

**REGULATION OF CDC25A IN HUMAN TUMOR CELLS BY CYCLIN-DEPENDENT
KINASE 2**

by

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Deregulation of normal cell cycle control is essential for malignant transformation. The Cdc25A dual-specificity phosphatase promotes cell cycle progression by dephosphorylating and activating the cyclin-dependent kinases. Cdc25A has oncogenic and anti-apoptotic activity and is overexpressed in many human tumors. The mechanisms by which Cdc25A is overexpressed in human cancer are unknown. Cdc25A protein levels are downregulated by cell cycle checkpoints in response to genotoxic stress; cell cycle checkpoints are frequently compromised in tumor cells. In addition, under normal physiologic conditions, the half-life of Cdc25A protein is short. Alterations to physiologic Cdc25A regulatory mechanisms could be sufficient to result in oncogenic overexpression of this cell cycle regulatory protein. While Cdc25A downregulation in response to genotoxic stress occurs through defined signal transduction pathways, regulation of Cdc25A protein levels in non-stressed cells is poorly understood. The purpose of this thesis was to examine the physiological regulation of Cdc25A protein levels in human tumor cells. The goals of our studies were: 1) to investigate regulatory mechanisms of Cdc25A protein levels in non-stressed human tumor cells; 2) to understand how Cdk2 kinase activity regulates Cdc25A protein levels; and 3) to explore the mechanism by which Cdk2 kinase

activity regulates Cdc25A protein turnover. The results of our studies revealed that Cdc25A protein half-life in non-stressed interphase cells is regulated, in part, by Cdk2 kinase activity, and that Cdk2 does not regulate Cdc25A turnover by affecting several known signal transduction pathways that control Cdc25A protein stability. Recent reports on the role of ubiquitin ligases in physiologic Cdc25A turnover have identified several phosphorylation sites that are necessary for efficient Cdc25A recruitment to ubiquitin ligases. The kinase(s) responsible for phosphorylating these serine residues remain to be identified, although Cdk2 could be one prime candidate. While initial reports of the interactions between Cdc25A and Cdk2 focused on an auto-amplification feedback loop that results in increased catalytic activity of both proteins, it now appears that Cdk2 also regulates Cdc25A stability and plays an important role in regulating Cdc25A protein levels during interphase progression.

FORWARD

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PREFACE

The following chapters have been modified from their original manuscripts to provide greater detail and a consistent format throughout this dissertation:

Chapter 1: Lyon, M. A., Ducruet, A. P., Wipf, P. and Lazo, J. S. (2002) Dual-specificity phosphatases as targets for antineoplastic agents. *Nat. Rev. Drug Discov.* **1**: 961-976.

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ABBREVIATIONS

AP	alkaline phosphatase
APC/C	anaphase promoting complex/cyclosome
Ask1	apoptosis signal-regulating kinase 1
Asn	asparagine
Asp	aspartic acid
ATM	ataxia-telangiectasia-mutated
ATR	ATM- and Rad3-related
C-TAK1	cdc twenty-five C associated protein kinase
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
Chk	checkpoint kinase
CHX	cycloheximide
CPD	cdc4 phosphodegron
DIG	digoxigenin
DMSO	dimethylsulfoxide (Me ₂ SO ₄)
DN	dominant-negative
DSPase	dual-specificity phosphatase
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
Erk	extracellular regulated kinase
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione-S-transferase
HDAC1	histone deacetylase 1
HECT	homologous to E6-AP C Terminus
HEK	human embryonic kidney
HPV	human papillomavirus
IPTG	isopropyl β -D-1-thiogalactopyranoside
IR	ionizing radiation
JNK	jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
Mg ²⁺	magnesium
MOI	multiplicity of infection

NES	nuclear export sequence
NLS	nuclear localization signal
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PKB	protein kinase B
PTPase	protein tyrosine phosphatase
Rb	retinoblastoma
Redox	reduction-oxidation
RING	really interesting new gene
RT-PCR	reverse transcription-polymerase chain reaction
SAPK	stress-activated protein kinase
SCF	skp1/cullin or cul1/f-box
Ser	serine
siRNA	small interfering RNA
TGF- β	transforming growth factor beta
Thr	threonine
Tyr	tyrosine
UV	ultraviolet
VHR	vaccinia H1-related phosphatase

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Cancer is defined as the unregulated proliferation of previously normal cells that have undergone specific alterations to their genomes, enabling them to acquire the necessary capabilities to evade the homeostatic regulation of cell division, invade nearby tissues and metastasize to distal sites in the body (1). Cancer is the second leading cause of mortality in the United States, contributing to ~23% of all deaths in 2001¹. Furthermore, half of all men and one third of all women in the United States will develop cancer in their lifetime². A central theme of the efforts to control and eliminate cancer, the stated goal of the National Cancer Institute, is to promote basic science research in an effort to better understand the fundamental biology of cancer with the hope of generating novel therapeutic interventions that can be translated into clinical treatments for cancer³. While human cancers possess numerous genotypic alterations that can occur in any of the >100 different human tumor types, these mutations can be classified according to six essential alterations in normal cellular processes that enable malignant cell growth. These processes are "...self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (2)." To fully understand how instability in the human genome contributes to the malignant phenotype, it is

¹ The American Cancer Society: Cancer Statistics 2004 (<http://www.cancer.org>)

² The American Cancer Society: Cancer Statistics 2004 (<http://www.cancer.org>)

³ The National Cancer Institute: "The Nation's Investment in Cancer Research: A Plan and Budget Proposal for Fiscal Year 2004." Prepared by the Director, National Cancer Institute

important to understand the fundamental underlying biological processes before they go awry. The work presented herein is an effort to understand the biology of the cell division cycle and the regulation of the cell division machinery. These studies were undertaken to provide insight on the mechanisms leading to deregulation of the cell division machinery and to further the understanding of how deregulated cell biology contributes to the development of cancer.

1.2. THE MAMMALIAN CELL DIVISION CYCLE

The mammalian cell division cycle, or cell cycle, consists of 2 major processes, namely the replication of genetic material and the uniform segregation of this newly duplicated genetic material into two daughter cells (3). Progression through the cell cycle occurs in response to the activation of mitogenic signaling cascades, which results in the synthesis and activation of proteins necessary for operation of the cell cycle machine (4). The cell cycle consists of four distinct phases: G1, the first gap phase, during which the cell senses signals from its external environment and commits to cell division; S, the DNA synthesis phase, during which the genome is replicated; G2, the second gap phase, in which the cell can correct any errors in DNA replication and prepare for cell division; and M, or mitosis, in which the cell physically divides and segregates its identical copies of genetic material into two daughter cells (5, 6). Progress from one phase of the cell cycle to the next requires movement through cell cycle transitions between each cell cycle phase; the major catalysts of movement through cell cycle transitions are the cyclin-dependent kinases (Cdks). The Cdks consist of a catalytic kinase subunit (the Cdk itself) and a regulatory subunit known as a cyclin (4). Cdk activity is primarily regulated in a temporal manner through the regulated expression of cognate cyclins, whose expression levels oscillate with specific phases of the cell cycle (1). Cyclin D is expressed throughout the cell

cycle in response to mitogenic stimulation; cyclin E expression increases in late G1, peaks at the G1/S transition, and decreases in S phase; cyclin A expression increases throughout S phase, peaking in late G2 and decreasing before the G2/M transition; and cyclin B expression emerges in late S and early G2 and peaks in mitosis (7). This expression pattern results in cell cycle phase specific activity of various Cdks. The D-type cyclins associate with Cdk4 and Cdk6 in G1 to promote G1 progression and contribute to the activity necessary for the G1/S transition; E-type cyclins associate with Cdk2 and also contribute to the G1/S transition and S phase progression; A-type cyclins associate with Cdk2 in S phase to promote S phase progression and with Cdk1 in late S phase and in G2 to promote progress through G2; and B-type cyclins associate with Cdk1 in late G2 and in mitosis to promote the G2/M transition and enable mitotic progression (Figure 1.1) (1, 7). The Cdks, therefore, serve as the major promoters of progress through the cell division cycle and, as such, are subject to precise regulation to ensure a proper sequence of activation.

1.3. CDK REGULATION

As the driving force behind cell cycle progression, Cdk catalytic activity is constrained by several layers of regulation: 1) association with cognate cyclins results in catalytic activation of the Cdk kinase subunit; 2) Cdk activity is regulated both positively and negatively by phosphorylation events; and 3) Cdk activity is negatively regulated by small protein Cdk inhibitors (CDKI). Temporal regulation of cyclin expression results in precise timing of Cdk activation; this is the primary mechanism regulating Cdk activation (7, 8). The Cdks are also positively regulated by phosphorylation of a threonine (Thr) residue in the T loop, an extended loop in the Cdk protein that impedes substrate binding in the absence of phosphorylation (8).

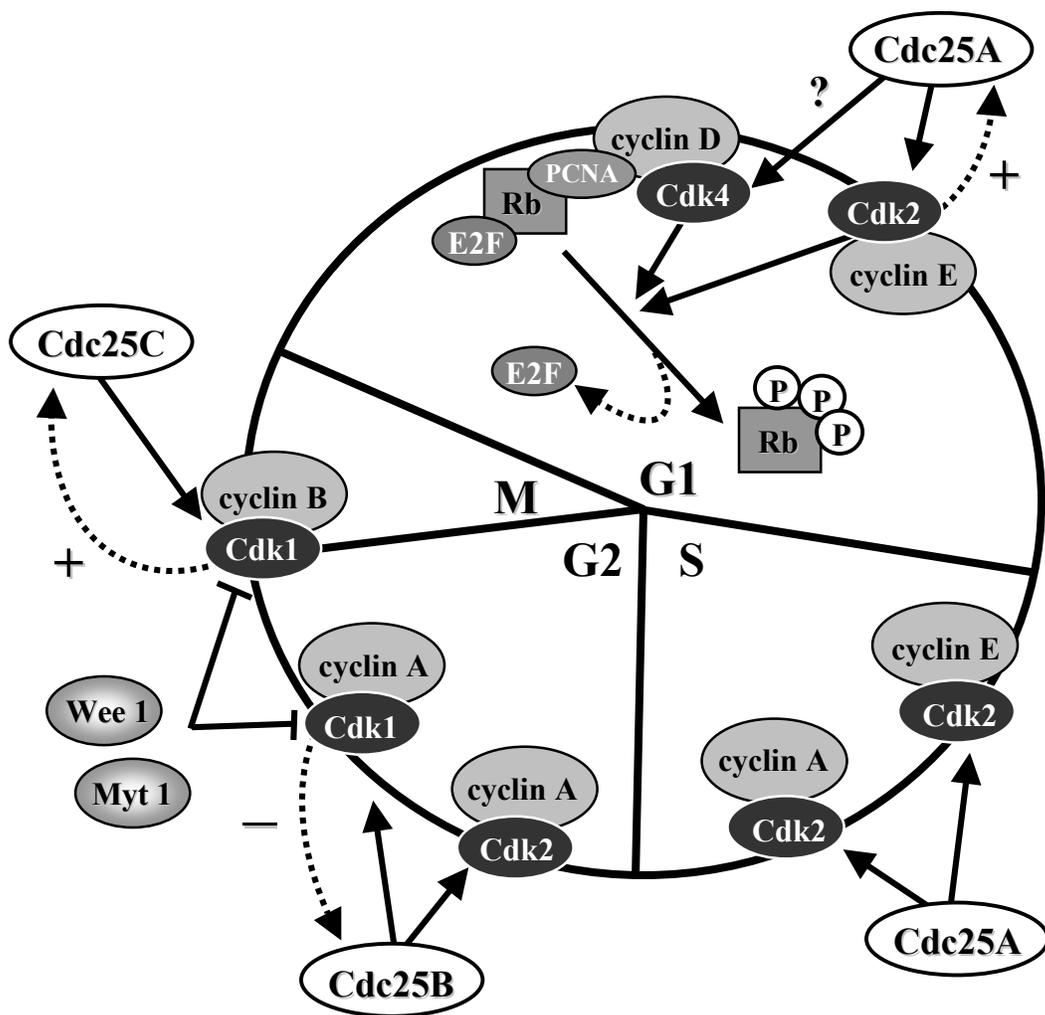


Figure 1.1: Regulation of the Mammalian Cell Cycle by Cdks and Cdc25s.

Cdc25 DSPases promote mammalian cell cycle progression by dephosphorylating and activating the Cdks. Solid arrows indicate activation by Cdc25 while ---| indicates inhibition. Dotted arrows represent known feedback loops, either positive (+) or negative (-) as indicated. It is unclear whether Cdk4/cyclin D is a *bona fide* substrate of Cdc25A.

Cdks are phosphorylated at this threonine residue (Thr 161 in Cdk1 and Thr 160 in Cdk2) by the Cdk activating kinase (CAK) (Figure 1.2) (9). On the other hand, the Cdks are negatively regulated by phosphorylation at Thr 14 and tyrosine (Tyr) 15 (in Cdk1 and Cdk2) in the ATP-binding domain of the Cdk, which prevents phosphorylation of its substrates (8). While inhibitory phosphorylation in the ATP-binding site has been clearly defined for Cdk1 and Cdk2, it is still unclear whether comparable negative regulatory phosphorylation plays a role in Cdk4 regulation, although several reports correlate decreased Cdk4 catalytic activity with increased tyrosine phosphorylation of Cdk4 (10, 11). Cdk phosphorylation at Thr 14 and Tyr 15 is catalyzed by Wee1 and Myt1 protein kinases (Figure 1.2). Wee1 is a tyrosine-specific protein kinase that localizes to the nucleus, whereas Myt1 encodes a dual-specific protein kinase that resides outside of the nucleus and localizes to the Endoplasmic Reticulum (ER) and Golgi complex (12, 13). Cdk activity can also be inhibited by a family of proteins known as CDKIs, which bind to Cdk/cyclin complexes to inhibit their activity. The CDKIs comprise two families, the INK4 family, consisting of p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D}, which selectively target the G1 specific Cdk4/cyclin D and Cdk6/cyclin D, and the Cip/Kip family, consisting of p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, which are more promiscuous Cdk inhibitors, inhibiting the activity of multiple Cdks throughout the cell cycle (1, 14, 15). Full catalytic activation of the Cdks is only achieved when the triply phosphorylated (Thr 14, Tyr 15, and Thr 161) Cdk/cyclin complex is dephosphorylated at both Thr 14 and Tyr 15. Dephosphorylation of Cdks at these sites to promote cell cycle progression is catalyzed by the activity of the Cdc25 dual-specificity phosphatases (DSPases) (Figure 1.2).

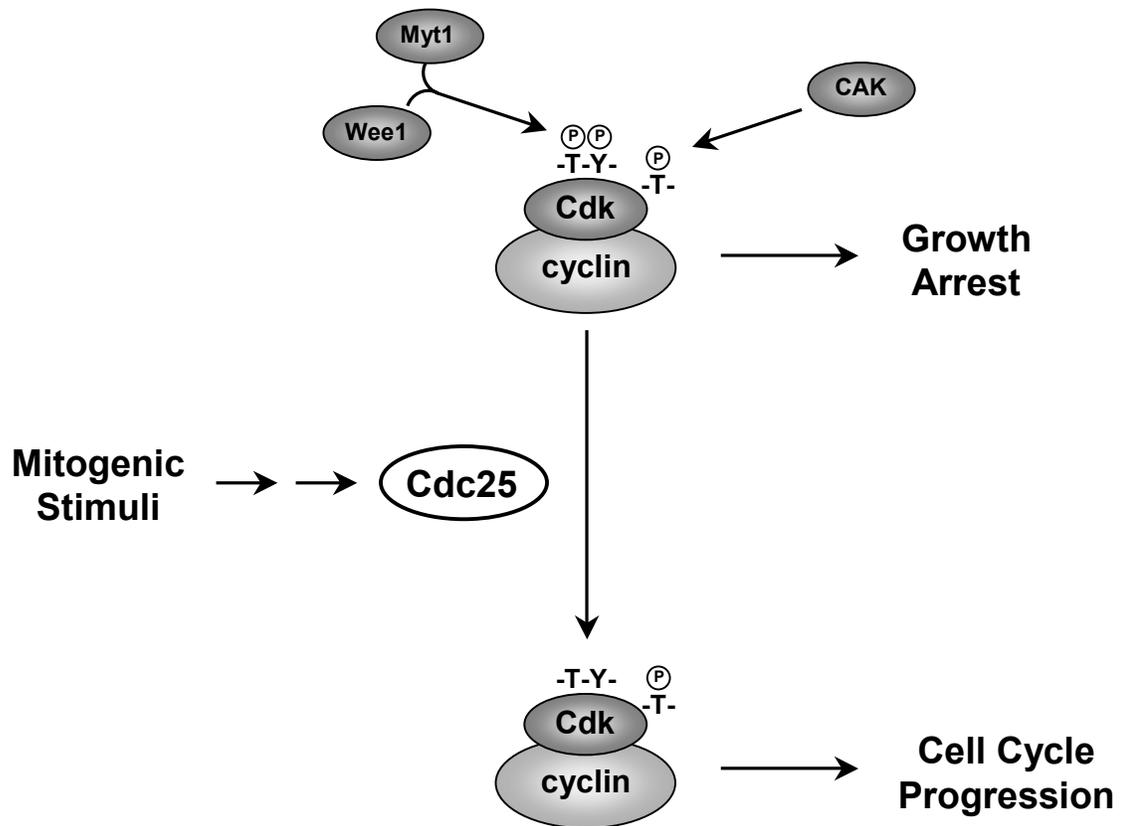


Figure 1.2: Cdk Activation by Cdc25 Phosphatases.

Mitogenic signaling promotes the activation of Cdc25 phosphatases to initiate cell cycle progression. Cdc25s activate Cdks by removing inhibitory phosphorylations from threonine 14 and tyrosine 15 in the ATP binding domain of the kinase. Cdk activity requires threonine 161 phosphorylation by CAK. Growth inhibitory signals promote Cdc25 inactivation and inhibitory Cdk phosphorylation catalyzed by Wee1 and Myt1 kinases.

1.4. CDC25 PHOSPHATASES

cdc25 was first identified in *S. pombe* fission yeast as the 25th protein that influenced the yeast cell division cycle (16). It was noted that upon mutation of *cdc25*, cells assumed the opposite of a *wee1* phenotype, namely that they failed to divide and grew to an enlarged state. This suggested that *cdc25* functioned antagonistically to *wee1*, which was known to encode a protein kinase that inhibited yeast cell division (16, 17). Several lines of evidence suggested that *cdc25* encoded a protein phosphatase: 1) phenotypic evidence that *cdc25* was antagonistic to *wee1*, a known kinase; 2) phosphotyrosine content increased in cells lacking *cdc25*; 3) the protein product of an additional cell cycle regulatory factor, *cdc2*, was tyrosine phosphorylated to inhibit its activity; and 4) a tyrosine to phenylalanine mutation in *cdc2* rendered yeast cells resistant to disruption of *cdc25* (17). Moreover, the *cdc25* gene product demonstrated the ability to dephosphorylate phosphotyrosine, phosphoserine and phosphothreonine residues *in vitro* (18). Three human homologs of *cdc25*, Cdc25A, Cdc25B, and Cdc25C (Figure 1.3), were identified using a degenerate oligonucleotide primer-based PCR cloning strategy and by genetic complementation of a *cdc25^{ts}* temperature-sensitive yeast strain (19-21). While all three human homologs successfully complemented the *cdc25^{ts}* temperature-sensitive yeast mutant, we now know that their expression is cell cycle specific in mammalian cells: Cdc25C mRNA is predominantly expressed in G2 and M phase, Cdc25B mRNA is expressed throughout the cell cycle and is elevated in G2, and Cdc25A mRNA is also expressed throughout the cell cycle, with peak expression during late G1 and S phases (20-22).

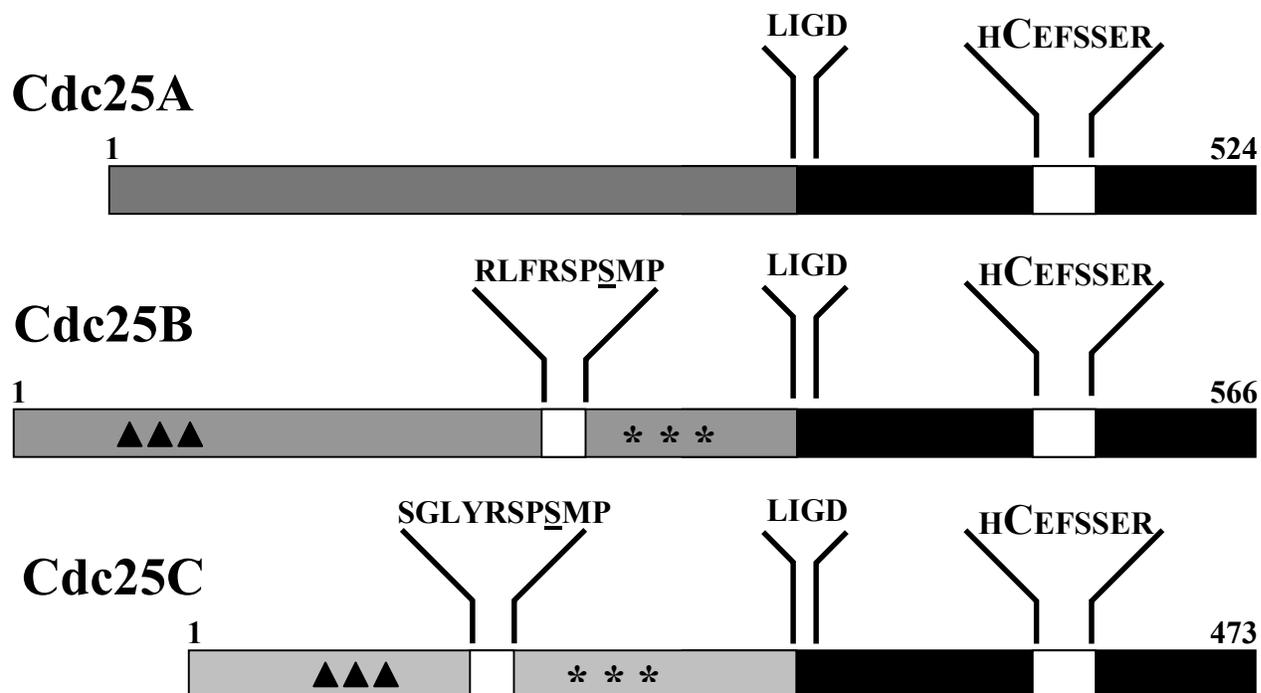


Figure 1.3: Mammalian Cdc25 Phosphatases.

The mammalian Cdc25A, Cdc25B, and Cdc25C phosphatases are encoded by three distinct genes and have specific roles in cell cycle regulation. They comprise an amino terminal domain, which serves a regulatory role and is the site of multiple phosphorylations, and a carboxy terminal domain, marked by a conserved -L-I-G-D- motif. The carboxy terminal domain contains the canonical PTPase active site motif -H-C-X₅-R- (where X is any amino acid). Single letter amino acid abbreviations are used. Demarkated sequences in Cdc25B and Cdc25C identify 14-3-3 binding sites (serine phosphorylation site underlined) that facilitate checkpoint-mediated cytoplasmic sequestration by interfering with the proximal NLS (NLS sequences are indicated with asterisks). NES sequences are indicated with triangles and the catalytic cysteine is marked by larger font. Cdc25A phosphorylation sites and 14-3-3 interactions are detailed in Figure 5.1.

1.4.1. MAMMALIAN CDC25 DUAL-SPECIFICITY PHOSPHATASES

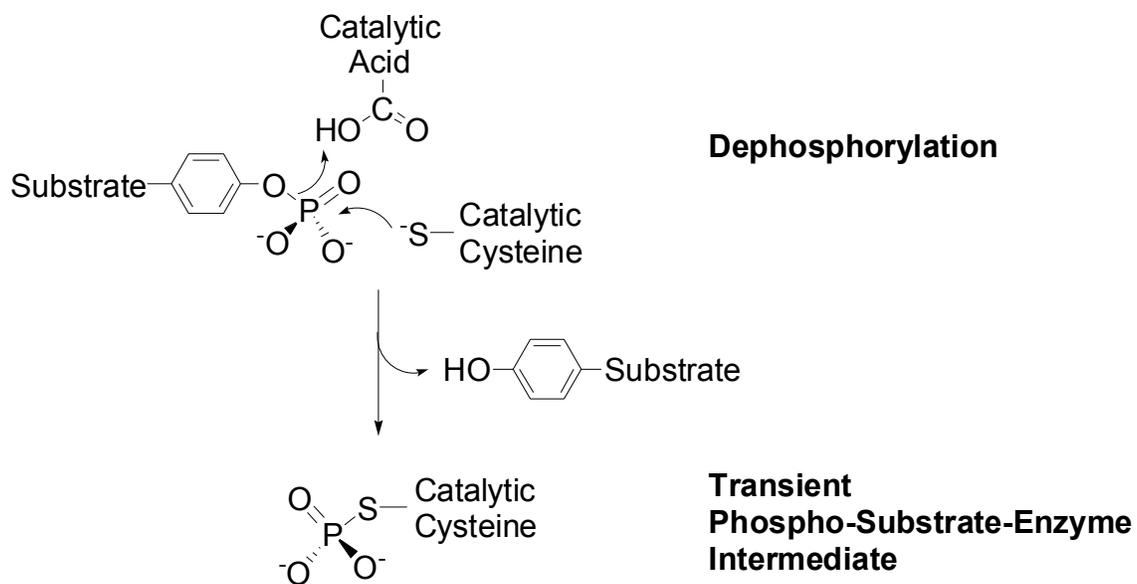
The three human Cdc25 isoforms, while sharing functional and sequence homology, are encoded by distinct genes that localize to different chromosomes: Cdc25A is found on 3p21, Cdc25B on 20p13, and Cdc25C on 5q31. The human Cdc25 family is further complicated by splicing to generate multiple variants of each isoform: 2 for Cdc25A, 5 for Cdc25B and 5 for Cdc25C (23, 24). While the function of the Cdc25 splice variants remains unknown, it has been speculated that the Cdc25A and Cdc25C splice variants may have different roles in different cell lines or may differ in their cell cycle phase distribution. Furthermore, alternative splicing may eliminate specific consensus phosphorylation sites, potentially subjecting the different splice variants to differential regulation; deletion of such phosphorylation motifs decreases Cdc25B phosphatase activity, and Cdc25C activity has been reported to increase following phosphorylation (24-27). Of the Cdc25B splice variants, only 2 of the 5 seem to be predominantly expressed in mammalian cells: Cdc25B2 and Cdc25B3 (23).

At the protein level, Cdc25 DSPases are structurally divided into two major domains: a highly conserved carboxy terminal domain, which is delineated by a conserved -L-I-G-D- motif and comprises approximately 30% of the protein, and an amino terminal domain, which varies in length and shares little homology between the three human isoforms (Figure 1.3) (28). The Cdc25 catalytic site, with the canonical PTPase active site motif -H-C-X₅-R- (where X is any amino acid), resides in the carboxy terminal domain (29). In mammalian cells, the Cdc25 phosphatases function as dual-specificity phosphatases, a protein tyrosine phosphatase (PTPase) sub-class uniquely able to hydrolyze phosphate ester bonds on both a tyrosine and either a serine or threonine located in the same protein substrate (30). While the canonical PTPase active site is

typically a deep ~ 9 Å cleft, the DSPase active site is a shallow ~ 6 Å cleft; the more shallow nature of the DSPase active site is presumably necessary to enable DSPases to dephosphorylate both the less extended phosphoserine and phosphothreonine motifs as well as the more extended phosphotyrosine motif (30-32). Like the PTPases, DSPases employ a two-step catalytic mechanism to dephosphorylate their protein substrates (Figure 1.4). This mechanism involves formation of a transient phospho-enzyme intermediate by transferring the phosphate from the substrate amino acid to the DSPase catalytic cysteine residue. The dephosphorylated substrate is then expelled from the active site following protonation of the tyrosine phenolic oxygen by an acidic amino acid residue; phosphoserine/phosphothreonine hydrolysis is catalyzed by the same active site and is believed to proceed via a similar mechanism (30, 33-35). The active phosphatase is regenerated when a basic amino acid residue activates a proximal water molecule, allowing hydrolysis of the phospho-enzyme intermediate and resulting in the release of inorganic phosphate (30, 36). It is not clear, however, whether the Cdc25 DSPases employ a general acid in their catalytic mechanism, as an amino acid functioning as the catalytic acid has not been convincingly identified and may even reside on the protein substrate (37-40). While the carboxy terminal domain of the Cdc25 DSPases houses the enzyme's catalytic machinery, the amino terminus is thought to be the major regulatory region of the protein, containing multiple phosphorylation sites that may positively or negatively regulate Cdc25 activity (28, 41-43).

The crystal structures for the Cdc25A and Cdc25B catalytic domains have been reported at 2.3 Å and 1.9 Å resolutions, respectively, but no crystal structure for a full length Cdc25 protein is available. Both phosphatases contain the canonical -H-C-X₅-R- PTPase catalytic site motif nestled in the P-loop structural motif, a characteristic of all tyrosine phosphatases (36, 38, 44). While the overall structures of the catalytic domains of the two Cdc25 phosphatases are

A.



B.

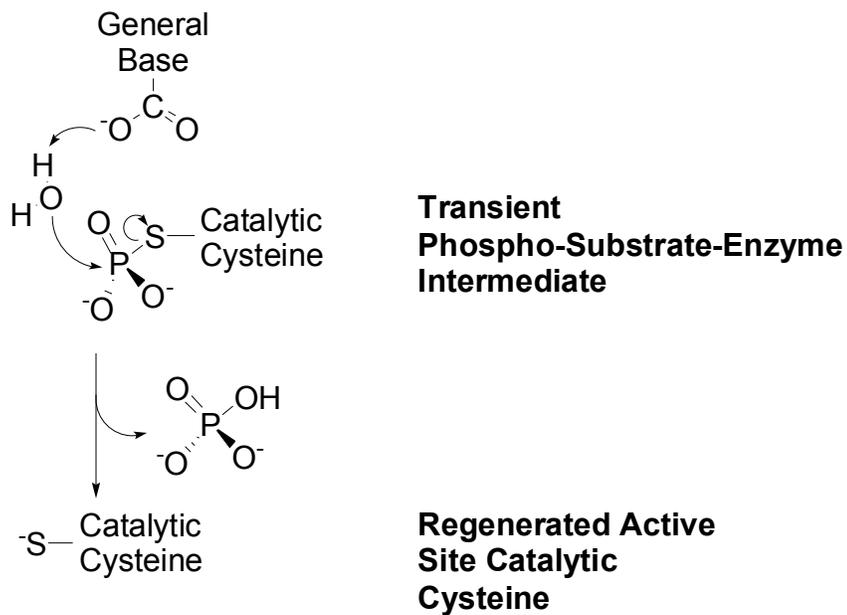


Figure 1.4: Catalytic Mechanism of Cdc25 Phosphatases.

Cdc25 phosphatases employ the general PTPase catalytic mechanism to dephosphorylate protein substrates. In a two step mechanism, the phosphate from a phosphoamino acid substrate is transferred to the catalytic cysteine to form a phospho-enzyme intermediate. The dephosphorylated substrate is then expelled from the active site by protonation of the tyrosine phenolic oxygen (Panel A). Phosphoserine/phosphothreonine hydrolysis is catalyzed by the same active site and is believed to proceed via a similar mechanism. The active phosphatase is regenerated by means of a proximal water molecule that hydrolyzes the phospho-enzyme intermediate, facilitating release of inorganic phosphate (Panel B) (33). The catalytic cysteine in Cdc25A is cysteine 430 and a residue with the potential to function as the catalytic acid is glutamic acid 431, although the requirements for a catalytic acid in the dephosphorylation mechanism employed by the Cdc25s is still a matter of debate (33, 37-40, 44).

similar, Cdc25A failed to bind oxyanions in its catalytic site, whereas Cdc25B readily bound tungstate and sulfate in its catalytic site in a mode akin to other PTP- and DSPases (44). This may stem from the more shallow nature of the Cdc25A active site when compared to the active site architecture of Cdc25B, which is more reminiscent of other DSPases active sites; the Cdc25A catalytic domain also lacks any loops proximal to the active site that may facilitate substrate binding (38, 44). A comparison of the two crystal structures also revealed that the C-terminal tail of Cdc25B folds back upon its active site, whereas the C-terminal tail of Cdc25A is directed away from the active site cleft, resulting in a more open structure (44). These structural data lend credence to biochemical data arguing that the final 17 carboxy terminal residues of Cdc25B function to confer its substrate specificity and the reports in the literature suggesting a higher degree of promiscuity for Cdc25A substrate selection (45-48). Interestingly, while Cdc25A has been functionally compared to other PTP- and DSPases because of its canonical -H-C-X₅-R- motif in its active site, it unexpectedly shares topology with the bacterial sulfur transferase protein rhodanese; the significance of this homology is unclear, but suggests that the two enzyme families may share a common evolutionary origin (38, 49). In contrast, Cdc25B compares more favorably with other PTP- and DSPases (44).

1.4.2. CELL CYCLE CONTROL BY CDC25 PHOSPHATASES

The roles for Cdc25A, Cdc25B, and Cdc25C in different phases of the cell cycle have been well studied. Because Cdc25C is the human Cdc25 isoform most homologous to yeast and *Xenopus* Cdc25, most investigators believe that it functions primarily in mitosis and catalyzes mitotic progression by activating Cdk1/cyclin B (18, 50-52). Microinjection of antibodies against Cdc25C into HeLa cells blocked entry into mitosis, substantiating this hypothesis (53).

However, Cdc25C may also be involved in regulating DNA synthesis, as Cdc25C phosphatase activity was detected in S phase cell extracts and downregulation of Cdc25C was accompanied by S phase inhibition (54). A potential role for Cdc25C in S phase, however, remains unknown. Since Cdc25B also activates Cdk1/cyclin B, it was initially believed to be functionally redundant to Cdc25C; microinjection of Cdc25B antibodies also inhibited entry into mitosis (55, 56). However, Cdc25B and Cdc25C activity appear to be separated temporally, with Cdc25B activity peaking prior to that of Cdc25C. Cdc25B has been identified as the “mitotic trigger”, activating a pool of Cdk1/cyclin B to promote the onset of mitosis (55, 57-62). Moreover, Cdc25B can also activate Cdk2/cyclin A in S phase and Cdk1/cyclin A in G2 (25, 63, 64). The primary function attributed to Cdc25A was promoting the G1/S cell cycle transition and S phase progression (Figure 1.1). The original Cdk substrate identified for Cdc25A was Cdk2/cyclin E, overexpression of Cdc25A accelerated S phase entry with premature Cdk2 activation, and microinjection of Cdc25A antibodies prevented S phase entry in cells induced by serum stimulation (22, 65, 66). However, Cdc25A protein levels and activity remain elevated past S phase and increase as cells enter mitosis (67); in fact, Cdk1/cyclin B phosphorylates Cdc25A and increases its stability in mitotic cell populations (68). While the precise function of Cdc25A in G2 and mitosis remains unclear, Cdc25A appears to be rate limiting for the G2/M transition and mitotic progression by regulating Cdk1/cyclin B activity (Figure 1.5) (67-70). This should not come as a complete surprise because, when Cdc25A was first identified, it was found to associate with cyclin B and Cdk1 and was hypothesized to function in mitosis (19). As Cdc25A is expressed and active for all the major cell cycle transitions, targeted deletion of Cdc25B or Cdc25C would be predicted to have no significant impact on cell cycle progression. In fact, mouse embryonic fibroblasts (MEFs) harvested from mice with targeted deletions of Cdc25B or

Cdc25C have normal cell cycle profiles when compared to wild type MEFs, suggesting a dominant role for Cdc25A in cell cycle control (71, 72).

1.4.3. CDC25S AS TARGETS OF CELL CYCLE CHECKPOINTS

The function of the cell cycle is to faithfully replicate (S phase) and separate (M phase) the genome into two identical daughter cells (1). To that end, cells possess highly evolved checkpoint mechanisms to terminate cell cycle progression in an effort to ensure high fidelity DNA replication and distribution (73). As the major promoters of Cdk activation and cell cycle progression, Cdc25 phosphatases are targets of cell cycle checkpoints. Cdc25s were first implicated in the yeast DNA damage checkpoint, which was characterized by increased Cdc2 Tyr 15 phosphorylation (Cdc2 is equivalent to Cdk1 in mammalian cells), implying Cdc25 inhibition (74). In response to DNA damage, Chk1 phosphorylated Cdc25, promoting 14-3-3 binding, nuclear exclusion and functional Cdc25 inactivation; 14-3-3 proteins bind specifically to phosphoserine motifs in signal transduction and cell cycle regulatory proteins and can affect the subcellular localization of target proteins (75, 76). Inhibition of Cdc25 phosphatases at cell cycle checkpoints is a conserved phenomenon from yeast to mammals and these checkpoints are activated in response to diverse stimuli (76-81). While Cdc25B and Cdc25C are targets of the G2/M cell cycle checkpoint, Cdc25A is targeted by all the major cell cycle checkpoints, reinforcing findings from the Cdc25B and Cdc25C knockout mouse studies that Cdc25A plays a major regulatory role in mammalian cell cycle progression (68, 71, 72, 82-90). Nevertheless, Cdc25B and Cdc25C must functionally contribute to cell cycle progression, as they are inactivated in cell cycle checkpoints by Chk1-, Chk2-, and p38 mitogen-activated protein kinase

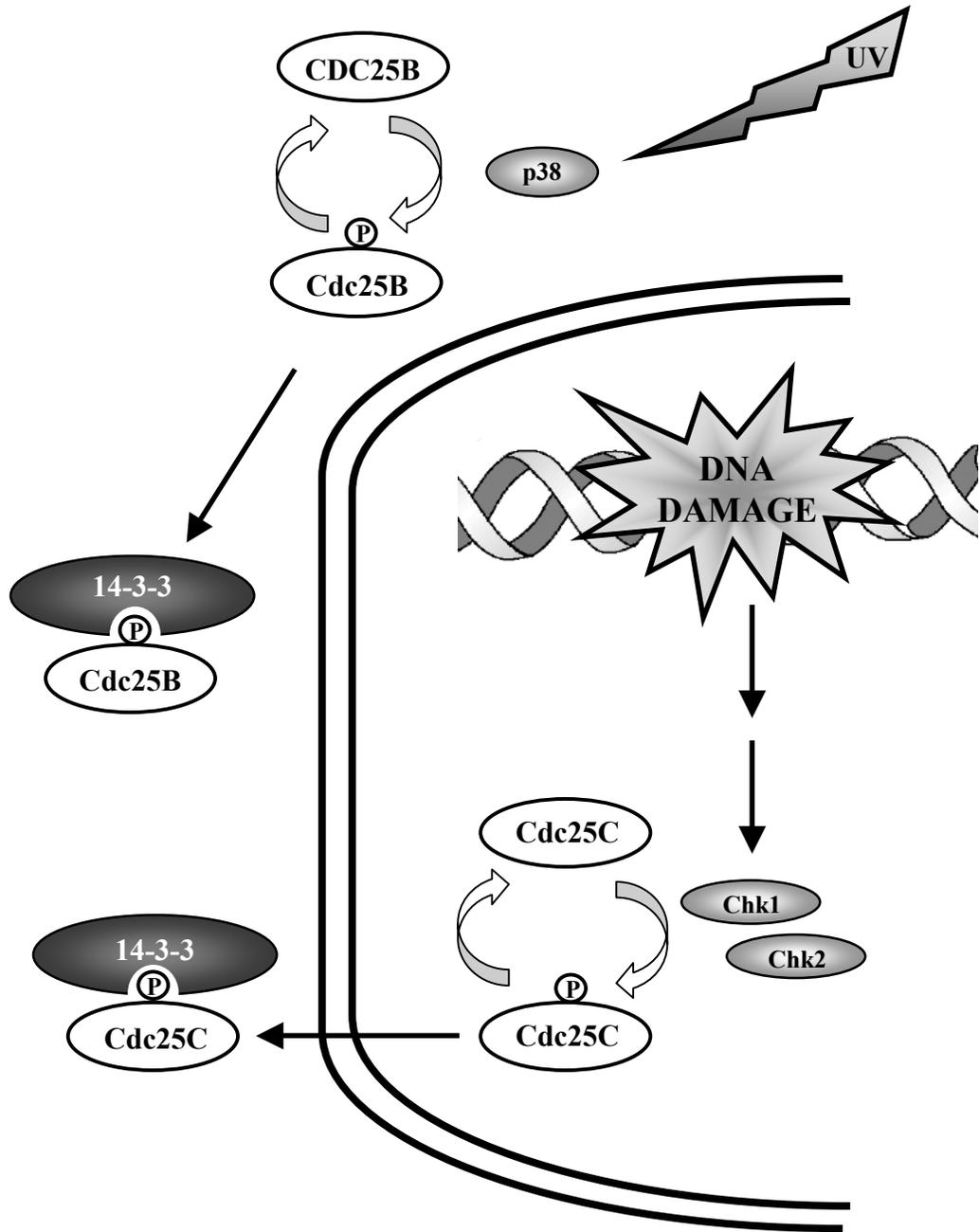


Figure 1.6: Cdc25B and Cdc25C are inactivated by Cell Cycle Checkpoints.

Cdc25B and Cdc25C are targets of cell cycle checkpoints, resulting in their inactivation by 14-3-3 binding and cytoplasmic sequestration (see text for details).

(MAPK)-mediated phosphorylation and cytoplasmic sequestration by 14-3-3 proteins; incidentally, these phosphorylation sites are localized to the amino termini of Cdc25B and Cdc25C (see section 1.4.1) (Figure 1.6) (23, 41, 91-94). Cdc25B and Cdc25C inactivation by cytoplasmic sequestration occurs as a result of 14-3-3-dependent obstruction of their nuclear localization signals (NLS), blocking nuclear entry (79, 80, 92-96). In addition, there is some evidence that phosphorylation of Cdc25 by Chk1 results in decreased Cdc25 phosphatase activity, independent of 14-3-3 (78). The cell cycle checkpoints targeting Cdc25A are independent of 14-3-3 and involve proteolytic degradation (Figure 1.7). Cdc25A does not appear to have validated NLS or NES consensus sequences, and its subcellular localization is a matter of debate, as Cdc25A has been reported to localize in the nucleus, the cytoplasm and the plasma membrane and to interact with proteins that reside in each of these cellular compartments (42, 45, 48, 66, 97-100). Following any number of genotoxic stresses, including DNA damaging chemotherapeutics, UV or IR irradiation, oxidative stress, or disruption of DNA synthesis, cells enact p53-independent cell cycle checkpoint programs that lead to rapid ubiquitin-mediated proteolysis of Cdc25A at the G1/S, intra-S and G2/M checkpoints (67, 68, 82-90). These p53-independent checkpoints serve as a rapid response to genetic insults; p53-dependent checkpoints can then be enacted to maintain cell cycle arrest, if necessary, to preserve genomic integrity (87). Phosphorylation of Cdc25A at Ser 123 was found to be necessary for promoting Cdc25A ubiquitination in response to genotoxic stress (84). It is now apparent, however, that Cdc25A is phosphorylated at a number of additional amino terminal serine residues, including Ser 75, and that Cdc25A ubiquitination and proteolytic degradation in response to cell cycle checkpoints is the result of a complicated and poorly understood interaction between multiple phosphorylation sites and effector proteins (discussed in detail in Chapter 5). Because of its critical role in

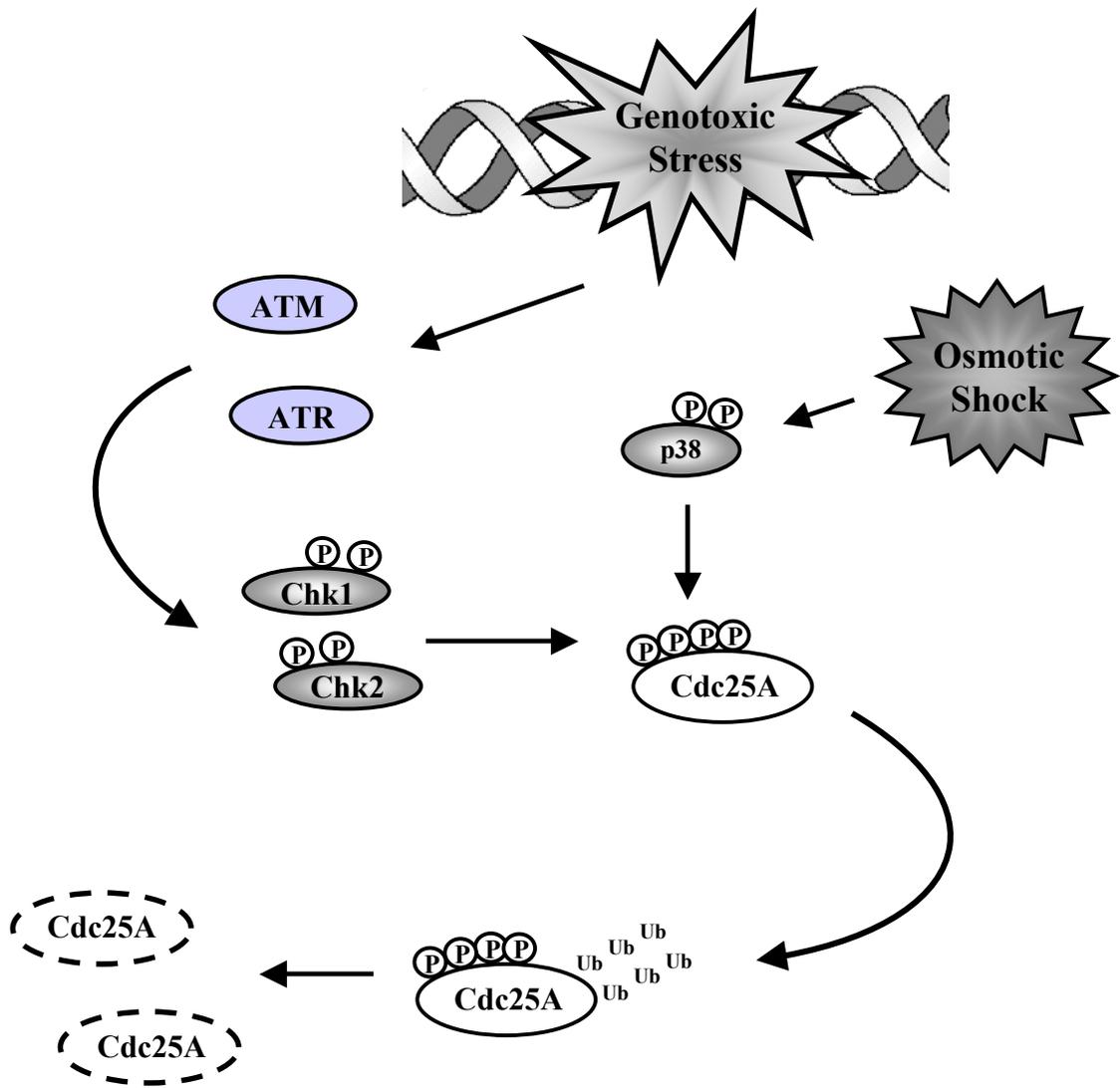


Figure 1.7: Cell Cycle Checkpoints Target Cdc25A for Proteolytic Degradation.

Following DNA damage and other genotoxic stresses, Cdc25A is phosphorylated by checkpoint kinases, resulting in poly-ubiquitination, proteolytic processing and irreversible Cdc25A inactivation (see text for details). Dotted lines represent degraded protein.

promoting all the major cell cycle transitions, it is not surprising that the cell possesses precise regulatory mechanisms targeting Cdc25A for destruction to arrest the cell cycle and maintain genomic integrity.

1.4.4. CDC25A IS A COMPONENT OF MULTIPLE SIGNALING PATHWAYS

In addition to its critical role in regulating cell cycle progression by dephosphorylating the Cdks, Cdc25A has been implicated in several signaling pathways that mediate cell growth and cell survival (Figure 1.8). Growth factor stimulation of cells initiates signal transduction cascades that culminate in activation of the cell cycle machinery. This signaling paradigm relies on a functional connection between mitogenic signal transduction pathways and cell cycle proteins. Such a connection exists between the MAPK signaling cascade and Cdc25A (Figure 1.8). Cdc25A interacts with Raf1, a mitogen-activated protein kinase kinase kinase that transmits signals from growth factor receptors to effector MAPKs, such as extracellular regulated kinase (Erk). Raf1 co-precipitated with Cdc25A and co-localized with Cdc25A at the cellular level in immunofluorescence microscopy studies, and Raf1 was shown to phosphorylate Cdc25A, leading to increased Cdc25A catalytic activity (98). This provided a clear link between mitogenic stimulation and the cell cycle, as Raf1 is activated in response to mitogenic stimulation and Cdc25A promotes cell cycle progression. This functional relationship, however, appears to exist in an equilibrium, as Cdc25A also dephosphorylated Raf1 at phosphotyrosine residues, downregulating Raf1 kinase activity and attenuating mitogenic signaling through the MAPK signaling pathway (48). This functional interaction provides the cell with a mechanism for terminating mitogenic signaling once the threshold for activating cell cycle progression has been crossed; while transient mitogenic stimuli are clearly necessary to promote cell growth,

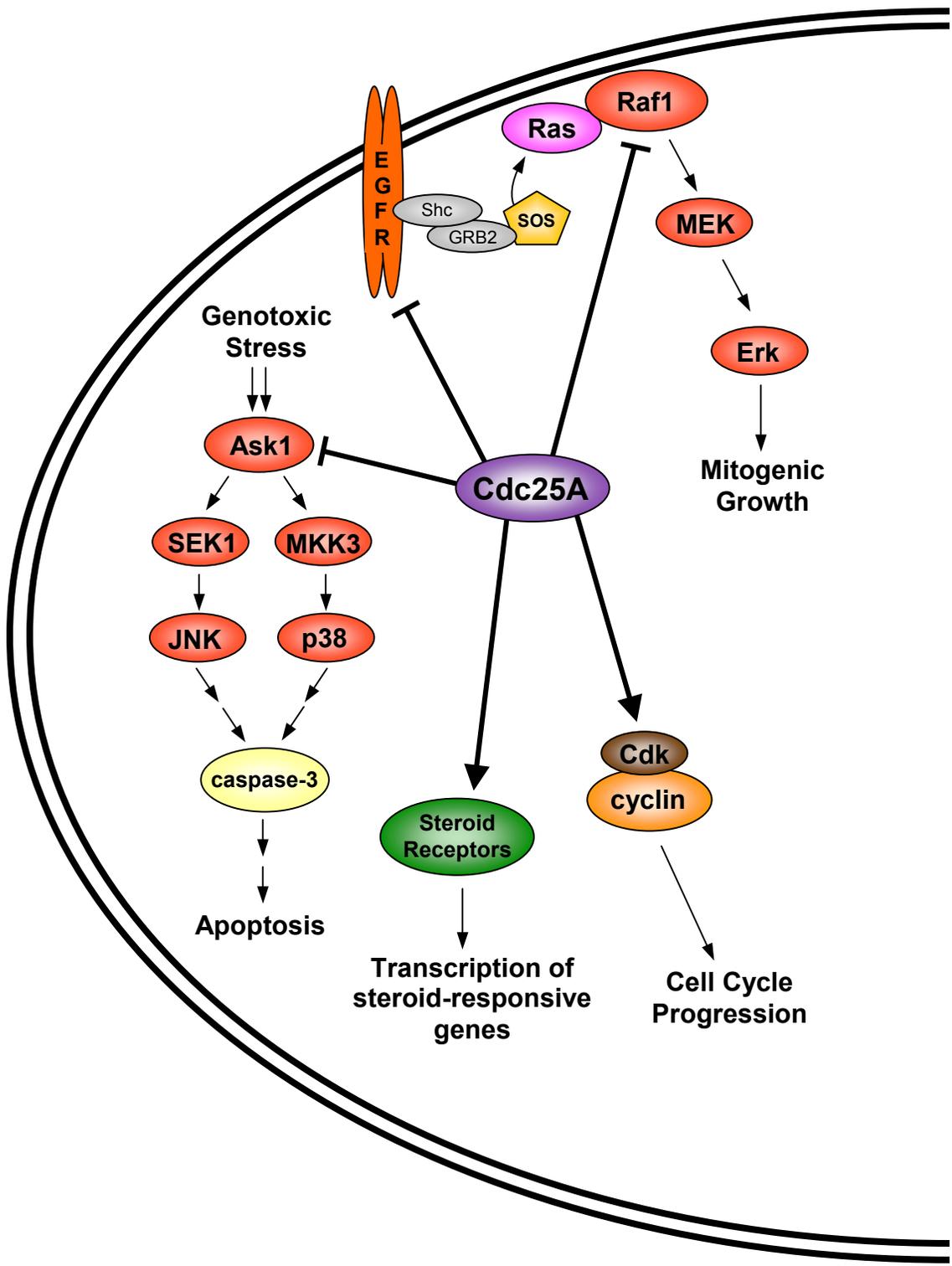


Figure 1.8: Cdc25A Regulates Multiple Signaling Pathways.

Cdc25A plays an important role in multiple signal transduction pathways. Cdc25A downregulates mitogenic signaling by dephosphorylating Raf1 and the EGFR, promotes cell cycle progression by activating multiple Cdk complexes, upregulates hormone-responsive gene expression by acting as a cofactor for steroid hormone receptors, and downregulates apoptotic stimuli by blocking Ask1 dimerization, which is required for Ask1 activation.

prolonged mitogenic stimulation can have deleterious effects on cells (101-104). Cdc25A can also serve as an epidermal growth factor receptor (EGFR) phosphatase. By dephosphorylating and inactivating the EGFR, Cdc25A further contributes to the regulatory feedback mechanism that downregulates mitogenic signaling following initiation of the cell cycle (Figure 1.8) (45). The redundant nature of feedback regulation between the cell cycle and mitogenesis emphasizes the importance of maintaining these two processes in balance. Moreover, Cdc25A is a component of cytokine-mediated mitogenic signaling pathways that involve Pim1. Pim1 is an oncogenic serine/threonine protein kinase whose expression is rapidly upregulated following cytokine stimulation and is required for cytokine-mediated cell proliferation (105). Cdc25A lies downstream of Pim1, and phosphorylation of Cdc25A by Pim1 results in increased Cdc25A phosphatase activity (99). Furthermore, proliferation in response to cytokines is impaired in *pim1*-deficient cells, implying that activation of Cdc25A by Pim1 is necessary for cytokine-mediated initiation of cell proliferation (106, 107). Therefore, Cdc25A activation is a culminating point for mitogenic signaling pathways that result in initiation of cell cycle progression.

Cell proliferation depends on timely and faithful gene expression in response to environmental stimuli. In addition to growth factor-mediated gene expression, cell proliferation can be promoted in response to steroid hormone stimulation. Steroid hormone stimulation results in sequence specific binding of steroid hormone receptors to palindromic response elements, recruitment of coactivators and transcription factors, and transcription of steroid hormone-responsive genes (108, 109). Cdc25A can function as a steroid receptor coactivator in a hormone-dependent manner, leading to increased expression of steroid hormone-responsive genes (Figure 1.8); Cdc25A can also enhance hormone-independent steroid receptor

transactivation, albeit modestly, presumably as a result of enhancing ligand-independent steroid receptor activation (110). Cdc25A is believed to affect steroid receptor activity by a mechanism similar to that of Cdc25B, which promotes steroid receptor transactivation through protein-protein interactions independent of Cdc25 phosphatase activity (110). By enhancing expression of proliferation-promoting genes through its ability to function as a steroid receptor coactivator, in addition to its ability to mediate activation of the cell cycle following mitogenic stimulation, Cdc25A bridges growth-inducing stimuli to the cell cycle.

Cdc25A also participates in the cellular response to stress. Cells have complex signaling mechanisms that trigger apoptosis in response to DNA damage, environmental stresses and changes in reduction-oxidation (redox) potential, which involve signaling cascades culminating in stress-responsive gene activation (111, 112). Apoptosis signal-regulating kinase 1 (Ask1) is an upstream kinase in the signal transduction cascade activated by multiple stress-induced signals that culminates in activation of the stress-responsive MAPKs, p38 and SAPK/JNK, resulting in execution of cellular apoptosis (113, 114). Critical to the activation of Ask1 is its ability to homo-oligomerize; inhibition of Ask1 homo-oligomerization is a mechanism by which cells avoid unscheduled apoptotic signaling (115-119). Cdc25A promotes cell survival by inhibiting Ask1 activity and activation of signaling pathways downstream of Ask1 that culminate in initiation of the apoptotic program (Figure 1.8) (120). Interestingly, Cdc25A-mediated inhibition of Ask1 is independent of its phosphatase activity; the carboxy terminal domain of Cdc25A, whether proficient or deficient in phosphatase activity, as a result of mutation of the catalytic cysteine, interacted with the carboxy terminal domain of Ask1 and prevents Ask1 homo-oligomerization (120). Furthermore, this interaction in cells suppressed apoptosis following oxidative stress, validating this role for Cdc25A in cell survival (120). Therefore,

Cdc25A not only plays a critical role in promoting cell cycle progression but also provides a link between external growth stimuli and cell cycle activation and contributes to cell survival. The multitude of biological processes involving Cdc25A require that it is precisely regulated and suggest that loss of this regulation can result in aberrant cell cycle progression in the absence of external stimuli and inappropriate cell survival, hallmarks of malignant transformation.

1.4.5. ROLE IN MALIGNANCY

Deregulation of cell cycle control is an essential alteration to cell biology typical of malignant transformation (2). Accelerated cell cycle progression in tumor cells can be promoted through multiple mechanisms, including loss of tumor suppressor genes, such as the Retinoblastoma gene (Rb) and p53, whose physiological function is to regulate cell cycle progression, or through amplification or gain-of-function mutations of proto-oncogenes, whose normal physiological role is to promote and relay growth signals from the cell surface to the nucleus. It is therefore not surprising that deregulated expression of Cdc25A can promote malignant transformation. Indeed, Cdc25A has been reported to have oncogenic properties, transforming normal mouse embryonic fibroblasts in cooperation with an activated isoform of Ras (Ha-Ras^{G12V}) or in an Rb^{-/-} background (121). Overexpression of Cdc25A has been documented in numerous human cancers, including breast cancer, colorectal carcinoma, esophageal squamous cell carcinoma, gastric carcinoma, hepatocellular carcinoma, ovarian cancer, squamous cell carcinoma of the head and neck, non-small cell lung cancer, non-Hodgkin's lymphoma and thyroid neoplasms (121-136). Cdc25A overexpression in tumors is most likely due to the deregulation of multiple processes, as increased Cdc25A expression has been documented in human cancers at both the mRNA and protein levels; the chromosomal

location of Cdc25A maps near an area frequently involved in karyotypic abnormalities in renal carcinomas, small cell lung carcinomas and benign salivary gland tumors (65, 137). Furthermore, Cdc25A overexpression has been associated with a poor prognosis and decreased survival in multiple tumor types.

Several hypotheses exist to explain the oncogenic potential of Cdc25A, based on its regulatory roles in the cell cycle and its involvement in multiple cellular processes. The proliferation of normal human cells is constrained by a phenomenon known as senescence, which limits the number of population doublings to a finite number (138). This constraint to replicative potential may serve as a tumor suppression mechanism; immortal cells that are immune to senescent arrest have an increased potential to accumulate genetic errors and progress to the malignant stage (139). Senescent human mammary epithelial cells are arrested in G1, in part due to downregulation of Cdc25A, which contributes to Cdk2/cyclin E inhibition (66, 140). Overexpression of Cdc25A in senescent mammary epithelial cells might enable escape from senescent arrest to an inappropriate proliferative state, contributing to malignant progression. This hypothesis is supported by the observation that elimination of Cdc25A expression by antisense oligonucleotides resulted in decreased Cdk2 activity and inhibition of S-phase progression in MCF-7 breast cancer cells (124). Cdc25A may also exert its oncogenic potential by affecting the MAPK signaling cascade (Figure 1.8) (45, 48, 98, 104). Cdc25A activation by Raf1 promotes cell cycle activation and Cdc25A quenches mitogenic signaling by dephosphorylating and inactivating Raf1 and EGFR. (45, 48, 97, 98). This may, in fact, serve as a common mechanism for multiple mitogenic signaling pathways that culminate in initiation of the cell cycle. Since constitutive activation of the MAPK cascade has been reported to have cytostatic and cytotoxic effects, overexpression of Cdc25A to downregulate MAPK activity may

be functionally significant in cells transformed by upstream components of the MAPK signal transduction cascade, enabling them to escape the lethal consequences of constitutive MAPK signaling (101-104). Therefore, deregulated Cdc25A expression accelerates cell cycle progression, contributing to malignant transformation, but may also protect transformed cells from the toxic effects of constitutive mitogenic stimulation by downregulation of MAPK signaling.

Cdc25A may also contribute to oncogenic transformation by decreasing cellular responsiveness to genotoxic stress via downregulation of the pro-apoptotic signaling kinase Ask1. Cdc25A inhibits Ask1 activation by a non-catalytic, protein-protein interaction mechanism that prevents Ask1 dimerization, a necessary event for Ask1 activation (Figure 1.8) (120). Therefore, tumor cells overexpressing Cdc25A may become refractory to apoptotic stimuli as a result of Cdc25A/Ask1 hetero-dimer formation competing with the formation of Ask1/Ask1 homo-dimers, leading to loss of Ask1 apoptotic signaling and the acquisition of a selective growth advantage (120). Overexpression of Cdc25A can also provide a growth advantage through resistance to cell cycle checkpoints, enabling cell cycle progression in the presence of compromised genetic material that would otherwise result in cell cycle arrest. Because Cdc25A is a central target of cell cycle checkpoints enacted in response to genetic insults (Figure 1.7), cells overexpressing Cdc25A will circumvent anti-growth signals in response to DNA damage, proceed with genomic replication under compromised conditions, and progress through the cell cycle with damaged or altered DNA. In fact, in cells engineered for inducible Cdc25A expression, overexpression of Cdc25A to levels that saturated the cellular capacity for proteolytic Cdc25A degradation resulted in loss of cell cycle checkpoint capacities (84, 87). These results support the hypothesis that cells overexpressing Cdc25A are more prone

to propagating genetic abnormalities and acquiring a growth advantage (84, 87). Because of its oncogenic potential and the multiple pathways by which Cdc25A can contribute to the malignant phenotype, cells possess multiple regulatory mechanisms to control homeostatic Cdc25A expression.

1.4.6. BIOCHEMICAL REGULATION OF CDC25A

Under normal physiological conditions, Cdc25A expression is subject to tight regulation at both the transcriptional and post-translational levels. Transcription of Cdc25A mRNA is regulated by E2F and c-Myc transcription factors and seems to be cell cycle dependent, with Cdc25A mRNA expression peaking in late G1 and S phase, consistent with its role in promoting the G1/S transition (22, 66, 141-144). Cdc25A mRNA expression can also be transactivated by the high-risk human papillomavirus (HPV) E7 oncoprotein (87, 144, 145). Furthermore, Cdc25A mRNA expression is downregulated by an E2F4/p130/histone deacetylase 1 (HDAC1)-dependent mechanism in response to transforming growth factor-beta (TGF- β), which contributes to inhibition of Cdk activity and cell cycle arrest (146, 147). In addition to transcriptionally-mediated regulatory mechanisms, Cdc25A protein levels are tightly controlled by post-translational phosphorylation, predominantly in the amino terminus (discussed in detail in Chapter 5; see Table 5.1 and Figure 5.1). The reported phosphorylation sites predominantly target Cdc25A for ubiquitin-mediated proteolysis through a complex and poorly understood mechanism. While Cdc25A is phosphorylated by Chk1, Chk2 and p38 MAPK in response to cell cycle checkpoints to target it for destruction by the proteasome, it is now known that rapid proteolytic Cdc25A degradation is not restricted to the cell cycle checkpoint responses (67, 68, 84-87). In fact, ubiquitin-mediated Cdc25A proteolysis appears to be the predominant

mechanism for maintaining physiologic Cdc25A protein levels, and Cdc25A is a highly labile protein throughout interphase (70). Only as cells approach and enter mitosis does Cdc25A stability increase, as Cdc25A is rate-limiting for the G2/M transition and mitotic progression; mitotic stabilization of Cdc25A has been attributed to phosphorylation by Cdk1/cyclin B, which seems to uncouple Cdc25A from its ubiquitin-proteasome degradation pathway (68). Cdc25A catalytic activity can also be modified by phosphorylation; while these phosphorylation sites are presumed to be in the amino terminal regulatory domain, they have not yet been identified. Cdc25A activity is upregulated following phosphorylation by Cdk2/cyclin E, Raf1 and Pim1 kinases (see section 1.4.4.) (66, 98, 99). These phosphorylation events enable the cell to control Cdc25A activity and promote cell cycle progression in response to appropriate mitogenic stimuli. The phosphorylation status of Cdc25A has also been reported to affect its association with 14-3-3 proteins. While 14-3-3 association with Cdc25B and Cdc25C enforces cytoplasmic sequestration in cell cycle checkpoints (Figure 1.6), 14-3-3 association with Cdc25A has not been reported to affect Cdc25A subcellular localization but to prevent Cdc25A association with cyclin B. Phosphorylation of Cdc25A by Chk1 promotes 14-3-3 binding to prevent premature activation of Cdk1/cyclin B and inappropriate mitotic division, thus preserving genomic integrity (69). 14-3-3 has also been hypothesized to facilitate Cdc25A interactions with Raf1, though the details of this interaction are unknown; formation of a Cdc25A/14-3-3/Raf1 complex may be important for facilitating Cdc25A activation by Raf1 to translate mitogenic signals to the cell cycle machinery (48, 97). Cdc25A activity can also be negatively regulated by p21. Cdc25A has a cyclin binding motif in its amino terminus that favors cyclin E and cyclin A binding; this motif is similar to the cyclin binding motif of p21. p21 inhibits Cdc25A activity by competing for a common binding site in the cyclin subunit of Cdk2/cyclin E or Cdk2/cyclin A, thus

preventing Cdc25A from dephosphorylating and activating Cdk2 (148). Because of its roles in multiple signaling pathways and the deleterious consequences of its deregulated expression, Cdc25A is subject to precise regulatory mechanisms to ensure the faithful execution of normal cell processes.

1.5. STATEMENT OF PROBLEM AND HYPOTHESIS

While Cdc25A is rapidly degraded by cell cycle checkpoints following genotoxic stress, Cdc25A is also a highly labile protein throughout interphase (70). Overexpression of Cdc25A has been documented in multiple human cancers. Deregulated expression of Cdc25A in malignant tumors could be attributed to a failure to downregulate Cdc25A in response to genotoxic stress, as several components of the cell cycle checkpoints targeting Cdc25A can be lost in tumors (149-151). Deregulated Cdc25A expression in tumors could also be attributed to loss of the normal regulatory mechanisms that maintain physiological Cdc25A protein levels. The following studies were undertaken to explore the mechanisms regulating physiologic Cdc25A protein levels in the absence of genetic insults; while Cdc25A has a short half-life in interphase in the absence of genotoxic stress, the mechanisms controlling its proteolysis under these conditions remain poorly understood. Cdc25B protein levels are downregulated by a mechanism involving one of its proximal downstream effectors, Cdk1/cyclin A (61), and regulatory feedback loops involving proximal downstream effectors are a common mode of regulation for the Cdc25 DSPases (28, 61, 62, 66, 152, 153). Therefore, the hypothesis underlying the objective of these studies is that a candidate for interphase regulation of Cdc25A protein levels in the absence of genetic insults is a proximal downstream effector of Cdc25A, namely, a cyclin-dependent kinase.

2. EXPERIMENTAL METHODS

2.1. MATERIALS

Roscovitine (2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine), olomucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) and cycloheximide were purchased from Calbiochem (La Jolla, California) (see Appendix A). Plasmids expressing dominant-negative (DN) mutants of Cdk1, Cdk2 and Cdk3 were generously provided by Dr. Sander van den Heuvel (154) and obtained from Dr. Richard Steinman (University of Pittsburgh). These mutants were generated by point mutation of Asp to Asn in the amino acid sequence KLADFGLAR (Asp¹⁴⁶ for Cdk1, Asp¹⁴⁵ for Cdk2, and Asp¹⁴⁵ in Cdk3). This Asp residue plays an essential role in the phosphotransfer reaction by chelating Mg²⁺ and orienting the Mg²⁺ATP phosphates in the catalytic site of the kinase (154). Plasmids encoding wild-type Cdc25A and the catalytically inactive C430S mutant of Cdc25A in pcDNA3 were provided by Dr. Thomas M. Roberts (Harvard Medical School) (48). A plasmid encoding GST-Cdc25C (200-256) in pGEX-2T was generously provided by Dr. Yves Pommier (National Cancer Institute) (155). *E. coli* strain BL21(DE3) was from Novagen (Madison, WI). Lipofectamine PLUS™ was from Invitrogen (Carlsbad, CA). Primary antibodies specific for Cdc25A (F6), Cdc2 p34 (17), Cdk2 (M2), Cdk3 (Y-20), p21 (C-19), p27 (C-19) Chk1 (G4), cyclin B1 (H-433), cyclin D1 (A-12) and vinculin (H-300) were from Santa Cruz (Santa Cruz, CA); Cdc25B and β -catenin antibodies were from BD Transduction Labs (Lexington, KY); p38 MAPK, phospho-p38 MAPK (Thr180/Thr182), Chk1 and phospho-Cdc25C (S216) antibodies were from Cell

Signaling Technology (Beverly, MA); β -tubulin antibodies were from Cedarlane Laboratories (Hornby, Ontario); and p21 WAF1 antibodies were from Oncogene Research Products (San Diego, CA). Antibody species and dilutions are provided in Appendix B. Peroxidase-conjugated goat-anti-mouse and goat-anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein A/G-PLUS agarose conjugates and normal mouse IgG were from Santa Cruz. Digoxigenin (DIG)-labeled RNA Molecular Weight Marker I, anti-DIG-AP Fab fragments, CDP-*Star* ultra-sensitive chemiluminescent substrate for AP, and RNase A were from Roche Applied Science (Indianapolis, IN). Isopropyl β -D-1-thiogalactopyranoside (IPTG), glutathione and propidium iodide were purchased from Sigma (St. Louis, MO). 10x Kinase Assay Buffer and 10 mM ATP were from Cell Signaling Technology (Beverly, MA). Glutathione-sepharose 4B was from Amersham Pharmacia Biotech (Piscataway, NJ).

2.2. CELL CULTURE

HeLa human cervical carcinoma cells, MCF-7 human mammary adenocarcinoma cells, and NIH 3T3 murine embryonic fibroblasts (American Tissue Culture Collection, Manassas, VA) were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO₂ at 37°C. Wild type, p53^{-/-} and p21^{-/-} HCT-116 human colorectal carcinoma cells, generously provided by Dr. Bert Vogelstein (Johns Hopkins University) and obtained from Dr. Lin Zhang (University of Pittsburgh), were maintained in McCoy's 5A with L-glutamine supplemented with 10% fetal

bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO₂ at 37°C.

2.3. TRANSIENT TRANSFECTION OF HUMAN TUMOR CELLS

HeLa, HCT-116, and NIH 3T3 cells were transfected at 50-60% confluence using Lipofectamine PLUS™ according to the manufacturer's instructions. Maximum transfection efficiency was achieved in the presence of serum for HeLa and NIH 3T3 cells and in serum-free conditions for HCT-116 cells. Unless otherwise indicated, cells were processed for experiments 48 hr following removal of DNA:lipid complexes and addition of complete medium.

2.4. WESTERN BLOTTING

Cells were harvested and lysed in a HEPES lysis buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM MgCl₂, 25 mM NaF, 1 mM EGTA, pH 8.0, 10 mM NaCl, 2 mM Na₃VO₄, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 100 µg/ml 4-(2-aminoethyl)benzenesulfonylfluoride, and 6.4 mg/ml Sigma 104 phosphatase substrate), incubated on ice for 30 min, and centrifuged at 13,000 x g to clear the lysates. Protein content was determined by the Bradford method. Total cell lysates (20 to 50 µg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated in blocking solution for at least 1 hr and then probed with primary antibodies overnight. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Renaissance, NEN, Boston, MA). To reprobe membranes with different primary antibodies, a conventional

membrane stripping protocol was followed. Membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol) for 30 min at 50°C, washed multiple times, re-incubated in blocking solution and probed with a different primary as described above. For experiments involving quantitation of protein expression levels, X-ray films were scanned on a Molecular Dynamics personal SI densitometer and analyzed using the ImageQuant software package (Version 4.1, Molecular Dynamics, Sunnyvale, CA).

2.5. RNA ISOLATION, NORTHERN BLOTTING AND RT-PCR

Total RNA was isolated from cells using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically using a DU640 Spectrophotometer (Beckman Instruments, Fullerton, CA).

Northern blotting was performed using NorthernMax™ system (Ambion, Austin, TX) according to the manufacturer's instructions. Briefly, 5 µg total RNA was separated on 1% denaturing agarose gel containing 2.2 M formaldehyde, transferred to Nytran® SuPerCharge membrane (Schleicher & Schuell BioScience, Keene, NH), UV crosslinked and processed for detection of mRNA. A 711 base, DIG-labeled, single strand anti-sense DNA probe was generated by asymmetric PCR amplification using the PCR DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Briefly, the template for probe synthesis was a 711 bp PCR product at the 3' end of human Cdc25A cDNA; this template was generated by conventional PCR methodology using the following primers:

5'–AAGAGGAGGAAGAGCATGTC–3' (Primer A)

5'–TCAGAGCTTCTTCAGACGAC–3' (Primer B)

The DIG-labeled probe was generated by asymmetric PCR from this template using primer B. β -actin mRNA was detected with a DIG-labeled β -actin RNA probe (Roche Applied Science, Indianapolis, IN). Overnight hybridization of probes to the immobilized RNA was carried out in ULTRAhyb™ Ultrasensitive Hybridization Buffer (Ambion, Austin, TX) and the membrane was processed for detection of the hybridized probes using the DIG Wash and Block Buffer Set (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Hybridized probes were detected using anti-DIG-AP Fab fragments; the hybridized probe/anti-DIG-AP complex was visualized on X-ray film (Kodak, Rochester, NY) after incubation of the membrane with CDP *Star*. Relative intensities of the hybridization signals were quantified as described above for western blotting (see section Experimental Methods 2.4).

RT-PCR analysis of gene expression was performed using the Advantage RT-for-PCR Kit according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA). cDNA synthesis was performed using 1 μ g total RNA and random hexamer primers. The resulting cDNA was used to amplify Cdc25A and Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) with the following primers:

Cdc25A (126):

5'-GAGGAGTCTCCACCTGGAAGTACA-3' (forward)

5'-GCCATTCAAAACAGATGCCATAA-3' (reverse)

G3PDH:

5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (forward)

5'-CATGTGGGCCATGAGGTCCACCAC-3' (reverse)

The PCR conditions for amplification consisted of 30 cycles of denaturing at 95°C for 30 sec, annealing at 56°C (Cdc25A) or 60°C (G3PDH) for 30 sec, and extension at 72°C for 1 min,

followed by a final incubation at 72°C for 10 min. PCR reaction conditions were in the linear range of amplification as determined using a standard curve. Amplified PCR products were resolved using 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

2.6. FLOW CYTOMETRY

HeLa cells were transfected with the DN Cdk2 mutant or an empty pcDNA3.1 vector as described in Experimental Methods 2.3. Cells were harvested by trypsin treatment, washed with ice cold phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol overnight at –20°C. The following day, fixed cells were washed twice in PBS and stained with 250 µg/ml RNase A and 50 µg/ml propidium iodide for 1 hr at room temperature. Flow cytometry analysis of DNA content in 20,000 cells per sample was conducted with a Becton Dickinson FACSCalibur (BD Pharmingen, San Diego, CA).

2.7. GENERATION OF RECOMBINANT GST-CDC25C (200-256)

GST-Cdc25C (200-256) in pGEX-2T was transformed into *E. coli* strain BL21(DE3) according to the manufacturer's instructions. Protein induction was achieved using IPTG and GST-Cdc25C (200-256) was purified using glutathione-sepharose 4B beads as described in Appendix C.

2.8. CHK1 KINASE ASSAY

HeLa cells or HCT-116 cells were transfected with dominant-negative Cdk2 mutant or an empty vector as described in Experimental Methods 2.3. Cells were harvested in lysis buffer

supplemented with 2 mM DTT and protein content determined as described in Experimental Methods 2.4. 500 µg whole cell lysates were precleared for 30 min with normal mouse IgG and protein A/G-PLUS agarose beads at 4°C on an orbital rocker platform. Chk1 was immunoprecipitated from precleared lysates using anti-Chk1 antibodies and protein A/G-PLUS agarose beads overnight at 4°C on an orbital rocker platform. Chk1 immunoprecipitates were washed twice in PBS supplemented with protease and phosphatase inhibitors and twice in 1x Kinase Assay Buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na₃VO₄, 10 mM MgCl₂) supplemented with protease inhibitors. Beads were then resuspended in 40 µl 1x Kinase Assay Buffer supplemented with 200 µM ATP and 2 µg GST-Cdc25C (200-256) and incubated at 30°C for 30 min. Reactions were terminated by addition of 4x SDS sample buffer followed by boiling. Samples were analyzed by 12% SDS-PAGE and immunoblotted for phospho-Cdc25C (S216) as a measure of Chk1 kinase activity.

2.9. DOMINANT-NEGATIVE CDK2 ADENOVIRUS

A recombinant adenovirus expressing the dominant-negative Cdk2 mutant (Ad.DN Cdk2) under control of the CMV promoter was generously provided by Dr. Michael D. Schneider (Baylor College of Medicine) (156). Amplification of the recombinant adenovirus to prepare high-titer stocks was performed by Jaculyn Duke (Department of Molecular Genetic & Biochemistry, University of Pittsburgh). Briefly, high-titer adenoviral stocks were prepared using HEK 293 cells. Adenoviruses were harvested from the media and from cell supernatants by freeze-thawing the cell pellet. Adenoviruses were purified by multiple rounds of cesium chloride density gradient centrifugation and viral titer was determined by absorbance at 260 nm.

HCT-116 cells were infected with adenoviruses at indicated MOI for 1 hr at 37°C in a minimal volume of phosphate-buffered saline (PBS), pH 7.4. Following the 1 hr incubation with virus, complete medium was added to the plates. Infected cells were incubated at 37°C for 48 hr and harvested as described in Experimental Methods 2.4.

3. REGULATION OF CDC25A PROTEIN LEVELS IN HUMAN TUMOR CELLS

3.1. INTRODUCTION

By dephosphorylating and activating the Cdks, Cdc25A promotes the G1/S cell cycle transition and S phase progression, and plays a non-redundant role in the G2/M transition (22, 66-68).

Cdc25A overexpression transformed normal mouse embryonic fibroblasts in cooperation with an activated isoform of Ras (Ha-Ras^{G12V}) or in an Rb^{-/-} background (121) and Cdc25A overexpression has been documented in numerous human cancers (42); Cdc25A regulatory mechanisms are, therefore, of considerable interest. Cdc25A mRNA expression is regulated in a cell cycle-dependent manner (22, 66, 141-144). Cdc25A protein levels are tightly regulated by proteasome-mediated degradation pathways that may involve multiple ubiquitin ligases (67, 70, 83, 84, 87). While Cdc25A protein stability is negatively regulated in a cell cycle checkpoint-dependent manner by poly-ubiquitination and subsequent proteasome-mediated degradation (67, 87), Cdc25A is also rapidly turned over during interphase, but the mechanisms regulating Cdc25A protein stability in the absence of genetic insults remain unclear (67, 84, 87).

Due to the highly labile nature of Cdc25A protein, candidates for interphase regulation of Cdc25A protein levels in the absence of genetic insults would be proximal downstream effectors. By analogy, the stability of Cdc25B is decreased following phosphorylation by one of its proximal downstream effectors, Cdk1/cyclin A (61). To test the hypothesis that Cdk activity contributes to Cdc25A instability in interphase, Cdc25A protein levels were measured in human

tumor cells following inhibition of Cdk activity using complementary pharmacologic and genetic approaches.

3.2. RESULTS

A common regulatory mechanism for the Cdc25 DSPases involves feedback loops that integrate the activity of proximal downstream effectors to regulate Cdc25 activity; for example, Cdc25B protein levels are downregulated by a mechanism involving Cdk1/cyclin A, a proximal downstream effector of Cdc25B activity (61, 62). Phosphorylation of Cdc25B by Cdk1/cyclin A is a critical step in proteasome-mediated Cdc25B degradation, and Cdc25-Cdk feedback loops appear to be a common regulatory feature of cell cycle biology (28, 61, 62, 66, 152, 153). A similar regulatory mechanism may therefore be responsible for controlling Cdc25A protein levels. In support of this hypothesis are studies indicating higher expression levels of a catalytically inactive Cdc25A mutant (Cdc25A C430S) compared to the wild type Cdc25A protein (Figure 3.1). This suggests that the catalytic activity of Cdc25A may contribute to its protein expression levels by affecting the activity of downstream targets and lends credence to the hypothesis that Cdk activity, as a downstream effector of Cdc25A activity, may function in a feedback loop contributing to regulation of Cdc25A protein levels.

3.2.1. INHIBITION OF CDK ACTIVITY INCREASES CDC25A PROTEIN LEVELS

To probe the role of Cdk activity in regulating Cdc25A protein levels, asynchronous HeLa human cervical carcinoma cells were treated with the Cdk inhibitor roscovitine.

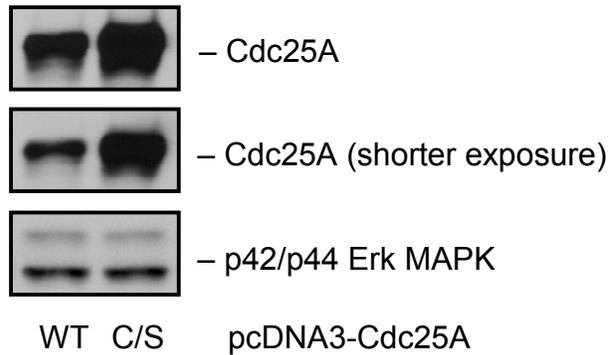


Figure 3.1: Cdc25A Catalytic Activity Contributes to Cdc25A Expression.

Representative results from NIH 3T3 cells transfected with pcDNA3-Cdc25A wild type (WT) or pcDNA3-Cdc25A catalytically inactive C430S mutant (C/S). Cells were harvested 24 hr after transfection and Cdc25A and total p42/p44 Erk MAPK levels (loading control) were examined by western blot. Catalytically inactive Cdc25A protein is expressed at higher levels than catalytically competent Cdc25A, supporting the hypothesis that a downstream target of Cdc25A contributes to the regulation of its expression.

Roscovitine is a potent and selective Cdk inhibitor that inhibits Cdk1, Cdk2, Cdk3 and Cdk5 but does not significantly inhibit Cdk4 and Cdk6 (Appendix A). Roscovitine (10 μ M) treatment of HeLa cells resulted in a marked increase in Cdc25A protein levels at 24 hr (Figure 3.2). In agreement with previously published results (61), roscovitine treatment resulted in an increase in Cdc25B levels (Figure 3.2), presumably due to inhibition of Cdk1/cyclin A-mediated targeting of Cdc25B for proteasome-mediated degradation. Roscovitine treatment had no effect on Cdc25C protein levels (Figure 3.2), whose activity is predominantly regulated by cytoplasmic sequestration and inactivation (78, 81, 96). Cdc25A protein levels in HeLa cells increased in a concentration- and time-dependent manner following treatment with roscovitine (Figures 3.3.A and 3.4), suggesting that this increase was due to the specificity of roscovitine as a Cdk inhibitor; similar results were obtained when HeLa cells were treated with olomucine, a structurally distinct Cdk inhibitor with a similar selectivity profile but with reduced potency (Figure 3.3.C). Because Cdc25A protein levels were elevated rapidly, namely within one hour of roscovitine treatment (Figure 3.4), it seems unlikely that the increased Cdc25A protein levels were due to cell cycle perturbation.

3.2.2. CDK INHIBITION INCREASES CDC25A PROTEIN LEVELS INDEPENDENT OF p53 AND HPV STATUS

Regulation of Cdc25A protein levels by DNA damage checkpoints is a p53-independent phenomenon (87). Cdc25A expression is also affected by the high-risk human papillomavirus (HPV) E7 oncoprotein (87, 144, 145). To test whether the increase in Cdc25A protein levels in HPV-positive HeLa cells following Cdk inhibition was dependent on p53 or HPV status, MCF-7 human mammary adenocarcinoma cells, wild type for p53 and HPV-negative, were treated with

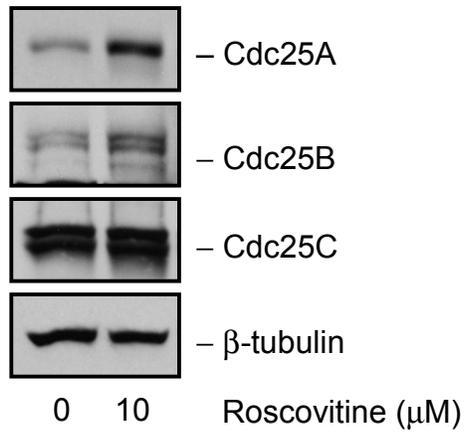
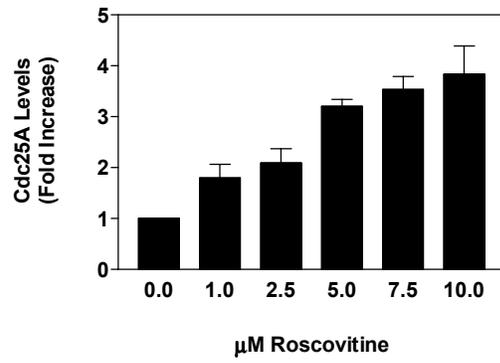
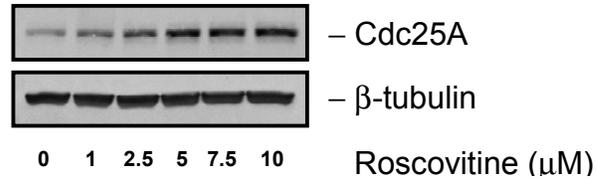


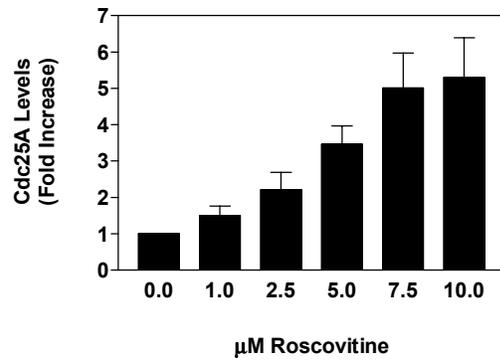
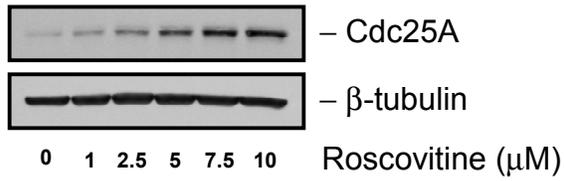
Figure 3.2: Roscovitine Treatment Increases Cdc25A Protein Levels.

HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μ M roscovitine. Cdc25A, Cdc25B, Cdc25C and β -tubulin levels (loading control) were examined by western blot. Inhibition of Cdk activity in HeLa cells resulted in increased Cdc25A and Cdc25B protein levels, while Cdc25C protein levels remain unchanged.

A.



B.



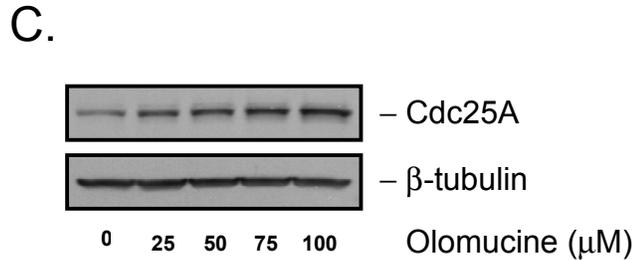


Figure 3.3: Cdk Inhibition Increases Cdc25A Protein Levels in a Concentration-Dependent Manner.

HeLa cells (Panels A & C) and cells MCF-7 (Panel B) were treated for 24 hr with vehicle control (DMSO) or increasing concentrations of roscovitine (Panels A & B) or olomucine (Panel C). Cdc25A and β -tubulin (loading control) levels were examined by western blot. Cdc25A levels are expressed as fold increase over vehicle control \pm S.E.M. (n = 3-5) (Panels A & B). Increasing concentrations of roscovitine significantly increased Cdc25A protein levels (ANOVA $p < 0.05$). Olomucine, a structurally distinct Cdk inhibitor with a similar selectivity profile but reduced potency, also increased Cdc25A protein levels in a concentration-dependent manner in HeLa cells (Panel C).

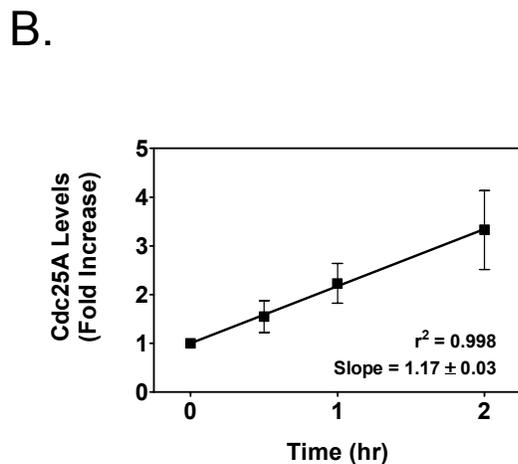
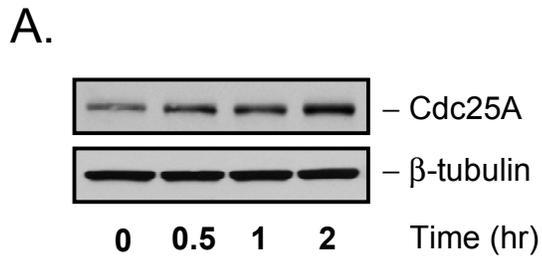


Figure 3.4: Roscovitine Treatment Increases Cdc25A Levels in a Time-Dependent Manner.

HeLa cells were treated with 10 μ M roscovitine for 0 to 2 hr. Cdc25A and β -tubulin (loading control) levels were examined by western blot (Panel A). Cdc25A levels from roscovitine treated cells are expressed as fold increase over control (0 hr) \pm S.E.M. (n = 5 to 7) (Panel B). Cdc25A protein levels significantly increased with increasing length of roscovitine treatment (ANOVA $p < 0.05$).

increasing concentrations of roscovitine for 24 hr. Similar to the results obtained in HeLa cells, Cdc25A protein levels in MCF-7 cells increased in a concentration-dependent manner following roscovitine treatment, indicating that increases in Cdc25A levels resulting from Cdk inhibition were independent of p53 activity or HPV status (Figure 3.3.B).

3.2.3. CDC25A PROTEIN LEVELS ARE INCREASED BY INHIBITION OF CDK2 KINASE ACTIVITY AND ARE NOT SECONDARY TO CELL CYCLE PERTUBATION

Because roscovitine is a broad-spectrum Cdk inhibitor, the relative contribution of roscovitine-sensitive Cdks to the regulation of Cdc25A protein levels was examined. HeLa cells were transfected with dominant-negative mutants of Cdk1, Cdk2 and Cdk3, as these are the predominant roscovitine-sensitive Cdks in HeLa cells. These dominant negative Cdk mutants are believed to inhibit endogenous Cdk kinase activity by competing for essential interacting molecules, including cyclins, creating specific loss-of-function phenotypes (157). The specific loss-of-function phenotype associated with a dominant-negative Cdk mutant could be complemented by overexpression of the corresponding wild type Cdk, revealing the specificity of Cdk inhibition (154). Only genetic inhibition of Cdk2 kinase activity resulted in increased Cdc25A protein levels, whereas genetic inhibition of Cdk1 or Cdk3 had no effect on Cdc25A levels (Figure 3.5). In addition, no significant alteration in the HeLa cell cycle profile was observed after transfection with the dominant-negative Cdk2 mutant (Figure 3.6), consistent with the recent reports that cancer cells can proliferate in the absence of Cdk2, that the Cdk2^{-/-} mouse is fully viable with no developmental abnormalities, and that Cdk2^{-/-} MEFs display no defects in mitotic cell cycle progression (158-160). Thus, the increase in Cdc25A protein levels after

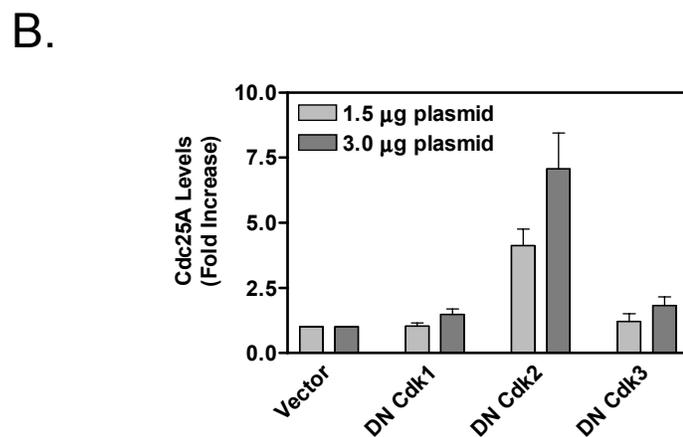
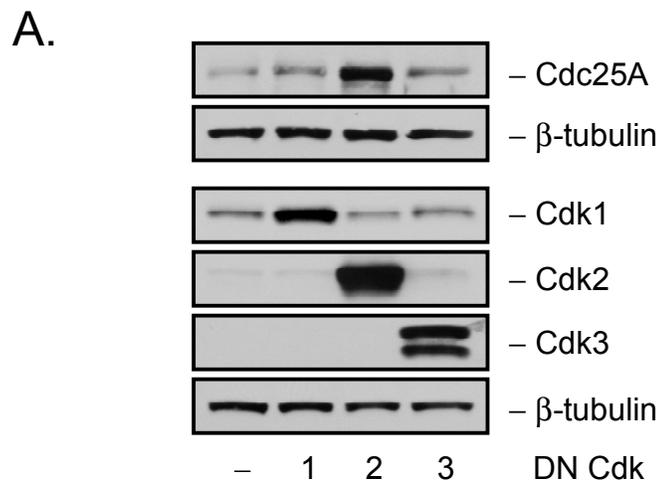


Figure 3.5: Dominant-Negative Cdk2 Mutant Expression Increases Cdc25A Levels in HeLa Cells.

HeLa cells were transfected with pcDNA3.1 (empty vector control) or vectors encoding dominant-negative (DN) Cdk1, DN Cdk2, or DN Cdk3 mutants. 48 hr after transfection, Cdc25A, Cdk1, Cdk2, Cdk3 and β -tubulin (loading control) levels were examined by western blot (Panel A). Cdc25A levels are expressed as fold increase over vector control \pm S.E.M (n = 3) (Panel B). DN Cdk2 expression significantly increases Cdc25A protein levels in HeLa cells (ANOVA $p < 0.05$).

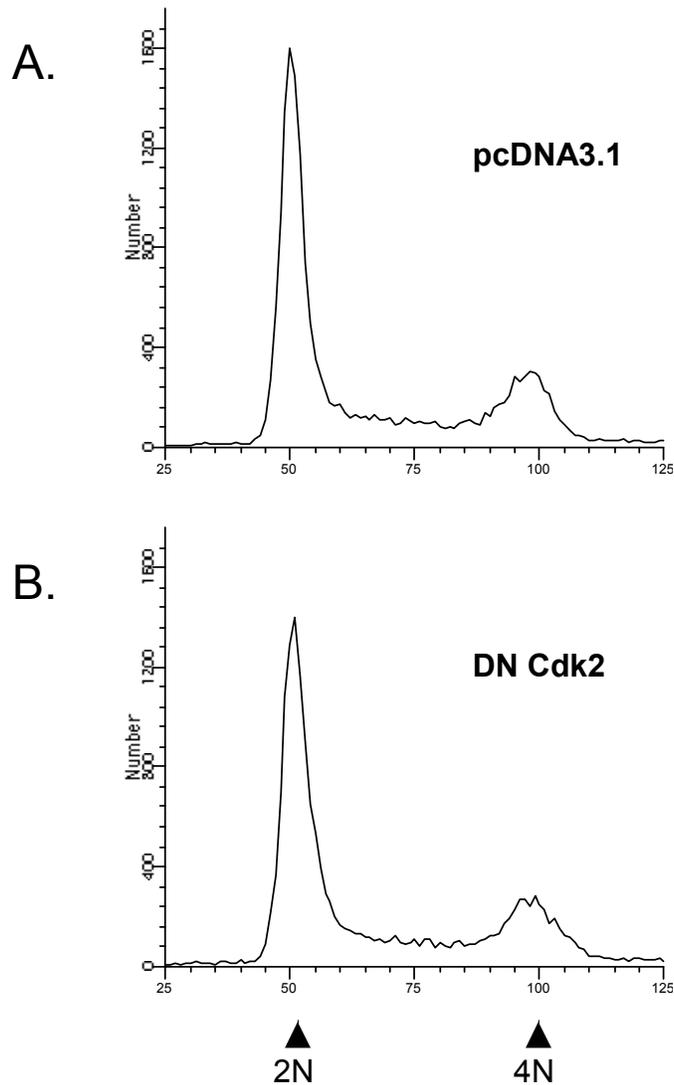


Figure 3.6: Dominant-Negative Cdk2 Mutant Expression does not Alter Cell Cycle Progression in HeLa Cells.

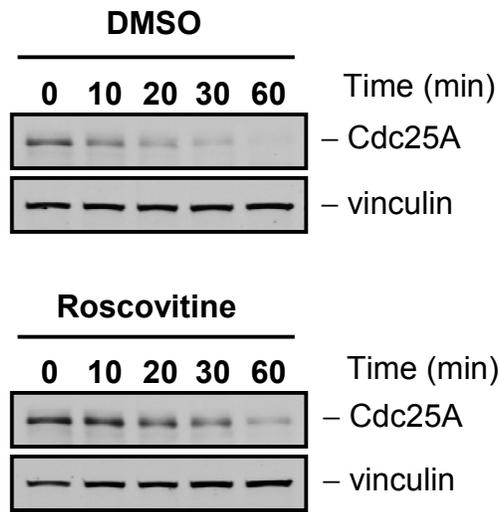
HeLa cells were transfected with either pcDNA3.1 (empty vector control) (Panel A) or DN Cdk2 mutant (Panel B). 48 hr after transfection, cells were fixed and stained with propidium iodide. DNA content was analyzed by flow cytometry to monitor cell cycle progression. Genetic inhibition of Cdk2 kinase activity did not result in cell cycle perturbation.

ectopic expression of the dominant-negative Cdk2 mutant was not secondary to cell cycle arrest. These results indicate that Cdk2 kinase activity plays an important role in regulating Cdc25A protein levels in asynchronous cells.

3.2.4. CDK2 INHIBITION INCREASES CDC25A PROTEIN HALF-LIFE

Since Cdc25A expression can be regulated at both the transcriptional and post-translational levels, the mechanism by which inhibition of Cdk2 kinase activity increases Cdc25A levels was investigated. In response to genetic insults or inhibition of DNA synthesis, Cdc25A is phosphorylated and targeted for rapid ubiquitin-mediated proteolytic degradation by the checkpoint kinases Chk1 and Chk2 (67, 84, 87). Furthermore, Cdc25A is a labile protein throughout interphase independent of genotoxic stress (70). Cdc25A expression can also be regulated at the transcriptional level by E2F and c-Myc transcription factors (142-144). Because Cdc25A has a short half-life in interphase and an increase in the half-life of a labile protein would result in a significant accumulation of that protein, the protein half-life of Cdc25A in asynchronous cells was explored following inhibition of Cdk2 kinase activity. Following a 24 hr treatment with roscovitine or DMSO (vehicle control), HeLa cells were treated with 10 $\mu\text{g/ml}$ cycloheximide for 0 to 60 min and Cdc25A levels were examined by western blotting. The basal half-life of Cdc25A was 6.26 ± 0.78 min, which is in agreement with previous reports (67, 70). Roscovitine-mediated inhibition of Cdk2 kinase activity doubled the half-life of Cdc25A (Figure 3.7); this could readily account for the observed time-dependent increase in Cdc25A protein levels. To confirm that the increased Cdc25A protein levels were not affected by a transcriptionally-mediated mechanism, Cdc25A mRNA levels were examined by northern blotting. Roscovitine treatment of HeLa cells did not significantly increase Cdc25A mRNA

A.



B.

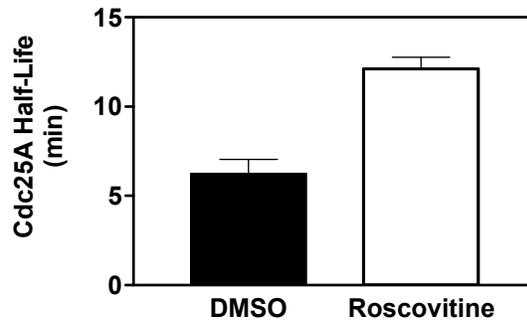
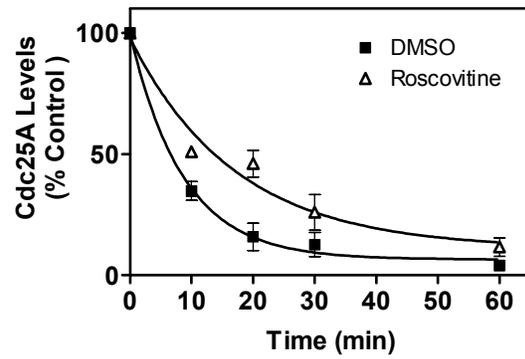


Figure 3.7: Inhibition of Cdk2 Activity Increases Cdc25A Half-Life in Asynchronous Cells.

HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μ M roscovitine followed by 10 μ g/ml cycloheximide (CHX) for 0-60 min. Cdc25A and vinculin (loading control) levels were examined by western blot (Panel A). Cdc25A levels are expressed as percent of control \pm S.E.M (n = 3) (Panel B). Roscovitine treatment significantly increased the half-life of Cdc25A (Student's t-test $p < 0.05$).

levels (Figure 3.8.A, upper two panels and quantified in 3.8.B), confirming that Cdk2 kinase activity affects Cdc25A protein levels by a post-transcriptional mechanism. These results were independently confirmed by RT-PCR (Figure 3.8.A, lower two panels).

3.3. DISCUSSION

Cdc25A biology is undergoing a paradigm shift, from a narrow role as the critical regulator of the G1/S checkpoint to a more broad responsibility in the cell cycle with an essential function in mitosis. Specifically, it is now known that Cdc25A levels peak for the G2/M transition and mitotic progression and that degradation of Cdc25A is necessary for the G2/M checkpoint in response to DNA damage (67, 68). The original models describing the modes of regulation for Cdc25A are now being refined to focus on protein stability as a key regulatory mechanism (67, 68, 70, 83, 84, 87). The relationship between Cdc25A and Cdk2 was originally thought to be that of an auto-amplification feedback loop where Cdc25A contributed to the activation of Cdk2 and Cdk2 contributed to the activation of Cdc25A to sufficiently amplify the activities of both proteins to enable progression through the G1/S cell cycle transition (66). The results presented herein indicate that Cdk2 kinase activity also contributes to the labile nature of Cdc25A in interphase, and this kinase activity may in fact be the same Cdk2 kinase activity originally reported to activate Cdc25A phosphatase activity. These results contribute to the understanding of this Cdc25A-Cdk feedback loop and support a mathematical model that suggests hyperphosphorylation of Cdc25A by Cdk2 may contribute to its degradation (66, 161). By directly linking Cdc25A stability to the activity of its substrates, physiologic levels of Cdc25A can be maintained in a tight feedback loop to prevent catastrophic deregulation of Cdc25A protein levels or activity. This relationship between increased activity and decreased

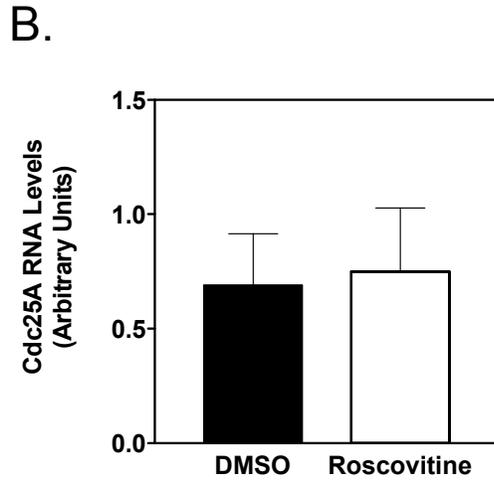
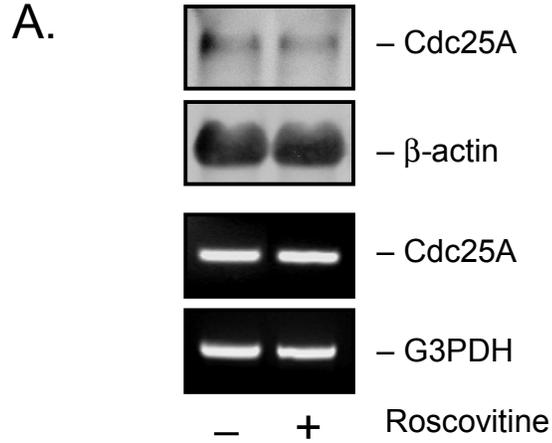


Figure 3.8: Roscovitine Treatment does not Increase Cdc25A mRNA Levels in HeLa Cells.

HeLa cells were treated for 24 hr with vehicle control (DMSO) or 10 μ M roscovitine. Total RNA was extracted as described in Experimental Methods 2.5. Cdc25A and β -actin levels were examined by northern blot (Panel A, upper two panels) and Cdc25A and G3PDH levels were examined by RT-PCR (Panel A, lower two panels). Cdc25A mRNA levels from northern blots (Panel A, upper two panels) are expressed as arbitrary units \pm S.E.M (n = 3) (Panel B). Inhibition of Cdk2 activity by roscovitine treatment does not significantly increase Cdc25A mRNA levels.

protein stability has been described for another protein phosphatase, PTEN, and may in fact serve as a common regulatory mechanism for many enzymes. PTEN phosphorylation maintains the phosphatase in a stabilized state with decreased catalytic activity; upon loss of phosphorylation in the C-terminal PTEN tail, catalytic activity is increased and protein stability is decreased (162). Although the results presented herein do not specifically detail the nature of the Cdk2/cyclin complex that contributes to the inherent instability of Cdc25A in interphase or the detailed molecular mechanism involved, there are several testable hypotheses. Cdc25A was recently reported to associate with elements of the SCF ubiquitin ligases and may be a target of the APC/C^{cdh1} and SCF ubiquitin ligases (70). However, it remains uncertain how Cdc25A might be targeted to these ubiquitin ligases. Other cell cycle regulatory proteins, notably p27 and cyclin E, are targeted by the SCF ubiquitin ligase for proteolytic degradation by a phosphorylation-dependent mechanism, while conversely, phosphorylation may not be necessary for p21 and cyclin D degradation mediated by SCF ubiquitin ligases (163, 164). It remains unclear whether phosphorylation of Cdc25A is a necessary event preceding ubiquitin ligase association, as Cdc25A phosphorylation can have both positive and negative effects on its ubiquitination and proteolytic degradation. Cdc25A phosphorylation promotes its degradation in response to genetic insults while, on the other hand, Cdc25A is rescued from proteolytic degradation in mitosis by Cdk1/cyclin B-mediated phosphorylation (68). The results presented herein support a role for Cdk2-mediated phosphorylation of either Cdc25A itself or a specific effector protein(s) necessary for the rapid degradation of Cdc25A. While Chk1 and Chk2, given their role in phosphorylating Cdc25A and targeting it for degradation in response to genotoxic stress, cannot be ruled out as possible downstream effectors of the Cdk2 kinase activity responsible for Cdc25A degradation, their role may be unique to proteasomal targeting of

Cdc25A following cellular stress and may not play a role in regulating Cdc25A levels in the absence of cellular stress. This would conform to the regulatory model for Cdc25C; Cdc25C is inactivated in the G2/M checkpoint by Chk1- and Chk2-dependent phosphorylation, 14-3-3 association and cytoplasmic sequestration (78-81, 93, 165, 166). Cdc25C, however, is maintained inactive and sequestered in the cytoplasm by 14-3-3 association during interphase in the absence of cellular stress as a result of phosphorylation by Cdc twenty-five C associated protein kinase (C-TAK1), a non-checkpoint kinase (167).

The results presented herein, together with the above mentioned studies, support the following model for DNA-damage-independent regulation of Cdc25A: Cdc25A levels are upregulated by transcription in G1 to a level that enables sufficient Cdk2/cyclin E activation to promote the G1/S transition. Cdc25A protein levels are then carefully maintained via Cdk2/cyclin E and Cdk2/cyclin A kinase activity through S phase and into late G2 phase. Only once Cdk1/cyclin B has been activated in advance of the G2/M transition are Cdc25A protein levels released from this strict regulatory loop, permitted to increase and reach their maximal levels, which are required to for catalyzing the G2/M transition and promoting mitotic progression (67, 68).

3.1 CONCLUSIONS

Inhibition of Cdk activity with small molecule Cdk inhibitors increases Cdc25A protein levels in a concentration- and time-dependent manner in human tumor cells. Cdc25A protein levels are increased by specific inhibition of Cdk2, as demonstrated using a dominant-negative Cdk2 mutant, whereas Cdk1 and Cdk3 are not involved. Cdk2 regulates Cdc25A protein levels

by a post-transcriptional mechanism; inhibition of Cdk2 kinase activity increases Cdc25A protein half-life but not Cdc25A mRNA levels.

4. MOLECULAR MECHANISM OF CDK2-MEDIATED REGULATION OF CDC25A PROTEIN HALF-LIFE

4.1. INTRODUCTION

Protein ubiquitination and proteasome-mediated degradation is an irreversible mechanism for regulating protein abundance (168). Cdc25A degradation by the ubiquitin-proteasome pathway is a central component of the rapid G1/S, S and G2/M cell cycle checkpoint responses to genotoxic stresses (67, 68, 84, 87, 89, 90). While Cdc25A was originally relegated to promoting the G1/S transition, it is now known that Cdc25A has dramatically increased stability in mitosis to catalyze Cdk1/cyclin B activation and mitotic progression, is rapidly degraded with kinetics similar to Cyclin B following mitotic exit and is, in fact, also a highly labile protein throughout interphase (22, 66, 68, 70). Two ubiquitin ligase complexes have been reported to ubiquitinate Cdc25A and target it for proteasome-mediated degradation; the APC/C (Anaphase Promoting Complex or Cyclosome) ubiquitin ligase complex regulates Cdc25A degradation following mitotic exit and the SCF (Skp1/Cullin or Cul1/F-box) ubiquitin ligase complex regulates Cdc25A turnover throughout interphase (70).

Protein ubiquitination is the result of a stepwise mechanism catalyzed by 3 protein complexes. It involves the catalytic activation of ubiquitin by the E1 ubiquitin-activating enzyme (E1), the transfer of activated ubiquitin to the E2 ubiquitin-conjugating enzyme (E2), and the conjugation of activated ubiquitin to the target protein by the E3 ubiquitin ligase (E3), which is a complex of several regulatory and scaffolding proteins (169). E3 ubiquitin ligases, which ultimately unite the target protein with the ubiquitin machinery, are responsible for target

specificity and are made up of two main classes, the HECT (Homologous to E6-AP C Terminus) ubiquitin ligases and the RING (Really Interesting New Gene) finger ubiquitin ligases, among which are the APC/C and the SCF ubiquitin ligases (169).

The APC/C ubiquitin ligases control mitotic cell cycle regulatory proteins, such as cyclin B, while the SCF ubiquitin ligases have a broader role in interphase cell cycle regulation and catalyze the ubiquitination of p27, cyclin D, p21 and cyclin E (164, 169-172). Substrate recognition by E3 ubiquitin ligases is facilitated by adaptor proteins, either Cdc20 or Cdh1 in the APC/C (173), or the F-box proteins in SCF, of which more than one hundred have been identified (174, 175). Throughout mitosis and upon mitotic exit, Cdc25A protein levels appeared to parallel those of cyclin B, a known APC/C substrate, suggesting that Cdc25A degradation upon mitotic exit could be catalyzed by a mechanism similar to the one regulating cyclin B expression (70, 176). In fact, overexpression of the APC/C adaptor protein Cdh1, but not Cdc20, decreased Cdc25A protein levels in transfected cells, confirming that Cdc25A, like cyclin B, could indeed be an APC/C substrate (70). Cdc25A contains 3 APC/C targeting motifs, one of which is required in APC/C targets to ensure specificity of substrate recognition (169). Cdc25A contains one putative D-box sequence, first identified in cyclin B as a destruction targeting motif, and two Cdc25A KEN-box motifs, identified by virtue of their role in targeting destruction of the APC/C adaptor Cdc20 (177, 178). One of the two KEN-box motifs appeared to be conserved throughout all human Cdc25s and Cdc25s of other species, and mutation of this KEN-box motif blocked Cdc25A degradation by APC/C^{Cdh1} following mitotic exit, confirming the role of APC/C^{Cdh1} in Cdc25A turnover (70). However, this KEN-box-mutated Cdc25A was still unstable in interphase, indicating that APC/C^{Cdh1}-independent ubiquitin ligases must be involved; the SCF E3 ubiquitin ligase is active in interphase and is responsible for regulating cell

cycle proteins, making it an attractive candidate. Indeed, inhibition of SCF ubiquitin ligase activity using a dominant-negative mutant of Cull1, a bridging structural protein component of the SCF ubiquitin ligase complex, resulted in increased Cdc25A protein levels (70). The same result was observed for p27, a known SCF substrate, confirming that expression of the dominant-negative Cull1 mutant inhibited SCF ubiquitin ligase activity, whereas the APC/C substrate cyclin B was unaffected by expression of the dominant-negative Cull1 mutant (70, 179). Cdc25A interacted with both Skp1 and Cull1, components of the SCF ubiquitin ligase complex, in cells, further substantiating Cdc25A as a SCF substrate (70). The F-box protein responsible for SCF ligase-mediated ubiquitination of Cdc25A during interphase, however, remains a mystery. Specificity of F-box-mediated target recognition is often governed by phosphorylation of the target proteins (169).

The destruction of Cdc25A following DNA damage is mediated by Chk1 and Chk2 protein kinases, which phosphorylate Cdc25A at S123 to facilitate its ubiquitin-mediated proteolysis (84, 87). While Chk1 and Chk2 activity was assumed to require activation by upstream DNA damage-activated kinases ATM (Ataxia-Telangiectasia-Mutated) and ATR (ATM- and Rad3-related), Chk1 possess basal kinase activity independent of DNA damage-associated stimuli (180-182). This led to the identification of Chk1 as a regulator of physiologic turnover of Cdc25A protein levels in the absence of DNA damage, in addition to its role in checkpoint-mediated Cdc25A degradation (183). Proteolytic turnover of Cdc25A involves three novel Chk1 phosphorylation sites (Ser 178, 278, and 292), in addition to Ser 123, both under physiological conditions and in response to cell cycle checkpoints; however, the precise function and relative contribution of each of these phosphorylation sites to Cdc25A degradation is not clear (183). Chk2, on the other hand, is DNA damage-dependent and does not appear to possess

significant basal kinase activity in the absence of upstream stimuli and, as such, is not involved in the physiological regulation of Cdc25A turnover (181-184).

The presence of multiple phosphorylation sites in the amino terminus of Cdc25A that cooperate in facilitating its turnover suggests a more complex regulatory mechanism for Cdc25A proteolysis than first believed. In fact, recent studies have identified another serine phosphorylation site, Ser 75, in the amino terminus of Cdc25A that contributes to its turnover in response to UV irradiation (85, 86). Ser 75 was phosphorylated by Chk1 following UV exposure, targeting Cdc25A for proteolytic degradation as previously described in response to UV irradiation (85-87). In addition, following osmotic shock, p38 MAPK was responsible for Ser 75 phosphorylation to promote Cdc25A degradation; whether p38 MAPK contributes to physiological Cdc25A turnover in the absence of osmotic stress, however, is not clear (85). That a kinase other than Chk1 or Chk2 can facilitate stress-induced turnover of Cdc25A introduces the possibility that non-checkpoint serine/threonine kinases can participate in the regulation of Cdc25A turnover. The individual contributions of each amino-terminal Cdc25A serine phosphorylation site and any novel, as of yet unidentified phosphorylation sites, remain to be elucidated; only once the individual roles of each phosphorylation site are understood will a cumulative model of the regulation of Cdc25A stability be feasible.

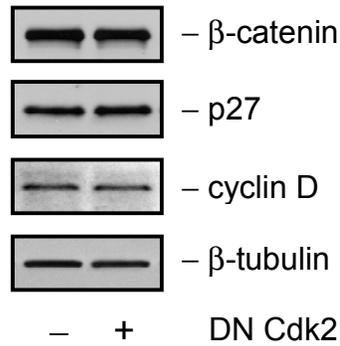
Therefore, in an effort to understand the molecular mechanism(s) by which Cdk2 contributes to the physiological turnover of Cdc25A, the effect of inhibiting Cdk2 kinase activity on the known mechanisms governing Cdc25A protein stability, namely SCF and APC/*Cdh1* ubiquitin ligase activities, Chk1 activity, and p38 activity, was examined.

4.2. RESULTS

4.2.1. INHIBITION OF CDK2 KINASE ACTIVITY DOES NOT INHIBIT SCF OR APC/C UBIQUITIN LIGASE ACTIVITIES

Based on the known association of Cdc25A with components of the SCF ubiquitin ligases *in vivo* (70), several substrates of SCF ubiquitin ligases were examined to determine whether inhibition of Cdk2 kinase activity by ectopic expression of the dominant-negative Cdk2 mutant downregulated SCF ubiquitin ligase activities. β -catenin, p27, and cyclin D (164, 179, 185) protein levels were not increased in cells transfected with the dominant-negative Cdk2 mutant, suggesting that the increased Cdc25A protein half-life due to Cdk2 inhibition was not due to downregulation of SCF ubiquitin ligase activities (Figure 4.1.A). While p27 has been identified as a Cdk2 substrate, with Cdk2-mediated p27 phosphorylation promoting p27 degradation by the ubiquitin-proteasome pathway, p27 turnover can also be catalyzed by Erk MAPKs (186-188). Furthermore, Erk MAPK-mediated p27 turnover was not impaired by Cdk2 inhibition (186), which is consistent with our finding that p27 turnover does not appear to be significantly perturbed by the dominant-negative Cdk2 mutant. Cdc25A has also been reported to be a substrate of the APC/C ubiquitin ligase complex during mitosis and in early G1 (70). To determine whether inhibition of Cdk2 kinase activity downregulated the activity of the APC/C ubiquitin ligase, cyclin B levels were examined in cells transfected with the dominant-negative Cdk2 mutant; cyclin B is a known substrate of the APC/C ubiquitin ligase (176). Cyclin B levels were not increased following inhibition of Cdk2 kinase activity, suggesting that the increase in Cdc25A protein half-life following Cdk2 inhibition was not due to perturbation of APC/C ubiquitin ligase activities (Figure 4.1.B).

A.



B.

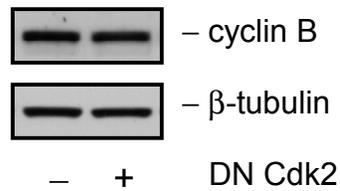


Figure 4.1: Effect of Cdk2 Inhibition on SCF and APC/C Ubiquitin Ligase Activities.

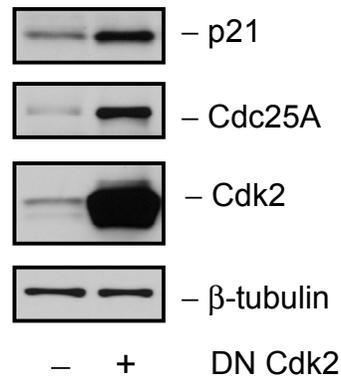
HeLa cells were transfected with pcDNA3.1 (empty vector control) or DN Cdk2. 48 hr after transfection, β -catenin, p27, cyclin D (Panel A), cyclin B (Panel B) and β -tubulin (loading control) levels were examined by western blot. DN Cdk2 expression in HCT-116 cells does not decrease SCF ubiquitin ligase activity (Panel A) or APC/C ubiquitin ligase activity (Panel B).

While SCF and APC/C ubiquitin ligase activities appear to be unaltered by inhibition of Cdk2 kinase activity, p21 protein levels were increased following inhibition of Cdk2 kinase activity with the dominant-negative Cdk2 mutant in both HeLa cells (Figure 4.2.A) and in HCT-116 colorectal carcinoma cells (Figure 4.2.B), which are p53 wild type. Increased p21 protein levels following Cdk2 inhibition was not an artifact of p21 sequestration by ectopic expression of the dominant-negative Cdk2 mutant protein, as p21 levels were not increased by overexpression of other Cdk mutant proteins (Figure 4.3.A). Moreover, p21 levels were increased following roscovitine-mediated inhibition of Cdk2 kinase activity, further supporting the hypothesis that p21 levels were specifically elevated as a consequence of inhibiting Cdk2 kinase activity (Figure 4.3.B).

4.2.2. p21 IS NOT INVOLVED IN CDK2-MEDIATED REGULATION OF CDC25A PROTEIN HALF-LIFE

p21 protein levels can be regulated by p53-dependent and p53-independent transcriptional mechanisms and by post-transcriptional mechanisms, including phosphorylation by p38 α MAPK, JNK1, and AKT/PKB and protein-protein interactions with PCNA, all of which enhance the half-life of p21 (189-192). p21 has been reported to associate with Cull1, a component of the SCF ubiquitin ligase machinery and has recently been confirmed as a substrate of the SCF ubiquitin ligase coupled to a specific F-box protein, Skp2 (164, 193). The relationship between p21 levels and Cdc25A is unclear, but p21 appears to have a negative effect on Cdc25A function. p21 and Cdc25A compete for Cdk2/cyclin E and Cdk2/cyclin A binding, creating a dynamic equilibrium between active and inactive Cdk2/cyclin complexes (148). In addition, Cdc25A levels were found to be elevated in p21^{-/-} mouse embryonic fibroblasts (MEF)

A.



B.

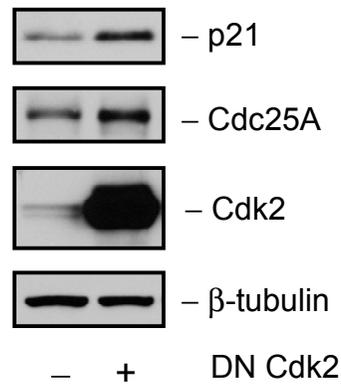
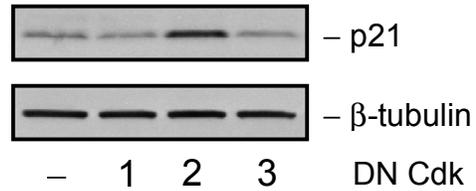


Figure 4.2: Cdk2 Inhibition Increases p21 Protein Levels.

HeLa cells (Panel A) and HCT-116 cells (Panel B) were transfected with pcDNA3.1 (empty vector control) or DN Cdk2. 48 hr after transfection, p21, Cdc25A, Cdk2, and β -tubulin (loading control) levels were examined by western blot. Inhibition of Cdk2 activity in HeLa cell and HCT-116 cells results in increased p21 protein levels.

A.



B.

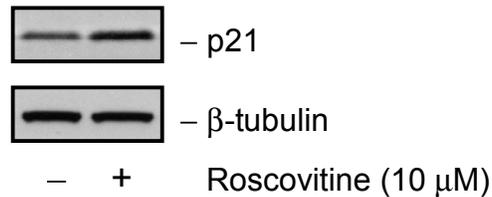


Figure 4.3: p21 Protein Levels are Specifically Increased by Inhibiting Cdk2 Activity.

HeLa cells were transfected with pcDNA3.1 (empty vector control) or vectors encoding DN Cdk1, DN Cdk2, or DN Cdk3 mutants (Panel A). 48 hr after transfections, p21 and β -tubulin (loading control) levels were examined by western blot. In a separate experiment, HeLa cells were treated with 10 μ M roscovitine or DMSO for 24 hrs (Panel B). p21 and β -tubulin (loading control) levels were examined by western blot. Increased p21 protein levels are specifically due to inhibition of Cdk2 kinase activity and not due to non-specific p21 sequestration by overexpression of dominant-negative Cdk proteins.

due to increased Cdc25A mRNA levels; the mechanism by which targeted deletion of p21 upregulates Cdc25A mRNA levels is unknown (194). The unexpected increase in p21 protein levels following inhibition of Cdk2 kinase activity prompted an investigation into whether p21 was involved in or necessary for Cdk2-dependent regulation of Cdc25A protein half-life. To that end, wild type, p53^{-/-}, or p21^{-/-} isogenic HCT-116 cells were transfected with the dominant-negative Cdk2 mutant and examined for effects on Cdc25A protein levels. Inhibition of Cdk2 kinase activity by overexpression of the dominant-negative Cdk2 mutant resulted in increased Cdc25A protein levels in both the wild type (Figure 4.4, left-most panel) and the p21^{-/-} HCT-116 (Figure 4.4, right-most panel) cells, indicating that p21 is not necessary for Cdk2-mediated regulation of Cdc25A protein half-life. In addition, Cdc25A protein levels were increased in the p53^{-/-} HCT-116 cells following overexpression of the dominant-negative Cdk2 mutant (Figure 4.4, center panel), confirming previous results that Cdk2-mediated regulation of Cdc25A protein levels is a p53-independent event (Results 3.2.2) (87).

4.2.3. CHK1 ACTIVITY IS NOT RESPONSIBLE FOR CDK2-MEDIATED REGULATION OF CDC25A PROTEIN HALF-LIFE

Because Chk1 was recently identified as a physiological regulator of Cdc25A stability in the absence of genotoxic stress (183), Chk1 protein levels and Chk1 kinase activity were examined following inhibition of Cdk2 kinase activity in HeLa cells using the dominant-negative Cdk2 mutant. Inhibition of Cdk2 kinase activity did not decrease Chk1 protein levels or Chk1 kinase activity, as measured by *in vitro* phosphorylation of GST-Cdc25C (200-256), a surrogate Chk1 substrate (Figure 4.5.A), indicating that Cdk2 regulates Cdc25A protein half-life through a Chk1-independent pathway. Similar results were obtained in HCT-116 cells (p53 wild type and

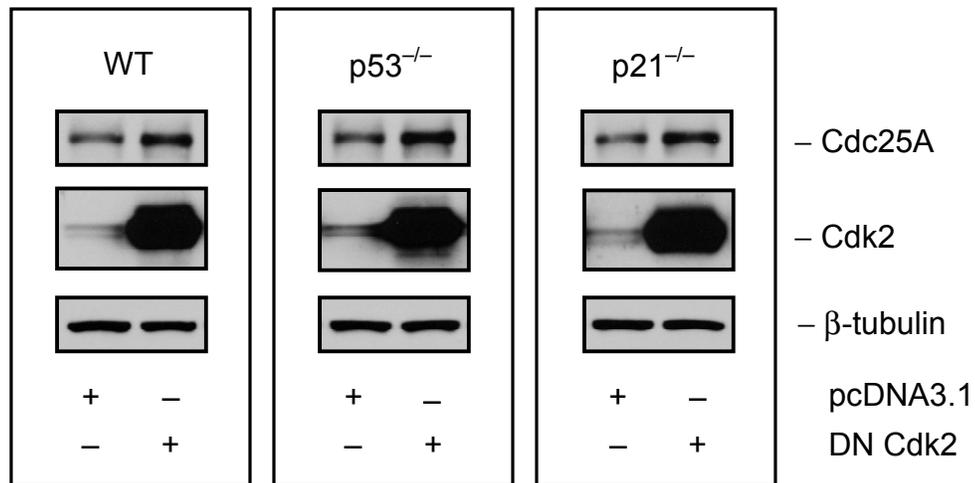
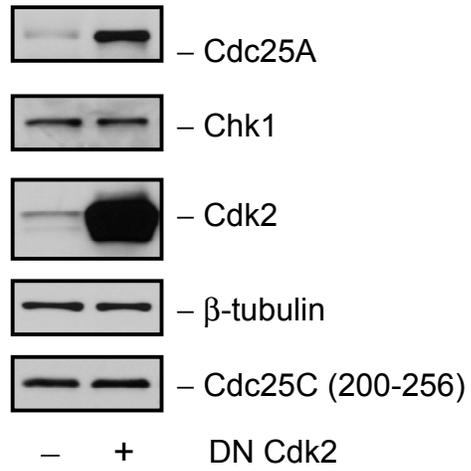


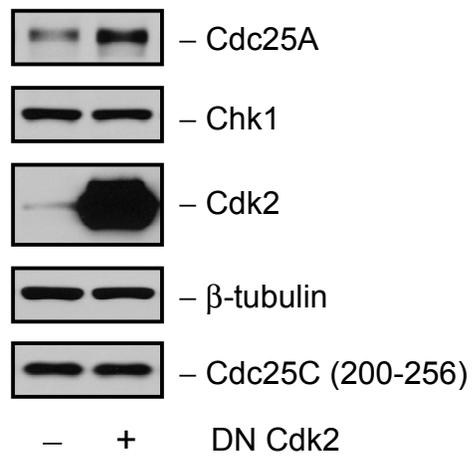
Figure 4.4: p21 is not necessary for Cdk2-mediated Regulation of Cdc25A Protein Half-Life.

Wild type (left-most panel), p53^{-/-} (center panel), or p21^{-/-} (right-most panel) isogenic HCT-116 cells were transfected with pcDNA3.1 (empty vector control) or DN Cdk2 mutant. 48 hr after transfection, Cdc25A, Cdk2, and β-tubulin (loading control) levels were examined by western blot. Inhibition of Cdk2 activity increased Cdc25A protein levels in all three isogenic HCT-116 cell lines, indicating that p21 is not necessary for Cdk2-mediated regulation of Cdc25A protein half-life.

A.



B.



C.

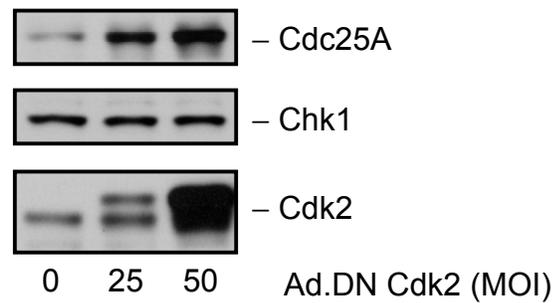


Figure 4.5: Cdk2 Inhibition does not Downregulate Chk1.

HeLa cells (Panel A) and HCT-116 cells (Panels B) were transfected with pcDNA3.1 (empty vector control) or DN Cdk2. 48 hr after transfection, Cdc25A, Chk1, Cdk2, and β -tubulin (loading control) levels were examined by western blot and Chk1 kinase activity was determined by immunoprecipitation of Chk1 followed by an *in vitro* kinase assay (Experimental Procedures 2.8). Inhibition of Cdk2 kinase activity in HeLa cells and HCT-116 cells does not decrease Chk1 kinase activity or Chk1 protein levels.

HCT-116 cells were infected with an adenovirus expressing the DN Cdk2 mutant (Ad.DN Cdk2) at 0, 25, and 50 M.O.I. (Panel C). 48 hr after infection, Cdc25A, Chk1, and Cdk2 levels were examined by western blot. Inhibition of Cdk2 activity causes a concentration-dependent increase in Cdc25A protein levels without affecting Chk1 protein levels.

HPV-negative) transfected with the dominant-negative Cdk2 mutant (Figure 4.5.B). These results confirm that Cdk2 regulates Cdc25A protein half-life in a Chk1-independent manner. In addition, HCT-116 cells infected with an adenovirus expressing the dominant-negative Cdk2 mutant resulted in a concentration-dependent effect of Cdk2 inhibition on Cdc25A protein levels that was independent of effects on Chk1 (Figure 4.5.C). This confirms previous results showing a concentration-dependent effect of small molecule Cdk inhibitors (Figure 3.3.B) and of dominant-negative Cdk2 mutant expression on Cdc25A protein levels in transient transfections (Figure 3.5.B). Taken together, these results suggest that Cdk2 does not lie upstream of Chk1 in a signaling cascade that maintains the physiological regulation of Cdc25A protein half-life.

4.2.4. p38 MAPK IS NOT INVOLVED IN CDK2-MEDIATED REGULATION OF CDC25A PROTEIN HALF-LIFE

p38 MAPK activity has been reported to regulate Cdc25A protein stability following osmotic stress; p38 MAPK phosphorylated Cdc25A at Ser 75, promoting Cdc25A degradation (85). However, a role for p38 MAPK activity in regulating Cdc25A protein levels in the absence of osmotic stress has not been thoroughly investigated. It was therefore hypothesized that Cdk2 could regulate Cdc25A protein half-life by affecting p38 MAPK activity. To determine whether p38 MAPK activity was downregulated by Cdk2 inhibition, HCT-116 cells were transfected with the dominant-negative Cdk2 mutant and p38 MAPK phosphorylation at threonine 180 and threonine 182, a widely accepted surrogate for p38 MAPK activity, was analyzed. Inhibition of Cdk2 kinase activity did not downregulate p38 MAPK phosphorylation (Figure 4.6), suggesting that p38 MAPK activity is not involved in Cdk2-mediated regulation of Cdc25A protein half-life.

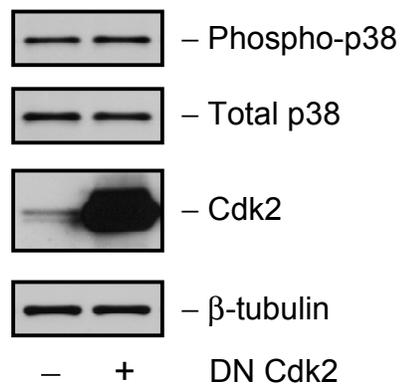


Figure 4.6: Cdk2 Inhibition does not Downregulate p38 MAPK.

HCT-116 cells were transfected with pcDNA3.1 (empty vector control) or DN Cdk2. 48 hr after transfection, phospho-p38 MAPK, total p38 MAPK, Cdk2, and β -tubulin (loading control) levels were examined by western blot. Inhibition of Cdk2 kinase activity by DN Cdk2 expression in HCT-116 cells does not decrease p38 MAPK phosphorylation or total p38 MAPK protein levels.

4.3. DISCUSSION

Conventional cell cycle dogma assigns Cdc25A the limited role of dephosphorylating and activating Cdk2/cyclin E at the G1/S transition and Cdk2/cyclin A during early S phase. Recent work has revealed that Cdc25A also plays a critical, non-redundant role in mitosis by contributing to the Cdk phosphatase activity that regulates Cdk1/cyclin B (68, 70, 89, 90). Cdc25A levels are subject to precise regulatory mechanisms that maintain strict control over Cdc25A protein levels through proteolytic degradation; while this mechanism is employed for cell cycle checkpoint-mediated downregulation of Cdc25A, it is now apparent that proteolytic degradation is also a major regulatory mechanism for Cdc25A expression in a cell cycle-dependent manner in the absence of genetic insults (67, 68, 70, 84, 87). Chk1, which contributes to cell cycle checkpoint-dependent Cdc25A proteolytic degradation, was identified as a physiologic regulator of Cdc25A turnover in interphase, and Cdc25A ubiquitination and degradation appears to be facilitated by APC/C^{Cdh1} in mitosis and early G1 and by SCF ubiquitin ligases in interphase (70, 183).

4.3.1. REGULATION OF CDC25A BY UBIQUITIN LIGASES

In accordance with the rapid turnover of Cdc25A in interphase catalyzed by the SCF ubiquitin ligase, it was hypothesized that decreased Cdc25A turnover following Cdk2 inhibition may be a result of downregulating SCF ubiquitin ligase activity, which targets multiple cell cycle regulatory proteins for proteolysis (70, 164, 169, 179, 193). Indeed, the studies confirming Cdc25A as an SCF substrate made use of a dominant-negative Cull1 mutant to inhibit SCF ubiquitin ligase activity, which resulted in increased p27 protein levels, an SCF substrate that

provided a convenient surrogate for monitoring SCF ubiquitin ligase activity (70, 179). p27 protein levels and protein levels of other SCF substrates, such as cyclin D and β -catenin, could therefore serve as useful endpoints for monitoring SCF activity to determine whether inhibiting Cdk2 kinase activity increased Cdc25A protein half-life by non-specific downregulation of SCF ubiquitin ligase activities. Inhibition of Cdk2 kinase activity with the dominant-negative Cdk2 mutant clearly did not affect SCF ubiquitin ligase activities because, while Cdc25A protein levels and half-life are elevated following Cdk2 inhibition, p27, cyclin D, and β -catenin, three well established SCF substrates, were unaffected.

Cdc25A turnover is subject to an alternate regulatory mechanism in mitotic exit and early G1, as proteolysis in this segment of the cell cycle is the domain of the APC/C ubiquitin ligases (169, 195). Cdc25A degradation upon mitotic exit was reminiscent of cyclin B degradation and Cdc25A contained a conserved KEN-box motif; this led to the identification of Cdc25A as a target of the APC/C^{Cdh1} ubiquitin ligase (70, 169, 176). To determine whether Cdk2 inhibition led to downregulation of APC/C^{Cdh1} ubiquitin ligase activity, we examined cyclin B levels in cells following inhibition of Cdk2 kinase activity with the dominant-negative Cdk2 mutant. Cdk2-mediated regulation of Cdc25A half-life did not involve decreasing APC/C^{Cdh1} ubiquitin ligase activity, as cyclin B levels were unaffected following Cdk2 inhibition. Collectively, inhibition of Cdk2 kinase activity did not downregulate general ubiquitin-proteolytic machinery, suggesting that an alternate mechanism was responsible for Cdk2-mediated regulation of Cdc25A protein half-life.

4.3.2. THE ROLE OF p21 IN CDK2-MEDIATED REGULATION OF PHYSIOLOGIC CDC25A TURNOVER

While Cdk2 inhibition did not alter SCF or APC/C^{Cdh1} ubiquitin ligase activities, p21 levels were increased in cells transfected with the DN Cdk2 mutant. Dominant-negative mutant proteins function by competing for essential substrates, thus inhibiting endogenous protein function (157). Because p21 can associate with multiple Cdks (1, 192), we compared p21 protein levels in cells transfected with dominant-negative Cdk1, dominant-negative Cdk2, or dominant-negative Cdk3 mutants to rule out elevation of p21 protein levels as an artifact of sequestration by ectopic expression of a dominant-negative Cdk mutant protein. p21 levels were not elevated following expression of DN Cdk1 or DN Cdk3 mutants, suggesting that the elevated p21 levels were not an artifact of sequestration by overabundance of Cdk protein; in addition, inhibition of Cdk2 catalytic activity with roscovitine also resulted in increased p21 protein levels, further supporting a direct link between Cdk2 catalytic activity and p21 protein levels. Because increases in p21 levels mirrored increases in Cdc25A levels following inhibition of Cdk2 kinase activity, we hypothesized that increased p21 levels may be a causative mechanism for Cdk2-mediated increases in Cdc25A half-life; moreover, all three cell lines (HeLa, MCF-7, HCT-116) in which Cdk2 inhibition increased Cdc25A protein levels have functional p21. To test the p21 hypothesis, we transfected isogenic HCT-116 cells, in which p21 or p53 gene expression was eliminated via *in vitro* targeted deletion, with the dominant-negative Cdk2 mutant. Cdc25A protein levels were elevated in all 3 isogenic cell lines following Cdk2 inhibition, indicating that p21 was not necessary for Cdk2-mediated regulation of Cdc25A turnover. While it is unclear how inhibition of Cdk2 kinase activity increased p21 protein levels, it may be a result of

upregulating p73 activity or by modulating regulatory mechanisms that dictate p53-independent p21 expression (described in Results 4.2.2) (192, 196).

4.3.3. A CDK2-CHK1-CDC25A CONNECTION?

One possibility is that Cdk2-mediated regulation of Cdc25A proceeds through conventional pathways regulating Cdc25A turnover in the absence of genotoxic stress. Chk1 was recently identified as a physiological regulator of Cdc25A protein turnover in the absence of stresses (183). While Chk1 activity was originally believed to be checkpoint-dependent, Chk1 activity accumulates in parallel with Chk1 protein levels even in the absence of DNA damage or stress and Chk1 possesses basal kinase activity (180). While no formal link has been established between Cdk2 and Chk1, the effect of inhibiting Cdk2 kinase activity on Chk1 kinase activity was explored to determine whether there was a functional connection. Inhibiting Cdk2 kinase activity had no effect on total Chk1 protein levels or Chk1 kinase activity in HeLa cells expressing the dominant-negative Cdk2 mutant; similar results were observed in HCT-116 cells. Furthermore, a concentration-dependent increase in Cdc25A protein levels in HCT-116 cells was observed with increasing expression of the dominant-negative Cdk2 mutant using an adenovirus expression system (Ad.DN Cdk2); this increase in Cdc25A was independent of changes in Chk1 protein levels, which remained unchanged throughout these experiments. These results suggest that Cdk2 does not regulate Chk1 expression or Chk1 activity and, therefore, Cdk2-mediated regulation of physiological Cdc25A turnover occurs independently of Chk1. At this point, the data reported herein cannot support or refute the hypothesis that Cdk2 directly phosphorylates Cdc25A to regulate its stability, in a mode akin to Chk1-mediated regulation of Cdc25A stability; Cdk2 has indeed been reported to phosphorylate Cdc25A to upregulate its phosphatase

activity at the G1/S transition (66) and this or additional phosphorylation events may contribute to Cdk2-mediated regulation of Cdc25A stability.

4.3.4. p38 MAPK-MEDIATED REGULATION OF CDC25A TURNOVER

UV-mediated degradation of Cdc25A was recently shown to involve an additional Chk1 phosphorylation site, Ser 75, which is analogous to a phosphorylation site in *Xenopus* Cdc25A required for the mid-blastula transition (85, 86, 88, 197). This serine phosphorylation seems to contribute to Chk1-mediated Cdc25A protein turnover. In addition, p38 MAPK can promote Cdc25A downregulation by phosphorylating Cdc25A at Ser 75 in response to osmotic stress (85). This is not entirely unexpected, as p38 MAPK has been reported to phosphorylate Chk1 phosphorylation sites in Cdc25B and Cdc25C *in vitro* and p38 MAPK negatively regulates Cdc25B *in vivo* in response to cell cycle checkpoints (91). Furthermore, several layers of redundant regulatory mechanisms are expected for Cdc25A expression, as loss of Cdc25 regulation can have deleterious effects on cell biology (67, 68, 84, 87). Because Chk1 contributes to the physiologic regulation of Cdc25A and since p38 MAPK has been shown to be able to phosphorylate Chk1 phosphorylation sites, it was hypothesized that p38 MAPK may contribute to the physiologic regulation of Cdc25A turnover mediated by Cdk2, even though no evidence exists to suggest a functional link between the two. Such a model would predict that for Cdc25A stability to be increased, p38 MAPK activity should be decreased following Cdk2 inhibition. However, expression of the dominant-negative Cdk2 mutant had no effect on p38 MAPK phosphorylation, a common surrogate for MAPK activity, revealing that Cdk2 does not affect physiologic Cdc25A turnover by downregulating p38 MAPK activity.

4.3.5. β -TrCP IS THE F-BOX PROTEIN THAT REGULATES CDC25A UBIQUITINATION AND DEGRADATION BY THE SCF UBIQUITIN LIGASE

Ubiquitin-mediated proteolysis catalyzed by SCF ubiquitin ligases requires substrate recognition and binding, which is facilitated by the F-box protein subunit of the SCF ubiquitin ligase complex; F-box proteins determine the substrate specificity for the SCF ubiquitin ligase machinery (169). Constitutive and DNA damage-induced turnover of Cdc25A protein was recently reported to be catalyzed by the SCF ^{β -TrCP} ubiquitin ligase, which is known to catalyze the turnover of β -catenin, I κ B and several other proteins (185, 198, 199). β -TrCP is an F-box protein that interacts with proteins containing a DSG phosphodegron, a short phospho-peptide motif containing the sequence DS*G ϕ X_nS*, where S* is a phosphoserine, ϕ is a hydrophobic amino acid, and X is any amino acid (where $n \geq 1$) (200-202). While recognition of Cdc25A by the SCF ^{β -TrCP} ubiquitin ligase involved Chk1-mediated phosphorylation of Cdc25A at Ser 75, Chk1 activity by itself was insufficient for β -TrCP binding and, in fact, a Cdc25A S75A mutant still weakly associated with β -TrCP *in vivo*, questioning the necessity of this phosphorylation site for β -TrCP binding (198). Ser 75 is not part of the DSG phosphodegron recognized by β -TrCP but somehow appears to facilitate β -TrCP binding to the proximal upstream phosphodegron, which includes phosphorylated Ser 82 and either phosphorylated Ser 79, phosphorylated Ser 88, or both (198, 199). Incidentally, neither Ser 79, Ser 82, nor Ser 88 are Chk1 phosphorylation sites, nor are they phosphorylated by casein kinase I α , initially hypothesized as a potential alternate candidate kinase for these sites (198). While Chk1 appears to be important for Cdc25A proteolytic degradation in the presence and absence of genotoxic stress, Chk1-mediated Cdc25A phosphorylation does not form the SCF ^{β -TrCP} phosphodegron and Chk1-mediated phosphorylation of Ser 75 is dispensable for *in vivo* association between Cdc25A and the SCF ^{β -}

TrCP, which questions the role of Chk1-mediated phosphorylation of Cdc25A at Ser 75, Ser 123, Ser 178, Ser 278, Ser 292 in Cdc25A protein turnover. It has been hypothesized that the multiple Chk1-catalyzed Cdc25A phosphorylation sites could serve to enhance the association between Cdc25A and the SCF ^{β -TrCP} ubiquitin ligase machinery to enable efficient ubiquitination in a mode akin to that involved in proteasome-mediated destruction of the SCF substrate Sic1, a yeast G1 Cdk inhibitor. Efficient recognition of Sic1 by SCF^{Cdc4} required phosphorylation of at least six sites to generate a functional Cdc4 phosphodegron (CPD); while alone, each phosphorylation site formed a low-affinity CDP, the combination of 6 low-affinity CPDs enabled Sic1 recognition by SCF^{Cdc4}, creating a biological threshold for fine tuning this irreversible regulatory switch (203, 204). While this hypothesis is appealing for fine tuning Cdc25A protein stability, multi-site phosphorylation may play a different role in regulating Cdc25A, as phosphorylation of Ser 82 and either Ser 79 or Ser 88 appear to be the only necessary phosphorylations required for interaction with SCF ^{β -TrCP} (198, 199). Cdc25A mutants lacking the 4 Chk1 phosphorylation sites (Ser 123, Ser 178, Ser 278, Ser 292) were observed to be consistently labeled with shorter ubiquitin conjugates, suggesting that Chk1-mediated Cdc25A phosphorylation might serve to affect the sites of ubiquitin conjugation or the efficiency of polyubiquitination, which could impact proteasome recruitment and subsequent degradation of Cdc25A (198, 199, 205). The identity of the kinase that phosphorylates the Cdc25A DSG motif serine residues, Ser 79, Ser 82 and Ser88, is unknown and it is not clear if one or more kinases are involved. At least one of the serine phosphorylation sites in the β -TrCP phosphodegron, serine 88, constitutes the minimal consensus Cdk phosphorylation site, S/T*-P (206). Inhibition of Cdk2 kinase activity, therefore, could interfere with formation of the β -TrCP phosphodegron in Cdc25A, resulting in decreased recognition of Cdc25A by SCF ^{β -TrCP}, decreased Cdc25A turnover and an increase in Cdc25A

protein half-life without directly downregulating SCF ^{β -TrCP} ubiquitin ligase activity; this hypothesis would be consistent with the observation that inhibition of Cdk2 kinase activity did not increase β -catenin protein levels, which confirmed that Cdk2 inhibition does not downregulate SCF ^{β -TrCP} ubiquitin ligase activity. While the data presented herein cannot rule out the involvement of additional factors in Cdk2-mediated regulation of Cdc25A turnover, the hypothesis that Cdk2 is involved in generating the β -TrCP phosphodegron in Cdc25A is indeed plausible. Evaluation of this hypothesis, using site-directed mutagenesis to create phosphorylation site-deficient Cdc25A mutants and phospho-DSG motif-specific antibodies to probe the role of the DSG motif serine phosphorylation sites in Cdk2-mediated regulation of Cdc25A protein turnover, and resolution of the Cdk2-specific phosphorylation sites in Cdc25A remain future directions for this project.

4.4. CONCLUSIONS

Inhibition of Cdk2 kinase activity with a dominant-negative Cdk2 mutant did not decrease SCF or APC/C ubiquitin ligase activities. Inhibition of Cdk2 kinase activity increased p21 protein levels in both HeLa and HCT-116 cells; p21, however, is not involved in Cdk2-mediated regulation of Cdc25A protein half-life, as inhibition of Cdk2 kinase activity in p21^{-/-} HCT-116 cells resulted in increased Cdc25A protein levels. Chk1, recently described as a physiologic regulator of Cdc25A protein stability, is not involved in Cdk2-mediated regulation of Cdc25A protein half-life. p38 MAPK, recently described as a regulator of Cdc25A stability in an osmotic stress checkpoint, does not play a role in the physiologic regulation of Cdc25A stability by Cdk2.

5. DISCUSSION

Genomic instability is a hallmark of cancer that stems in part from illegitimate cell cycle progression (134, 207). The cell cycle machinery is a common target of oncogenic mutations, and overexpression of the proto-oncogene Cdc25A has been documented in clinical studies of multiple human cancers (121-129, 131-134, 208). As Cdc25A mediates cell cycle progression and is a target of cell cycle checkpoints, overexpression of Cdc25A can promote inappropriate cell cycle progression in the presence of damaged DNA or premature cell division prior to faithful completion of DNA replication, both of which contribute to genomic instability (42, 82). Due to the labile nature of Cdc25A during normal cell cycle progression in the absence of genotoxic stress, one possible explanation for increased Cdc25A protein levels in human tumors is a defect in physiologic protein turnover mechanisms. In support of this hypothesis, it was recently shown that in a panel of breast cancer cell lines, Cdc25A levels were elevated not as a result of increased mRNA expression but as a result of enhanced protein stability; while some cell lines were defective in DNA damage checkpoints, suggesting defects in Chk1 signaling could be responsible for enhanced Cdc25A protein stability, others were proficient in degrading Cdc25A following DNA damage, suggesting normal Chk1 function and supporting the existence of Chk1-independent mechanisms for regulating physiologic Cdc25A protein turnover (134). In addition, a gain-of-function mutation in the *Caenorhabditis elegans* Cdc25 gene (*cdc-25.1*) resulted in deregulated hyperproliferation of intestinal cells, reminiscent of neoplastic behavior (209). A mutant *Caenorhabditis elegans* strain was identified by the presence of extra intestinal cells, and the responsible mutation was traced back to the *cdc-25.1* gene. Sequence analysis

revealed that this gain-of-function Cdc-25.1 mutant protein encoded a serine to phenylalanine mutation at amino acid 46; this residue falls in within a SRDSG motif (the second serine residue being affected by the mutation) in the protein's amino terminus and was hypothesized to be a site of negative regulation for the protein (209). Furthermore, intestinal hyperplasia could be generated in a normal *Caenorhabditis elegans* strain by introducing the mutant *cdc-25.1* (Ser 46 Phe) as a transgene, confirming its direct oncogenic potential (209). This SRDSG motif in Cdc-25.1 is reminiscent of a putative DSG ubiquitin ligase binding motif; loss of this motif could enable a gain-of-function mutation as a result of compromised protein turnover. By analogy, deregulation of Cdc25A protein turnover as a result of mutations to its DSG ubiquitin ligase binding motif could be sufficient to induce a hyperplastic growth state as a result of Cdc25A overexpression. These findings argue in favor of deregulated physiologic Cdc25A turnover as a potential mechanism contributing to Cdc25A overexpression in malignancies and emphasize the need to better understand physiologic Cdc25A turnover mechanisms.

To more fully understand the physiologic regulation of Cdc25A turnover, we explored the molecular mechanisms that regulate cell cycle checkpoint-independent regulation of Cdc25A protein levels.

5.1. REGULATION OF CDC25A BY PHOSPHORYLATION

Cdc25A phosphorylation is catalyzed by multiple kinases (Table 5.1 and Figure 5.1). Preliminary studies on Cdc25A determined that phosphorylation by several different kinases served to upregulate its catalytic activity following mitogenic stimuli to promote cell cycle progression (66, 98, 99). Cdc25A phosphorylation is now known to also play a key role in

Table 5.1: Reported Human Cdc25A Phosphorylation Sites.

Residue	Amino Acid	Kinase	Function
17	Serine	Cdk1/cyclin B	Mitotic stabilization
75	Serine	Chk1	Phosphorylation site involved in degradation Promotes recognition by SCF ^{β-TrCP}
75	Serine	p38 MAPK	Phosphorylation site involved in degradation Promotes recognition by SCF ^{β-TrCP}
79	Serine	???	Phosphodegron formation SCF ^{β-TrCP} binding site
82	Serine	???	Phosphodegron formation SCF ^{β-TrCP} binding site
88	Serine	???	Phosphodegron formation SCF ^{β-TrCP} binding site
115	Serine	Cdk1/cyclin B	Mitotic stabilization
123	Serine	Chk1, Chk2	Phosphorylation site involved in degradation
178	Serine	Chk1, Chk2	Phosphorylation site involved in degradation Promotes 14-3-3 binding Prevents Cdk1/cyclin B activation
278	Serine	Chk1	Phosphorylation site involved in degradation
292	Serine	Chk1, Chk2	Phosphorylation site involved in degradation
507	Threonine	Chk1	Promotes 14-3-3 binding Prevents Cdk1/cyclin B activation
???	???	Cdk2	Increases Cdc25A catalytic activity
???	???	Cdk2	Decreases Cdc25A stability
???	???	Pim1	Increases Cdc25A catalytic activity
???	???	Raf1	Increases Cdc25A catalytic activity

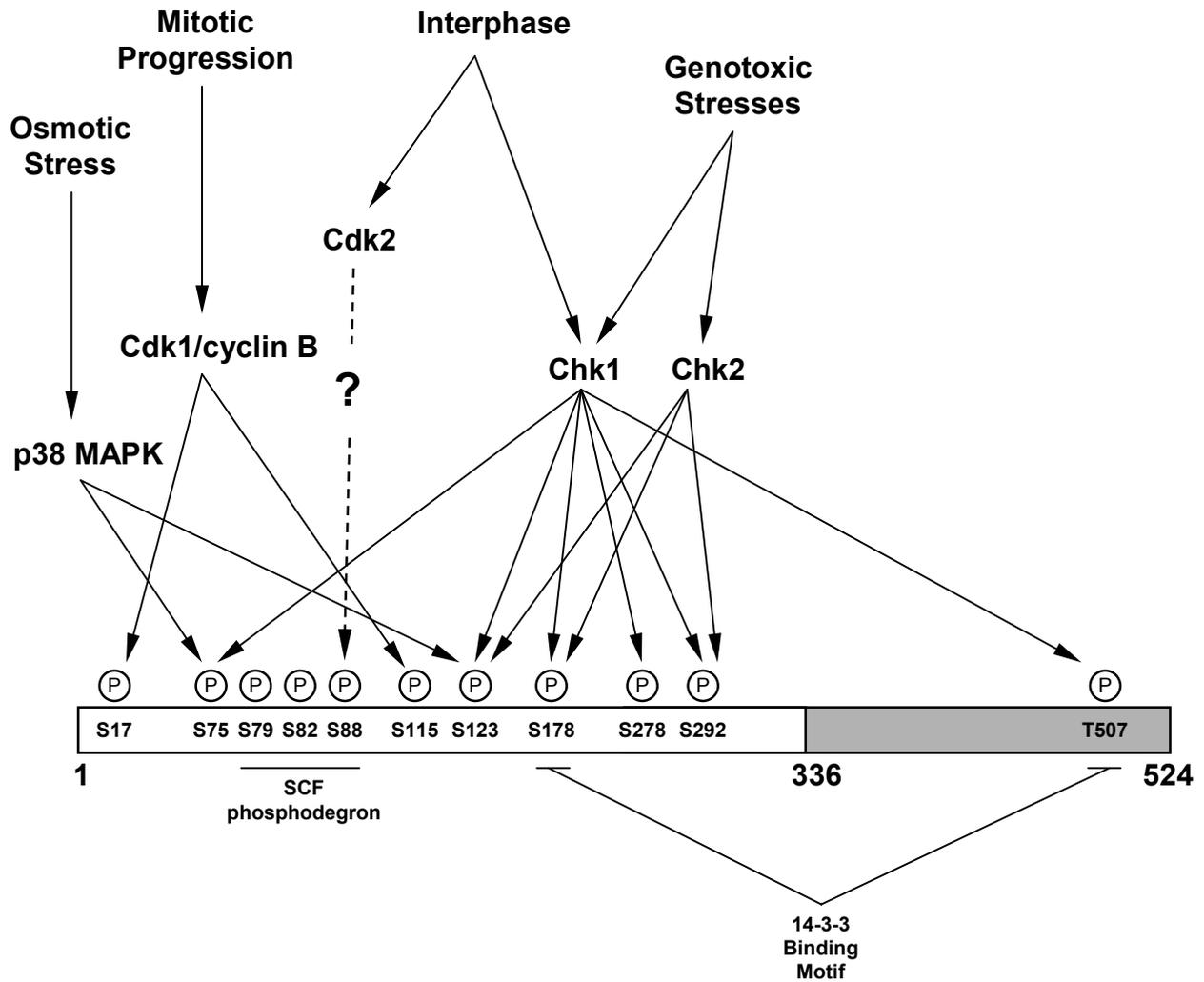


Figure 5.1: Regulation of Cdc25A by Phosphorylation.

Cdc25A is regulated by multiple phosphorylation events, the majority of which occur in the amino terminal regulatory domain. Several Cdc25A phosphorylation sites remain to be elucidated (Table 5.1). Moreover, candidate kinases for several phosphorylation sites also remain to be discovered.

regulating protein stability. Since the seminal report identifying Cdc25A as target of DNA damage checkpoints (87), Cdc25A phosphorylation studies have focused on how genotoxic stress responses are relayed to regulatory proteins that target Cdc25A for degradation. The mechanism that emerged has revealed that targeting Cdc25A for ubiquitin-mediated degradation requires more than the original model of checkpoint kinase-catalyzed Ser 123 phosphorylation (84, 87); Cdc25A degradation in response to genotoxic stresses is, in fact, the result of a previously unanticipated and complicated interaction between phosphorylation content and phosphorylation kinetics, with multiple amino terminal phosphorylation sites playing important and non-redundant roles (68, 70, 85, 86, 183, 199). The known Cdc25A phosphorylation sites are listed in Table 5.1 and depicted in Figure 5.1, and while the detailed mechanism by which these phosphorylation sites affect Cdc25A stability is unclear, recent studies support several conclusions. Cdc25A plays an essential role in catalyzing mitotic cell division. Cdc25A protein stability is significantly increased as cells approach and enter mitosis, and this is a result of Cdk1/cyclin B-catalyzed phosphorylation at Ser 17 and Ser 115. Phosphorylation at these sites uncouples Cdc25A from the rapid proteolytic degradation typical of physiologic interphase Cdc25A regulation and promotes the increase in Cdc25A protein levels essential for mitotic progression (68). While this mechanism promotes the onset of mitosis, the cell has also developed countermeasures to prevent premature initiation of mitosis. Mitotic progression is inhibited by Chk1-mediated phosphorylation of Ser 178 and Thr 507, which promotes 14-3-3 binding to Cdc25A (69). While 14-3-3 association with Cdc25B and Cdc25C results in obstruction of the Cdc25 NLS and concomitant nuclear exclusion, the interaction between 14-3-3 and Cdc25A has not been reported to affect Cdc25A subcellular localization; 14-3-3 binding effectively prevents Cdc25A from binding cyclin B by obstructing the cyclin B binding motif in

Cdc25A and blocks Cdk1/cyclin B activation that is required for mitotic progression (69). 14-3-3 association may facilitate Cdc25A degradation, as inhibiting the interaction between Cdc25A and Cdk1/cyclin B will prevent Cdk1/cyclin B from phosphorylating Cdc25A at Ser 17 and Ser 115 to increase its stability; the precise roles of Ser 178 and Thr 507 phosphorylation in Cdc25A turnover, however, are still unclear. Upon mitotic exit and cell cycle re-entry, Cdc25A lability returns, most likely as a result of cyclin B degradation and concomitant inactivation of Cdk1/cyclin B complexes. This results in a loss of the phosphorylations that stabilize Cdc25A and facilitates its degradation by the APC/C^{Cdh1} ubiquitin ligase (68, 70). Cdc25A regulation during interphase is also a complex matter. Chk1 phosphorylates Cdc25A at Ser 123, 178, 278 and 292 during unperturbed cell cycle progression, and these phosphorylation sites contribute to its labile nature (183). While Chk2 appears to phosphorylate all of these serine residues except Ser 278, Chk2 is only implicated in Cdc25A turnover following genotoxic stresses; increased lability of Cdc25A following DNA damage involves increased kinetics of Cdc25A phosphorylation, presumably reflecting the cumulative effect of Chk1- and Chk2-mediated phosphorylations (181-184). In addition, Cdc25A phosphorylation at Ser 75 also contributes to its labile nature both in the presence and absence of genotoxic stress (85, 198). Ser 75 phosphorylation is mediated by either Chk1 or p38 and appears to be one of the more critical phosphorylation sites for Cdc25A ubiquitination (198). While phosphorylation is clearly a prerequisite for Cdc25A degradation, the mechanism by which Ser 75, 123, 178, 278 and 292 phosphorylation contributes to Cdc25A lability is unknown. Furthermore, regulation of Chk1-dependent Cdc25A phosphorylation is complicated and poorly understood, as Chk1 phosphorylates Cdc25A at multiple sites to affect multiple processes at different points in the cell cycle (69, 85-87, 90, 183). Recently, interphase turnover of Cdc25A was reported to depend on

SCF ^{β -TrCP} ubiquitin ligase, both in the presence and absence of DNA damage (198, 199). While Chk1-dependent phosphorylation of Ser 75 appeared to be important for Cdc25A ubiquitination in the absence of genotoxic stress, Cdc25A could still associate with SCF ^{β -TrCP} ubiquitin ligase, albeit in a decreased manner, following mutation of Ser 75 to alanine, implying that alternate phosphorylation sites could facilitate Cdc25A recognition by the β -TrCP F-box protein in the absence of Chk1-dependent phosphorylation of Ser 75 (198). It appears that interphase ubiquitin-mediated turnover of Cdc25A by SCF ^{β -TrCP} depends on phosphorylation at Ser 82 and either Ser 79 or Ser 88, which together constitute a phosphodegron necessary for Cdc25A ubiquitination by SCF ^{β -TrCP} (198, 199). Ser 79, 82 and 88 are not Chk1 phosphorylation sites, confirming an essential role for kinases other than Chk1 in mediating physiological Cdc25A turnover. Chk1 phosphorylation of Cdc25A at Ser 75 is believed to ‘prime’ Cdc25A for phosphorylation at Ser 82 and either Ser 79 or Ser 88 to form the SCF ^{β -TrCP} phosphodegron (198). While the details of such a ‘priming’ mechanism are still unknown, Ser 75 phosphorylation could form a docking site for a specific protein kinase(s) or a protein complex containing one or more kinases with the ability to phosphorylate Cdc25A at Ser 79, 82 and/or 88. Candidate kinases for these phosphorylation sites remain to be identified, although one potential kinase for Ser 88 phosphorylation, based on primary amino acid sequence, could be Cdk2.

An additional layer of complexity is added to Cdc25A regulation by phosphorylation when interphase regulation of Cdc25A activity is considered. Cdc25A phosphatase activity increases following phosphorylation by Raf1, Pim1, and Cdk2/cyclin E (66, 98, 99). Cdc25A phosphorylation by Raf1 and Pim1 couples mitogenic stimulation to initiation of the cell cycle, whereas phosphorylation of Cdc25A by Cdk2/cyclin E, which requires prior Cdc25A activity, endows Cdc25A with sufficient phosphatase activity to promote progression through the G1/S

transition. The precise location of the Raf1-, Pim1-, and Cdk2/cyclin E-mediated Cdc25A phosphorylation sites, however, remains to be elucidated. Their impact on Cdc25A protein stability will also need to be considered. Regulation of Cdc25A by phosphorylation is clearly a complex interplay between positive and negative regulatory stimuli; the built in redundant and overlapping signals reflect the importance of Cdc25A in cell biology.

5.2. REGULATION OF CDC25A BY CDK2

The first reports of Cdk2 affecting Cdc25A described an enzyme/substrate positive feedback loop in which Cdc25A dephosphorylated Cdk2/cyclin E, resulting in Cdk2/cyclin E activation (66). Activated Cdk2/cyclin E would then, in turn, phosphorylate Cdc25A, increasing Cdc25A activity; this feedback loop would proceed until sufficient Cdc25A and Cdk2/cyclin E activities were achieved to promote the transition from G1 to S phase. Furthermore, the early literature maintained that Cdc25A was present and active in late G1 and early S phase, whereas cell cycle progression beyond these points was relegated to Cdc25B and Cdc25C activities (20-22, 28, 66). It is now believed that Cdc25A not only catalyzes the G1/S transition but it also plays critical roles throughout S phase, in G2, at the G2/M transition and in mitosis (67, 68, 89, 90). The relationship between Cdc25A and Cdk2 is also regarded differently, as Cdk2 appears to contribute to the lability of Cdc25A in interphase (210). While the details of this relationship remain to be fully elucidated, several points are clear. The kinase activity of Cdk2 is necessary for proper maintenance of Cdc25A protein half-life in the absence of genotoxic stress (Chapter 3.2.4). The retarded degradation of Cdc25A following inhibition of Cdk2 kinase activity is not due to downregulation of SCF or APC/C ubiquitin ligase activities, which are responsible for Cdc25A turnover in interphase and mitosis, respectively (Chapter 4.2.1). Furthermore, inhibiting

Cdk2 kinase activity does not downregulate one catalyst of physiologic Cdc25A turnover, Chk1 (Chapter 4.2.3), implying that Cdk2 does not lie upstream in a signaling pathway that culminates in Chk1 activation to regulate Cdc25A turnover. Based on the results presented herein and the current state of Cdc25A biology, an attractive hypothesis for Cdk2-mediated regulation of Cdc25A half-life in the absence of genotoxic stress is that Cdk2 phosphorylates Cdc25A at serine residues essential for formation of the phosphodegron that enables Cdc25A recognition by SCF ^{β -TrCP}. Inhibition of Cdk2 kinase activity would therefore result in retarded Cdc25A degradation by decreasing Cdc25A recognition and ubiquitination by SCF ^{β -TrCP}. Validating this hypothesis would entail analysis of 1) Cdc25A ubiquitination following inhibition of Cdk2 kinase activity, 2) Cdc25A/SCF ^{β -TrCP} association following inhibition of Cdk2 kinase activity, and 3) Cdk2-specific phosphorylation sites in Cdc25A. These constitute the future aims for this research project. One cannot rule out, however, that Cdk2 facilitates activation of an intermediate kinase that contributes to generation of the Cdc25A phosphodegron.

5.3. CELL CYCLE REGULATION AND CANCER

Oncogenic alterations of the cell cycle machinery occur frequently in human tumors, contribute to the disruption of normal growth control and override cell cycle checkpoints (2, 211, 212). Therefore, pharmacologic inhibitors of oncogenic cell cycle regulatory proteins are predicted to possess great theoretical value for treatment of human neoplastic disease (4). The current anticancer strategy of designing novel therapies against specific molecular targets to minimize effects on non-neoplastic tissues, however, depends on validating the suitability of molecular targets for therapeutic intervention (3, 213). The best example to date of the need for

target validation comes from recent studies revealing the suitability of Cdk2 as a target for therapeutic intervention of human cancers. While targeting Cdk2 emerged as a novel therapeutic strategy based on its apparent central role in promoting cell cycle progression (3, 214-219), cancer cells proliferated in the absence of Cdk2 activity and Cdk2 protein, indicating that in fact, Cdk2 is probably not a suitable target for cancer therapy (160). This unexpected result was further validated by targeted deletion of Cdk2 in mice, which confirmed that cells can progress through the cell cycle in the absence of Cdk2 (158, 159). However, it is still possible that genetic instability could result in tumor cells that acquire dependence upon Cdk2 activity. These studies emphasize the need for thorough target validation for novel therapeutic strategies. As oncogenic drivers of cell proliferation, Cdc25 phosphatases are a source of potential targets for the treatment of cancer, and many groups have endeavored to search for specific small molecule Cdc25 inhibitors (42). Much like the case for Cdk2, cells from mice that have undergone targeted deletion of Cdc25B or Cdc25C proliferate normally, implying that specific targeted inhibition of these two Cdc25s, if achievable, might not generate a useful therapeutic for human cancers (71, 72). Furthermore, targeted deletion of Cdc25A in mice is embryonic lethal (Peter J. Donovan, personal communication). However, these results may be attributed to essential roles for the Cdc25 DSPases in developmental biology and the ability of Cdc25A to functionally compensate for Cdc25B or Cdc25C, as inhibition of Cdc25B using antisense oligonucleotides (220) and inhibition of Cdc25A using antisense oligonucleotides or small interfering RNA (siRNA) (68, 124) results in inhibition of cell cycle progression in human tumor cells; moreover, inhibition of Cdc25 activity in human tumor cells using small molecule inhibitors also results in inhibition of cell cycle progression (42, 221). Therefore, interfering with Cdc25A activity could provide a novel therapeutic opportunity for the treatment of cancer. However, caution must be

taken with this approach because, as the efforts for inhibiting Cdk2 revealed, regulation of cell cycle progression is at the same time less intricate and perhaps also more complicated than previously assumed (222). Full target validation and a thorough understanding of novel target protein biology is an essential precursor to any successful therapeutic development efforts. The work presented herein contributes to the fundamental understanding of Cdc25A regulation in unperturbed interphase cells. This is important because, as in *Caenorhabditis elegans*, where loss of Cdc25 regulation led to a hyperproliferative phenotype, and in human tumors overexpressing cyclin B, which could lead to unscheduled activation of Cdk1/cyclin B complexes and increased Cdc25A stability, loss of Cdc25A regulation in human cells could promote deregulation of cell cycle proliferation, insensitivity to anti-growth signals and genetic instability, several essential alterations that are hallmarks of human cancer (2, 19, 198, 209). Therefore, understanding the basic biology of Cdc25A may provide insight into the mechanisms by which Cdc25A levels are overexpressed in tumors, how elevated Cdc25A levels contribute to the malignant phenotype and may generate novel approaches for the treatment of human cancers by therapeutic intervention targeting Cdc25A.

5.4. CONCLUSIONS

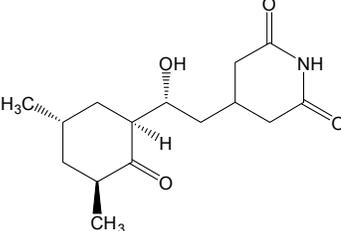
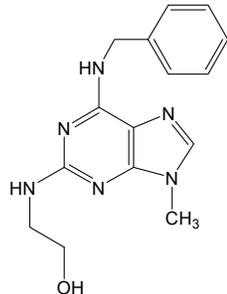
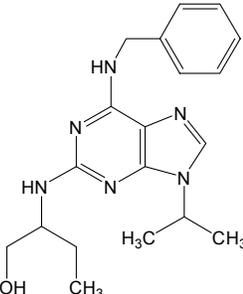
Inhibition of Cdk2 kinase activity in human tumor cells increased Cdc25A protein levels by decreasing Cdc25A protein turnover. Cdk2-mediated regulation of Cdc25A turnover in interphase was a p53- and p21-independent phenomenon. Cdk2 did not regulate Cdc25A turnover by inhibiting APC/C^{Cdh1} or SCF ^{β -TrCP} ubiquitin ligase activities. Cdk2 did not regulate Cdc25A turnover by modulating Chk1 or p38 MAPK, two known regulators of Cdc25A

stability. Cdk2 activity may play an essential role in facilitating Cdc25A recognition by SCF ^{β}
TrCP ubiquitin ligase, a hypothesis awaiting evaluation.

APPENDICIES

APPENDIX A

SMALL MOLECULES

NAME	STRUCTURE	ACTIVITY ^a
Cycloheximide		<ul style="list-style-type: none"> • Inhibitor of protein synthesis • Interferes with translocation step
Olomucine		<ul style="list-style-type: none"> • Cdk Inhibitor • ATP site competitive inhibitor • Cdk1/cyclin B (IC₅₀=7 μM) • Cdk2/cyclin A or E (IC₅₀=7 μM) • Cdk5/p35 (IC₅₀=3 μM) • p44 MAPK (IC₅₀=25 μM) • Cdk4/cyclin D1 (IC₅₀>1 mM) • Cdk6/cyclin D3 (IC₅₀>250 μM)
Roscovitine		<ul style="list-style-type: none"> • Cdk Inhibitor • ATP site competitive inhibitor • Cdk1/cyclin B (IC₅₀=0.65 μM) • Cdk2/cyclin A or E (IC₅₀=0.70 μM) • Cdk5/p35 (IC₅₀=0.20 μM) • Erk1 MAPK (IC₅₀=34 mM) • Erk2 MAPK (IC₅₀=14 mM)

^a Data obtained from Calbiochem (<http://www.calbiochem.com>)

APPENDIX B

ANTIBODIES FOR WESTERN BLOTTING

Antibody	Species	Dilution	Vendor
β-catenin	mouse	1:500	BD Transduction Labs
β-tubulin	mouse	1:5000	Cedarlane Laboratories
Cdc2 p34 (17)	mouse	1:1000	Santa Cruz
Cdc25A (F6)	mouse	1:100	Santa Cruz
Cdc25B	mouse	1:750	BD Transduction Labs
Cdk2 (M2)	rabbit	1:2000	Santa Cruz
Cdk3 (Y-20)	rabbit	1:500	Santa Cruz
Chk1 (G4)	mouse	1:250	Santa Cruz
Chk1	rabbit	1:500	Cell Signaling Technology
cyclin B1 (H-433)	rabbit	1:200	Santa Cruz
cyclin D1 (A-12)	mouse	1:200	Santa Cruz
p21 (C-19)	rabbit	1:500	Santa Cruz
p21 WAF1	mouse	1:100	Oncogene Research Products
p27 (C-19)	rabbit	1:500	Santa Cruz
p38 MAPK	rabbit	1:500	Cell Signaling Technology
phospho-p38 MAPK (Thr180/Thr182)	rabbit	1:1000	Cell Signaling Technology
phospho-Cdc25C (S216)	rabbit	1:5000	Cell Signaling Technology
vinculin (H-300)	rabbit	1:500	Santa Cruz

APPENDIX C

INDUCTION AND PURIFICATION OF GST-CDC25C (200-256)

The following protocol was adapted from Frangioni and Neel (223) for purification of recombinant GST fusion proteins:

Protein Overexpression in *E. coli*:

1. Inoculate 100 ml of LB media containing 100 µg/ml ampicillin with *E. coli* strain BL21(DE3) transformed with pGEX-2T-GST-Cdc25C (200-256). Incubate at 37°C overnight.
2. Inoculate 1L of LB media containing 100 µg/ml ampicillin with 100 ml starter culture (Step 1) and allow to grow 3-5 hr at 37°C while monitoring the OD₆₀₀.
3. When OD₆₀₀ = 0.6-0.8, induce with IPTG to a final concentration of 1 mM and shake at 37°C for 3 hr.
4. Pellet cells by centrifugation at 5000 x g for 15 min. at 4°C.

Sample Preparation/Protein Purification:

1. Resuspend pellet in STE buffer (see below), 25 ml per L culture, supplemented with 100 µg/ml lysozyme and protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml AEBSF).
2. Incubate on ice for 15 min.
3. Add DTT to final concentration of 10 mM.

4. Add N-laurylsarcosine (sarkosyl) [alkyl anionic detergent to aid in solubility] to final concentration of 1.5% from a 10% solution in STE buffer.
5. Vortex for 5 sec to mix.
6. Sonicate on ice for 5 X 5 sec. pulses at power level 4, 50% duty cycle. Save sample of sonicate for analysis by SDS-PAGE.
7. Centrifuge at 10,000 x g for 30 min at 4°C. Save samples of pellet and supernatant for analysis by SDS-PAGE.
8. Add Triton X-100 to final concentration of 2% from a 10% stock solution in STE buffer to supernatant.
9. Vortex for 5 sec.
10. Prepare glutathione-sepharose 4B beads.
 - a. Resuspend slurry in bottle. Pipet 1.33 ml of original slurry. (1.33 ml 75% slurry, which equals 1 ml bed volume in working 50% slurry suspension.)
 - b. Centrifuge at 500 x g for 5 min at 4°C. Decant the supernatant.
 - c. Wash the beads in 10 ml PBS (per 1 ml bed volume). Centrifuge at 500 x g for 5 min. Decant supernatant.
 - d. Resuspend beads in 1 ml STE buffer containing protease inhibitors. (1 ml bead volume + 1 ml buffer = 50% slurry)
11. Add 2 ml 50% glutathione-sepharose 4B beads (from step 10.d) to supernatant. Allow binding to occur on rocker at 4°C for 1hr.
12. Centrifuge at 800 x g (2000 rpm) for 5 min at 4°C. Save sample of unbound material for analysis by SDS-PAGE.

13. Wash beads twice with 10 bed volumes (10 ml) STE buffer supplemented with DTT and protease inhibitors (see Step 1). Spin at 800 x g (2000 rpm) for 5 min at 4°C between washes to pellet beads. Save washes for analysis by SDS-PAGE.
14. Wash beads twice with 10 ml elution buffer supplemented with DTT and protease inhibitors (see Step 1). Spin at 800 x g (2000 rpm) for 5 min at 4°C between washes to pellet beads. Save washes for analysis by SDS-PAGE.
15. After final wash, add 0.5 ml elution buffer to bead pellet. Resuspend and transfer to 1.5 ml microcentrifuge tube. Spin at 800 x g (2000 rpm) for 5 min at 4°C. Remove supernatant.
16. Elute protein 3X with 0.5 ml of 10 mM glutathione in elution buffer containing inhibitors. Spin at 800 x g (2000 rpm) for 5 min at 4°C between elutions. Allow elution to occur at 4°C for 30 min.
17. Determine protein concentration by Bradford method.
18. Analyze fractions and other samples by SDS-PAGE analysis.
19. Add 20% glycerol to each fraction.
20. Aliquot fractions and store at -80°C.

Buffers

- STE: 10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA
- Elution Buffer: 30 mM Tris (pH 8.0), 75 mM NaCl, 1 mM EDTA

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