation directly on cyclin F prevents its degradation. Consequently, a phosphatase would then be required to dephosphorylate cyclin F to enable its degradation.

In addition to measuring the half-life of cyclin F in the presence of calyculin A, we also measured the half-life of cyclin F following okadaic acid treatment. As expected based on our previous results demonstrating that only high levels of okadaic acid affect cyclin F phosphorylation status, low concentrations of okadaic acid (0.1 μM) had no effect on the half-life of cyclin F, but high concentrations (1 μM) prolonged cyclin F's half-life (Figure 3.7B). The stabilization of cyclin F in the presence of 1 μM okadaic acid was not as dramatic as with calyculin A, likely because calyculin A is a more potent inducer of cyclin F phosphorylation. Similar to calyculin A, we also found that okadaic acid modestly decreased the half-life of cyclin D as well and did not prevent its degradation, indicating that these inhibitors do not interfere with overall SCF-mediated proteasomal protein degradation (Figure 3.7B).

Given that calyculin A could prevent the degradation of cyclin F in normal cells, we tested whether calyculin A could also disrupt cyclin F downregulation in response to DNA damage. Cells were treated with etoposide in the presence or absence of calyculin A, and cyclin F levels were measured over time. In cells treated with only etoposide, cyclin F levels were dramatically decreased after 1.5 hours of etoposide treatment, but at

the same time point in the etoposide plus calyculin A treated cells, cyclin F levels were still very high (Figure 3.8A). Even after four hours of etoposide treatment the calyculin A treated cells had significantly higher levels of cyclin F as compared with the etoposide only treated cell (Figure 3.8A and B). This interesting finding led us to look for phosphatases that interact with cyclin F; if phosphorylated cyclin F cannot be degraded, then a phosphatase must be required to dephosphorylate cyclin F to allow for its degradation following DNA damage. As only high concentrations of okadaic acid could affect cyclin F phosphorylation status and stability, we hypothesized that PP1 is responsible for cyclin F dephosphorylation. To investigate whether cyclin F is dephosphorylated by PP1, we first looked for any interaction between cyclin F and PP1 in both untreated and etoposide treated cells. Endogenous cyclin F was immunoprecipitated, and PP1 interaction was determined by immunoblotting for different PP1 isoforms. Interestingly, the PP1 isoform PP1 β interacted with cyclin F in both untreated and etoposide treated cells (Figure 3.8C). This result suggests that PP1 β may regulate cyclin F phosphorylation (and potentially its stability) under basal conditions. To test if PP1 β plays a role in cyclin F degradation in response to DNA damage, we used siRNA targeted toward all three PP1 isoforms (pan-PP1) or PP1 β alone and measured cyclin F levels following etoposide treatment. Both siRNAs slightly increased cyclin F levels in untreated cells but did not prevent cyclin F downregulation following etoposide treatment (Figure 3.8D). However, the knockdown efficiency of

PP1 β was not 100%, and therefore we cannot rule out the possibility that PP1 β may still be required for cyclin F degradation in response to DNA damage. Further studies will be required to determine if PP1 β plays a role in DNA damage-induced cyclin F downregulation.

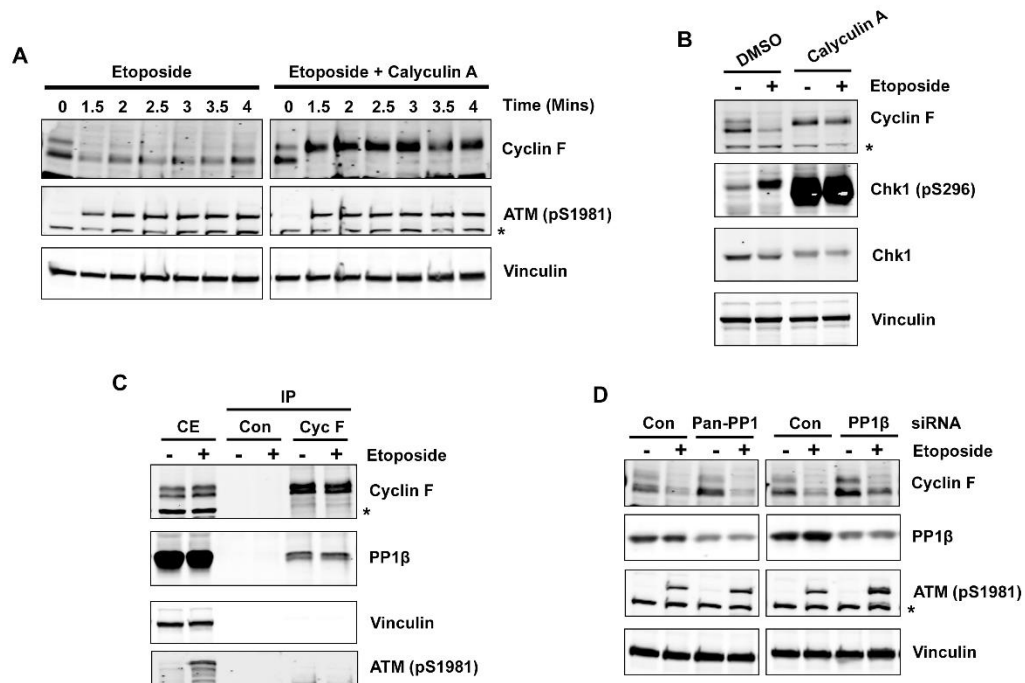


Figure 3.8: Calyculin A prevents DNA damage-induced cyclin F downregulation. (A) HeLa Tet-Off cells were treated with etoposide (50 μ M) in the presence or absence of calyculin A (20 nM), and cells were harvested at the indicated time points. (B) HeLa Tet-On cells were treated for 4 hours with etoposide (50 μ M) and DMSO or calyculin A (20 μ M). (C) HeLa Tet-On cells were pre-treated for 1 hour with MG132 (20 μ M) prior to treatment with DMSO or etoposide (50 μ M) for 4 hours. Cyclin F was immunoprecipitated from whole cell lysate. (D) HeLa Tet-Off cells were transfected with control (sc-37007), pan-PP1 (sc-43545), or PP1 β -2 siRNA. 48 hours later, cells were treated for 4 hours with etoposide (50 μ M) before harvesting. (A-D) Protein levels were detected by immunoblotting with the indicated antibodies. IP: immunoprecipitation; CE: cell extract. * denotes a non-specific band.

3.2.2 DNA damage-induced downregulation of cyclin F mRNA

3.2.2.1 Cyclin F mRNA levels are downregulated in response to DNA damage

In the previous section, we investigated DNA damage-induced downregulation of cyclin F at the protein level and found that the half-life of cyclin F protein is decreased following DNA damage. Given that cyclin F already has a very short half-life under basal conditions, we speculated that other factors may also be involved in the dramatic downregulation of cyclin F in response to DNA damage. Additionally, we observed that inhibiting the SCF E3 ubiquitin ligase that targets cyclin F protein for degradation only partially restored cyclin F levels following DNA damage, which suggests that other mechanisms contribute to cyclin F downregulation. Therefore, we hypothesized that cyclin F downregulation may also exist at the level of cyclin F mRNA transcription or stability.

We measured cyclin F mRNA levels by qPCR in untreated and etoposide treated cells, and determined that strikingly, cyclin F mRNA levels were dramatically decreased following etoposide treatment, suggesting that this decrease in cyclin F mRNA is likely a significant contributor to the decrease in cyclin F protein levels observed following etoposide treatment (Figure 3.9A). We next performed a time course of etoposide treatment to look at the decrease in cyclin F mRNA levels over time. We found that after just one hour of etoposide treatment, cyclin F mRNA levels were already decreased by nearly 40%, and after four hours of etoposide treatment, cyclin F mRNA levels were

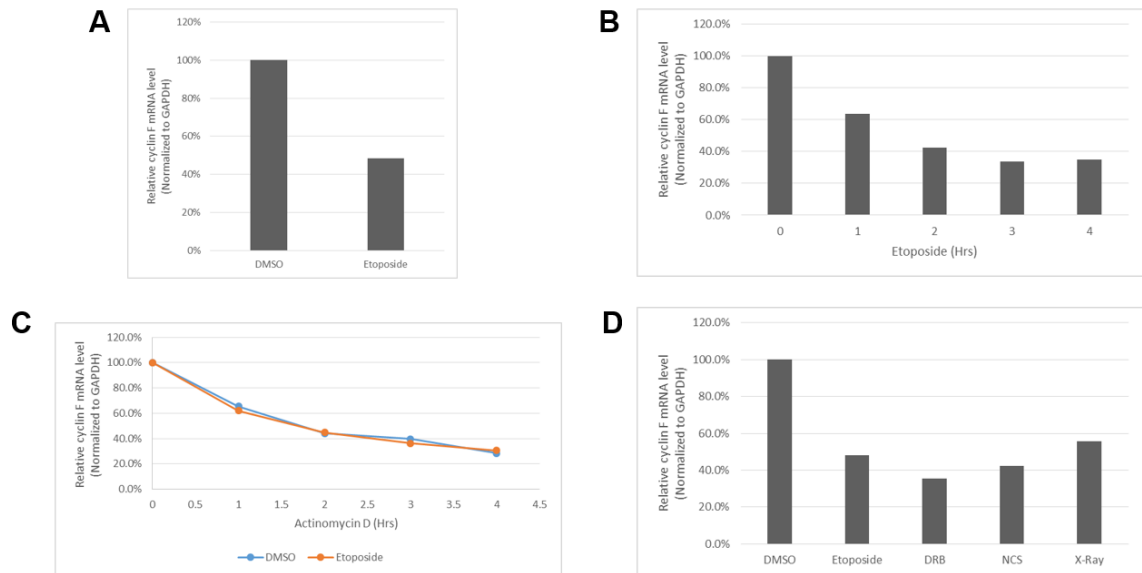


Figure 3.9: DNA damage induces cyclin F mRNA downregulation. (A-D) qPCR was used to measure cyclin F mRNA levels. Expression levels are shown relative to GAPDH and normalized to the DMSO sample. (A) HeLa Tet-On cells were treated with DMSO or etoposide (50 μ M) for 4 hours before harvesting. (B) HeLa Tet-Off cells were treated with etoposide (50 μ M) and collected at the indicated time points. (C) HeLa Tet-Off cells were treated with actinomycin D (5 μ g/mL) and DMSO or etoposide (50 μ M) and collected at the indicated time points. (D) HeLa Tet-On cells were treated for 4 hours with the indicated DNA damaging agents: etoposide (50 μ M), doxorubicin (DRB, 1 μ M), neocarzinostatin (NCS, 0.1 μ g/mL), or X-irradiation (X-ray, 5 Gy).

decreased by more than 60% (Figure 3.9B). The downregulation time course of cyclin F mRNA closely resembles the decrease in cyclin F protein levels following DNA damage, again suggesting that downregulation of cyclin F mRNA is a significant contributor to the observed decrease in cyclin F protein levels. To determine whether cyclin F mRNA downregulation was due to changes in mRNA stability, we used actinomycin D to halt transcription so we could assess the half-life of cyclin F mRNA. Cyclin F mRNA has a relatively short half-life of around 1.5 hours, and we did not observe any changes in the half-life of cyclin F mRNA following etoposide treatment (Figure 3.9C). Therefore, the

mRNA downregulation in response to etoposide treatment must be due to decreased transcription of cyclin F. We also tested whether other DNA damaging agents induced cyclin F mRNA downregulation and found that doxorubicin, neocarzinostatin, and X-irradiation also decreased cyclin F mRNA levels (Figure 3.9D). Therefore, cyclin F mRNA downregulation is likely a universal mechanism of cyclin F downregulation in response to many different types of DNA damage.

3.2.2.2 Chk1 inhibition prevents DNA damage-induced cyclin F mRNA downregulation

As shown in section 3.2.1.1, Chk1 inhibition prevents the downregulation of cyclin F protein following DNA damage. This result indicates that Chk1 kinase activity is required for cyclin F downregulation in response to DNA damage. To investigate if Chk1 mediates cyclin F mRNA downregulation following DNA damage, we used qPCR to measure cyclin F mRNA levels in cells treated with etoposide or neocarzinostatin (NCS) in the presence or absence of the Chk1 inhibitor, PF-477736. We found that Chk1 inhibition prevented the DNA damage-induced downregulation of cyclin F mRNA levels (Figure 3.10). Interestingly, we also found that in certain experiments, Chk1 inhibition increased cyclin F mRNA levels in unperturbed cells. As shown in Figure 3.10, even in the presence of DNA damage induced by NCS, Chk1 inhibition resulted in elevated mRNA levels as compared with the DMSO control. As mentioned in section 3.2.1.2, Chk1 inhibition alone increased cyclin F protein levels in certain cell lines. This

result may be partially due to increased cyclin F mRNA levels following Chk1 inhibition.

We also measured the effect of MLN4924 and MG132 treatment on cyclin F mRNA levels in response to DNA damage-induced by etoposide and neocarzinostatin, but neither of these inhibitors prevented the downregulation of cyclin F mRNA (Figure 3.10). This indicates that the SCF complex and the proteasome solely contribute to cyclin F protein degradation and not to mRNA downregulation. Interestingly, we observed that cyclin F mRNA levels were actually decreased by treating with MG132 alone, although treating with etoposide in addition to MG132 caused a further decrease in cyclin F levels (Figure 3.10). MLN4924 caused a slight decrease in cyclin F mRNA levels, but the effect of MG132 was much greater (Figure 3.10). This ability of MG132 to decrease cyclin F mRNA levels helps explain our earlier finding from section 3.2.1.1 in which MG132 did not elevate cyclin F protein levels as significantly as MLN4924. The increase in cyclin F protein level in the presence of MG132 is dampened because while MG132 prevented degradation of the protein, it also decreased cyclin F mRNA levels. While we do not know the reasoning behind this decrease in cyclin F mRNA levels following MG132, it may be the result of increased protein levels of a negative repressor of cyclin F transcription.

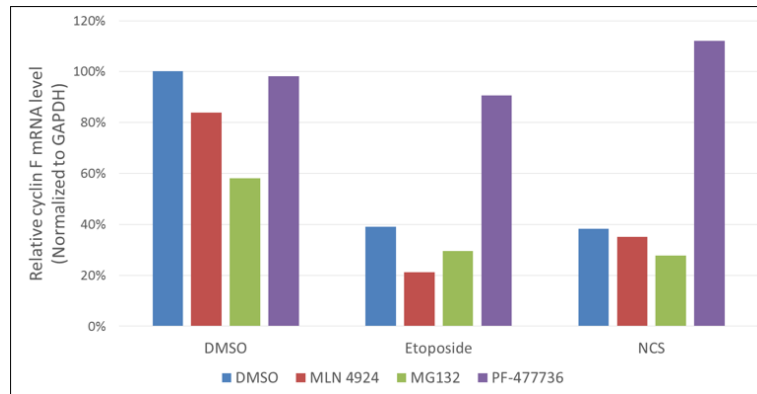


Figure 3.10: Chk1 inhibition prevents cyclin F mRNA downregulation following DNA damage. HeLa Tet-Off cells were pre-treated for 1 hour with MLN4924 (1 μ M), MG132 (20 μ M), or PF-477736 (0.4 μ M) and then treated for 4 hours with DMSO, etoposide (50 μ M), or neocarzinostatin (NCS, 0.1 μ g/mL). qPCR was used to measure cyclin F mRNA levels. Expression levels are shown relative to GAPDH and normalized to the DMSO alone sample.

3.2.3 Cell cycle regulation by cyclin F

3.2.3.1 Decreased cyclin F levels delay mitotic entry

In addition to the role cyclin F plays in the DDR, cyclin F has also been implicated in regulating the cell cycle during routine cell division. As described in section 1.4.1, overexpression of cyclin F has been shown to cause a significant increase in the percentage of G2 cells in a given population of cells, which indicates that excess cyclin F protein either (1) condenses the G1 or S-phases of the cell cycle or (2) prolongs the G2 phase.⁹⁵ Cyclin F null mouse embryonic fibroblasts (MEFs) have also been used to investigate the role of cyclin F in cell cycle regulation. Cyclin F null MEFs proliferate slower than wild-type MEFs, and they are also slower to re-enter the cell cycle following quiescence as a result of serum starvation or low density plating.⁹⁹ Taken together, these results suggest that cyclin F plays a role in regulating the proper timing of cell division.

To better understand the expression pattern of cyclin F in unperturbed cells, we first measured cyclin F levels at different stages of the cell cycle using synchronized cells. We arrested cells at the G1/S transition with a double thymidine block, released the cells into fresh media, and collected samples at different time points. Immunoblot and flow cytometry analysis were used to assess the cell cycle stages. MPM2, phospho-histone H3, and cyclin B levels all peaked at 12 hours post G1/S release, indicating that the majority of cells were in mitosis at that time point (Figure 3.11A). Figure 11B shows the cell cycle profile as assessed by flow cytometry at each time point. Cyclin F levels peaked at 10 hours post G1/S release, just prior to the peak in mitotic proteins, indicating

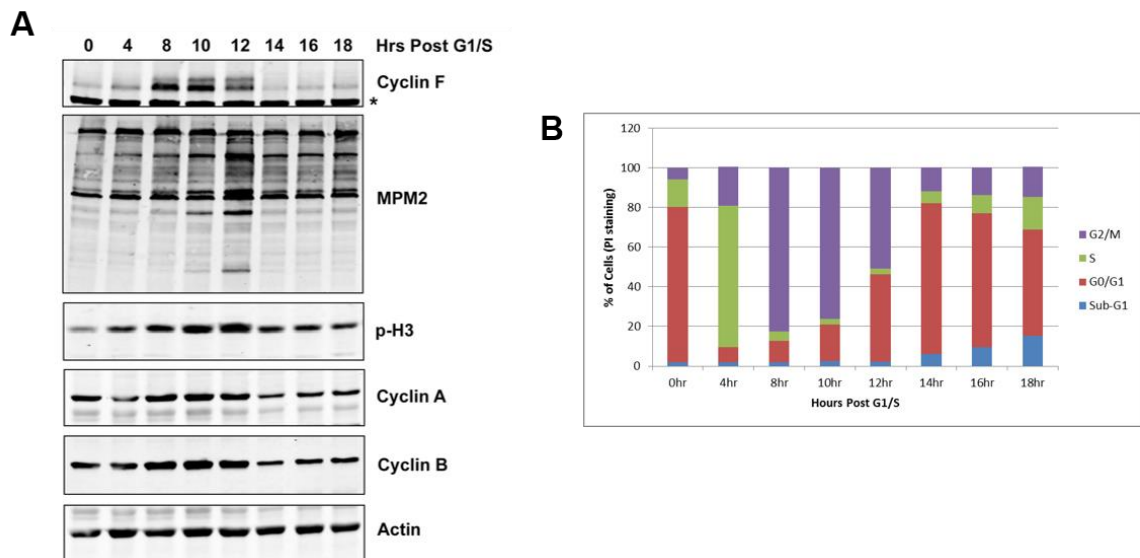


Figure 3.11: Cyclin F protein levels oscillate during the cell cycle and peak at G2/M. (A and B) HeLa Tet-Off cells were synchronized by a single thymidine block to arrest cells at G1/S, washed twice with PBS, and released into thymidine-free media. Cells were collected at the indicated time points. **(A)** Protein levels were detected by immunoblotting with the indicated antibodies. **(B)** Cell cycle stages were determined by propidium iodide (PI) staining and flow cytometry analysis.

that cyclin F levels are highest near the G2/M transition (Figure 3.11A). This expression pattern is suggestive of a role for cyclin F during the preparatory G2 phase or in promoting mitotic entry.

To explore the function of cyclin F in cell cycle regulation, we made use of cyclin F-targeted siRNA. For this experiment, we transfected cyclin F siRNA and then synchronized the cells with a double thymidine block before releasing into fresh media containing nocodazole. Interestingly, we found that knocking down cyclin F delayed mitotic entry as demonstrated by delayed accumulation of MPM2 and phospho-histone H3 (Figure 3.12A). We also analyzed cell cycle profiles using flow cytometry in synchronized cells that were released into fresh media without nocodazole. We observed that cyclin F knockdown cells spent more time in the G2/M stage of the cell cycle as compared with control knockdown cells, which is consistent with a delayed mitotic entry phenotype (Figure 3.12B). This delayed mitotic entry may be indicative of an additional role for cyclin F downregulation during the DDR. It is possible that cyclin F downregulation may play a role in the cell cycle arrest induced by the DDR to ensure that cells do not enter mitosis until all DNA damage has been repaired. Future studies will be required to determine the cell cycle effects of cyclin F expression following DNA damage.

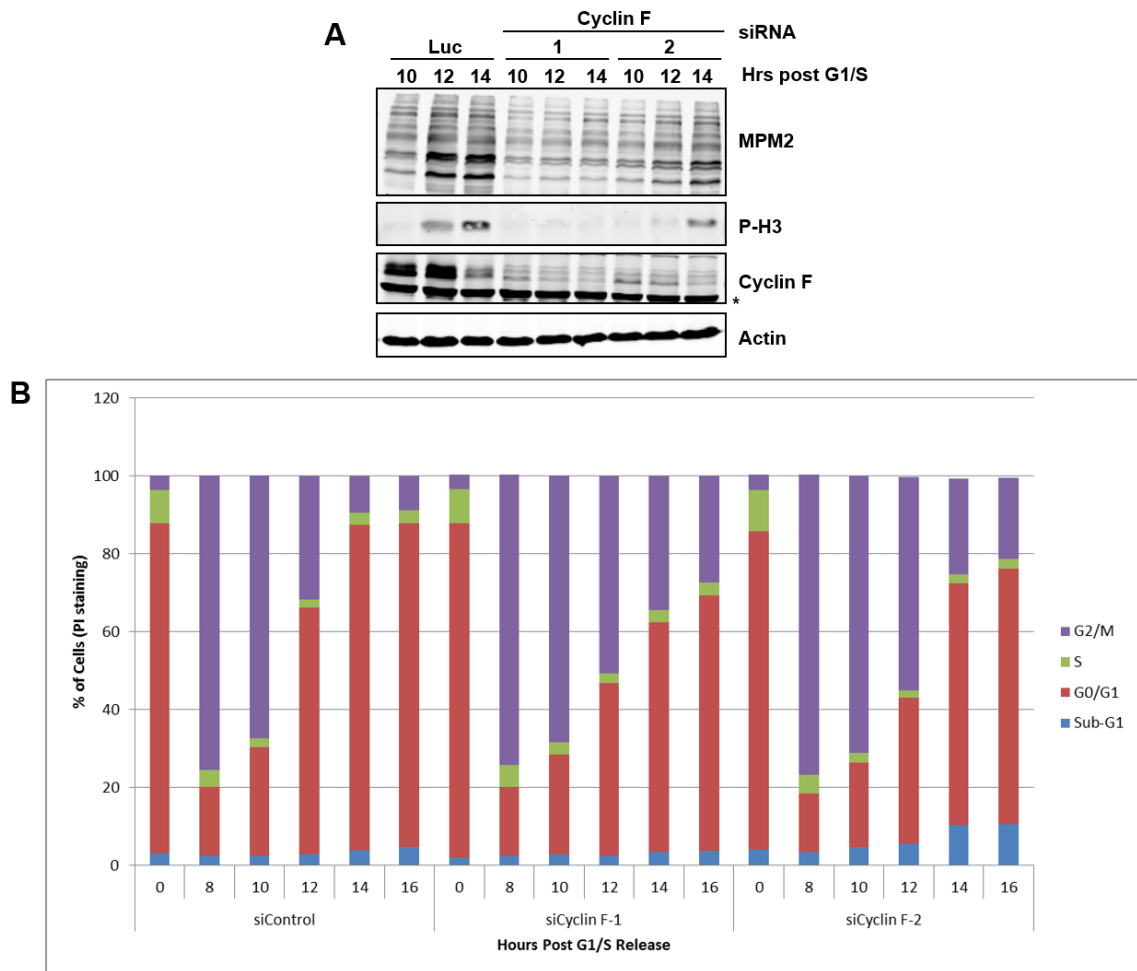


Figure 3.12: Knockdown of cyclin F delays mitotic entry. (A) HeLa Tet-Off cells were transfected with the luciferase (Luc) or 1 of 2 different cyclin F siRNAs and then synchronized at G1/S by a single thymidine block. Cells were released into nocodazole containing medium and collected at the indicated time points. Protein levels were detected by immunoblotting. (B) HeLa Tet-Off cells were transfected with the indicated siRNA and synchronized at G1/S by a single thymidine block. Cells were then released into thymidine-free media and collected at indicated time points. Cell cycle stages were determined by propidium iodide (PI) staining and flow cytometry analysis. * denotes a non-specific band.

3.2.3.2 Cyclin F overexpression delays mitotic entry

In addition to studying the role of cyclin F in cell cycle regulation by knocking down cyclin F, we also overexpressed cyclin F and assessed changes in cell cycle progression. We generated HeLa Tet-On cells with doxycycline-inducible mcherry-FLAG-cyclin F to allow us to induce cyclin F expression during specific stages of the cell cycle. As in previous experiments, we used a double thymidine block to synchronize the cells. Then we added doxycycline to induce cyclin F expression prior to release from G1/S. Interestingly, cells overexpressing cyclin F showed a similar phenotype to cyclin F siRNA cells. Overexpressed cyclin F caused cells to have a delayed entry into mitosis as

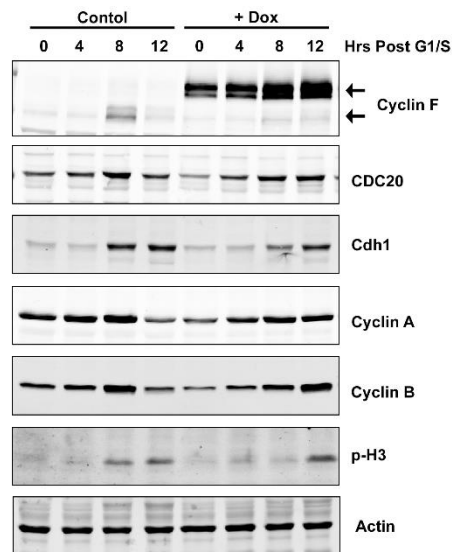


Figure 3.13: Cyclin F overexpression delays mitotic entry. HeLa S3 Tet-On cells containing doxycycline-inducible mCherry-FLAG-cyclin F were synchronized by a double thymidine block. Doxycycline (1 $\mu\text{g}/\text{mL}$) was added to the cells after the first thymidine block to induce expression. Cells were collected at the indicated time points, and protein levels were detected by immunoblotting with the indicated antibodies. The top arrow is mCherry-FLAG-cyclin F, and the bottom arrow is endogenous cyclin F.

demonstrated by a delayed accumulation of a number of mitotic markers (Figure 3.13). Because elevated cyclin F levels have been shown to induce RRM2 degradation, we hypothesized that this delayed mitotic entry may be due to an increase in the amount of time spent in S phase because of decreased availability of dNTPs for DNA replication.

3.2 Discussion

In this chapter, we investigated the signaling pathways involved in downregulating cyclin F in response to DNA damage and also in unperturbed cells. While DNA damage-induced decreases in cyclin F levels were previously thought to be regulated only at the protein level, we discovered that cyclin F is downregulated at both the protein and mRNA level in response to DNA damage. Downregulation by two independent mechanisms provides an efficient way to ensure that cyclin F levels are quickly decreased following DNA damage.

In studying the mechanism responsible for cyclin F mRNA downregulation in response to DNA damage, we determined that the stability of cyclin F mRNA does not change in the presence of etoposide. Therefore, it appears that DNA damage-induced cyclin F mRNA downregulation is the result of transcriptional repression. The DDR induces transcriptional changes of many genes involved in DNA repair, cell cycle arrest and apoptosis.¹⁰⁴ While the transcription factor p53 is known to mediate the transcriptional activation of many genes, very little is known about the mechanisms regulating transcriptional repression. A previous report demonstrated that cyclin A and

B are also transcriptionally repressed following DNA damage, but this repression is the result of Chk1 dissociation from chromatin. Following DNA damage, Chk1 has been shown to dissociate from chromatin and accumulate at the centrosomes, which leads to dephosphorylation of its target protein histone H3.¹⁰⁴ Dephosphorylation of histone H3 was shown to impair the binding of the acetylase GCN5, which leads to histone deacetylation and reduced transcription.¹⁰⁴ However, in the case of cyclin F, Chk1 activity is actually required for its downregulation in response to DNA damage, suggesting that Chk1 either activates a repressor or inactivates an activator.

We determined that downregulation of cyclin F at the protein level is mediated by proteasomal degradation in response to DNA damage. We also demonstrated that the SCF complex mediates cyclin F degradation under basal conditions and contributes to cyclin F downregulation following DNA damage. Interestingly, inhibiting the SCF complex with MLN4924 was more effective than MG132 at increasing cyclin F levels under basal conditions. However, we demonstrated that inhibiting proteasomal degradation with MG132 actually decreases cyclin F mRNA levels, which explains why MG132 treatment does not cause as much of an increase in cyclin F protein levels as MLN4924. MG132 was more effective than MLN4924 at preventing cyclin F downregulation in response to DNA damage, but this is likely due to the fact that MG132 prevented cyclin F protein degradation but also decreased cyclin F mRNA levels. Therefore, when cells are treated with both MG132 and etoposide, the relative

difference in cyclin F protein levels is not as dramatic as when cells are treated with MLN4924, which does not have as great an effect on cyclin F mRNA levels. The significant DNA damage-induced downregulation of cyclin F protein in the presence of MLN4924 is likely the result of cyclin F mRNA downregulation. It is unclear at this time how much of the DNA damage-induced downregulation of cyclin F is due to protein degradation versus transcriptional repression, but our data suggests that both mechanisms play a role.

Once we determined that cyclin F is targeted for degradation by the SCF complex, we attempted to identify the F-box protein that recognizes cyclin F and recruits it to the SCF complex. We demonstrated that the F-box protein β -TrCP appears to play a small role in targeting cyclin F for degradation, but as β -TrCP-directed siRNA could only partially rescue cyclin F levels following etoposide treatment, other F-box proteins must also be responsible for targeting cyclin F for degradation. Further investigations into SCF-dependent cyclin F degradation will be important for identifying the cyclin F targeting F-box proteins.

Although cyclin F downregulation in response to DNA damage was previously reported to be Chk1-independent by D'Angiolella, et al.⁹², we found that cyclin F downregulation in several cell lines is predominantly dependent on Chk1 kinase activity. Chk1 is activated mainly through ATR, although other kinases, such as ATM, likely contribute to Chk1 activation; ATR inhibition was not as effective as Chk1

inhibition at restoring cyclin F levels following DNA damage. Interestingly, we found that Chk1 is required for cyclin F mRNA downregulation in response to DNA damage. Chk1 inhibition fully restored cyclin F mRNA levels following neocarzinostatin and nearly completely rescued cyclin F mRNA levels in response to etoposide. Etoposide did not alter the half-life the cyclin F mRNA, and thus Chk1 is likely a repressor of cyclin F transcription.

We also observed that Chk1 inhibition could increase cyclin F protein levels in unperturbed cells, and Chk1 inhibition also appeared to increase cyclin F mRNA levels in certain experiments. This data points to a potential role for Chk1 regulation of cyclin F during the normal cell cycle, and it may be interesting to further explore cyclin F regulation by Chk1 during different phases of the cell cycle. In addition to Chk1's role in the DDR, Chk1 is also active during S-phase to monitor for genotoxic stress during DNA replication. Another potential function of Chk1 during S-phase may be to repress cyclin F transcription to allow for increased RRM2 levels and increased dNTP production. Then during G2 phase, when cyclin F levels increase until they peak at G2/M, we would expect that Chk1 transcriptional repression of cyclin F would decrease. We are currently working on developing a cyclin F luciferase reporter assay, which will be useful for identifying transcriptional regulators of cyclin F at different phases of the cell cycle and in response to DNA damage.

There are still several unanswered questions regarding how Chk1 rescues cyclin F downregulation in DNA damaged cells. We have shown that Chk1 mediates cyclin F mRNA downregulation in response to DNA damage, but the molecular mechanisms regulating this response are still unknown. Furthermore, it is possible that Chk1 may also promote DNA damage-induced cyclin F protein degradation in addition to cyclin F mRNA degradation. If Chk1 does promote cyclin F degradation, it could do so by either (1) directly phosphorylating cyclin F or (2) by phosphorylating another component of the cyclin F degradation machinery. We hypothesized that Chk1 directly phosphorylates cyclin F protein to induce its degradation and found that Chk1 can phosphorylate cyclin F *in vitro*. However, when we used mass spectrometry to try to identify etoposide-induced phosphorylation sites on cyclin F that may be mediating its degradation, we were not able to identify any phosphorylation sites that were required for cyclin F degradation. This negative result may simply be due to the fact that the cyclin F protein coverage was very low and so the phosphorylation status of large regions of the protein are still unknown. Therefore, Chk1-directed phosphorylation on cyclin F remains a possible mechanism of cyclin F protein degradation, and future studies will be important to determine if phosphorylation affects cyclin F protein degradation. We also observed that certain cell lines are less dependent on Chk1 for DNA damage-induced cyclin F downregulation, and so experiments investigating the mechanisms mediating

cyclin F downregulation in these cell lines will also be important to increase our understanding of the DDR signaling pathways.

Additionally, we demonstrated that cyclin F exists in cells as both a hypo- and hyper-phosphorylated protein. Although the hypo-phosphorylated protein is more abundant, cyclin F was rapidly phosphorylated following phosphatase inhibition by calyculin A. In the presence of calyculin A, cyclin F degradation was dramatically decreased in unperturbed cells as measured by changes in cyclin F half-life.

Additionally, calyculin A also prevented cyclin F downregulation in the presence of etoposide. These findings suggest that phosphorylation on cyclin F may inhibit its degradation. Thus, an unknown phosphatase must dephosphorylate cyclin F to target it for degradation. In searching for the cyclin F-targeted phosphatase, we found that PP1 β interacts with cyclin F both in the presence and absence of DNA damage. PP1 β knockdown resulted in slightly increased levels of cyclin F, but it did not affect cyclin F DNA damage-induced degradation. Future experiments are needed to determine the relevant cyclin F phosphatase and to determine its role in DNA damage-induced cyclin F degradation. Additionally, it will also be interesting to determine the kinase that is responsible for cyclin F phosphorylation as it may also be regulated by the DDR.

Although calyculin A-induced phosphorylation of cyclin F inhibits its degradation, Chk1 may still phosphorylate cyclin F on other residues to actually promote cyclin F degradation. In other words, we propose that cyclin F has a specific

phosphorylation code that can either trigger degradation or stabilization, depending on the specific residues that are phosphorylated. We know that cyclin F phosphorylation status is constantly in flux, and it appears to regulate cyclin F stability. It will be important for future studies to determine which phosphorylation sites and which enzymes are required to target cyclin F for degradation, both during routine cell cycle progression and in the presence of DNA damage.

Lastly, we determined that cyclin F has an important role in regulating cell cycle progression, particularly during mitotic entry. We found that both knocking down and overexpressing cyclin F delayed mitotic entry in synchronized cells released from G1/S arrest. Overexpression of cyclin F likely results in prolonged S-phase due to lower levels of RRM2 and less dNTP production thereby increasing the amount of time it takes for the cell to enter mitosis. The mechanism behind delayed mitotic entry resulting from cyclin F knockdown is still unknown and remains an interesting area to be explored. An intriguing possibility is that cyclin F downregulation following DNA damage may be important for delaying mitotic entry until the DNA damage has been repaired.

4. Regulation of the MCC by mitotic phosphatases

This chapter is adapted from a manuscript currently under review.

4.1 Introduction

The spindle checkpoint is an essential signaling network that protects the genomic integrity of a cell during mitosis. The spindle checkpoint prevents the separation of the sister chromatids by inactivating the anaphase-promoting complex/cyclosome (APC/C), the E3 ubiquitin ligase required for mitotic progression from metaphase to anaphase.⁵⁵ As described in section 1.2.2, the mitotic checkpoint complex (MCC), which consists of CDC20, Mad2, Bub3, and BubR1, is the major inhibitor of the APC/C while the spindle checkpoint is active.^{22,29,105} Once all of the chromosomes are properly aligned and engaged by the spindle, the checkpoint is satisfied, and the MCC disassembles and releases CDC20 to activate the APC/C.¹⁰⁶⁻¹⁰⁸ Activated APC/C then targets cyclin B and securin for degradation, which leads to CDK1 inactivation and sister chromatid segregation, respectively.²²

MCC formation and maintenance during the spindle checkpoint is essential for preventing premature entry into anaphase, but there are still many questions surrounding the signaling pathways involved in MCC regulation. As described throughout section 1.2, kinase activity, especially from MPS1 and Aurora B, is critical for MCC formation and effective spindle checkpoint signaling. Phosphatases, on the other hand, have only been shown to be required for silencing of the spindle

checkpoint.^{22,23,29,109-111} However, in this chapter, we describe our finding that phosphatases have an additional role during the spindle checkpoint.

Protein phosphatases can be classified into two general groups according to the specific residues they can dephosphorylate: serine/threonine phosphatases and tyrosine phosphatases. The serine/threonine phosphatases can be further divided into two different families: the phosphoprotein phosphatase (PPP) family and the protein phosphatase Mg²⁺-dependent (PPM) family.¹¹² The PPP family includes many of the most well-known phosphatases: PP1, PP2A, PP2B (calcineurin), PP4, PP5, PP6, and PP7.¹¹³ The PPM family consists of PP2C and pyruvate dehydrogenase phosphatase, in addition to other phosphatases that require Mg²⁺ for their catalytic activity.¹¹⁴ The PPP family members share a high degree of sequence similarity and are sensitive (to varying degrees) to some of the same small molecule inhibitors.¹⁰² Using two PPP family inhibitors, calyculin A and okadaic acid, we determined that a PPP family Ser/Thr phosphatase is required during the spindle checkpoint to prevent MCC disassembly and APC/C activation.

4.2 Results

4.2.1 Calyculin A and okadaic acid can override the spindle checkpoint to activate the APC/C.

To study the role of protein phosphatases during the spindle checkpoint, we used five small molecule inhibitors of the PPP family: okadaic acid, calyculin A, fostriecin, FK506, and cyclosporin A. This panel of inhibitors can be used to identify the

activity of the different PPP family members based on their differential sensitivities to the various inhibitors. Okadaic acid at low concentrations inhibits PP2A and PP4, while at higher concentrations, it inhibits PP1 and PP5.¹⁰² Calyculin A inhibits PP1, PP2A, PP4, and PP5. PP2B and PP7 are insensitive to both okadaic acid and calyculin A. The sensitivity of PP6 to these two compounds has not been tested, but it is likely similar to PP2A and PP4 based on their sequence similarity.¹⁰² Fostriecin inhibits PP2A, PP4, and likely PP6, but PP1, PP2B, PP5, and PP7 are very resistant to this compound.¹⁰² FK506 and cyclosporin A are both inhibitors of only PP2B.

In order to investigate whether protein phosphatases are involved in APC/C inhibition during the spindle checkpoint, we first needed to obtain cells arrested at the spindle checkpoint. Therefore, we treated cells with thymidine, which inhibits DNA replication and synchronizes cells in S phase. Once synchronized, the cells were released into media containing either nocodazole or taxol – two microtubule inhibitors that interfere with proper mitotic spindle formation. These two inhibitors cause cells to arrest during prometaphase at the spindle checkpoint, but they differ in their mode of action. Nocodazole inhibits microtubule polymerization, while taxol prevents depolymerization, which inhibits the dynamic movement of microtubules that is necessary for proper mitotic spindle formation.¹¹⁵

We then treated the synchronized nocodazole-arrested cells with our panel of phosphatase inhibitors and measured cyclin B levels in cell lysates. We discovered that

cells treated with okadaic acid (0.1 μ M), fostriecin, or the calcineurin inhibitors, FK506 and cyclosporin A, retained high levels of cyclin B in nocodazole-arrested cells, indicating that spindle checkpoint signaling was still inactivating the APC/C (Figure 4.1A). However, in cells treated with the phosphatase inhibitors calyculin A or a high concentration of okadaic acid (1 μ M), cyclin B levels were dramatically reduced, indicating that the APC/C had likely been activated as a result of phosphatase inhibition (Figure 4.1A). We also measured cyclin A levels, as it is another target of the APC/C. We observed that levels were high in nocodazole-arrested cells but were reduced following calyculin A or okadaic acid (1 μ M) treatment similar to cyclin B. Calyculin A treatment also accelerated cyclin B degradation during mitotic exit as compared to control cells or cells treated with fostriecin or cyclosporin A (Figure 4.1B). Based on the differential sensitivities of the PPP family members to okadaic acid and calyculin A, we hypothesized that PP1 was likely required during the spindle checkpoint to prevent cyclin A and B degradation. We will further explore the identity of the phosphatase responsible for maintaining elevated cyclin levels during the spindle checkpoint in section 4.2.5.

During normal mitotic progression, cyclin A and B are both ubiquitinated by the APC/C and then degraded via the proteasome. To determine whether the calyculin A-induced decrease in cyclin levels was also mediated by the proteasome, we pre-treated synchronized nocodazole-arrested cells with the proteasome inhibitor MG132 and then

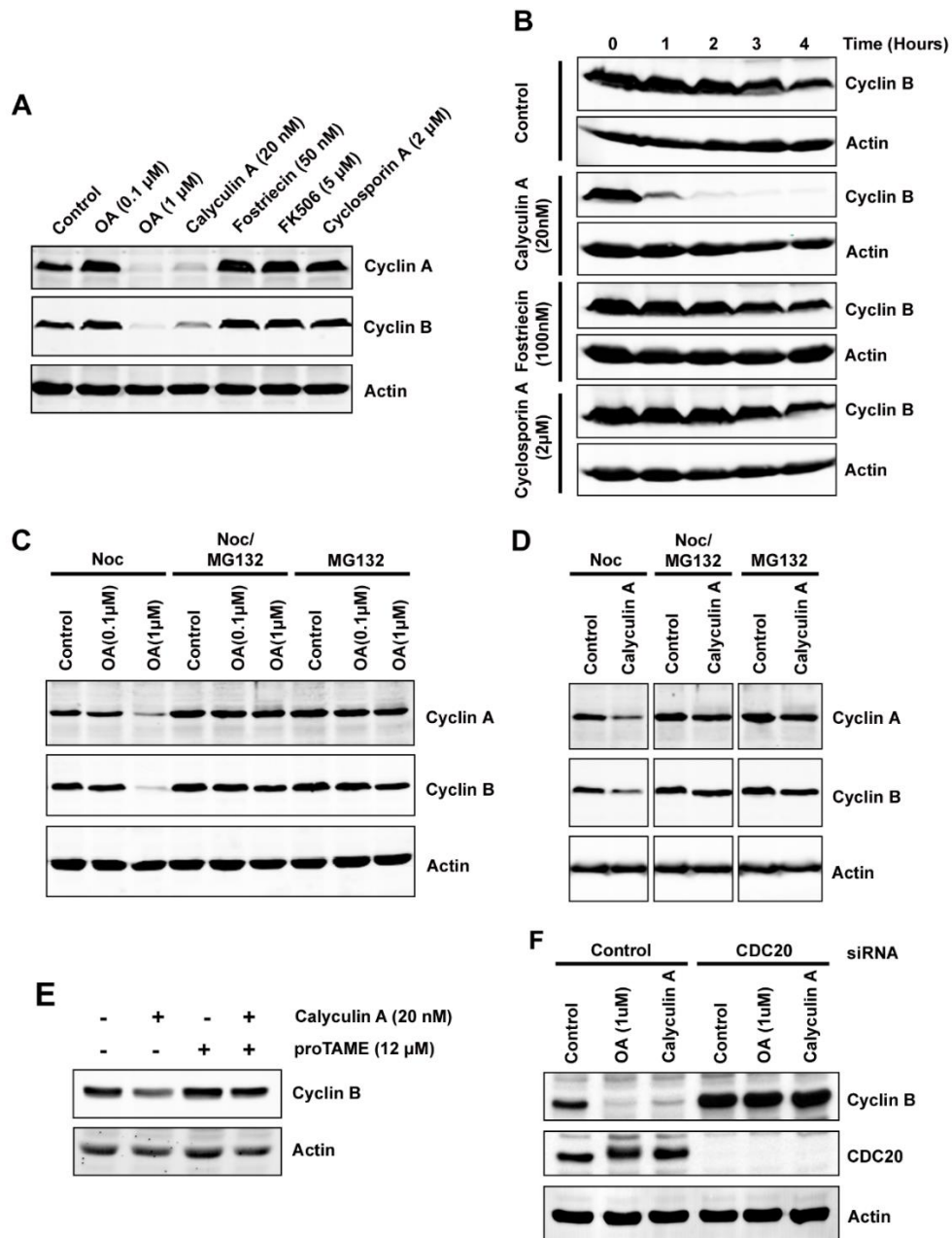


Figure 4.1: Calyculin A and okadaic acid (1 μ M) induce cyclin degradation in spindle checkpoint-arrested cells via the APC/C^{CDC20}. (A) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with the indicated phosphatase inhibitors for 2 hours.

(B) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with the indicated phosphatase inhibitors for 30 minutes prior to washing out nocodazole. Fresh media containing the phosphatase inhibitors was then added, and cells were harvested at indicated time points post nocodazole release. (C and D) Synchronized HeLa-Tet-Off

cells were treated with nocodazole for 16 hours and then treated with MG132 (20 μ M) for 1 additional hour prior to treatment with the indicated phosphatase inhibitors for 2 hours. (E) Synchronized nocodazole-arrested HeLa Tet-Off cells were pretreated with proTAME (12 μ M) for 1 hour and then treated with calyculin A (20 nM) for 2 hours. (F) HeLa Tet-Off cells were transfected with CDC20 or control siRNA, synchronized by a double thymidine block, and released into nocodazole. Cells were then treated with the indicated inhibitors for 2 hours. (A-F) Protein levels were detected by immunoblotting with the indicated antibodies. OA: okadaic acid; Noc: nocodazole.

measured cyclin levels following phosphatase inhibition. Pre-treatment with MG132 rescued cyclin A and B levels in okadaic acid and calyculin A-treated cells, confirming that okadaic acid and calyculin A induce proteasomal degradation of cyclin A and B (Figure 4.1C and D). We also tested the involvement of the APC/C in phosphatase inhibitor-mediated cyclin degradation and found that directly inhibiting the APC/C with the small molecule inhibitor proTAME or depleting CDC20 using siRNA restored cyclin B levels following calyculin A treatment (Figure 4.1E and F). Therefore, calyculin A-induced cyclin degradation appears to occur via the pathway normally activated upon mitotic exit and is dependent on both the APC/C^{CDC20} and the proteasome. Taken together, these results demonstrate that a calyculin A and okadaic acid (1 μ M)-sensitive phosphatase is necessary for APC/C inactivation during the spindle checkpoint.

4.2.2 Phosphatase activity is required for MCC maintenance during the spindle checkpoint.

As the MCC is the major inhibitor of the APC/C during the spindle checkpoint, we hypothesized that calyculin A and okadaic acid (1 μ M) may induce MCC disassembly. This dissociation would free CDC20 to activate the APC/C so that it could

ubiquitinate cyclin A and B to target them for degradation. To test this hypothesis, we examined the interaction of CDC20 with the other MCC components in synchronized taxol-arrested mitotic cells treated with our panel of phosphatase inhibitors. In control cells, endogenous CDC20 co-immunoprecipitated with Mad2, Bub3, and BubR1, indicating that the complex was intact. Interestingly, the binding between CDC20 and the other MCC components was greatly reduced in calyculin A and okadaic acid (1 μ M) treated cells (Figure 4.2A). Additionally, CDC20 still disassociated from the MCC in the presence of MG132, which rescued cyclin A and B degradation (Figure 4.2B). This finding indicates that calyculin A and okadaic acid-induced MCC disassembly is not dependent on the proteasomal degradation of cyclin A and B. When cells were arrested with nocodazole in place of taxol, calyculin A and okadaic acid (1 μ M) still induced MCC dissociation, while the other phosphatase inhibitors had no effect on the MCC (Figure 4.2C).

To investigate the timing of MCC dissociation with respect to cyclin A and B degradation, nocodazole-arrested cells were treated with calyculin A for varying amounts of time, followed by CDC20 immunoprecipitation and immunoblot analysis of the MCC components and cyclin A and B levels. CDC20 interaction with BubR1 and Bub3 began to weaken after just 15 minutes of calyculin A treatment while binding with Mad2 was noticeably decreased by 30 minutes and almost completely abolished by 60

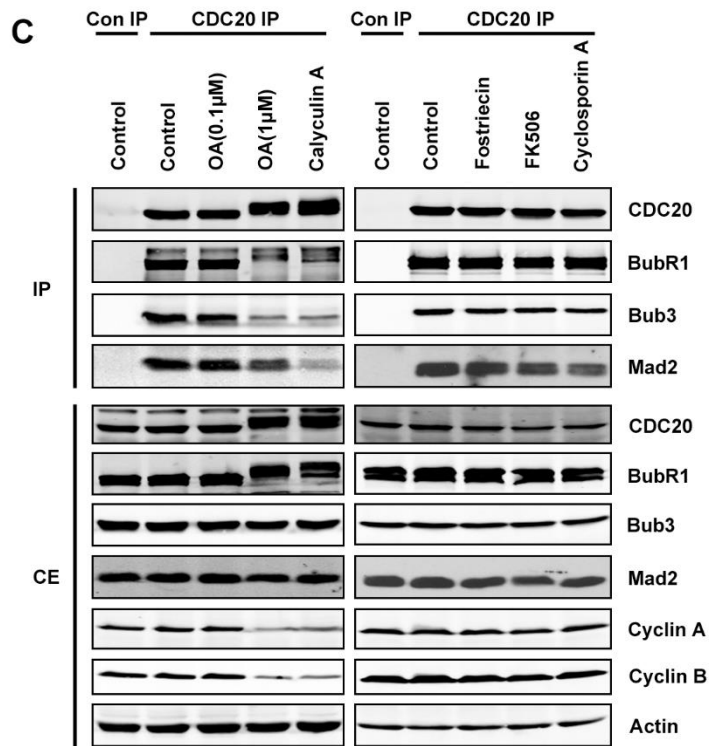
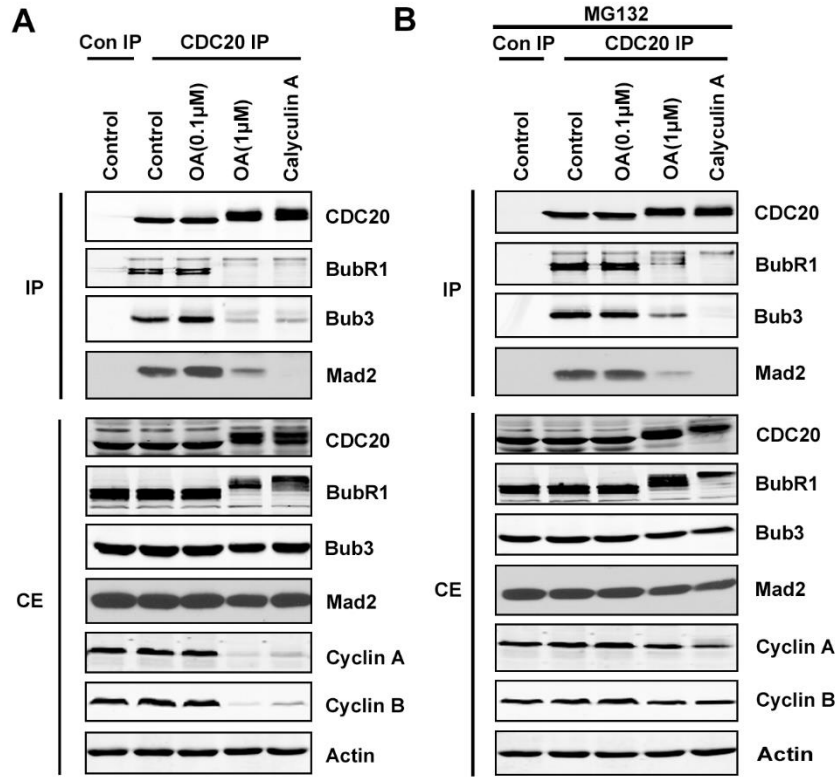


Figure 4.2: Phosphatase inhibition induces MCC dissociation during the spindle checkpoint. (A) Synchronized taxol-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors before cells were harvested and lysed. (B) Synchronized nocodazole-arrested cells were pre-treated with MG132 (20 μ M) for 1 hour prior to phosphatase inhibitor treatment. (C) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors before cells were harvested and lysed. (A-C) CDC20 was immunoprecipitated from lysates, and protein levels were detected by immunoblotting with the indicated antibodies. Con IP: IgG immunoprecipitation; OA: okadaic acid; IP: immunoprecipitation; CE: cell extract.

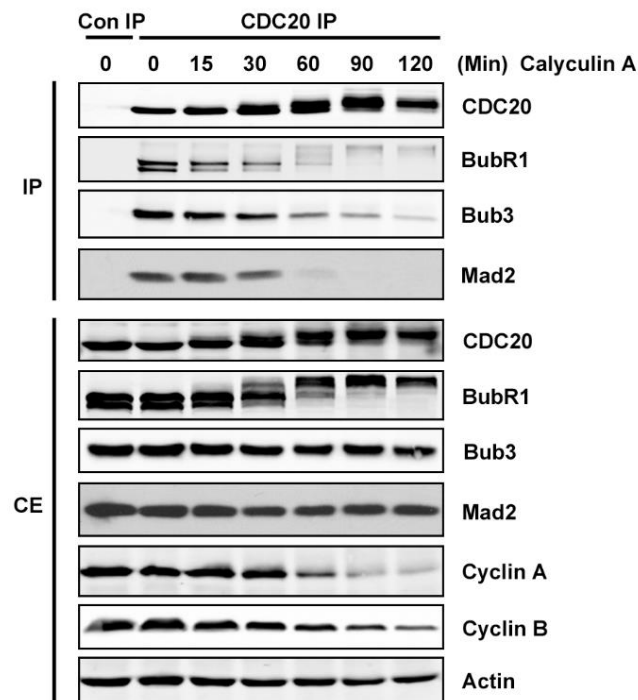


Figure 4.3: Calyculin A induces MCC dissociation prior to cyclin A and B degradation. Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with calyculin A (20 nM) and harvested at the indicated time points. CDC20 was immunoprecipitated from lysates, and protein levels were detected by immunoblotting with the indicated antibodies. Con IP: IgG immunoprecipitation; OA: okadaic acid; IP: immunoprecipitation; CE: cell extract.

minutes (Figure 4.3). Strikingly, the degradation of cyclin A and B began to occur shortly after CDC20 lost its interaction with the MCC components. These data further support our hypothesis that calyculin A-induced cyclin degradation is the result of MCC dissociation and subsequent APC/C activation.

4.2.3 BubR1 and CDC20 as potential targets of phosphatase activity

In carrying out these experiments, we observed a dramatic SDS-PAGE mobility shift of both CDC20 and BubR1 after treating with calyculin A. We verified these two proteins were hyperphosphorylated following calyculin A treatment by treating cell lysates with lambda protein phosphatase, which caused both CDC20 and BubR1 to downshift to their normal point of migration in the gel (Figure 4.4A and B). We speculated that the increased phosphorylation of CDC20 or BubR1 may be responsible for the MCC disassembly following calyculin A treatment. To test this hypothesis, we performed mass spectrometry analysis of overexpressed CDC20 and BubR1 in nocodazole-arrested cells and identified four calyculin A-induced phosphorylation sites on each protein (Figure 4.4C). Non-phosphorylatable mutants of CDC20 and BubR1, in which the identified serine and threonine residues were mutated to alanine, were transfected into cells to determine the importance of these phosphorylation events for calyculin A-induced cyclin degradation during the spindle checkpoint. No significant differences in cyclin levels were observed between cells expressing wild type protein and those expressing mutant proteins, indicating that phosphorylation on these four

sites is not required for MCC disassembly by calyculin A (Figure 4.4D and E). However, it should be noted that these mutations did not completely abolish the observed electrophoretic shift of BuBR1 and CDC20 proteins following calyculin A treatment, so it remains possible that unidentified phosphorylation sites on these proteins are responsible for the observed effects of calyculin A on the APC/C.

4.2.4 Calyculin A and okadaic acid-induced APC/C activation does not lead to mitotic exit.

We have shown that treating mitotic cells with calyculin A and okadaic acid (1 μ M) causes cyclin B degradation. As described in section 1.1.2, degradation of cyclin B leads to CDK1 inactivation, which is typically followed by dephosphorylation of CDK1 mitotic substrates and rapid exit from mitosis. However, as phosphatases (notably PP1 and PP2A) are necessary for CDK1 substrate dephosphorylation at mitotic exit, inhibiting PP1 and PP2A with calyculin A or okadaic acid prevents cells from exiting mitosis even though cyclin B has been degraded. To demonstrate that calyculin A and okadaic acid treated cells remain in mitosis, we measured CDK1 substrate phosphorylation using the MPM2 antibody in cell lysates from inhibitor-treated, nocodazole-arrested cells. As predicated, the MPM2 signal was present in all conditions, indicating that the cells remained arrested in mitosis (Figure 4.5A). The MPM2 signal was greatly enhanced in cells treated with calyculin A or okadaic acid (1 μ M), despite the fact that cyclin A and B were degraded, highlighting the necessity of phosphatase

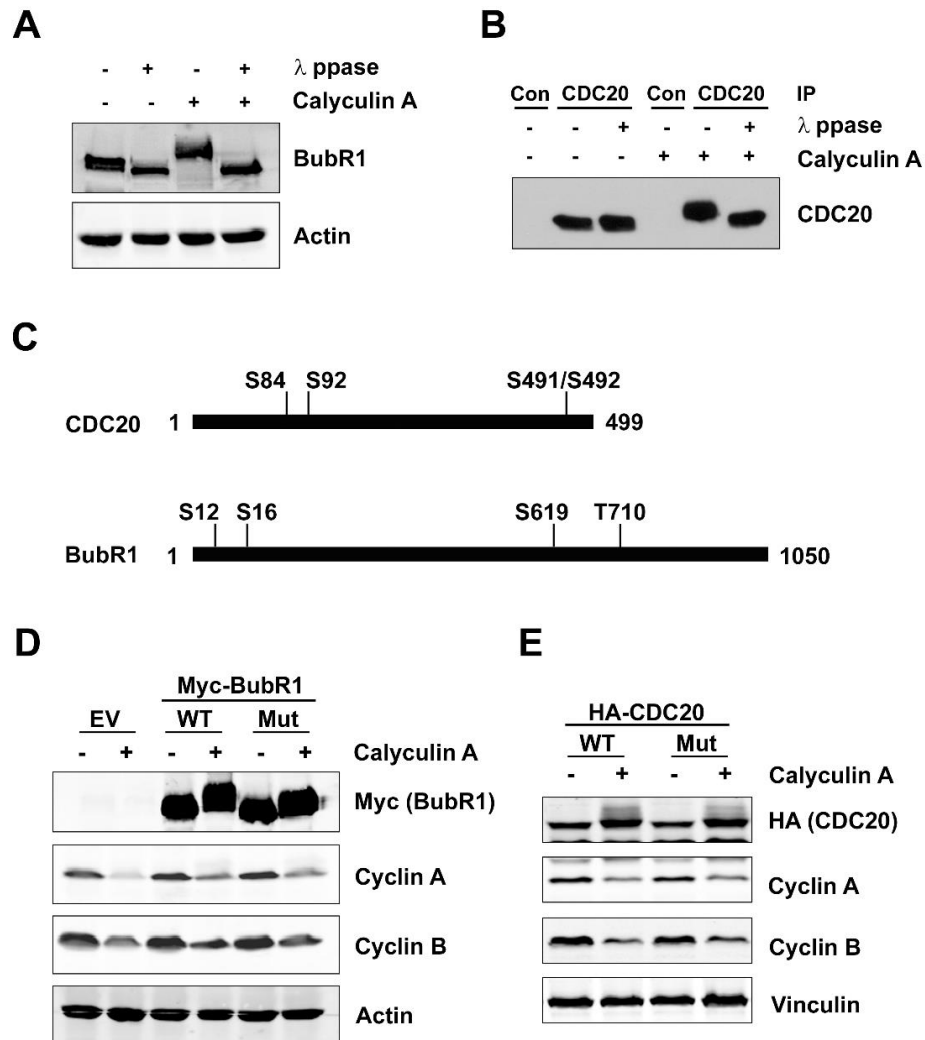


Figure 4.4: Calyculin A-induced phosphorylation of CDC20 and BubR1 at mass spectrometry-identified sites is not necessary for MCC dissociation. (A) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with calyculin A (20 nM) for 2 hours, and cell lysates were treated with lambda phosphatase (λ ppase). Protein levels and mobility shift were detected by immunoblotting with the indicated antibodies. (B) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with calyculin A (20 nM) for 2 hours. CDC20 was immunoprecipitated from whole cell lysates and then treated with lambda phosphatase (λ ppase). Protein levels and mobility shift were detected by immunoblotting with the indicated antibodies. (C) Schematic representation of the calyculin A-induced phosphorylation sites on CDC20 and BubR1 that were identified by mass spectrometry. (D and E) The 4 identified phosphorylation sites BubR1 and CDC20 were all fddfmuted to alanine and the wild-type (WT) and mutant (Mut) constructs were transfected into 293T cells. The cells were arrested at the spindle

checkpoint with nocodazole and then treated with calyculin A (20 nM) for 2 hours. Cells were harvested 48 hours post transfection, and protein levels were detected by immunoblotting with the indicated antibodies. Note: Actin immunoblot in (D) was cut.

Con: IgG immunoprecipitation; EV: empty vector; IP: immunoprecipitation.

activity for mitotic exit. As a positive control for mitotic exit, we treated cells with the CDK1 inhibitor roscovitine, which induced robust CDK1 substrate dephosphorylation (Figure 4.5B). Another marker of mitosis, phospho-histone H3 was also elevated in the inhibitor treated cells (Figure 4.5A). We also visualized chromosome condensation using chromosome spreading to further assess the mitotic state of the cells treated with calyculin A and okadaic acid. Using this technique, we could clearly see condensed chromosomes in the nocodazole-arrested cells treated with calyculin A and okadaic acid (1 μ M) further confirming the cells remained in mitosis (Figure 4.5C). Taken together, our data demonstrate that while calyculin A and okadaic acid (1 μ M) lead to MCC disassembly and APC/C activation, the cells are not able to exit mitosis.

4.2.5 Investigating the PPP family phosphatases in spindle assembly maintenance

Lastly, in an effort to identify the specific phosphatase(s) required for MCC maintenance during the spindle checkpoint, we used siRNA or shRNA to knockdown the PPP family phosphatases that are inhibited by calyculin A and okadaic acid (PP1, PP2A, PP4, PP5, and PP6) and have a potential role in the spindle checkpoint. Following siRNA-mediated knockdown of the phosphatases, we measured cyclin levels in nocodazole-arrested cells to determine if the APC/C had been inactivated. As mentioned

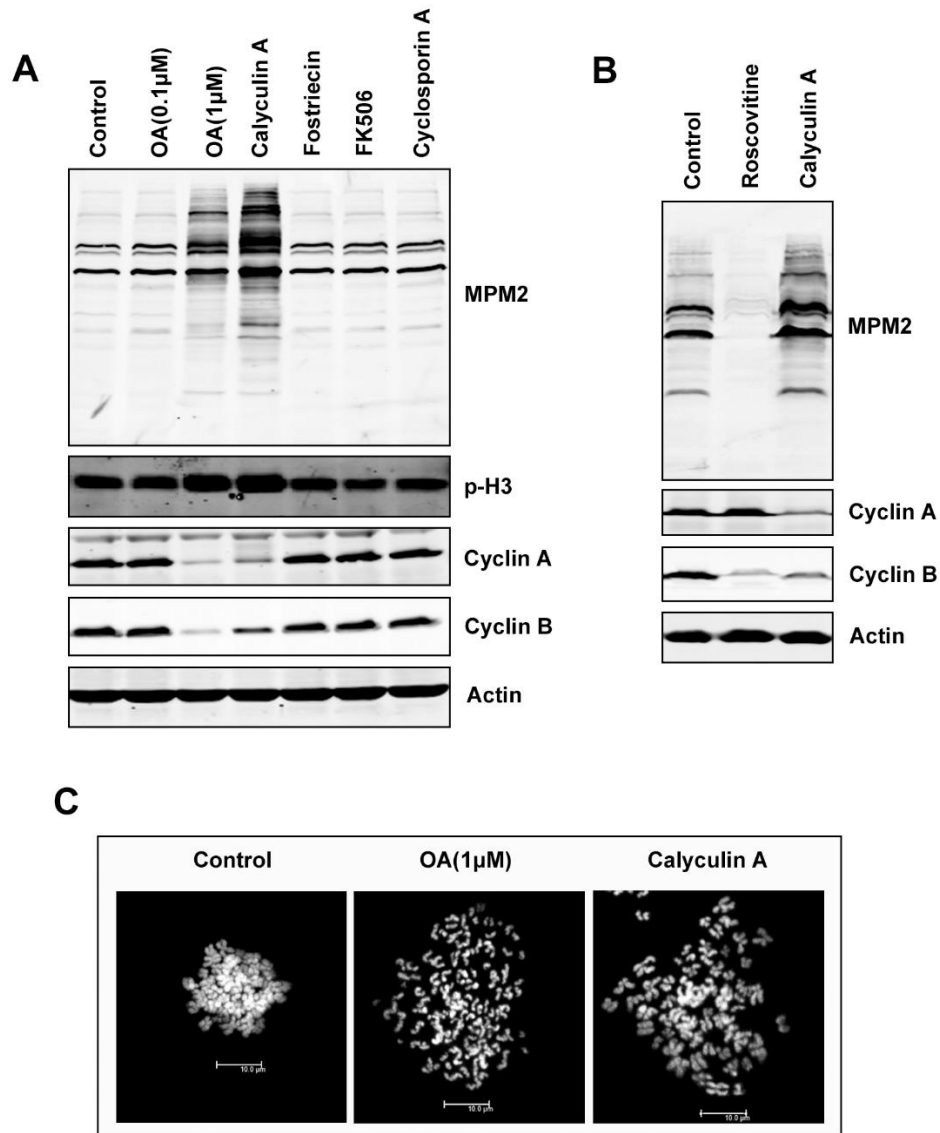


Figure 4.5: Calyculin A and okadaic acid (1 μ M) do not induce mitotic exit despite APC/C activation. (A and B) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors. Protein levels were detected by immunoblotting with the indicated antibodies. **(C)** Synchronized nocodazole-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors. Cells were then harvested and chromosome spreads were performed. OA: okadaic acid; p-H3: phospho-histone H3 (Ser10).

previously, low concentrations of okadaic acid (0.1 μ M) are known to inhibit PP2A, PP4, and likely PP6.¹⁰² As we did not observe any changes in cyclin A or B levels at low okadaic acid concentrations, we hypothesized that the phosphatase involved in MCC maintenance during the spindle checkpoint would likely be PP1, which is only inhibited by higher concentrations of okadaic acid.¹⁰² PP5's okadaic acid sensitivity is in between PP1 and PP2A, but its sensitivity to calyculin A is several fold higher than PP1 and PP2A¹⁰², which further supports our hypothesis that PP1 is the relevant phosphatase for MCC maintenance. However, none of the PPP phosphatase siRNAs or shRNAs, including PP1 siRNAs, decreased cyclin B levels in synchronized nocodazole-arrested cells (Figure 4.6A-G). We actually observed increased levels of cyclin A and B when knocking down each of the three isoforms of PP1 (α , β , and γ), indicating that PP1 knockdown cells may have arrested cells in mitosis prior to nocodazole treatment. To investigate if there is redundancy between the three PP1 isoforms, all three isoforms were knocked down together, but cyclin A and B levels were still slightly elevated in the nocodazole-arrested cells (Figure 4.6D). However, there was residual PP1 protein remaining after gene knockdown, which may have been sufficient to dephosphorylate the targets required to maintain an intact MCC. Therefore, we cannot rule out the possibility that PP1 is the relevant calyculin A target and is responsible for MCC maintenance during the spindle checkpoint.

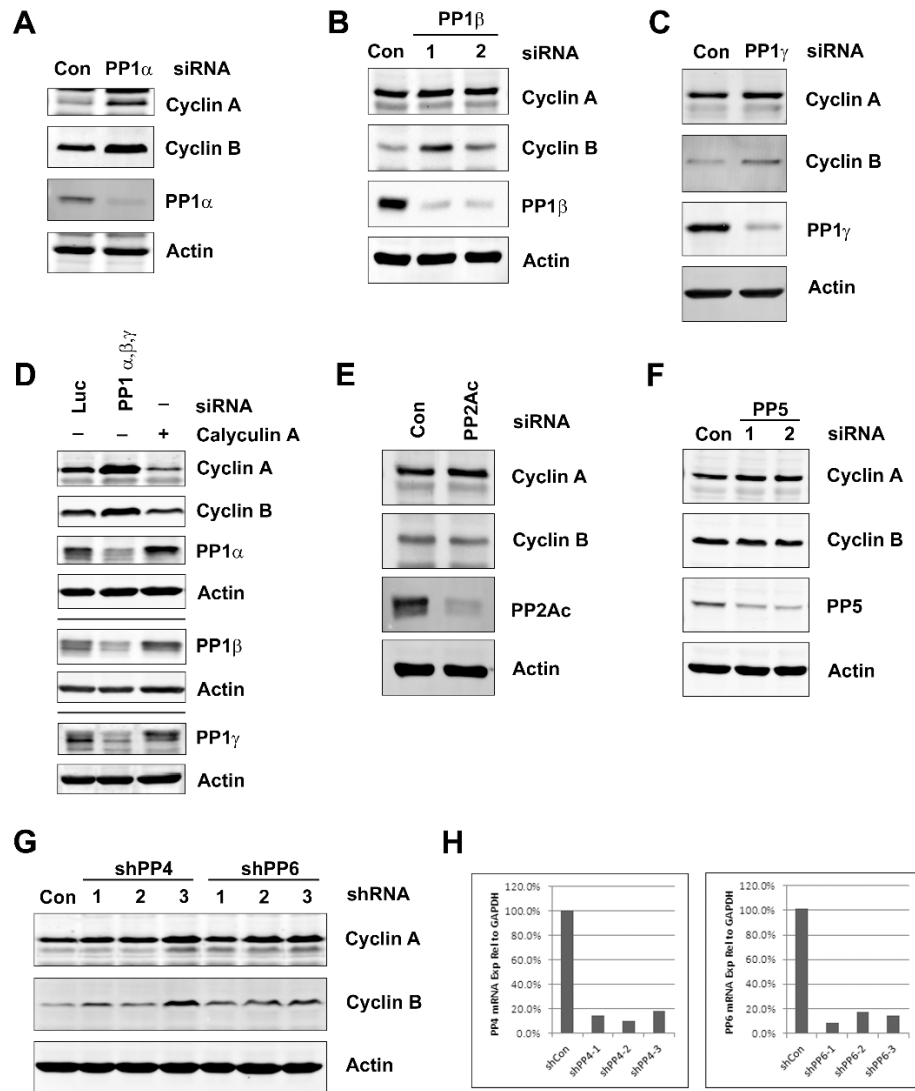


Figure 4.6: Knockdown of selected PPP family phosphatases did not induce cyclin degradation. (A-F) HeLa Tet-Off cells were transfected with the indicated siRNAs, synchronized by a double or single thymidine block, and released into nocodazole for 16 hours. Mitotic cells were harvested, and protein levels were detected by immunoblotting with the indicated antibodies. Where indicated, calyculin A (20 nM) was added for 2 hours prior to harvesting as a positive control. (G) HeLa Tet-Off cells stably expressing control shRNA or shRNA targeting PP4 or PP6 were synchronized as described above. Three different shRNA sequences were used for PP4 and PP6. (H) mRNA levels of PP4 and PP6 were measured by qPCR in shRNA expressing HeLa Tet-Off cells. Expression levels are shown relative to GAPDH (#2) and normalized to control cells. Con: control; Luc: luciferase.

To further investigate which phosphatase is responsible for MCC maintenance during the spindle checkpoint, we sought to identify MCC interacting phosphatases using mass spectrometry. Myc-tagged Mad2 and BubR1 were transfected into cells and then immunoprecipitated from nocodazole-arrested cell lysates following protein crosslinking to stabilize protein interactions. Mass spectrometry was then used to identify the Mad2 and BubR1 binding proteins. CDC20 and Bub3 were co-immunoprecipitated with both proteins, confirming that our overexpressed proteins were binding with the other MCC components. Thirteen phosphatases and phosphatase regulatory subunits were identified by mass spectrometry, including PP1 α , PP2Ac, PP5, a PP1 regulatory subunit, and three PP2A regulatory subunits (Table 4.1). We also

Table 4.1: A subset of mass spectrometry results showing Myc-Mad2 and Myc-BubR1 binding proteins. 293T cells transfected with pCS2-Myc-Mad2 or pcDNA3-Myc-BubR1 were arrested at the spindle checkpoint with nocodazole. Cells were harvested 48 hours after transfection, protein crosslinking was performed, and Myc-Mad2 and Myc-BubR1 were immunoprecipitated from cell lysates. Binding proteins were identified by mass spectrometry. NT: No Treatment; GN: Gene Name; PE: Protein Existence; SV: Splice Variant.

Protein ID	Accession ID	Exclusive Unique Peptide Count		
		NT	Mad2	BubR1
Cell division cycle protein 20 homolog GN=CDC20 PE=1 SV=2	CDC20_HUMAN	0	4	4
Mitotic checkpoint protein BUB3 GN=BUB3 PE=1 SV=1	BUB3_HUMAN	0	3	9
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform GN=PPP2R1A PE=1 SV=4	2AAA_HUMAN	0	4	10
Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform GN=PPP2CA PE=1 SV=1	PP2AA_HUMAN	0	2	4
Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform GN=PPP2R2A PE=1 SV=1	2ABA_HUMAN	0	2	2
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit GN=PPP1CA PE=1 SV=1	PP1A_HUMAN	0	2	3
Inorganic pyrophosphatase GN=PPA1 PE=1 SV=2	IPYR_HUMAN	0	1	3
dCTP pyrophosphatase 1 GN=DCTPP1 PE=1 SV=1	DCTP1_HUMAN	0	1	1
ADP-sugar pyrophosphatase GN=NUDT5 PE=1 SV=1	NUDT5_HUMAN	0	4	0
Protein phosphatase 1G GN=PPM1G PE=1 SV=1 (PP2C-gamma)	PPM1G_HUMAN	0	2	0
Serine/threonine-protein phosphatase 5 GN=PPP5C PE=1 SV=1	PPP5_HUMAN	0	0	1
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit gamma isoform GN=PPP2R5C PE=1 SV=3	2A5G_HUMAN	0	0	1
Protein phosphatase 1 regulatory subunit 12A GN=PPP1R12A PE=1 SV=1	MYPT1_HUMAN	0	0	1
Dual specificity protein phosphatase 9 GN=DUSP9 PE=1 SV=1	DUS9_HUMAN	0	0	1
Bifunctional polynucleotide phosphatase/kinase GN=PNKP PE=1 SV=1	PNKP_HUMAN	0	1	0

identified PP2C-gamma as an MCC binding protein, but we did not investigate this phosphatase further because it is not inhibited by calyculin A. Because we already tested the knockdown effect of PP1, PP2A, and PP5, we tested the effect of knocking down the remaining phosphatases and regulatory subunits on APC/C activation. However, we found no detectable differences in cyclin A or B levels during the spindle checkpoint following knockdown of each of these phosphatases (Figure 4.7A and B). Again, it is possible that residual levels of phosphatase activity following gene knockdown were responsible for inhibiting the APC/C or that multiple phosphatases contribute to this activity. Even so, our results clearly indicate that in addition to kinases, phosphatases are required for maintenance of the MCC during spindle checkpoint function.

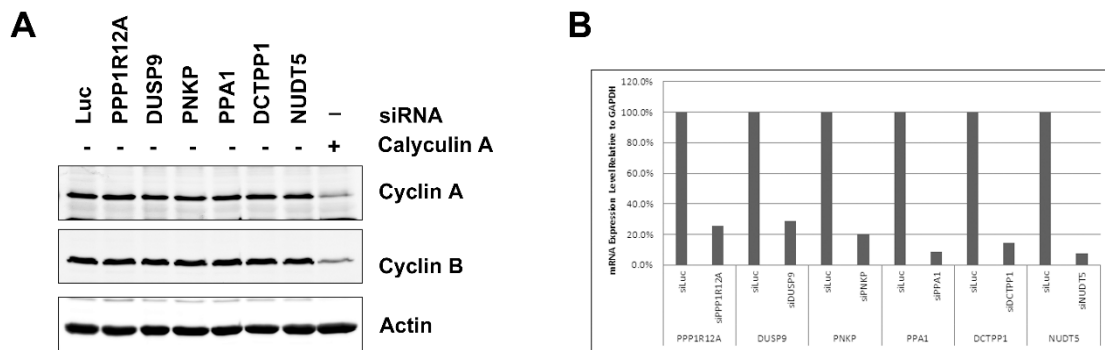


Figure 4.7: Knockdown of mass spectrometry-identified phosphatases did not induce cyclin degradation. (A) HeLa Tet-Off cells were transfected with the indicated siRNA, synchronized for 24 hours in thymidine, and released into nocodazole for 16 hours. Mitotic cells were harvested and divided in half for immunoblot (A) and qPCR (B) analysis. Protein levels were detected by immunoblotting with the indicated antibodies. Where indicated, calyculin A (20 nM) was added for 2 hours prior to harvesting. (B) qPCR was used to measure mRNA levels of the indicated phosphatases. Expression levels are shown relative to GAPDH and normalized to control cells. Luc: luciferase.

4.3 Discussion

In previous reports that examined the phosphoregulation of spindle checkpoint signaling, phosphatases had only been implicated in activities promoting checkpoint silencing (e.g., microtubule-kinetochore stabilization and dephosphorylation of MPS1 targets), while the counteracting kinases have clearly been shown to be critical for checkpoint activation and maintenance.^{22,23} Using phosphatase inhibitors in cells arrested at the spindle checkpoint, we specifically explored the role of phosphatases during the checkpoint as opposed to entry or exit. In this chapter, we have demonstrated a novel role for phosphatases in maintaining the MCC during the spindle checkpoint. This finding also reveals that an as yet unknown kinase may be required for dissolving the MCC once the spindle checkpoint has been satisfied. This apparent role reversal of kinases and phosphatases highlights the complexity of spindle checkpoint signaling.

Our results clearly demonstrate that phosphorylation of an unknown target leads to MCC disassembly; therefore, we attempted to identify the relevant phosphatase substrate(s) that becomes phosphorylated and drives MCC dissociation following calyculin A and okadaic acid (1 μ M) treatment. Two attractive potential substrates were the MCC components CDC20 and BubR1 because phosphorylation of both proteins is significantly increased following calyculin A treatment. However, despite identifying a number of calyculin A-induced phosphorylation sites on these two proteins by mass spectrometry and performing mutational analysis of these sites, we were unable to

observe any reversal of calyculin A's ability to cause cyclin degradation during the spindle checkpoint. Nevertheless, we cannot rule out these two proteins as relevant phosphatase targets during the checkpoint because there was still a calyculin A-induced upshift of the mutant CDC20 and BubR1 proteins. This result indicates there are other calyculin A-induced phosphorylation sites that were not identified by mass spectrometry, and these phosphorylation sites may be sufficient to induce MCC disassembly. It is also possible that phosphorylation of multiple substrates contributes to MCC dissociation following calyculin A treatment.

Lastly, we attempted to identify the relevant phosphatase required for MCC maintenance by using siRNA or shRNA to systematically deplete cells of the PPP family phosphatases that are inhibited by calyculin A and okadaic acid. Based on the phosphatases known to be inhibited by 1 μ M okadaic acid and calyculin A but not 0.1 μ M okadaic acid or fostriecin, we speculated that PP1 would likely be the responsible phosphatase. Given that PP1 is required for checkpoint silencing, the notion that it may also be required for checkpoint maintenance may seem contradictory. However, PP1 does not appear to be heavily recruited to kinetochores until metaphase, where it promotes stable kinetochore-microtubule attachments.¹¹⁶ An interesting possibility to consider is that during prometaphase when the spindle checkpoint is highly active, a pool of cytosolic PP1 not localized at the kinetochores may be responsible for maintaining the MCC in an intact complex so it can effectively inhibit the APC/C.

However, we did not detect any decreases in cyclin A or B levels following knockdown of any of the phosphatases, including all three isoforms of PP1 in tandem, in nocodazole-arrested cells. This negative result could be due to an insufficient knockdown of the PP1 protein. Alternatively, it is possible there is redundancy among the phosphatases and that calyculin A and okadaic acid cause such a dramatic decrease in cyclin A and B levels because they inhibit multiple phosphatases at once. Future studies investigating the precise phosphatase(s) responsible for MCC maintenance along with the specific targets of that phosphatase will be important for improving our understanding of MCC maintenance and spindle checkpoint signaling.

5. Conclusions and Perspectives

In this dissertation, we explored the molecular mechanisms regulating two important signaling networks that protect the genomic integrity of the cell: the DNA damage response (DDR) and the spindle checkpoint. The DDR alerts the cell to errors in the DNA sequence and signals the proper cellular machinery to halt the cell cycle and repair the altered DNA. The spindle checkpoint protects chromosomal integrity by ensuring that when a cell divides, it passes on an intact, complete set of chromosomes to each daughter cell. Failures in either of these two mechanisms can result in genomic errors, which can have devastating consequences for the larger organism. This is evidenced by cancers that often have insufficiencies in one of these signaling networks.¹¹⁷ Defects in the DDR may result in mutations in the DNA sequence, and if these mutations activate an oncogene or inactivate a tumor suppressor gene, they may promote tumorigenesis. Alterations in the spindle checkpoint may lead to cells with an incorrect number of chromosomes, a condition known as aneuploidy, which is a common feature of tumors and also the cause of certain birth defects such as Down syndrome. The work presented in this dissertation furthers our knowledge of how these two essential signaling networks are regulated.

5.1 Downregulation of cyclin F at both the protein and mRNA level in response to DNA damage

Our DNA is fragile and thus prone to damage by many intrinsic and extrinsic factors. In order for our cells to survive and reproduce in such a hostile environment, our cells have evolved the DDR to sense DNA lesions, halt the cell cycle, and facilitate DNA damage repair so an accurate set of genetic instructions can be maintained and passed on to the next generation of cells. Alternatively, if the damage is too severe for the cell to repair, the DDR will activate signaling pathways that cause the cell to die via apoptosis. The DDR is activated in response to several different types of DNA damaging agents, including many chemotherapeutics used in the treatment of cancer. The goal in treating cancer patients with DNA damaging chemotherapeutics is to damage the cancer cell DNA so that the cell initiates the DDR and cannot recover. However, these chemotherapeutics also damage the DNA of the healthy cells, and thus they are often quite toxic. Studying the signaling pathways activated by DNA damage will not only advance our understanding of this essential cellular response but may also reveal new targets for anti-cancer therapies.

In Chapter 3, we explored how the DDR mediates the downregulation of cyclin F. Cyclin F levels rapidly and substantially decline in response to several types of DNA damage, including chemotherapeutics and X-irradiation. We discovered that cyclin F levels are regulated at both the protein and mRNA level following DNA damage to drive this sudden drop in cyclin F protein levels. While the full implications of this

downregulation of cyclin F in response to DNA damage must be further explored, one report has demonstrated that an important consequence of decreased cyclin F levels is increased RRM2 levels and dNTP production.⁹² There are likely additional SCF^{cyclin F} substrates that accumulate as a result of cyclin F downregulation following DNA damage, and future experiments will be important for identifying these substrates and their role in the DDR. Our data demonstrating that cyclin F depletion caused cells to delay entry into mitosis suggests that cyclin F downregulation may also be essential for halting cell cycle progression while the DNA is being repaired. As we continue to learn more about the functions of cyclin F, we will surely come to better understand the full ramifications of cyclin F downregulation following DNA damage. Additionally, the molecular mechanisms mediating delayed mitotic entry in cells with decreased cyclin F levels remains an interesting area of future study.

We also determined that cyclin F downregulation in response to DNA damage is downstream of the ATR/Chk1 pathway. However, there was some variation among cell lines in their level of dependency on Chk1 for cyclin F downregulation. Therefore, other mechanisms must be in place to regulate cyclin F downregulation in cell lines less dependent on Chk1. Understanding the molecular basis for the variations in cyclin F downregulation pathways among different cell lines will be an interesting area of future study. In exploring potential mechanisms through which Chk1 may mediate cyclin F downregulation, we found that Chk1 could phosphorylate cyclin F *in vitro*, and we

hypothesized that Chk1 phosphorylates cyclin F to mark it for degradation. However, we were not able to identify specific phosphorylation sites that target cyclin F for degradation. Unexpectedly, we found that inhibiting phosphatases with calyculin A, which induces rapid phosphorylation of cyclin F, actually stabilized cyclin F protein levels. Furthermore, treating cells with calyculin A prevented cyclin F degradation in response to etoposide. Although this data is not conclusive, it suggests that cyclin F phosphorylation might actually prevent its degradation, as opposed to promoting its degradation. It will be interesting for future experiments to test if hyper-phosphorylation of cyclin F decreases its interaction with the SCF complex. Furthermore, identifying the other phosphorylation sites on cyclin F will be helpful to better define the effect of cyclin F phosphorylation on its stability and overall function. An interesting possibility is that there may be a specific pattern of phosphorylation that designates whether or not cyclin F is degraded, with certain phosphorylation sites inhibiting degradation while others promote degradation. In this scenario, Chk1 phosphorylation on cyclin F may still play a role in targeting cyclin F for degradation. However, because we discovered that Chk1 inhibition prevents DNA damage-induced downregulation of cyclin F mRNA, it is possible that Chk1 mediates cyclin F downregulation solely at the mRNA level.

Another finding of our work is that cyclin F is degraded via its own E3 ubiquitin ligase, the SCF complex. We found that cyclin F levels dramatically increased when the

SCF complex was inhibited in unperturbed cells. However, in the presence of DNA damage, inhibiting the SCF complex only marginally rescued cyclin F levels; while SCF inhibition prevents cyclin F protein degradation, there is still downregulation occurring at the mRNA level. Therefore, treating cells with DNA damaging agents in the presence of an SCF inhibitor results in an intermediate amount of cyclin F protein. The F-box protein that targets cyclin F degradation via the SCF complex is still unknown, but our studies indicate that β -TrCP may play a small role in targeting cyclin F for degradation. We hypothesized that cyclin F may serve as its own F-box protein to target itself to the SCF complex. In order to test this hypothesis, a mutant version of cyclin F that cannot bind to the SCF complex is needed. Unfortunately, in our studies with an F-box mutant of cyclin F (LP/AA) previously reported to prevent binding to its SCF complex binding partner Skp1, the LP/AA mutant protein still bound a significant amount of Skp1, even when endogenous cyclin F was depleted. Therefore, we have not been able to test our hypothesis, and future studies will be needed to investigate the F-box protein responsible for cyclin F degradation.

Ongoing studies in our lab are aimed at elucidating the mechanism responsible for the Chk1-dependent downregulation of cyclin F mRNA in response to DNA damaging agents. We have demonstrated that cyclin F mRNA stability does not change following etoposide treatment, and therefore we propose that DNA damage induces transcriptional repression of cyclin F. Using the cyclin F promoter sequence in a

luciferase reporter assay, our lab will investigate the transcriptional regulation of cyclin F in the presence of DNA damage. With this tool, we will be able to search for transcriptional regulators of cyclin F in the presence of DNA damage and explore the role of Chk1 in cyclin F transcriptional regulation. As Chk1 inhibition prevented downregulation cyclin F mRNA levels, it will be interesting to investigate whether Chk1 is activating a cyclin F transcriptional repressor or inhibiting an activator.

Currently, very little is known about the significance of cyclin F in cancer, but emerging evidence has led some to speculate that cyclin F may be a tumor suppressor gene.⁹⁷ One study found that cyclin F levels were decreased in hepatocellular carcinoma tissue compared with levels in normal tissue, and low cyclin F expression was significantly correlated with advanced disease and poor prognosis.⁹⁸ Furthermore, a new study was just released showing the cyclin F mutations were found in 20% of endemic Burkitt lymphoma.¹¹⁸ It is interesting to speculate that cyclin F expression or mutation status may one day be used as predictive or prognostic marker for patients with these types of cancers. Increasing our knowledge of cyclin F and its regulation may help us to better understand the role of cyclin F in human malignancies.

5.2 A novel role for mitotic phosphatase activity during the spindle checkpoint

Changes in protein phosphorylation regulate a countless number of events in the cell cycle, including the spindle checkpoint. The opposing actions of kinases and phosphatases regulate numerous proteins to control when the APC/C is inhibited by the

spindle checkpoint and when it becomes active following bi-orientation. Kinase activity is required for spindle checkpoint activation and maintenance, and until now, phosphatases were thought to only be required for signaling related to spindle checkpoint silencing. In chapter 4, we describe our finding that in addition to kinases, phosphatases are also required for a fully functional spindle checkpoint. We determined that phosphatase activity is necessary during the spindle checkpoint to promote MCC formation and maintenance and to prevent premature APC/C-mediated cyclin A and B degradation. In studying the kinetics of MCC disassembly and cyclin degradation following the inhibition of phosphatase activity, we observed that MCC disassembly occurs immediately prior to cyclin B degradation. Therefore, we believe that MCC dissociation is responsible for APC/C activation and subsequent cyclin B degradation in response to phosphatase inhibition.

This finding lead us to two pressing questions: (1) Which phosphatase(s) is/are required for MCC maintenance? and (2) What is/are the relevant substrate(s) that must be dephosphorylated to promote MCC maintenance and APC/C inhibition? In our search to identify the required spindle checkpoint phosphatase, we started with the phosphatase family known to be inhibited by okadaic acid and calyculin A: the PPP family of Ser/Thr phosphatases. Based on the known phosphatase sensitivities to the small molecule inhibitors that we tested, we identified PP1 as our top candidate because it is sensitive to low levels of calyculin A, but it requires high levels of okadaic acid to

inhibit its activity. PP1 is known to be involved in the regulation of the spindle checkpoint, however, as stated above, it has previously only been implicated in events promoting checkpoint silencing. We used siRNA to knock down PP1 and other PPP family phosphatases and then measured cyclin levels during the spindle checkpoint as a readout of APC/C activity. Unfortunately, we did not detect any differences in APC/C inhibition during the spindle checkpoint with any of the phosphatase siRNAs that we tested. We carried our investigation of the relevant phosphatase a step further and used mass spectrometry to identify phosphatases that interact with the MCC complex. This unbiased search identified several novel MCC interacting phosphatases. Using siRNA targeted to each phosphatase, we tested their involvement in APC/C inhibition during the spindle checkpoint, but again, we did not detect any differences in cyclin levels. As with any siRNA experiment, there remains the possibility that residual protein activity was sufficient to keep the APC/C inhibited during the checkpoint, or alternatively, that there is more than one phosphatase that can prevent APC/C activation during the checkpoint. Additionally, knocking down such important regulatory proteins as PP1 and PP2A affects many signaling pathways in the cell, which may confound our results. Therefore, future studies will be critical to determine the identity of the phosphatase required for MCC maintenance and APC/C inhibition during the spindle checkpoint.

To address our second question, we focused our investigation of relevant phosphatase substrates on the MCC components. Because MCC dissociation appears to

be mediating APC/C activation and cyclin degradation following phosphatase inhibition, we reasoned that a component of the MCC may need to be dephosphorylated for proper MCC formation and inhibition of the APC/C. Furthermore, both CDC20 and BubR1 become highly phosphorylated following calyculin A treatment, which is suggestive that phosphorylation of one or both of these proteins following phosphatase inhibition is the driving force behind MCC disassembly. However, when we identified calyculin A-induced phosphorylation sites on these two proteins and performed mutational analysis, we were not able to rescue the cyclin degradation resulting from calyculin A treatment. As mentioned in chapter 4, our mass spectrometry search did not identify all of the calyculin A-induced phosphorylation sites on these two proteins, and so it will be important for future experiments to try to identify the other calyculin A-induced phosphorylation sites on these two proteins. If all calyculin A-induced phosphorylation sites can be identified and mutated to non-phosphorylatable alanine residues, then it will be possible to make a clear conclusion about whether CDC20 and/or BubR1 phosphorylation is driving MCC dissociation and subsequent APC/C activation.

In addition to the MCC components, there are other possible phosphatase substrates that may mediate APC/C activation upon their phosphorylation. For instance, it is possible that a kinetochore component becomes phosphorylated following okadaic acid and calyculin A treatment and disrupts proper MCC formation at the kinetochore.

However, this possibility seems less likely because we know that the MCC can form in the cytosol away from the kinetochore, although this formation is less efficient. Another possibility is that calyculin A and okadaic acid induce APC/C activation by acting directly on the APC/C. Perhaps a subunit of the APC/C must be dephosphorylated in order for the APC/C to be inhibited by the MCC, such that once it becomes phosphorylated following okadaic acid or calyculin A treatment, the APC/C can no longer be inhibited by the MCC. However, as we believe the most likely cause of APC/C activation following phosphatase inhibition is the disruption of the MCC, our top candidate for the relevant substrate downstream of phosphatase inhibition is still a component of the MCC.

Our findings from chapter 4 change the canonical view of how kinases and phosphatases regulate the spindle checkpoint. The PPP family of Ser/Thr phosphatases have been canonically viewed as the proteins responsible for turning off and shutting down cellular responses. For example, we know that PP1 and PP2A-B56 silence the spindle checkpoint to allow cells to progress into anaphase, and dephosphorylation by PP1 and PP2A-B55 δ reverses cyclin B/CDK1 phosphorylation to turn off mitotic signals and promote mitotic exit. In light of our finding that phosphatase inhibition activates the APC/C, we must acknowledge a role for phosphatases in maintaining the spindle checkpoint. This also raises the question of what other functions phosphatases may have during mitosis. We know that the phosphatase Cdc25 promotes cyclin B/CDK1

activation, but perhaps the PPP family of phosphatases have other pro-mitotic functions as well. Our work lays a foundation for future studies to build upon as they investigate the diverse roles of phosphatases in the spindle checkpoint and throughout the cell cycle.

5.3 Concluding Remarks

The data presented in this dissertation have expanded our understanding of the complex cellular responses that safeguard our DNA from mutations and chromosomal abnormalities. We have detailed our discovery of a novel mechanism of cyclin F regulation in the DDR and have identified a noncanonical role for phosphatases in the regulation of the spindle checkpoint. The DDR and the spindle checkpoint are two processes that are essential for the survival and reproduction of healthy human cells. Identifying and characterizing the molecular mechanisms regulating these complex cell signaling networks may one day lead to the discovery of new approaches to treat or even prevent diseases such as cancer, in which these signaling networks have often gone awry.

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Biography

Kristen Marie Foss

Born Kristen Marie Nelson to Larry and Mary Nelson on September 29, 1983 in Goshen, Indiana.

Married to Kent Kristopher Foss on May 29, 2010 at Lake Poinsett, South Dakota.

Education

Ph.D. in Pharmacology and Cancer Biology, Certificate in Cell and Molecular Biology
Duke University, Durham, NC, December 2015

B.S. in Biology, *summa cum laude*
Minors in Microbiology, Chemistry, and Mathematics
South Dakota State University, Brookings, SD, December 2006

Publications

Foss K.M.*, Robeson A.C.*, Kornbluth S., Zhang L. Mitotic phosphatase activity is required for MCC maintenance during the spindle checkpoint. *Cell Cycle*. Accepted.

*Authors contributed equally to this work.

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Nelson K.M. and Weiss G.J. MicroRNAs and Cancer: Past, Present, and Potential Future. *Molecular Cancer Therapeutics* 7(12):3655-3660 (2008).

Honors and Awards

2013-2015: Ruth L. Kirschstein National Research Service Award, NIH/NCI Predoctoral Fellowship

2013-2014: Duke Scholar in Molecular Medicine, Oncology and Regenerative Medicine Track

2013: Fitzgerald Academic Achievement Award, Duke Department of Pharmacology and Cancer Biology