ELUCIDATING THE ROLE OF CDC25A IN HYPOXIA-MEDIATED CELL CYCLE ARREST

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M.S., University of Bath, 2002

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

School of Medicine

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Hypoxia represents an important element of the solid tumor microenvironment and contributes to tumorigenesis and resistance to chemo- and radiation therapy. Hypoxia can modulate cell cycle progression although the mechanisms involved remain unclear. The Cdc25A dual specificity phosphatase promotes cell cycle progression by dephosphorylating and activating cyclin-dependent kinases. Cdc25A is the master regulator of the cell cycle and its disruption induces cell cycle arrest in cancer cells. The recent observation that under hypoxic conditions, levels of Cdc25A protein and mRNA are decreased led to the hypothesis that hypoxia-mediated reduction in Cdc25A may represent a novel mechanism in the hypoxic regulation of the cell cycle. Given the prominent role of Cdc25A in regulating the cell cycle and the proposed changes in Cdc25A protein and mRNA under hypoxic conditions, it was hypothesized that Cdc25A plays an essential role in hypoxia-mediated cell cycle arrest in human tumor cells. The specific aims were to: 1) examine the mechanism of Cdc25A downregulation in response to hypoxia, 2) determine the role HIF-1α in Cdc25A regulation and cell cycle arrest, and 3) determine if Cdc25A downregulation is required for hypoxia-induced cell cycle arrest.

Under hypoxic conditions, Cdc25A protein levels were specifically and reversibly suppressed.

It was found that Cdc25A mRNA levels are significantly decreased by a p21-dependent mechanism. In addition, suppression of Cdc25A was independent of p53. Loss of Cdc25A protein occurred in the absence of checkpoint activation. Recent evidence has linked the microRNA miR-21 to Cdc25A and hypoxia. It is shown here that miR-21 was required for Cdc25A mRNA suppression in hypoxic colon cancer cells and miR-21 levels were increased under hypoxic conditions. The HIF-1α transcription factor was not required for suppression of Cdc25A but must be present for hypoxia-induced cell cycle arrest. Under hypoxic conditions, cells undergo p21- and miR-21- S-phase cell cycle arrest.

This study proposes a novel mechanism of transient regulation of Cdc25A via the p21- and miR-21-dependent regulation of mRNA levels in hypoxic cells leading to cell cycle arrest. This previously unknown mechanism may confer protection from hypoxic conditions, contributing to cell survival and the observed resistance to chemo- and radiation therapy.

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Theory guides. Experiment decides.

--Izaak Maurits Kolthoff

This thesis is dedicated to Jacqueline Santerre.

ACKNOWLEDGEMENT

I would first and foremost like to thank John Lazo for his guidance, support and friendship during my tenure in the laboratory. Dr. Lazo has taught me the value of independence, free thought and integrity. I am especially grateful for his continued support and encouragement to move forward with my ambitions.

I am grateful to the members of the Lazo laboratory, past and present. I am especially indebted to my former fellow graduate student Robert J. Tomko Jr. for always answering my questions, no matter how daft, and for his dedication to teaching and guiding his peers. I am also thankful to my junior colleagues Carolyn Kitchens and Yan Wang for their help and for always asking questions. I am also grateful to Andreas Vogt and Paul Johnston for their guidance over the last four years.

My gratitude goes out to the members of my thesis committee: Bruce Freeman, Lin Zhang, Yu Jiang and Bruce Pitt for their intellectual support, suggestions and constructive criticism that have helped shape this thesis.

I am indebted to Zhenghe Wang, Long Dang and Bert Vogelstein for the providing me with cell lines. Brian Zuckerbraun and Eric Kelley for assisting me with pO_2 measurements. I am grateful to the Department of Pharmacology and Chemical Biology, especially, Sharon Webb, Rhonda Toth, Jennifer Wong, Patricia Smith, Jim Kaczynski, Richard Smith, Jeanette McDew, Melanie McClain and Linda Sorch for administrative support.

I also extend my thanks to the INTBP Graduate Office staff, especially Cindy Duffy, Veronica Cardamone and Sandra Honick.

I would like to thank my parents Joëlle and João Queiroz de Oliveira for their unconditional love and support and for giving me opportunities to succeed and always encouraging me to pursue my passions. I am grateful to my brothers Jean and Philippe Queiroz de Oliveira for always standing by and keeping my feet firmly on the ground!

Last but not least my sincerest gratitude goes out to Maurizia Stock whose unwavering support, encouragement and dedication kept me going when things got tough and for always reminding me what is most important.

ABBREVIATIONS

7-AAD	7-actinomycin-D
APC/C	Anaphase-promoting complex/Cyclosome
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASK-1	Apoptosis signal-regulating kinase-1
ATM	Ataxia telangiectasia-mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related
β-ME	β-Mercaptoethanol
β-TrCP	β-Transducin repeat-containing protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAK	Cdk-activating kinase
Cdk	Cyclin-dependent kinase
CdkI	Cyclin-dependent kinase inhibitor
Chk	Checkpoint kinase
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
HA	Influenza virus hemaglutinin epitope
HIF-1α	Hypoxia inducible factor 1α
HRE	Hypoxia response element
HRM	Hypoxia-responsive miR
GSK3β	Glycogen synthase kinase 3 beta
IR	Ionizing radiation
NES	Nuclear export sequence
NLS	Nuclear localization signal
MAPK	Mitogen-activated protein kinase
MAPKAPK-2	Mitogen-activated protein kinase-activated protein kinase-2
MEF	Mouse embryonic fibroblast
miR	MicroRNA
MPF	Maturation promoting factor
PBS	Phosphate-buffered saline
PH3	Phospho-histone H3
PI	propidium iodide
pO_2	Partial pressure of oxygen
PTP1B	Protein tyrosine phosphatase 1B
pVHL	von Hippel-Lindau protein
Rb	Retinoblastoma
RIPA	Radioimmunoprecipitation assay
RPA	Replication protein A
SCF	Skp1/Cul1/F-box protein complex
SDS	Sodium dodecyl sulfate
TBS-T	Tris-buffered saline, 0.01% Tween-20
TGF-β	transforming growth factor beta
UV	Ultraviolet

1 INTRODUCTION

1.1 GENERAL INTRODUCTION – CANCER AND CELL DIVISION

In 2008, an estimated 1,400,000 new cases of cancer were diagnosed and 565,000 people were expected to die from cancer, equivalent to 1500 cancer-related deaths per day[1]. These staggering figures make cancer the second most common cause of death in the United States, responsible for 1 in 4 deaths. Despite a drop in cancer related deaths since 2005 (1), cancer is expected to surpass heart disease as the leading cause of death[2] in the United States. The American Cancer Society has predicted that half of all men and one-third of all women will develop cancer during their lifetime[3]. The National institutes of Health have estimated that the overall annual cost of cancer in the U.S. to be $219.2 billion in addition to the loss of human life. The need for new detection methods and improved therapies for the diagnosis and treatment of cancer is self-evident. To achieve this, we need to gain a greater understanding of the pathophysiology of cancer.

Cancer is, in fact, not a single disease but a multitude of diseases with diverse physical manifestations characterized by the uncontrolled proliferation of cells. These cells have acquired the ability to proliferate in the absence of growth signals or in the presence

[1] American Cancer Society: 2008 Facts and Figures (http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf)

[2] The Nation's Investment in Cancer Research: An Annual Plan And Budget Proposal Fiscal Year 2010 (http://plan.cancer.gov/)

[3] American Cancer Society: 2008 Facts and Figures (http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf)

signals intended to suppress cellular growth. This *proliferative advantage* is believed to arise from single or multiple mutations in the cells genomic DNA resulting from normal and abhorrent cellular processes and a variety of environmental factors. For the past three decades, cancer research has focused almost entirely on the cell itself without considering its surroundings. Recently, it has become clear that cancer development relies heavily on the tumor microenvironment. The present study attempts to relate the role of hypoxia, a component of the tumor microenvironment, with the complex cellular framework controlling cellular proliferation via modulation of the cell cycle.

1.2 THE MAMMALIAN CELL CYCLE

The cell division cycle or cell cycle allows for the transfer of genetic information to progenitor cells. The successful transmission of an organisms' genetic code is crucial for its survival. The cell cycle is divided into two parts: firstly the replication of genetic material followed by segregation into two, identical daughter cells. The mammalian cell cycle is divided into four distinct stages: G_1, S, G_2 and M phase. During G_1 the cell prepares itself for cell division, sensing extracellular signals prior to committing to cell division. Cellular DNA is synthesized and the genome is replicated during S phase along with the cellular organelles and structures required for cell division. In G_2, the cell confirms that its DNA has been successfully replicated without errors and prepares for cell division. During M phase, the cell segregates the duplicated DNA copies and organelles before physically splitting into two identical daughter cells, which share the

same genetic lineage as the parent cell. The progression of a cell through the phases of the cell cycle towards cell division is stringently regulated to ensure successful cell division.

1.3 CELL CYCLE REGULATION BY CYCLIN-DEPENDENT KINASES

Transition from one phase of the cell cycle to the next is tightly controlled by a series of checkpoints capable of sensing defects in DNA synthesis and chromosome segregation. The principal catalysts driving progression through these checkpoints and subsequently through the cell cycle are the cyclin dependent kinases (Cdk). Precise and timely regulation of Cdks is critical to ensure error-free cell division. Cell cycle checkpoints act to specifically modulate the activity of the Cdks thus ensuring progression of the cell cycle and integration of intracellular and extracellular signals and ensure the seamless coordination of cell cycle processes in the event of environmental change or mechanical failure (2). The Cdks are a group of serine/threonine kinases that form active heterodimeric complexes following binding to cyclins. Once activated, these Cdks phosphorylate cell cycle phase-specific proteins that promote cell cycle progression. Cdk activity requires binding of regulatory subunits known as cyclins. Cyclins are synthesized and destroyed in a specific circadian rhythm during the cell cycle thus controlling the timing of cell cycle progression. Although human cells contain multiple loci encoding Cdks and cyclins (3) only a limited number of Cdk-cyclin complexes are truly required for cell cycle progression.

Specific Cdk-cyclin complexes are responsible for the different cell cycle transitions (Figure 1). During G_1, mitogenic stimulation stimulates the expression of the D-type cyclins (D1, D2, and D3) that preferentially bind and activate Cdk4 and Cdk6 (4). Activation of these Cdk-cyclin complexes leads to the partial inactivation of the retinoblastoma proteins Rb, Rbl1 and Rbl2, allowing for the expression of cyclins E1 and E2 that bind to, and activate Cdk2 (5). This complex has been shown experimentally to be essential for the progression from G_1 to S phase (6). Once DNA replication nears completion, Cdk2 is activated once more but this time by cyclin A2 allowing the cell to progress from S-phase into the G_2 phase. Activation of Cdk1 by the A-type cyclins is thought to facilitate entry into mitosis but it is the association of Cdk1 with the B-type cyclins that actually drives the cell through mitosis (3). Cdk1 is the only Cdk required for cell cycle progression (7) with all others playing a redundant or overlapping role in the control of the cell cycle.

Cdk regulation is mediated by phosphorylation and protein-protein interactions leading to both activation and inactivation. The regulation of Cdk activity is initially mediated via the interaction with activating cyclins or Cdk Inhibitors (CdkI). Inhibition of Cdks or Cdk-cyclin complexes by CdkIs can result in sequestration of Cdks from cyclins or suppression of activity respectively. CdkIs can be divided into two classes based on their structural homology and Cdk targets. The first class, the INK4 family, includes the inhibitors p15, p16, p18 and p19 and specifically inhibits Cdk4 and Cdk6 (8). The second class, the Cip/Kip family, is compromised of $p21^{Cip1}$, $p27^{kip1}$ and $p57^{Kip2}$ and its members target the Cdk-cyclin-D, -E and -A complexes.

For the Cdks to become active they must first be paired with their cognate cyclins and CdkIs must be removed by sequestration or by proteasomal degradation (8). For proper catalytic activity, Cdks must be phosphorylated on a threonine residue located in the T-loop of the kinase (Thr161 on Cdk1 and Thr160 on Cdk2) by the Cdk activating complex (CAK; Cdk7-cyclin H), a serine/threonine kinase also involved in transcription and DNA repair (9). This results in the partial activation of the Cdk and is followed by the addition of two inhibitory phosphorylations on Thr14 and Tyr15 of the Human Cdk1 by the Wee1 and Myt1 kinases respectively (10-13). Complete catalytic activation of the Cdk-cyclin complex is achieved by dephosphorylation of Thr14 and Tyr15 by the Cdc25 dual specificity phosphatases (Figure 2).

1.3.1 THE CDC25 FAMILY OF DUAL-SPECIFICITY PHOSPHATASES

The *Cdc25* dual specificity phosphatase was first discovered in the fission yeast *Saccharomyces pombe* as the 25th gene discovered to regulate the **c**ell **d**ivision **c**ycle (14). It was discovered that upon mutation of the *Cdc25*$^{+}$ gene, yeast cells grew overly large and failed to undergo mitosis (14). Conversely, introduction of extra copies of the *Cdc25*$^{+}$ gene resulted in smaller than usual cells that underwent premature mitosis (15-17). Evidence for Cdc25 being a phosphatase emerged from several studies examining the Wee^{+} phenotype in which yeast cells undergo premature mitosis before reaching normal size. When both Wee and Cdc25A were mutated, yeast cells regained a normal mitotic phenotype providing evidence that Wee and Cdc25 might have the opposite effect

and antagonize each other physiologically (14). Arguably the most important evidence that Cdc25 was a phosphatase, however, came from studies of $p34^{Cdc2}$ showing that dephosphorylation of $p34^{Cdc2\text{-}Tyr15}$ was required for mitotic initiation (18). Furthermore, the levels of Cdc25 were found to peak during mitosis, consistent with a role in mitotic induction (19). This evidence was strengthened by the discovery that the Cdc25 genes from *Homo sapiens, Drosophila,* and *Xenopus* each encoded a protein with phosphatase activity against $p34^{cdc2}$ (20-23). The discovery that Cdc25 and its role in the eukaryotic cell cycle are highly conserved across species propelled the Cdc25 dual specificity phosphatases into the spotlight as regulators of the cell cycle.

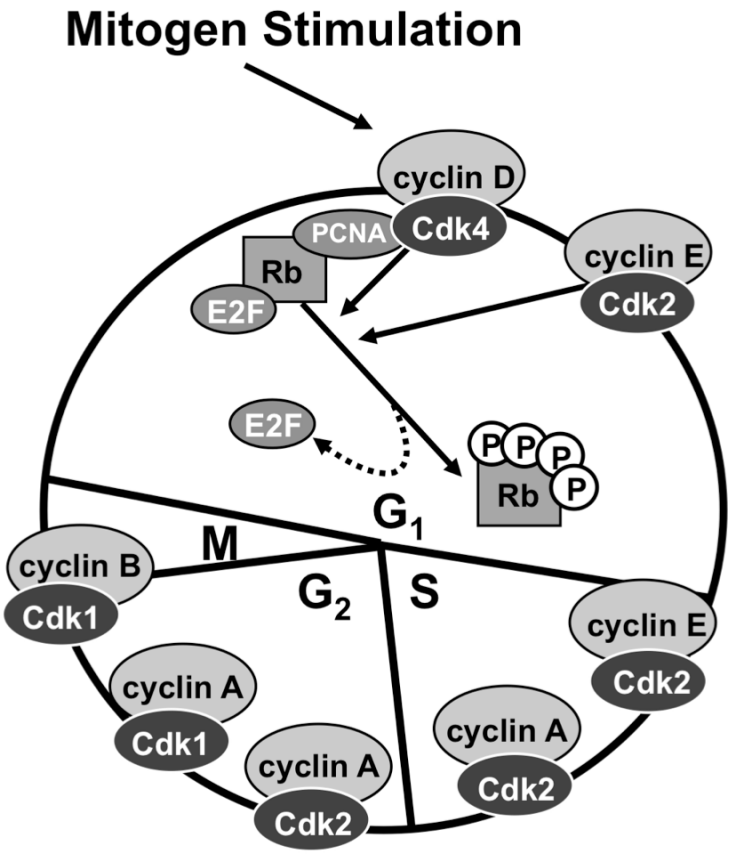

Figure 1: Cdks as drivers of the mammalian cell cycle.

The mammalian cell cycle is composed of four stages: G_1, S, G_2 and M phase. The cell cycle is initiated by mitogenic stimulation leading to increased expression of cyclin D. Cyclins form complexes with Cdks driving progression through the phases of the cell cycle. Activation of Rb by cyclin D/Cdk4 during the G1 phase prepares the cell for DNA replication during S phase.

1.3.2 THE HUMAN CDC25 PHOSPHATASES

Since the identification of Cdc25 as a phosphatase in humans, three isoforms of the Cdc25 have been identified: Cdc25A, Cdc25B and Cdc25C (24, 25). The Cdc25C isoform was discovered first using a degenerate oligonucleotide PCR approach based on the *Drosophila* Cdc25 and was found to share about 37% sequence homology with the fission yeast $Cdc25^{+}$ mitotic inducer (24). The isoforms Cdc25A and Cdc25B were identified by the same methodology in a different study (25). The three human isoforms were found to restore initiation of mitosis in temperature sensitive $cdc25^{+}$ mutants and were thus considered as human Cdc25s based on their homology and function (24).

The human Cdc25 isoforms are encoded by distinct genes, each located on different chromosomes: Cdc25A on 3p21, Cdc25B on 20p13 and Cdc25C on 5q31 (26-28). In addition several mRNA splice variants have been discovered for each isoform (2 for Cdc25A; 3 for Cdc25B; 5 for Cdc25C) (29, 30) but the determinants and expression patterns of the splice variants' protein products remain unknown. It also remains unclear why humans and mammals have evolved three isoforms but adds a layer of complexity to the function of the Cdc25 family in humans.

As mentioned above, there is some sequence homology between the human Cdc25 isoforms and the Cdc25 sequence in fission yeast. This homology is limited to the C-terminus of the protein. Because of this, the human Cdc25 isoforms are divided into two primary structural domains: the N-terminal, with no sequence homology, and the C-terminal, housing the catalytic domain (Figure 3). The conserved amino acid sequences LIGD mark the boundary between the amino and carboxy domains of the phosphatases.

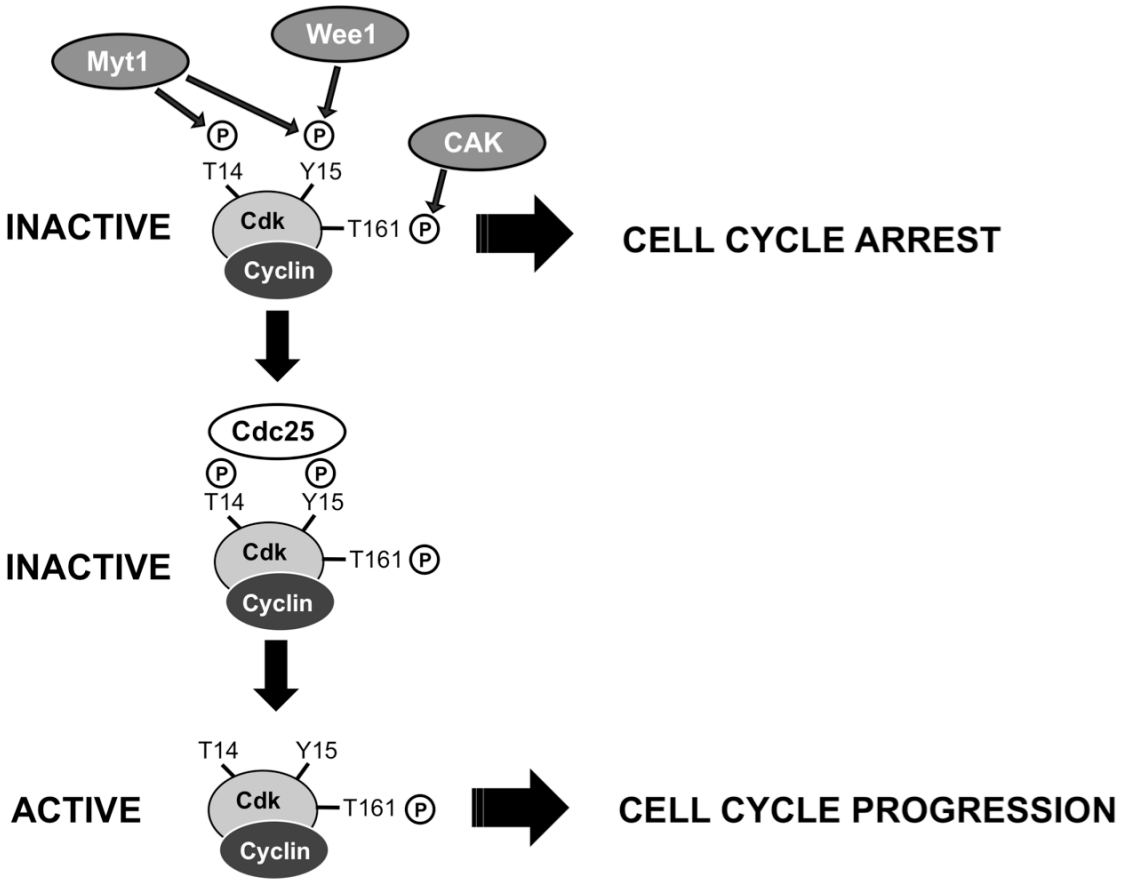

Figure 2: Multistep activation of Cdk/cyclin complexes by the Cdc25 phosphatases.

Myt1 and Wee1 kinases phosphorylate cyclins on T14 and Y15 rendering them inactive. CAK provides an activating phosphorylation on T161. Cdc25 phosphatases activate cyclins by dephosphorylating T14 and Y15 rendering the cyclin/Cdk complex active and allowing for cell cycle progression.

The N-terminus region of the human Cdc25 isoforms is not necessary for catalytic activity. Deletion experiments have shown that Cdc25s retain their catalytic activity in the absence of the N-terminus domain against small molecule substrate *O*-methylfluorescein in *in-vitro* experiments (31). As shown in Figure 3, the N-terminus region contains a nuclear localization sequence (NLS) as well as a nuclear export sequence (NES) that have been shown to play a role in the cellular localization the

protein in addition to multiple phosphorylation sites (32-35). These phosphorylation sites are believed to play a role in the determination on localization, stability and catalytic activity of the protein.

Despite the lack of homology in the N-terminus, all the human Cdc25s contain a consensus sequence that allows for interaction with the 14-3-3 family of proteins (36-38). These phosphothreonine and phosphoserine binding proteins regulate the intracellular localization of signaling proteins (39). In the case of the Cdc25s interactions with 14-3-3 occur primarily in response cellular stresses and upon induction of DNA damage (36, 37).

The C-terminus catalytic domain of the human Cdc25s has been extensively characterized (Figure 3). Crystal structures with better than 2.5 Å resolution have been determined for Cdc25A and Cdc25B (40, 41). These two structures have been found to have significant homology in the folding patterns within the conserved regions but with some variation in the extremity of the C-terminus (41). The structure of the catalytic domain varies from previously described phosphatases, including dual specificity phosphatases but resembles rhodanese, a sulfur-transfer protein (40). The active site of Cdc25 also differs from traditional phosphatase structures in the active site of the enzyme is extremely shallow and there are no auxiliary loops extending over the active site (40, 41). Despite differences in the active sites, the Cdc25s contain the signature motif found in protein tyrosine phosphatases and dual specificity phosphatases (HCxxxxxR) (41).

The catalytic mechanism of the Cdc25 phosphatases is similar to that of the dual specificity tyrosine phosphatases, however the Cdc25s preferentially dephosphorylate a monoprotonated phosphate in contrast to the more typical bisanionic phosphate substrate

(42). The reaction catalyzed by Cdc25 involves the transfer of a phosphate from the phosphoamino acid to water. The bisanionic phosphate is thought to bind in the loop of amide backbones and the positively charged arginine created by the HCxxxxxR motif (42). As with other protein tyrosine phosphatases, the dephosphorylation of the substrate occurs by a two-step mechanism involving the formation of a phosphocysteine intermediate by attack of the catalytic thiolate on the phosphate moiety and expulsion of the leaving group (42, 43). A catalytic acid is required to protonate the tyrosyl, threonyl or seryl oxygen's in order to produce a good leaving group.

1.3.3 CELL CYCLE CONTROL BY CDC25 PHOSPHATASES

The mammalian cell cycle is regulated by the coordinated activity of all three Cdc25 isoforms, their activity and expression levels being cell cycle-phase dependent (Figure 4). Initiation of the mammalian cell cycle occurs during G_1 in response to mitogenic stimulation resulting in increased expression of cyclin D and formation of Cdk4-cyclin D complexes (Figure 1). There is some evidence that Cdc25A may be involved in *starting* the cell cycle by dephosphorylating $Cdk4^{Tyr17}$ (44).

Cdc25A is the main regulator of the G_1/S transition by dephosphorylating and activating Cdk2/CylinE complexes, the rate limiting step for entry into S-phase (45). *In vitro* studies have shown that Cdc25A is able to increase Cdk4/6 kinase activity toward Rb (45) and microinjection of anti-Cdc25A antibodies has been shown to prevent the transition into S-phase (46) and mutation of $Cdk4^{Tyr17}$ negates the requirement for

Cdc25A (44). This evidence certainly points to a role for Cdc25A in activating Cdk4 and initiating the cell cycle.

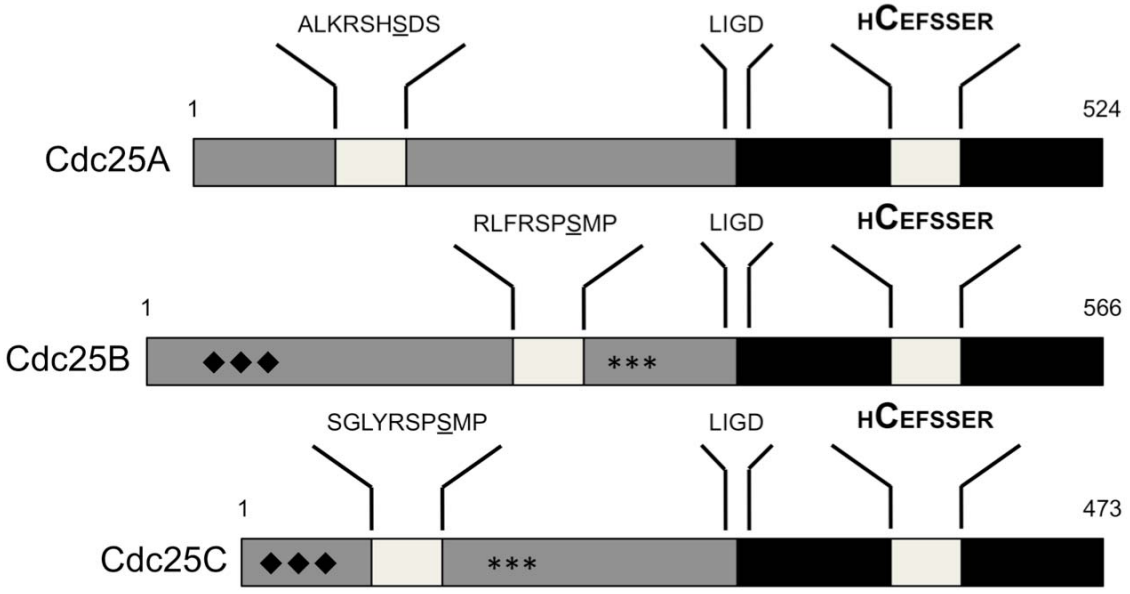

Figure 3: Mammalian Cdc25 phosphatases.

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 (grey), which serves a regulatory role and is the site of multiple phosphorylations, and a carboxy terminal domain (black), 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. Demarcated 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 nuclear localization signal sequences (NLS sequences are indicated with asterisks). Nuclear export signal sequences are indicated with diamonds and the catalytic cysteine is marked by larger font. Adapted from (31).

Experimentally, ectopic expression of Cdc25A results in an acceleration of the G_1/S transition and Cdc25A is able to activate Cdk/cyclin complexes that are functional during G_1 *in vitro* (45, 47). Cdc25A protein levels and activity (47) surge during G_1 and remain elevated through S-phase and have even been postulated to increase as cells enter mitosis (47, 48). The phosphatase activity of Cdc25A beyond the G_1/S transition appears to be driven by a positive feedback loop where Cdk2/cyclin E phosphorylates Cdc25A (47) however the phosphorylation site(s) on Cdc25A involved in potentiating its activity remain to be identified.

S-phase was initially thought to be regulated primarily by Cdc25A and Cdc25B activity has been shown to appear during late S-phase and peak during G_2 phase (Figure 4). Interestingly, downregulation of Cdc25B using antisense oligonucleotides in HeLa cells has been shown to cause S-phase delay (49) suggesting a role for the phosphatase in S-phase. The converse is also true: microinjection of antisense cdc25B oligonucleotides in Fs68 fibroblasts failed to abrogate S-phase (50). A similar discord exists with Cdc25C, which has been proposed to be a regulator of DNA replication (51) whereas other studies have found that microinjection of anti-Cdc25C antibodies had no effect on S-phase progression (47). These conflicting findings raise the question as to whether the observed roles of Cdc25B and Cdc25C are not in fact cell type specific and not *bona fide* regulatory mechanisms. This notion is supported by the fact that Cdc25B and Cdc25C knock out mice have no detectable defects in S-phase (52, 53).

Regulation of G_2 appears to me principally regulated by Cdc25A and Cdc25B (Figure 4). Overexpression of either phosphatase during S-phase or G_2 results in initiation of mitosis (54, 55) whereas decreased expression results in delayed G_2/M progression (55).

Mitotic initiation requires activation of Cdk1/Cyclin B and is regulated by all three Cdc25 isoforms in a distinct spatial and temporal fashion (Figure 4). Activation of Cdk1/Cyclin B complexes is believed to occur through a positive feedback loop and regulation of Cdk1/Cyclin B complexes at multiple levels ensures precise timing of the cells entry into mitosis. Cdc25B activity peaks during the G_2 phase (50) and performs the first wave of activation of Cdk1/Cyclin B complexes at the centrosome (55). This activation triggers the autocatalytic feedback loop with phosphorylation of Cdc25A and Cdc25C by Cdk1/Cyclin B culminating in full activation (20, 22, 56-59). This sequence of events is supported by studies showing that both Cdc25B and Cdc25C are shuttled from the cytoplasm, where they are sequestered during interphase, to the nucleus at the beginning of mitosis (33, 60). Cdc25A protein levels are stabilized during mitosis in contrast to the normally very rapid turnover (61). Functional studies have also demonstrated that all three phosphatases are employed during for mitosis. siRNA-mediated depletion of Cdc25A inhibits entry into mitosis (61) and depletion of Cdc25B by the same method results in delayed entry into mitosis (55). Finally the use of dominant negative Cdc25C mutants inhibit entry into mitosis (62). The phenotypes of the various Cdc25 knockouts are discussed in Chapter 1.4.5.

The Cdc25s are also postulated to have roles in several critical processes during cell division in addition to regulating Cdk/cyclin complex activity. Cdc25A may regulate chromatin condensation because siRNA depletion of the phosphatase extends the period between centrosome separation and DNA condensation (55). Cdc25B has been shown to regulate centrosome replication as overexpression of Cdc25B results in overreplication of centrosomes. Furthermore, studies have shown that Cdc25B accumulation in the

cytoplasm causes centrosomal microtubule nucleation (62) Whereas Cdc25B inhibition results in altered microtubule spindle assembly leading to centrosomal separation delay (55, 63, 64).

The Cdc25 phosphatases participate in the complex regulation of the mammalian cell cycle. Experimental studies have provided important information into the function of each isoform and the role that they play in cell cycle transitions. The mechanism of regulation of the Cdc25 phosphatases themselves adds a further level of complexity to the process of cell cycle progression but allows cells to respond to cellular insults and the extracellular *milieu*.

1.3.4 REGULATION OF CELL CYCLE CHECKPOINTS BY CDC25 PHOSPHATASES

The replication of a cells genome and its segregation into daughter cells is essential for the survival of an organism. One of the major obstacles in this process is the constant genomic insult: it is now known that the primary structure of DNA can be altered extensively by reacting with molecules in the normal cellular environment. For example, at 37°C, about 18,000 purine residues are lost (65), 100 to 500 cytosines are deaminated (66) and over 1200 methylation event occur (67) per cell, each day.

In addition, oxygen-free radicals, frequent by-products of cellular metabolism, react readily with DNA altering its structure and subsequently its coding sequence (68). There are also a vast array of natural and man-made agents and sources that can assault the

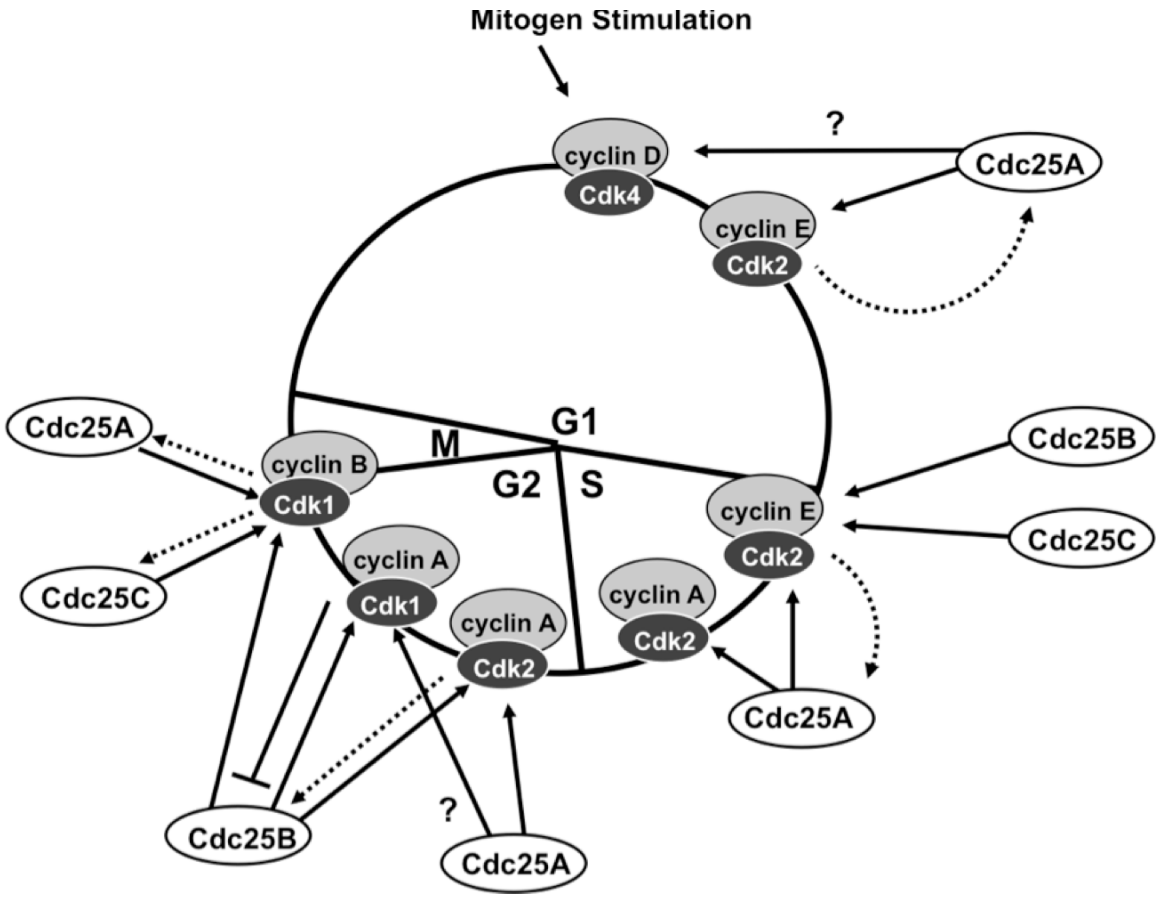

Figure 4: Cell cycle regulation by the human Cdc25 phosphatases.

The Cdc25 phosphatases dephosphorylate and activate cyclin/Cdk complexes at distinct but overlapping times during the cell cycle. There is speculation that Cdc25A is required for initial activation of Cdk4/Cyclin D and Cdk2/Cyclin E complexes. Cdc25A is predominantly responsible for the G_1/S transition though recent evidence suggests that Cdc25B and Cdc25C may also be involved in this transition. Cdc25A and Cdc25B coordinate progression through G_2. All three phosphatases regulate the G_2/M transition and entry into mitosis. Question marks indicate activities that are supported by limited experimental evidence, and dotted arrows illustrate known positive feedback phosphorylations by Cdk/cyclin complexes. The T-bar from Cdk1/cyclin A to Cdc25B represents inhibitory phosphorylation that targets Cdc25B for ubiquitin-proteasomal degradation, thus limiting its activity.

structure of DNA such as ultraviolet (UV) radiation from the sun, radiation, chemicals and environmental insults such as cigarette smoke and automobile exhaust. If left undetected and unrepaired, the resulting mutations are passed down to the cells progenitors. To avoid this, cells have developed mechanisms or checkpoints, that act to stop or slow the progression of the cell cycle of cells containing damaged DNA or when cells are under subject to physiological stresses such as nucleotide depletion, replication fork stalling, aberrant DNA structures or failure of the chromosomal kinetichores to attach to the mitotic spindle. By slowing or halting the cell cycle, cells have a finite period of time in which to repair or rectify damage and either resume cycling or undergo apoptosis.

The cell cycle checkpoints are positioned at the transitions between each phase of the cell cycle and are generally designated as the G_1, S-phase, G_2/M and M-phase checkpoints (Figures 1 and 4). These checkpoints have evolved complex surveillance systems detecting either specific DNA structures indicative of ongoing repair or replication, or the presence of protein complexes engaged in repair or replication (69). These checkpoints serve to link up the cellular mechanisms that detect and screen for anomalies in the cells DNA with the cell cycle drivers thus ensuring high-fidelity DNA replication and distribution (69). Signal transduction pathways are then triggered that prevent cell cycle progression by maintaining Cdk phosphorylation.

The Cdc25 phosphatases are at the center of the checkpoint mechanism. Cell cycle checkpoints are triggered by the detection of aberrant DNA, which in turn leads to recruitment of mediator proteins and the activation of checkpoint (Chk) kinases. The Cdks are the principal drivers of the cell cycle and they are in turn activated by

dephosphorylation of Thr14 and Tyr15 by Cdc25 (Figure 2). Upon checkpoint activation the Chk kinases phosphorylate Cdc25, inactivating the phosphatase and ultimately resulting in hyperphosphorylation of Cdks and induction of cell cycle arrest (Figure 5).

The checkpoint-mediated inactivation of Cdc25s appears to be biphasic with a rapid phase, initiated within an hour and a sustained phase taking several hours to initiate. The initial rapid phase is mediated by phosphorylation. There are a number of Chk kinases involved in the checkpoint response. They include Glycogen synthase kinase 3β (GSK3β) (70), the Chk1 and Chk2 kinases (71, 72), and p38MAPK/MK2 (73, 74).

The mechanism of checkpoint-mediated inactivation of Cdc25 is isoform specific. Cdc25A is regulated at the level of protein stability and rapidly degraded upon checkpoint activation by a p53 independent mechanism (48, 70-72, 75-78) (Figure 5). Cdc25A levels are rapidly decreased by polyubiquitylation and proteasome-mediated degradation. Conversely, Cdc25B and Cdc25C are phosphorylated on serine residues and sequestered in the cytoplasm by 14-3-3. More specifically, Cdc25B is phosphorylated on Ser309 (79) and Cdc25C on Ser216 (36).

Changes in Cdc25 protein stability are the not the only mechanisms of regulation in mammalian cells. Sustained suppression of the Cdc25 phosphatases is mediated at the level of gene transcription. Several studies have shown that the Cdk inhibitor p21 binds the Cdc25A promoter following DNA damage (80, 81) and in response to hypoxia where p21 has been shown to displace c-Myc from the Cdc25A promoter (82). p53 has also been shown to transcriptionally repress Cd25A by binding to its promoter in a p21-independent fashion in response to DNA damage (83). Cdc25C is transcriptionally repressed by p53 following DNA damage by two distinct mechanisms: the first involving

direct binding of p53 to the Cdc25C promoter and secondly via binding to a CDE/CHR (84). To date there is no evidence for the transcriptional regulation for Cdc25B but recently several studies have proposed mechanisms of post-translational regulation of Cdc25B in response to checkpoint activation (85, 86).

1.4 CDC25A: MASTER REGULATOR OF CELL CYCLE PROGRESSION

Cell cycle regulation is a complex process and as described above requires the coordinated activities of all three Cdc25 isoforms. To gain a better understanding of the roles of each phosphatase, knock out models have been developed. However, attempts to generate a Cdc25A knock out mouse have been unsuccessful suggesting that Cdc25A is essential for cell cycle progression and cell division (87). Studies with $Cdc25A^{+/-}$ mice have revealed no appreciable developmental defects and $Cdc25A^{+/-}$ mouse embryonic fibroblasts (MEF) exhibit normal cell cycle kinetics but have a shortened proliferative lifespan and prolonged G_2/M arrest indicative of a possible role for Cdc25A in cell cycle resumption (87). With the development of inducible knock out systems, mice have been generated where Cdc25A is knocked in adulthood thus overcoming the embryonic lethality of traditional Cdc25A knock out attempts. Cdc25A -/- adult mice survived for approximately 1 week before dying from weight loss as a result dramatically decreased absorption in the small intestine (88). The same study also established that Cdc25A is required in early stages of post implantation.

Cdc25B and Cdc25C have been successfully knocked out with no apparent defects suggesting that their roles in the cell cycle regulation are nonessential to ensure cell cycle progression and normal cell division. Cdc25B null mice develop normally and have no detectable phenotypes with the exception of sterility in females due to the absence of Cdc25B-mediated activation of the maturation-promoting factor (MPF) resulting in meiotic arrest (89). Cdc25C null mice undergo normal cell cycle progression and cell division (52). Furthermore, Cdc25B/Cdc25C null mice (53) have been found to exhibit normal cell cycle progression and checkpoint responses. These findings provide strong evidence that Cdc25A is the only *essential* Cdc25 phosphatase and that is able to compensate functionally for the loss of Cdc25B and Cdc25C. The precise regulation of Cdc25A levels and phosphatase activity is paramount for progression through the cell cycle as well as for modulation of the cell cycle under cellular stress.

1.4.1 REGULATION OF CDC25A ACTIVITY

As the master regulator of the cell cycle, Cdc25A activity must be stringently controlled. Aberrant Cdc25A activity suppresses apoptosis (90), promotes genomic instability (91), DNA damage (92) and tumorigenesis (61, 87).

Cdc25A levels are regulated in response to intracellular and extracellular stimuli. Transcription of *CDC25A* is controlled by several transcription factors that stimulate transcription (c-Myc, STAT3 and E2F) and repress transcription (p53, p21, STAT3 and HIF-1α) by binding to the *CDC25A* promoter. Expression of Cdc25A is cell cycle stage-

specific. Cdc25A levels begin to increase during G_1 and *CDC25A* transcription is mediated by E2F in response to mitogen stimulation (93). In the absence of mitogen stimulation, E2F is bound by members of the Rb family (p107 and p130) restricting its ability to induce transcription (94). c-Myc alone, (95) and in combination with STAT3 also drives the transcription of *CDC25A* in response to growth factor stimulation (96). In combination with Rb, STAT3 results in the transcriptional repression of *CDC25A* (96). As mentioned earlier, p53 is also able to induce repression of *CDC25A*, albeit by a mechanism dependent on p53 binding but not to the promoter (83). Transcriptional repression of *Cdc25A* has also been described via the displacement of c-Myc from the *CDC25A* promoter by p21 in response to DNA damage and by HIF-1α (82, 97) under hypoxic conditions. Expression patterns of Cdc25A fluctuate during the different phases of the cell cycle. It has been suggested that Cdc25A levels initially peak during the G_1 phase and then again during mitosis (46, 54).

The regulation of Cdc25A half-life is an important mechanism in the regulation of the cell cycle by intracellular and extracellular signals and is modulated upon checkpoint activation during interphase. Degradation of Cdc25A in response to checkpoint activation and in the G_1, S and G_2 phases of the cell cycle is mediated by Skp1-Cul1-Fbox (SCF) β-Transducin repeat containing protein (β-TrCP), a member of the SCF family of E3 ubiquitin ligases. This process has been shown to be extremely rapid, in the order of minutes (98, 99).

To be ubiquitinalated, Cdc25A must first be phosphorylated, but on different residues than those phosphorylated by Cdk/Cyclin complexes resulting in increased phosphatase activity. The responsible kinases and the phosphorylated residues vary depending on the

phase of the cell cycle. A number of residues have been identified within the N-terminus that must be phosphorylated in order for $SCF^{\beta\text{-}TrCP}$ to bind and ubiquitinate Cdc25A. The process of phosphorylation is believed to occur in several stages: The first stage primes Cdc25A for degradation: Ser 76 is phosphorylated by GSK-3β (70) during G_1 and by Chk1 during S and G_2 phases (75). There is evidence that during S-phase, GSK-3 β and Chk1 cooperate to regulate Cdc25A degradation in response to DNA damage (70) (Figure 5). Mutagenesis studies have revealed that Ser 82 and Ser 88 must be phosphorylated in a second stage, in order for $SCF^{\beta\text{-}TrCP}$ to bind to Cdc25A during S-phase in response to DNA damage. (76, 77). These serine residues form part of a conserved DSG motif ($TDS_{82}GFCLDS_{88}PGPLD$) that is recognized by $SCF^{\beta\text{-}TrCP}$ (77). The kinases responsible for Cdc25A phosphorylation in response to DNA damage and environmental stress include Chk1, Chk2, GSK-3β, and p38 (100). Each of these kinases has been shown to phosphorylate additional serine residues though the functions of these phosphorylations remain unclear. The kinase(s) responsible for cell cycle phase-dependent regulation of Cdc25A have yet to be identified. Chk1 is thought to play a role in both the basal regulation and checkpoint-mediated regulation of Cdc25A (101, 102). It has also been suggested that Cdk2 may be the homeostatic regulator of Cdc25A based on its expression and activity profiles during the cell cycle (99).

During mitosis, Cdc25A takes on a markedly stable form due to phosphorylation on Ser17 and Ser115 by Cdk1/Cyclin B, uncoupling Cdc25A from $SCF^{\beta\text{-}TrCP}$-mediated ubiquitin-proteasome turnover (54). Cdc25A in turn increases Cdk1/Cyclin B activity through a positive feedback loop, raising the activation threshold required for irreversible entry into mitosis.

At the end of mitosis Cdc25A levels decrease rapidly as a result of protein degradation by the ubiquitin-proteasome pathway. This process is mediated by the anaphase-promoting complex/cyclosome (APC/C^{cdhI}), a large multi-protein complex that functions as a ubiquitin ligase (103). The *CDHI* portion of the complex recognizes a KEN box motif located on the N-terminus of the phosphatase (98). The degradation of Cdc25A in the final stages of mitosis and the early stages of G_1 is critical for attenuation of Cdk1 activity helping the cell prepare for the next cell division cycle (100).

1.4.2 CDC25A IN CANCER

Malignancy is generally believed to arise either through the loss of tumor suppressor genes, the main function of which is to negatively regulate the cell cycle, or through the amplification of proto-oncogenes the main function of which is to promote and relay growth signals to the nucleus or a combination of both. It is thought that most cancers develop as a result of the acquisition of a set of functional capabilities described by Hanahan and Weinberg: the self sufficiency from growth signals; insensitivity to anti-growth signals; limitless replicative potential; evasion from apoptosis; sustained angiogenesis; and finally invasion and metastasis (104). Deregulation of the cell cycle embodies several of these capabilities. As a critical regulator of the cell cycle, Cdc25A has the potential to promote malignant transformation. Indeed Cdc25A has been shown to have oncogenic properties, transforming normal MEFs with an oncogenic form of Ras or in an Rb-null background (61).

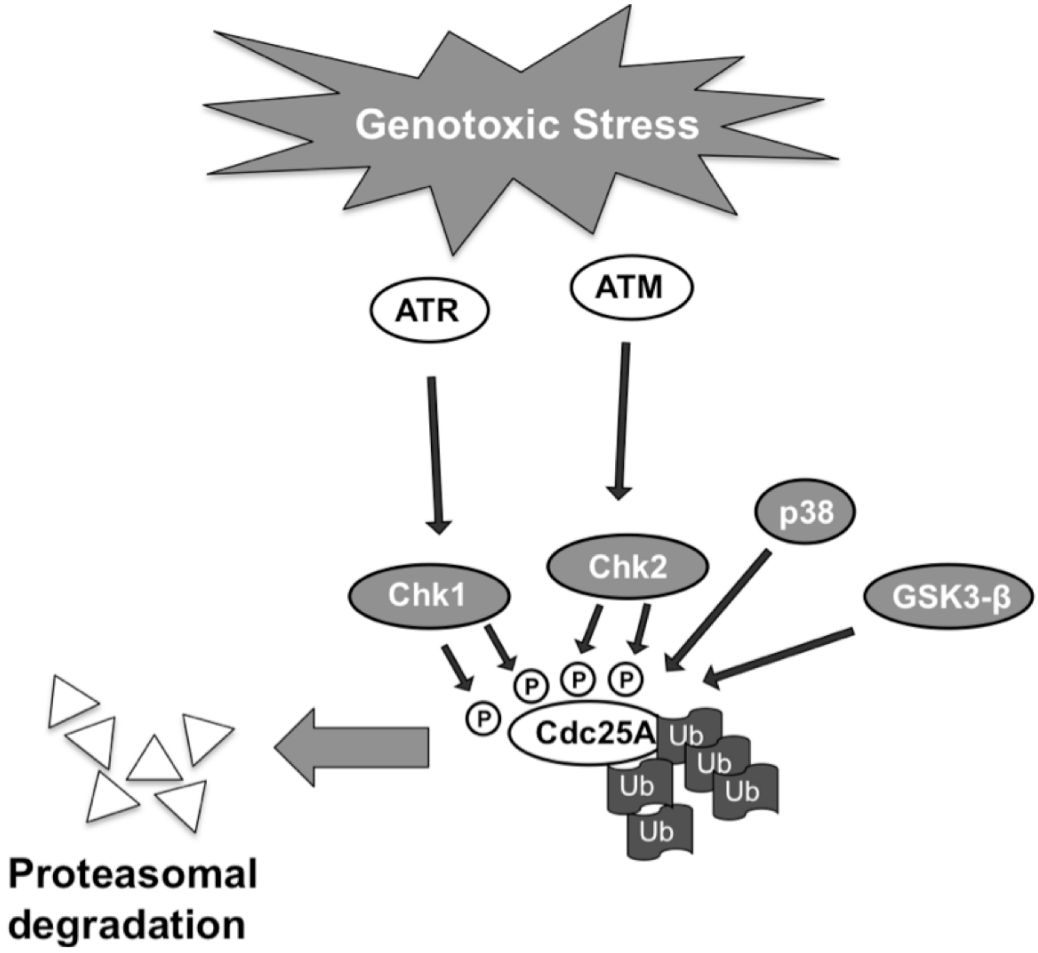

Figure 5: Checkpoint-mediated regulation of Cdc25A in response to genotoxic stress.

DNA damage and genotoxic stresses result in activation of the Chk kinases, p38 and GSK3-β. Cdc25A is phosphorylated by these kinases, resulting in poly-ubiquitination by the $SCF^{\beta\text{-}TrCP}$ complex and proteasomal degradation.

Since Cdc25A overexpression was first described by Nagata (105), Cdc25A overexpression has been reported in numerous human cancers including colon (106), breast (107, 108), gastric (109), head and neck (110), lung cancers (111), esophageal carcinoma (112), non Hodgkin's lymphoma (113) and neuroblastoma (114). In addition to being overexpressed, Cdc25A levels in breast carcinomas are predictive of poor survival (107).

As mentioned above, the proto-oncogenic properties of Cdc25A overexpression may lie in its ability to drive the cell cycle, promote cell division and drive malignancy. This is compounded by the finding that Cdc25A may interact with the proto-oncogene *Raf1* (61, 115) as well as by regulation of the MAPK signaling cascade (116). Indeed, Raf1 and Cdc25A have been shown to co-localize within the cytoplasm and the former has been shown to be able to phosphorylate and activate Cdc25A (117). Cdc25A has also been shown to dephosphorylate Raf1 following its activation by growth factor stimulation (115). These interactions may form a negative-feedback loop where the Ras-Raf-MAPK pathway promotes activation of Cdc25A which in turn negatively regulates Raf1, terminating MAPK activation and priming the cell for cell cycle progression (31). In cells transformed by Raf1, Cdc25A overexpression may play a role in decreasing the cytotoxic and cytostatic effects of aberrant MAPK activation while simultaneously promoting cell cycle progression (116, 118-120).

From the perspective of the cell cycle, overexpression of Cdc25A may allow cells to bypass the growth-inhibitory signals transmitted by the Chk1 and Chk2 kinases in response to DNA damage, promoting genetic instability, and increasing the likelihood of

the cell acquiring genetic mutations leading to malignant transformation and acquiring growth advantage (71, 72).

The spatial and temporal regulation of transitions between the different phases of the cell cycle must be strictly regulated in order to maintain genomic stability. It therefore places Cdc25A at critical position, where, in the event of a disruption of normal activity could result in a malignant phenotype of rapid proliferation and an insensitivity to cell cycle checkpoints (72). Cdc25A provides a direct link not only between mitogenic signaling and the cell cycle but also the cellular microenvironment and is therefore ideally situated to facilitate cell transformation and malignant development.

1.5 THE TUMOR MICROENVIRONMENT

The tumor microenvironment is characterized by a poorly developed vasculature, leading to regions of hypoxia, decreased pH and inadequate nutrient levels as well as an accumulation of metabolic waste products (for example, carbonic and lactic acid produced during glycolysis) resulting in regions of acidosis in the tumor mass and surrounding areas.

Tumor hypoxia constitutes a clinically relevant problem. Direct evidence of a relationship between hypoxia and cancer was established by Vaupel and colleagues using oxygen electrodes (121, 122). Low oxygen tension in tumors was associated with increased metastasis and poor survival in patients with solid tumors. Resistance to chemo- and radiation therapy (123, 124) is also common feature of hypoxic tumors and

contributes to the difficulty in treating these tumors and decreased survival (125). It is therefore highly pertinent to expand our understanding of the role of hypoxia in tumor development and resistance to treatment.

Hypoxia is characterized by a reduction in the normal levels of tissue oxygen tension and occurs during acute and chronic vascular disease, pulmonary disease and cancer (125). Both chronic and acute hypoxia arises from an abnormal vasculature in tumors. Chronic hypoxia arises due to the limited diffusion of oxygen from blood vessels into tissues whereas acute hypoxia arises from the dynamic nature of the aberrant tumor vasculature, such that vessels may become temporarily occluded down causing transient hypoxia in the surrounding tissue (126).

1.5.1 TUMOR HYPOXIA

Hypoxic regions are a common feature of solid tumors (127-129) and develop as a result of inefficient vascular development or an abnormal vascular architecture (126). More specifically, the primary features of tumor physiology that lead to the development of hypoxia are limited arteriolar supply and arteriolar deoxygenation (130), relatively low vascular density and disorderly vascular architecture (131), oxygen consumption that is out of balance with oxygen supply (132) and an unstable blood supply (133). Multiple studies have demonstrated that hypoxia is an independent adverse prognostic factor from tumor grade or treatment modality and positively correlates with tumor aggressiveness and poor disease survival (134-139).

Mammalian cells have evolved to utilize molecular oxygen for energy production. Cells can respond to a variety of oxygen concentrations via alterations in both their metabolic states and growth rates. Under hypoxic conditions, glucose is converted to lactate through the activity of glycolytic enzymes and lactate dehydrogenase A, a much less efficient means of generating ATP than via the complete oxidation of glucose by glycolytic enzymes, pyruvate dehydrogenase, the tricarboxylic acid cycle enzymes and the electron transport chain. The switch from oxidative to glycolytic metabolism in hypoxic cells is mediated by HIF-1α, which will be discussed later in this section. The absence of HIF-1α in hypoxic cells and blockade of the metabolic shift described above results in the toxic accumulation of reactive oxygen species and cell death (140).

It has been proposed that hypoxia can alter cell proliferation in two ways: via apoptosis or through growth arrest (141). In response to hypoxia, transformed cells undergo apoptosis via the p53 pathway (142) whereas non-transformed cells have been shown to undergo cell cycle arrest via the G_1/S checkpoint without alterations in long-term viability (143). During tumor growth, hypoxia can act as a selective pressure for the elimination of cells with wild-type p53 and the clonal expansion of cells with mutant or otherwise inactive p53 (144). Cell cycle arrest under hypoxic conditions was originally attributed to the inhibition of nucleotide synthesis (145, 146). However, it has since been found that inactivation of enzymes responsible for nucleotide synthesis occurs only under severe hypoxia (0.01% oxygen) or anoxia, but not under moderate hypoxia (0.1 to ~ 1% oxygen) (143). An increasing number of studies are shedding light on the molecular consequences of moderate hypoxia and have shown similarities with classic genotoxic stresses in terms of the cellular responses observed (141, 142, 147, 148). Several of these

studies have described the induction of DNA stress checkpoints in response to hypoxia resulting in cell cycle arrest. Studies have also shown that under moderate hypoxia, G_1 cell cycle arrest is induced via inhibition of Cdk/cyclin activity via an increase in CDKI such as p21 and p27 (143, 149) or through a reduction in critical cell cycle proteins such as Cyclin D, Cyclin E and Cdk4 (150).

Another important element in the hypoxic response is the family of hypoxia-inducible factors (HIFs), major regulators of the cellular response to hypoxia. HIF-1α is the most ubiquitously expressed of these transcription factors and acts as the master regulator of oxygen homeostasis in many cell types. HIF-1α is sensitive to decreased oxygen levels and is degraded rapidly by the ubiquitin proteasomal pathway under normoxic conditions. Hypoxia reduces the availability of HIF-1α to the Von Hippel-Lindau protein (pVHL), the recognition component of an E3 ubiquitin ligase complex, blocking HIF-1α degradation (151-153) and resulting in nuclear accumulation, and enhancement of transcriptional activity via binding to enhancer elements in target genes. Under hypoxic conditions, oxygen-dependent prolyl hydroxylase domain proteins are inhibited, preventing the hydroxylation of prolines required for pVHL binding to HIF-1α (152). Once it stabilized under hypoxic conditions, HIF-1α forms a heterodimer with ARNT (aryl hydrocarbon receptor nuclear translocator) and with the cofactor p300 to regulate the transcription of a diverse group of hypoxia response element (HRE) -containing genes involved in pathways in systemic, local and intracellular oxygen homeostasis (154-156).

Many of the genes regulated by HIF-1α allow a cell or tissue to adapt to the hypoxic conditions but also promote the survival of the tumorigenic phenotype found in cancer. Of the more than 60 targets of HIF-1α, many of these are involved in angiogenesis,

anaerobic glucose metabolism, metastasis, cell motility, iron metabolism, growth and survival, apoptosis, telomere maintenance and drug export mechanisms (157).

Hypoxia induces the expression of various growth factors known to promote cell proliferation such as transforming growth factor β (TGF-β) and platelet derived growth factor (158-160). In addition HIF-1α itself is activated by the p42/p44 mitogen-activated protein kinases in response to extracellular growth factors leading to the transcriptional activation of HIF-1α target genes (161). Hypoxia has been shown to increase the activity of several other mediators of cell proliferation including phosphatidylinositol 3-OH kinase (PI3K), and the oncogenic RAS pathway (162, 163). HIF-1α involvement in the hypoxia-mediated effects of these growth factors has come primarily from HIF-1α null models, where the absence of HIF-1α negatively affects tumor growth. Cells undergo a variety of biological response under hypoxic conditions including cell proliferation, angiogenesis, shifting from aerobic to anaerobic respiration, decreased apoptosis, migration and immortalization and cell cycle regulation (125, 155, 164). Hypoxic regulation of the cell cycle will now be discussed in greater detail.

1.5.2 HYPOXIC REGULATION OF THE CELL CYCLE

The precise role of hypoxia and HIF-1α in cell cycle regulation under hypoxic conditions remains unclear. It has been suggested that HIF-1α may be the primary determinant of cell cycle modulation under hypoxic conditions. Cdk2 activity is completely inhibited in hypoxic cells (143, 165) and possibly mediated by a HIF-1α-dependent increase in p27

(141). Elucidation of the role(s) of HIF-1α in the regulation of the cell cycle under hypoxic conditions has been complicated by a number of differing observations in both transformed and non-transformed cell lines. It is not inconceivable that the role of HIF-1α may differ in malignant cells compared with normal cells under hypoxic conditions. There is also evidence that hypoxia can regulate the cell cycle via activation of cell cycle checkpoints. Severe hypoxia has been shown to induce ATR-dependent S-phase arrest in the absence of detectable DNA damage (or by a form of chromatin disruption not detectable by traditional comet assays) (166). Although hypoxia does not appear to induce *bone fide* DNA damage, regions of single stranded DNA have been identified in hypoxic cells indicative of stalled replication forks (167). Hypoxia is frequently followed by periods of reoxygenation that elicit activation of an ATM and Chk2-dependent checkpoint response arresting cells at the G_2/M checkpoint presumably due to reoxygenation-induced oxidative DNA damage (168). In this study, the induction of cell cycle arrest also appears to be dependent on the presence of Cdc25C. The activation of ATM and Chk2 has also been observed under hypoxic conditions in the absence of detectable DNA damage (169). The activation of cell cycle checkpoints in cells under hypoxic conditions has also been linked to the activation of a number of DNA damage repair proteins including MLH1, NBS1 and BRCA1, indicative that hypoxia is causing alterations in DNA structure of not DNA damage itself (170, 171). The Cdk inhibitors p21 and p27 have also been linked to the regulation of the cell cycle under hypoxic conditions. Although neither is required for hypoxia-induced S-phase arrest, both p21 and p27 appear to play a role in maintaining cell cycle arrest under hypoxic conditions (149). Interestingly, it has been shown that p21 expression can be induced by HIF-1α by

a mechanism involving the displacement of c-Myc from the p21 promoter, presenting another level of regulation of genes lacking the canonical HRE and possibly regulation of the cell cycle by hypoxia (172). A recent study has shown that *Cdc25A* mRNA levels are decreased under hypoxic conditions and that this decrease may occur through a similar mechanism of displacement of c-Myc from the Cdc25A promoter (82).

The studies discussed above suggest that under hypoxic conditions, several regulatory pathways exist to drive tumorigenesis and control cell cycle progression in cancer cells. Recently, microRNAs (miRs) have been associated with tumor progression and several are upregulated under hypoxic conditions (173, 174). In fact several of these miRs appear to be involved in the pathogenesis of cancer as well as being regulated by hypoxia. Of particular interest is miR-21, which is overexpressed in many cancers and increased by hypoxia (175). This miR has recently been proposed as a putative regulator of Cdc25A (176, 177).

1.6 GENE REGULATION BY MICRORNAS

MiRs were originally discovered in *Caenorhabditis elegans* as essential regulators of larval development and have since been indentified in numerous organisms including vertebrates (178). To date, over 6000 miRs have been identified in humans (179) representing approximately 2% of human genes. Based on *in silico* studies (180-182), it is estimated that up to 30% of human genes may be regulated by miRs, however, the role of miRs in the regulation of many of these genes remains to be validated.

miRs are a family of short (usually about 21-24 nucleotides) non-coding RNA molecules involved in the regulation of at least thirty percent of all translated genes (183, 184). These species act predominantly by blocking the translation of mRNA targets by a mechanism similar to that utilized by siRNA. miRs are initially expressed as a primary transcript that is processed by the Drosha RNAase to generate a hairpin-RNA precursor (pre-miR). Pre-miR precursors are then further processed by the RNAase III Dicer, resulting in the formation of mature duplexes (185). One of the mature strands becomes associated with the RNA-induced silencing complex (RISC), after which it is capable of interfering with gene expression at the transcriptional level (186).

Much of the early work into the functions of miR was conducted in invertebrates where several miRs (*Lin4* and *Let7*) were found to be important regulators of larval development in *Caenorhabditis elegans*. When suppressed, these miRs promoted aberrant cell division without normal differentiation (187-189). Studies in *Drosophila melanogaster* have identified miRs involved in tissue growth and suppression of apoptosis (190, 191). Studies in mice and human cell lines have associated miRs with cellular differentiation and division functionally linking miR clusters to human cancers (192).

1.6.1 MICRORNAS AND CANCER

A number of miRs have been identified that can act as *bona fide* oncogenes (miR-17-92, miR-10b, miR-21) and tumor suppressors (miR-15-16 clusters). miR-21 is upregulated

in a number of tumor types including breast, colon, lung, pancreas, stomach (174) as well as in a number cancer cell lines of various origins (174). miR-21 levels have also been found to be high in a number of pathological conditions where aberrant cell growth and cellular stress are pathophysiological features such as in cardiac hypertrophy (193, 194).

A single gene located on chromosome 17q23.2, which overlaps with the protein-coding gene VMP-1, encodes the mature miR-21. The amplification of the 17q chromosomal region is associated with a number of cancers, however the genomic locus encoding miR-21 is not amplified in most cancers including those expressing high levels of miR-21 (176). This suggests that high levels of miR-21 is likely not due to gene amplification. Analysis of the consensus sequences within the miR-21 promoter region has identified several conserved enhancer elements, including binding sites for AP-1, C/EBP-α, nuclear factor-1, p53 and STAT3 (195). Oncogenic transformation is frequently associated with the enhancement of endogenous AP-1 activity through various signal transduction pathways so it is possible that increased miR-21 levels may reflect increased AP-1 activity in certain cancers. Interestingly, miR-21 is consistently induced in response to hypoxia in breast and colon cancer cells (173) and both HIF-1α and AP-1 sites have been identified on the miR-21 promoter, and which are both upregulated by hypoxia. This presents some clues as to the possible mechanism of hypoxia-dependent regulation of miR-21.

1.6.2 REGULATION OF CDC25A BY MICRORNAS

Cell cycle regulation is tightly regulated by several mechanisms in response to mitogenic stimulation and extracellular conditions. The regulation of Cdc25A by modulation of protein stability and gene expression has been discussed earlier. Two recent studies have shown that Cdc25A may be a target for miR-21 (196). Cdc25A has been identified from f *in silico* approaches and validated in recent studies. Cdc25A was found to be a target for miR-15a in human polycystic kidney and liver cells from humans and mice as a well as a number of other miRs (miR-34a, 103, 107, 195, 204, 326 and 331) (88). Another study has identified miR-21 as a regulator of Cdc25A in human colon cancer cell lines (Wang, P. unpublished). Both miR-15a and miR-21 are known to be overexpressed in cancer and miR-21 ahs also been indentified as a hypoxia-responsive miR (HRM).

1.6.3 MICRORNA REGULATION BY HYPOXIA

As mentioned above, hypoxia is major feature of the tumor microenvironment and appears to play an important role in tumorigenesis through the regulation of a plethora of cellular functions. To date, HIF-1α has been described as the key regulator of the cellular hypoxic response. Recent studies have uncovered that in addition to the gene induction under hypoxic conditions, gene repression also appears to be playing a role in the hypoxic response (82, 197, 198). The potential role of miRs in these repression events is

reinforced by the relative selectivity of repression, given that many genes continue to be expressed at normoxic levels under hypoxic conditions (199).

Several studies have been performed to identify hypoxia-induced miRs providing a link between the control of gene expression and the response to hypoxia in cancer cells. These hypoxia-regulated miRs (HRMs) include miR-21, 23a, 23b, 24, 26a, s6b, 27a, 30b, 93, 103, 106a, 107, 125b, 181a, 181b, 181c, 192, 195, 210 and 213 and have been found to be upregulated in breast and colon cancer cells in response to hypoxia (173, 199). It must be noted that certain HRM appear to be cancer and cell type specific adding complexity to the role of these miRs in the hypoxic adaptation in cancer cells.

The mechanism of miR regulation remains unclear although it has been suggested that miRs may be regulated by transcription factors. Indeed, c-Myc and the E2F transcription factor family have been found to regulate the expression of the miR-17-92 cluster (200, 201). Furthermore, identification of miR promoter regions is in its infancy with only a handful of studies being performed (202, 203). An *in silico* study (173) has shown that in the predicted and known promoter regions of HRMs, the incidence of potential HIF-1 binding sites was significantly higher than in those of miRs not regulated by hypoxia. miR-21 has been found to not only be upregulated in cancer but also in hypoxic cells (176, 177). Given the potential role of miR-21 in regulating Cdc25A and the fact that it is upregulated by hypoxia, gaining a better understanding of the role of miR-21 in control of the cell cycle under hypoxic conditions is warranted.

1.7 STATEMENT OF THE PROBLEM AND HYPOTHESIS

Hypoxia-mediated induction of cell cycle arrest is established but the mechanism of cell cycle arrest in tumor cells exposed to hypoxia remains unclear and a number of conflicting theories exist based on the heterogeneity of hypoxic conditions and cellular models used.

Cdc25A is the master regulator of the cell cycle and its disruption has been shown to induce cell cycle arrest in cancer cells. With the recent observation (82) that under hypoxic conditions, the levels of Cdc25A protein and mRNA are decreased has led to the hypothesis that hypoxia-mediated reduction in Cdc25A may represent a yet undescribed mechanism in the hypoxic regulation of the cell cycle. It is possible that Cdc25A suppression may enable cancer cells to gain increased resistance against chemo- and radio- therapy, a phenomenon described in the treatment of numerous cancers (123, 124). Given the prominent role of Cdc25A in regulation of the cell cycle and the proposed changes in Cdc25A protein and mRNA under hypoxic conditions, I hypothesized that **Cdc25A plays an essential role in the hypoxia-mediated cell cycle arrest in human tumor cells**. The specific aims of this dissertation were to 1) examine the mechanism of Cdc25A downregulation in response to hypoxia, 2) to determine the role HIF-1α in Cdc25A regulation and cell cycle arrest and 3) determine if downregulation of Cdc25A is required for the observed hypoxia-induced cell cycle arrest.

2 EXPERIMENTAL METHODS

2.1 REAGENTS

Nocodazole, hydroxyurea, caffeine and doxycycline were purchased from Calbiochem. G418 and hygromycin B were obtained from Invitrogen. Antibodies against Cdc25A, Cdc25C and Chk2 were obtained from Santa Cruz Biotechnology, Cdc25B and HIF-1α from BD Transduction Laboratories. Antibodies against Chk1, phosphorylated Chk1 (Ser^{345}), and phosphorylated Chk2 (Thr^{68}) were from Cell Signaling Technologies; the β-tubulin antibody was obtained from Cederlane Labs and anti-HA tag antibody from Covance. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch.

2.2 CELL CULTURE

HCT116 human colon cancer cells were obtained from the American Type Culture Collection. The p53-/-, Chk2-/- and p21-/- HCT116 cells were a gift from Dr. Bert Vogelstein (Johns Hopkins University), and HIF-1α -/- HCT116 cells were a gift from Dr. Long Dang (University of Michigan). Cells were maintained in McCoy's 5A medium

supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, and 2 mmol/L L-glutamine in a humidified incubator at 37°C with 5% carbon dioxide. The DLD WT and DLD1 miR-21 KO cells, a gift from Dr. Zhenghe Wang (Case Western Reserve University), were also maintained in the above-mentioned conditions. HTA-21 cells, HeLa cells expressing HA-Cdc25A under a tetracycline repressor promoter were maintained in DMEM supplemented with FBS, 100 μg/mL G-418, 100 μg/mL hygromycin B, and 100 ng/mL doxycycline in a humidified incubator at 37°C with 5% carbon dioxide.

2.3 TRANSFECTION OF CELLS WITH PRE-MIR-21

Pre-miR21 and pre-miR-224 complexes were obtained from Ambion. Prior to transfection, $8x10^5$ cells were plated on 6-well tissue culture dishes and allowed to adhere overnight. Lipofectamine 2000 reagent (Invitrogen) was combined with Optimem medium (Gibco) and incubated at room temperature for 10 minutes. Pre-miR complexes were added and the transfection mixture was allowed to incubate for 30 minutes at room temperature. Cells were transfected with complexes for 6 hours in complete reduced-serum media (1% FBS). Following transfection, cells were incubated in complete media for 24 hours.

2.4 TREATMENTS AND INDUCTION OF HYPOXIA

Cells exposed to hypoxia were incubated for the indicated periods of time in an InVivO2 400 hypoxic workstation (Ruskinn Technology Ltd). Cells were maintained under 1% oxygen and 5% carbon dioxide at 37°C in humidified conditions. Hypoxic conditions were confirmed using an iSTAT blood gas analyzer (Abbott). For ultraviolet irradiation, cells were washed once with PBS before UV irradiation at 60 J/m^2 using a UVC Crosslinker (Stratagene), followed by addition of fresh medium before incubation for 2 hours and cell harvesting.

2.5 WESTERN BLOTTING

For Western blotting, cells were harvested by scraping into a modified RIPA buffer (50 mM Tris pH 7.6, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 12 mM β-glycerol phosphate, 10 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 100 μg/mL 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride, 10 μg/mL soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride). Cells were lysed either by incubation on ice for 30 minutes with frequent vortexing or by sonication at 50% amplitude for 6 x 2 seconds on ice with a 2 second pause between pulses using a GEX-130 ultrasonic processor with a VC-50 2 mm microtip (Gene Q). Lysates were cleared by centrifugation at 13,000x g for 15 minutes, and protein content of the supernatant was determined by the method of Bradford. Total cell lysates (30 – 50 μg

protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes using an i-blot semi-dry transfer system (Invitrogen). The quality of transfer was confirmed by Ponceau staining of membranes followed by washing with ddH_2O and TBS-T. Membranes were then blocked in 5% non-fat milk or 5% BSA as recommended by the primary antibody manufacturer, and incubated with primary antibodies overnight at 4°C. After washing 3 times for 5 minutes with 25 mL TBS-T, horseradish peroxidase-conjugated secondary antibodies were added for one hour at room temperature before 3 additional TBS-T washes and visualization with Pierce Enhanced Chemiluminescence Western Blotting Substrate (Pierce). Chemiluminescence was detected using a LS3000 luminescent image analyzer and analyzed using Multiguage software (Fujifilm).

2.6 RNA ISOLATION AND QRT-PCR

Total RNA was extracted with an RNeasy RNA isolation kit (Qiagen) and small RNA species for microRNA analysis were extracted using a *mirVANA* RNA isolation kit from Ambion. Isolated RNA was quantified using a SpectraMax M5 spectrophotometer (Molecular Devices).

Quantitative PCR was performed using a one step protocol. Briefly, total RNA was subjected to reverse transcription with iScript reverse transcriptase (BioRad) and amplification of cDNA with iTaq DNA polymerase with SYBR green (BioRad). All reactions were performed on an iQ5 Real-Time PCR Detection System (BioRad). The following primers (IDT Technologies) were used: Cdc25A forward 5'-

CAAAATCCGTCTAGGAGCTGC-3', reverse 5'-GTCTCTCCTTACCTGCCCAG-3'; CDC25B forward 5'-CACTCGGTCCCAGTTTTGTT-3', reverse 5'GTTTGGGTATGCAAGGCACT-3'; β-actin forward 5'-GTGTCACCAAAGCAAAGGCTTGGA-3', reverse 5'-ACAGGTTCTCTTCTGTGCTTGGGA-3'. β actin expression was used for normalization of each experiment. Total RNA extracted using the *mirVANA* kit was subjected to reverse transcriptase PCR to generate cDNA with primers specific for miR-21 and RNU-24 (Applied Biosystems). PCR products were subsequently amplified from cDNA using a TaqMan Micro RNA assay (Applied Biosystems) according to the manufacturers protocol. The micro RNA RNU-24 was used as a control in these reactions and for normalization during data analysis. The $2^{-\Delta\Delta C}{}_t$ method (31) of relative quantification was used to determine changes in mRNA and microRNA levels. All experiments were conducted in triplicate and results presented as the mean ± SEM.

2.7 FLOW CYTOMETRY

Identification of mitotic cells was carried out by simultaneous staining of cells with propidium iodide (PI) (BD Pharmingen) and phospho-histone H3 using an anti-phospho-histone H3 antibody (Upstate). Cells were fixed overnight in 70% ethanol and permeabilized with 0.25% Triton X100 in PBS for 8 minutes on ice. After washing with 1% bovine serum albumin (BSA) in PBS, cells were incubated with 1 μg anti-phospho-histone H3 antibody for 2 hours. Cells were subsequently washed with PBS containing

1% BSA before incubation with 1 μg anti-rabbit IgG Alexa 488 for 40 minutes in the dark. Finally cells were resuspended in 500 μl PI/RNAase staining buffer for 15 minutes. 20,000 cells were analyzed by fluorescence activated cell sorting (FACS) using an EasyCyte Plus flow cytometer (Guava Technologies). ModFit LT cell-cycle analysis software (Verity Software House) was used to determine cell cycle phase distribution based on cellular DNA content. S-phase cells were identified using a BrdU flow kit (BD Pharmingen). Briefly, staining with 10 μM bromodeoxyuridine (BrdU) was performed 30 min prior to harvesting. After harvesting, cells were permeabilized with the detergent saponin and fixed with 4% paraformaldehyde. Cells were then treated with DNase to expose BrdU epitopes. Immunofluorescence staining was achieved using a FITC-conjugated anti-BrdU antibody followed by staining with 7-actinomycin-D for staining of total DNA content. Cells were then resuspended in staining buffer and analyzed by FACS. WinMDI software (TSRI) was used for analysis and to determine both phospho-histone positive cells and BrdU positive cells.

2.8 STATISTICAL ANALYSIS

Results are expressed as mean ± SEM of at least three independent experiments. Anova and *t* tests were carried out using Graphpad Prism 5 (Graphpad Software).

3 REGULATION OF CDC25A PROTEIN LEVELS IN HUMAN TUMOR CELLS BY HYPOXIA

3.1 INTRODUCTION

Cdc25A plays a critical role in cell cycle regulation by acting as the link between intracellular and extracellular stimuli and the cell cycle. Regulation of Cdc25A is mediated at the level of protein stability but also at the level of gene transcription. In response to genotoxic stress such as DNA damage, ATM and ATR become active and phosphorylate the Chk kinases (Chk1 and Chk2), which in turn phosphorylate Cdc25A leading to its polyubiquitination and proteasomal degradation. There is some evidence that this pathway is activated under hypoxic conditions and Cdc25A protein levels are suppressed in response to hypoxia. Several studies have shown that hypoxia does not induce *bone fide* DNA damage but instead can induce replication fork stalling and replication stress, which is known to activate a checkpoint response. The data presented below attempts to clarify the role of the Chk kinases in the regulation of Cdc25A under hypoxic conditions as well as the role of the CdkI p21, which is activated in response to replication stress.

Whereas the mechanisms of regulation of Cdc25A protein stability have been extensively described, regulation of *Cdc25A* gene expression and transcription is poorly understood.

There is some evidence that *Cdc25A* gene expression is driven by c-Myc and that p21 and HIF-1 can negatively regulate *Cdc25A.* Recently Cdc25A was found to be a putative target for miR regulation by miR-21.

The following study examines the regulation of Cdc25A by hypoxia. I demonstrate that under hypoxic conditions, Cdc25A protein levels were depressed and that this was not due to activation of a checkpoint response. Cdc25A mRNA levels were also decreased in a p21-dependent fashion. Examination of the role of miR-21 in this process revealed that miR regulation of Cdc25A mRNA levels represents a novel mechanism of regulation of Cdc25A in response to hypoxic stress.

3.2 RESULTS

3.2.1 CELL CULTURE MODEL OF HYPOXIA

Tumor hypoxia was modeled by incubating immortalized cancer cells under reduced oxygen conditions. There is a tremendous amount of heterogeneity in the simulation of hypoxia in cell culture models both from the perspective of the oxygen tension used and the types of cells studied. Experiments have been performed with oxygen concentrations ranging from 0.01 to 5% oxygen. For this study, I chose to define hypoxia as 1% oxygen in a mixture of nitrogen and carbon dioxide because at extremely low oxygen conditions (< 0.01%) and anoxia (0%), the lack of molecular oxygen causes the shutdown of

nucleotide production and the cells hypoxic response is thought to be primarily mediated by the mitochondria resulting of this is rapid S-phase arrest and induction of cell death (204). Moderate hypoxia allows for the study of hypoxic stress in the absence of mitochondrial sensing of hypoxia and ensures proper function of nucleotide synthesis pathways.

The hypoxia-induced stabilization of HIF-1α was used a marker for a cellular response to hypoxic stress. Figure 6 shows that within 1 hour of exposure to hypoxia (1% oxygen), HIF-1α protein levels were detectable and sustained for up to 24 hours of hypoxia. As would be predicted, HCT116 cells under normoxic conditions did not have detectable levels of HIF-1α (Figure 6, 0 hours).

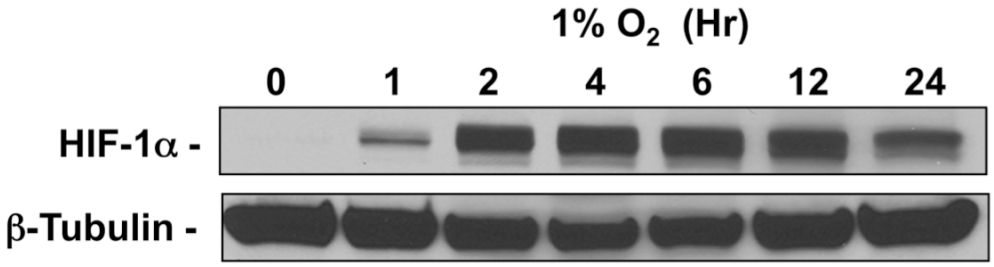

Figure 6: Stabilization of HIF-1α protein with hypoxia.

HCT116 cells were exposed to hypoxia (1% oxygen) for increasing time periods prior to cell lysis. Whole-cell lysates were resolved by SDS PAGE and HIF-1α levels were determined by Western blotting. β-tubulin levels were used as a loading control. The Western blot shown is representative of three independent experiments (N=3).

To maintain conditions as close to those found in a normoxic cell culture incubator, the InVivo$_2$ 400 chamber was maintained at 5% carbon dioxide 37°C, and humidified. Chamber oxygen levels were maintained at 1% oxygen but this did not provide any indication of the oxygen levels within the culture media of cells being exposed to hypoxia or maintained under normoxic conditions. To verify dissolved oxygen (pO_2) levels in the culture medium, an iSTAT portable blood gas analyzer (Abbott) was used to measure pO_2 levels. Cells maintained at 1% oxygen were found to have significantly lower oxygen levels (~5%) compared with identical cells maintained under normoxic conditions (~20%) (Figure 7).

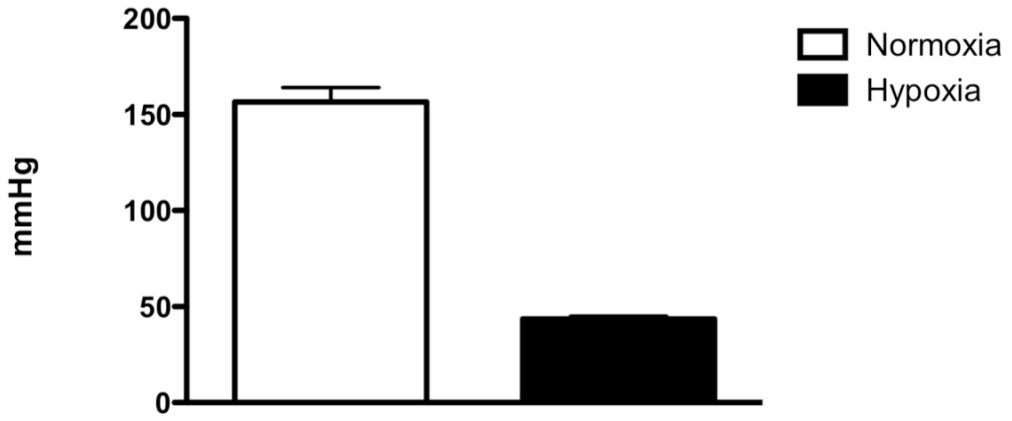

Figure 7: pO_2 levels are significantly decreased in cells exposed to hypoxia.

HCT116 cells were maintained under hypoxic conditions for 24 hours or maintained under normoxic conditions for 24 hours. Media samples were tested using an iSTAT portable gas analyzer and cG8+ cartridges. Results shown represent the mean ± SEM of three independent experiments.

3.2.2 REDUCTION IN CDC25A PROTEIN LEVELS BY HYPOXIA

Cdc25A protein levels were analyzed in HCT116 cells exposed to hypoxia for 0 to 24 hours. Cdc25A levels decreased with increasing duration of exposure to hypoxia and were undetectable after 24 hours by Western blot (Figure 8).

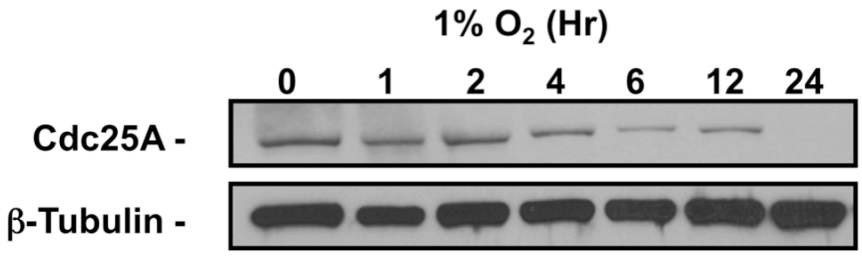

Figure 8: Cdc25A levels are decreased under hypoxic conditions.

HCT116 cells were incubated under hypoxic conditions (1% O_2) for 1-24 hr or maintained under normoxic conditions for 24 hours (0). Cells were lysed and whole cell lysates were analyzed by Western blot for Cdc25A protein levels. The above western blot is representative of three independent experiments (N=3). Blots were probed for β-tubulin as a loading control. Cdc25A protein levels were quantified relative to β-tubulin by densitometry using Multiguage software and are indicated below the Western blot.

3.2.3 LOSS OF CDC25A IS SPECIFIC UNDER HYPOXIC CONDITIONS

The cellular response to hypoxia is aimed at the conservation of resources including amino acids required for protein translation. With this in mind the levels of Cdc25B and

Cdc25C were analyzed in HCT116 cells exposed to hypoxia for 24 hours or maintained under normoxic conditions for 24 hours (Figure 9). Whereas Cdc25A protein levels were decreased, Cdc25B and Cdc25C levels appear to be unaltered by a 24-hour exposure to hypoxia. This finding indicates that the loss of hypoxia observed in not due to a non-specific reduction in protein translation, which would result in global decrease in protein expression levels.

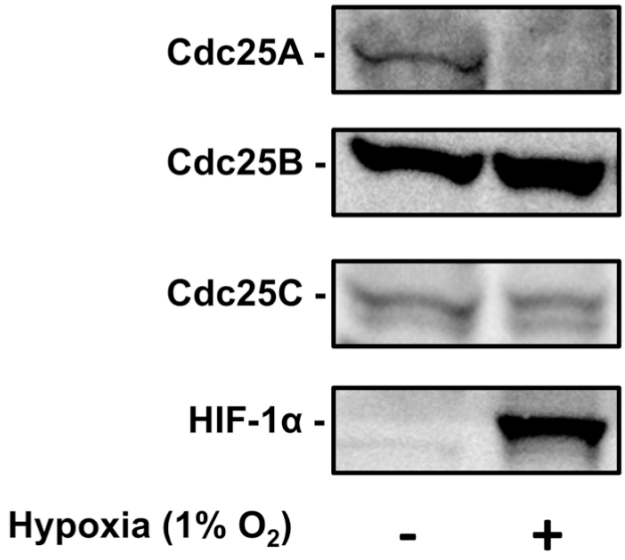

Figure 9: Hypoxia does not affect Cdc25B and Cdc25C phosphatase levels.

HCT116 Whole cell lysates were analyzed by Western blotting for Cdc25A, Cdc25B and Cdc25C levels following a 24-hour exposure to hypoxia or following 24 hours under normoxic conditions. The above Western blot is representative of three independent experiments (N=3).

3.2.4 LOSS OF CDC25A PROTEIN UNDER HYPOXIC CONDITIONS IS REVERSIBLE

The hypoxia-mediated decrease in Cdc25A protein appears to be specific and similar decreases were not observed in the other Cdc25 phosphatase isoforms Cdc25B and Cdc25C. Cdc25A levels were analyzed in cells that were initially exposed to hypoxia for 24 hours and then returned to normoxic conditions for 0 to 24 hours to further understand the nature of the repression of Cdc25A under hypoxic conditions. Cdc25A protein levels were detectable after 12 hours of reoxygenation suggesting that hypoxia-mediated loss of Cdc25A protein is reversible upon reoxygenation (Figure 10).

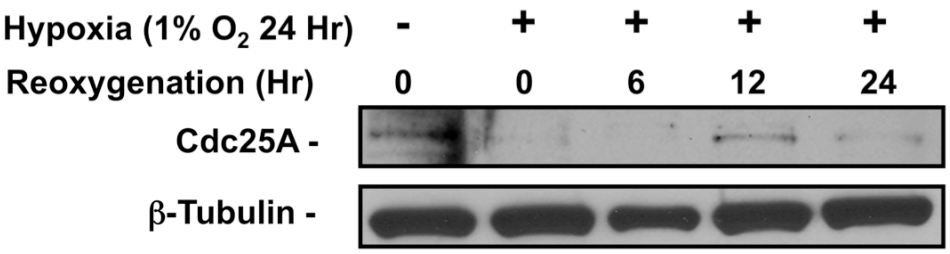

Figure 10: Hypoxia-mediated loss of Cdc25A is reversible upon reoxygenation.

HCT116 cells were incubated under hypoxic conditions for 24 hours followed by increasing periods of reoxygenation (20% oxygen) before cells were lysed and analyzed by Western blotting for Cdc25A levels. (N=1).

3.2.5 P21 BUT NOT P53 IS REQUIRED FOR HYPOXIA-MEDIATED LOSS OF CDC25A

p53 is known as the "guardian of the genome" and is stabilized following DNA damage and in response to cellular stress. As a major component of the tumor microenvironment, hypoxia represents a significant cellular stressor. Exposure to cellular stresses and DNA damage results in an increase in p53 levels, which in turn drives the expression of p21 in these cells. Both p53 and p21 have been demonstrated to suppress Cdc25A protein levels (80, 83) and p53 stabilization also activates Chk1 (205), known to be responsible for the phosphorylation-dependent degradation of Cdc25A. The role of p53 in the modulation of the cell cycle by hypoxia and ultimately cell survival remains unclear as cellular stress is believed trigger a p53 response (205) but studies have shown that hypoxia-mediated modulation of the cell cycle does not require p53 (165). An isogenic HCT116 cell line lacking p53 (p53 -/-) was subjected to 24 and 48 hours of hypoxia to establish whether p53 could be playing a role in the observed decrease in Cdc25A. Analysis of Cdc25A protein levels by Western blotting revealed that in the absence of p53, cells retain the capability to down regulate Cdc25A in response to hypoxia (Figure 11). These findings suggest that regulation of Cdc25A in response to hypoxia is independent of p53 and is in agreement with the work of others (148).

The CdkI p21 is capable of transcriptionally repressing Cdc25A in response to DNA damage (80) and has been linked to the maintenance of hypoxia-induced cell cycle arrest and resumption (149). Using an isogenic cell line that does not express p21 (HCT116 p21 -/-), the role of the CdkI in the regulation of Cdc25A in hypoxic cells was examined.

As can be seen in Figures 12 and 21, the absence of p21 abrogated the loss of Cdc25A in hypoxic cells after 24 hours confirming that p21 played a role in this process.

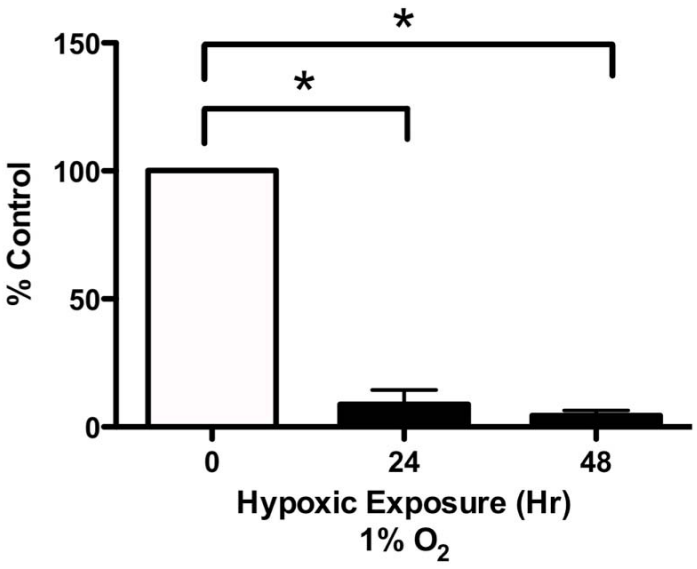

Figure 11: Hypoxia induced loss of Cdc25A occurs independently of p53.

p53-/- HCT116 cells were exposed to hypoxia (1% O_2) for 24 hr or maintained under normoxic conditions. Whole cell lysates were analyzed for Cdc25A expression. Relative expression of Cdc25A was quantified by densitometry as described above and normalized to β-tubulin (N=3),* = $p < 0.01$.

3.2.6 LOSS OF CDC25A OCCURS IN THE ABSENCE OF CELL CYCLE CHECKPOINT ACTIVATION

Regulation of Cdc25A following checkpoint activation has been extensively described. As shown in Figure 5, Cdc25A is phosphorylated and targeted for proteasomal degradation by a number of checkpoint kinases including Chk1 and Chk2 that have been shown by others to be involved in hypoxia-mediated cell cycle arrest (167, 169). I analyzed levels of Chk1 and Chk2 phosphorylation in cells exposed to hypoxia to determine if the observed loss of Cdc25A was due to checkpoint activation. Hypoxia did not induce an increase in the phosphorylation of Chk1 or Chk2 at Ser345 and Thr68 respectively or change overall kinase levels, compared with UV irradiation that increased both Chk1 and Chk2 phosphorylation levels (Figure 12). UV irradiation is known to induce a checkpoint response and cause the degradation of Cdc25A (206) and was used as a positive control for DNA damage-induced checkpoint activation and loss of Cdc25A. As expected in response to UV irradiation, Chk1 and Chk2 phosphorylation levels were significantly increased, indicative of checkpoint activation, and Cdc25A levels were suppressed by 2 hours after treatment, consistent with increased degradation of the phosphatase (Figure 12).

These findings indicate that 1) loss of Cdc25A under hypoxic conditions did not require Chk kinase activation and 2) that HCT116 cells were competent to activate the checkpoint response following induction of genotoxic stress and DNA damage by UV irradiation. In the absence of a viable Chk1 null cell line, a pharmacological blockade of

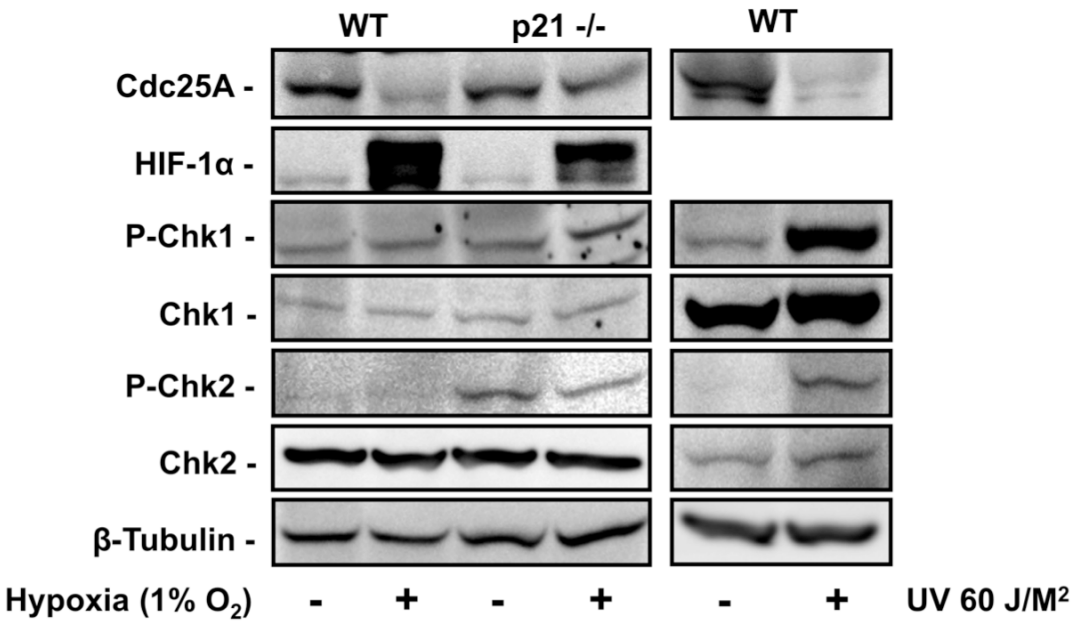

Figure 12: Cdc25A downregulation by hypoxia does not require Chk1 or Chk2 activation.

Cell lysates from WT and p21-/- HCT116 cells exposed to hypoxia were analyzed for increases in Chk1 and Chk2 phosphorylation. WT HCT116 cells were treated with UV irradiation (60 J/m^2) and then incubated for 2 hours prior to harvesting cells to confirm that HCT116 cells have a functional checkpoint response. The above Western blot is representative of three independent experiments (N=3).

Chk1 was used to confirm that Chk1 was not required for the observed loss of Cdc25A under hypoxic conditions. HCT116 cells were exposed to hypoxia in the presence of the ATM/ATR-Chk1 inhibitor caffeine (5 mM) (207) for the duration of the hypoxic exposure. As can be seen in Figure 13, caffeine treatment did not prevent the loss of Cdc25A under hypoxic conditions indicative of Chk1 independence. Interestingly, caffeine alone appeared to repress Cdc25A levels under both normoxic and hypoxic

conditions (lanes 2 and 4), which was not expected following treatment with an agent that prevents checkpoint kinase activation. Inhibition of ATM and ATR by caffeine would on the contrary be expected to have increased Cdc25A protein levels.

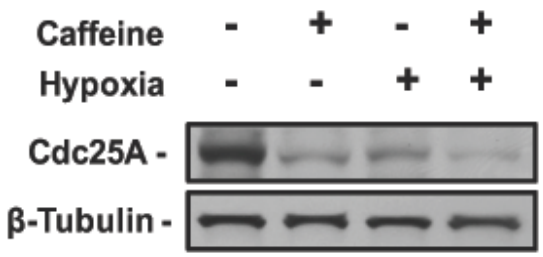

Figure 13: Caffeine treatment does not block hypoxia-mediated Cdc25A loss.

HCT116 cells were pretreated with 5 mM caffeine for 30 minutes to block ATM/ATR Chk1 basal activity and then exposed to hypoxia (24 hours) in the presence of caffeine at the same concentration. The above Western blot is representative of three independent experiments (N=3).

The role of Chk2 in the hypoxia-induced loss of Cdc25A was determined thanks to the existence of the isogenic Chk2 -/- HCT116 cell line. Upon exposure to hypoxia, Chk2 -/- cells downregulated Cdc25A to the same extent as the WT cells albeit with much slower kinetics of downregulation as can be seen in Figure 14.

Checkpoint activation in response to DNA damage or replication stress results in a dramatic decrease in Cdc25A protein levels via increased degradation (71). I utilized a cell line capable of ectopically expressing HA-tagged Cdc25A to test whether hypoxia

increases Cdc25A protein degradation. The HTA-21 cells are HeLa cells expressing HA-Cdc25A under a tetracycline repressor promoter. Upon removal of doxycycline, these cells readily overexpress HA-tagged Cdc25A under control of a constitutive promoter. These cells were exposed to hypoxia for 24 hours in the presence or absence of doxycycline. As expected, the cells cultured without doxycycline had high levels of HA-tagged Cdc25A that was degraded when these cells were exposed to UV irradiation (60 J/m^2) (Figure 14). However, when overexpressing cells were cultured under hypoxic conditions, levels of HA-tagged protein did not decrease. This finding suggested the mechanism of hypoxia-induced suppression of Cdc25A did not involve increased Cdc25A protein degradation as would be expected with checkpoint activation or via a mechanism leading to the phosphorylation of Cdc25A and subsequent degradation.

Taken together the finding that neither Chk1 nor Chk2 were required for hypoxia-mediated loss of Cdc25A along with the absence of Chk1 or Chk2 activation or increased degradation of HA-Cdc25A in response to hypoxic stress suggested that this phenomenon might be occurring by a mechanism that did not require checkpoint activation and did not affect Cdc25A stability.

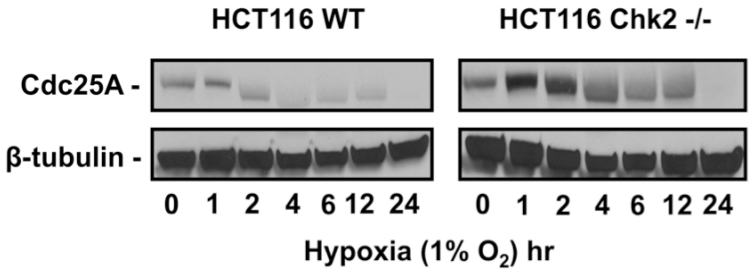

Figure 14: Chk2 is not required for hypoxia-mediated loss of Cdc25A.

Lysates from WT and Chk2-/- HCT116 cells exposed to increasing time periods of hypoxia were analyzed by Western blot for Cdc25A protein levels. The above Western blot is representative of three independent experiments (N=3). Cdc25A protein levels were quantified relative to β-tubulin by densitometry using Multiguage software and are indicated above the Western blot.

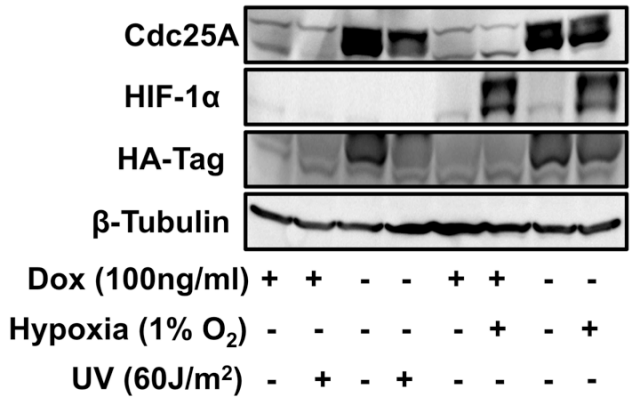

Figure 15: Hypoxia does not increase protein degradation.

HTA-21 cells containing a Tet-repressor expression system allowing for ectopic expression of Cdc25A in the absence of doxycycline. Cells were exposed to hypoxia (24 hours) or maintained under normoxic conditions in the presence or absence of doxycycline followed by harvesting of whole cell lysates and analysis of Cdc25A protein levels by Western blot. HIF-1α levels were analyzed to confirm induction of hypoxia. Cells exposed to UV irradiation were treated with 60 J/m^2 and incubated for 2 hours prior to harvesting of protein. The above Western blot is representative of three independent experiments (N=3).

3.2.7 SUPRESSION OF CDC25A MRNA LEVELS BY HYPOXIA

Regulation of Cdc25A under hypoxic conditions does not appear to be regulated by changes in protein stability mediated as a result of cell cycle checkpoint activation. There are reports that Cdc25A expression can be controlled by several mediators including c-Myc, which has been described to drive Cdc25A expression and p21, which has been shown to repress Cdc25A expression by displacing c-Myc (80, 81) and is essential for observed hypoxia-mediated loss of Cdc25A.

Cdc25A mRNA levels were analyzed by real time quantitative PCR and expression was normalized to β-actin. Analysis of mRNA levels was done using the comparative C_T method (208) and relative reaction amplification efficiencies were calculated to validate the analysis and reliability of the data.

Cdc25A mRNA levels were examined in WT and p21 -/- HCT116 cells after a 24 hour exposure to hypoxia and compared with mRNA levels in the same cells under normoxic conditions. Cdc25A mRNA levels were significantly reduced after 24 hours of hypoxia compared with normoxic WT cells (Figure 16A). However, in the p21 -/- HCT116 cells, no statistically significant change in Cdc25A mRNA levels was observed (Figure 16A). This finding was consistent with the requirement of p21 for loss of Cdc25A under hypoxic conditions. Cdc25B mRNA levels were analyzed in WT HCT116 to determine the specificity of the observed repression of Cdc25A mRNA and as expected there was no change in Cdc25B mRNA levels with hypoxic exposure compared with normoxic cells (Figure 16B).

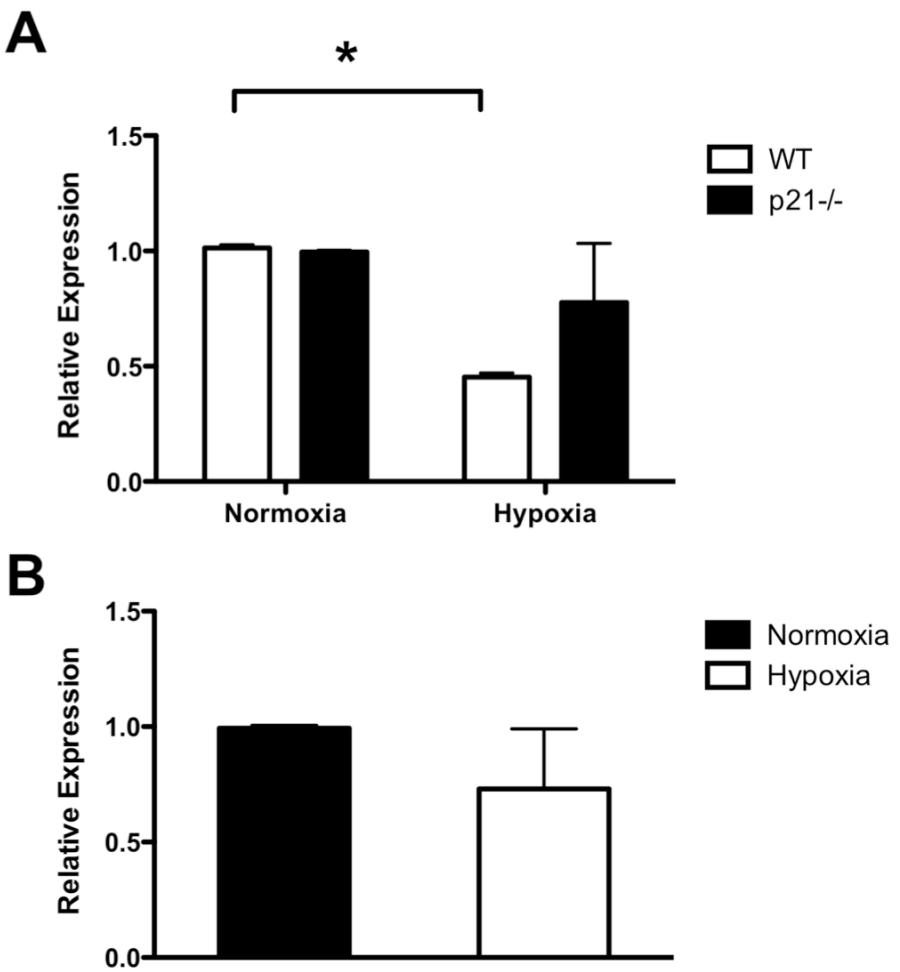

Figure 16: Hypoxia-mediated decrease in Cdc25A mRNA levels.

Total RNA was isolated from cells exposed to hypoxia (1% O_2) or maintained under normoxic (20% O_2) conditions. (A) Cdc25A and (B) Cdc25B expression levels were analyzed by quantitative real-time PCR. Expression was normalized to β-actin and relative expression determined by the Livak method. * = $P<0.05$; N=3.

3.2.8 MIR-21 AS A REGULATOR OF CDC25A IN CANCER CELLS UNDER HYPOXIC CONDITIONS

Cdc25A has been proposed as a target for miR-21. miR-21 has recently been shown to be upregulated in hypoxic cells although the consequences of elevated mir-21 are not established (173). To test the hypothesis that miR-21 may represent a novel regulator of Cdc25A under hypoxic conditions I examined the effect of hypoxia on Cdc25A protein levels in WT and miR-21 knock out (KO) cells. As can be seen in Figure 17, WT DLD1 cells have decreased Cdc25A protein levels after 24 hours of hypoxia whereas the DLD mir-21 KO cells did not exhibit a decrease in Cdc25A protein levels. This finding suggested that miR-21 was indeed a regulator of Cdc25A and was required for the suppression of Cdc25A by hypoxia.

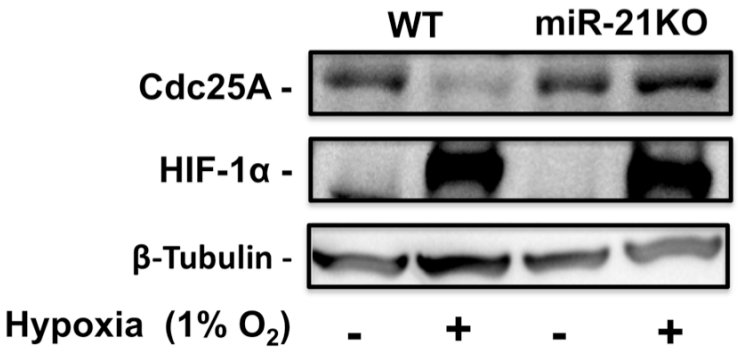

Figure 17: Hypoxia-mediated loss of Cdc25A in DLD1 cells requires miR-21.

WT and miR-21 KO cells were exposed to hypoxia (1% O_2) for 24 hours. Cells were lysed and whole-cell lysates were examined by Western blot for Cdc25A protein expression. Cdc25A protein levels in WT and miR-21 KO DLD1 cells under normoxic were used as control. The above Western blot is representative of three independent experiments (N=3).

miRs regulate their targets by degrading mRNAs. I analyzed Cdc25A mRNA levels to further explore the role of miR-21 as a regulator of Cdc25A under hypoxic conditions in WT and miR-21 KO DLD1 cells following exposure to hypoxia. WT cells were found to have significantly reduced Cdc25A mRNA levels compared to normoxic cells whereas the miR-21 KO cells had no detectable decrease in Cdc25A mRNA levels (Figure 18).

I performed real time quantitative PCR to ascertain if hypoxia was inducing an increase in miR-21 levels in HCT116 cells using primers specific for miR-21 and as a control the miR RNU-24. It was found that with a 24 hour exposure to hypoxia, miR-21 levels were increased 3-fold in the WT HCT116 cells whereas levels of the control miR RNU-24

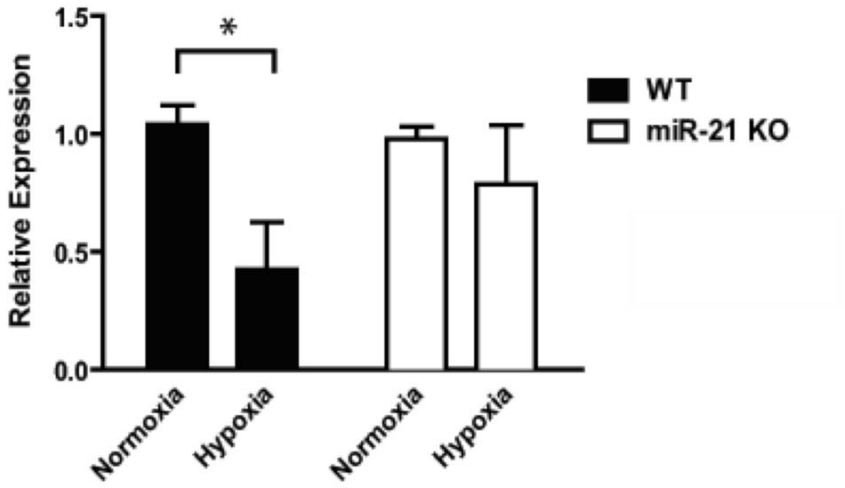

Figure 18: Decreased Cdc25A mRNA levels in WT but not miR-21 KO DLD1 cells.

DLD1 WT and DLD1 miR-21 KO cells were incubated under hypoxic conditions for 24 hours and mRNA levels were determined by quantitative real-time PCR. Expression was normalized to β-actin and relative expression determined by the Livak method. * = $P<0.05$; N=3.

remained unchanged by hypoxia (Figure 19). This finding is in agreement with the work of others and establishes a possible role for miR-21 in the hypoxic regulation of Cdc25A in two independent colon cancer cell lines.

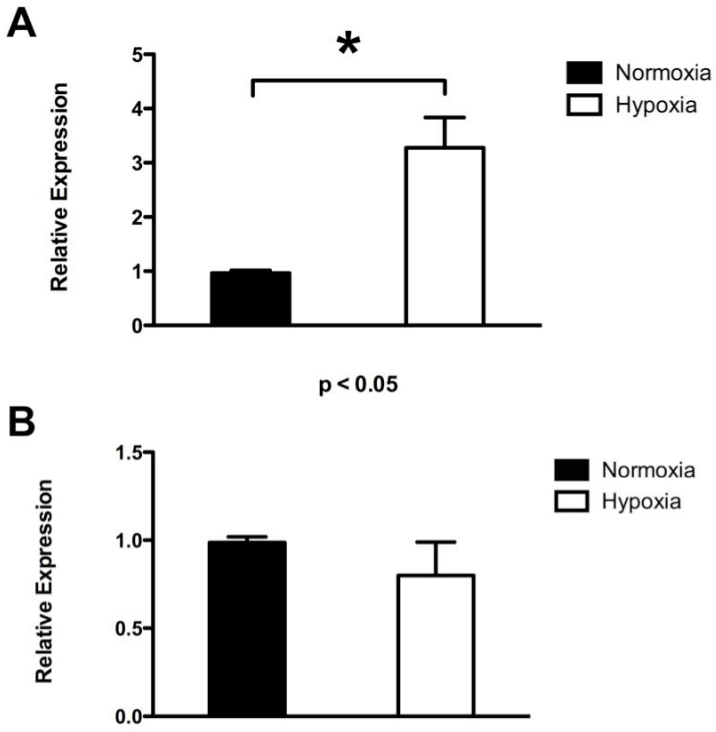

Figure 19: Increased levels of miR-21 in hypoxic HCT116 cells.

miR-21 levels were determined using a TaqMan real-time PCR microRNA assay. WT HCT116 cells were cultured under normoxic conditions or hypoxic conditions for 24 hours followed by harvesting of total RNA using the *mir*VANA RNA isolation assay. miR-21 (A) and RNU-24 (B) were analyzed using TaqMan probes by quantitative real-time PCR and relative expression was analyzed by the Livak method. * = $p < 0.05$.

To determine if restoration of miR-21 in miR-21 null could restore the phenotype of hypoxia-mediated Cdc25A loss, I transfected miR-21 KO DLD1 cells with a pre-miR-21 complex or a pre-miR-224 complex as control and then exposed these cells to hypoxia. As can be seen in Figure 20, cells transfected with the control pre-miR-224 did not repress Cdc25A under hypoxic conditions but cells transfected with pre-miR-21 had decreased Cdc25A levels under both hypoxic and normoxic conditions. These results indicate that reintroduction of miR-21 into cells restores the cells ability to repress Cdc25A under hypoxic conditions.

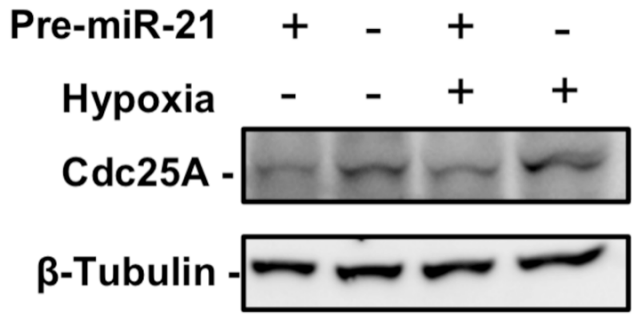

Figure 20: Expression of miR-21 in mir-21 KO cells restores cdc25A-loss phenotype.

DLD1 miR-21 KO cells were transfected with a pre-miR-21 complex followed by exposure to hypoxia for 24 hours or maintained under normoxic conditions for 24 hours. Control cells were transfected with pre-miR-224 to control for non-specific effects of transfection. Cdc25A levels were determined by Western blotting. The above western blot is representative of three independent experiments (N=3).

3.3 DISCUSSION

Numerous studies have described the modulation of the cell cycle in primary and immortalized cells exposed to hypoxia (141, 143, 148, 149, 155, 165-167, 169-171). The majority of studies have focused on severe hypoxic conditions (<0.1% oxygen) and have proposed that modulation of the cell cycle under hypoxic conditions is mediated by checkpoint activation in response to DNA damage and aberrant structures located on DNA such as stalled replication forks. Although activation of ATM, ATR and their downstream mediators has been described in response to hypoxia, the role of the Cdc25 phosphatases has not been extensively studied. In the present study, Cdc25A but not Cdc25B or Cdc25C was repressed under hypoxic conditions.

I developed a cell culture model of hypoxia in colon cancer cells to address the issue of hypoxia-mediated regulation of Cdc25A. HCT116 cells exposed to 1% oxygen for up to 48 hours resulted in a significant decrease in pO_2 levels in the cell culture medium and HIF-1α levels increased within 1 hour of hypoxic exposure and remained elevated for over 24 hours. Moderate hypoxia caused a reversible decrease in Cdc25A protein levels in the colon cancer cell lines HCT116 and DLD1. The observed repression was specific to Cdc25A as the Cdc25B and Cdc25C phosphatase levels were not affected by hypoxia. This finding indicates that repression of Cdc25A in response to hypoxia is not the result of a global suppression in protein translation or gene expression that has been reported in cells exposed to cellular stresses including hypoxia (209).

This study has shed some light on the mechanism of regulation of Cdc25A under hypoxic conditions. Previous studies have described the regulation of Cdc25A through activation of checkpoints resulting in the phosphorylation, polyubiquitination and degradation of the phosphatase. This study demonstrates that colon cancer cells exposed to moderate hypoxia did not activate cell cycle checkpoints as indicated by the absence of Chk1 and Chk2 activation in addition to the fact that Cdc25A loss is not suppressed in Chk2 -/- cells or following pharmacological inhibition of the ATR-Chk1 pathway by caffeine. The absence of checkpoint activation was not entirely unexpected given that a number of studies have failed to show that hypoxia induces DNA damage but instead causes replication fork stalling (166-169). The cells used in this study were however, capable of inducing degradation of Cdc25A following induction of DNA damage with UV irradiation, indicative of responsive checkpoint pathways. Others have shown that under the same conditions no significant DNA damage is detected until cells are returned to normoxic conditions upon which substantial induction of DNA damage is observed (148). Reoxygenation-induced DNA damage can be induced by reactive oxygen species, which are dramatically increased in reoxygenated cells. This phenomenon may also account for the delayed return of Cdc25A upon reoxygenation and slightly decreased levels at 24 hours post reoxygenation.

p21 and p53 play an important role in the cellular response to genotoxic stress. Their involvement in regulation of Cdc25A and the cell cycle remains to be fully characterized. Isogenic cell lines lacking p21 (p21 -/-) or p53 (p53 -/-) were cultured under hypoxic conditions to test the involvement of p21 and p53. The absence of p53 did not affect the loss of Cdc25A but the absence of p21 abolished the hypoxia-mediated loss of Cdc25A.

p21 has been shown to repress Cdc25A gene expression by displacing c-Myc from the Cdc25A promoter (80, 81) in response to genotoxic stress and has been shown to be upregulated under hypoxic conditions (147). It is therefore possible that p21 may regulate the expression of Cdc25A under hypoxic conditions in addition to genotoxic stress.

Given the absence of checkpoint activation after exposure to hypoxia and no detectable increase in Cdc25A protein degradation as determined by ectopic expression of HA-Cdc25A, the regulation Cdc25A mRNA levels was examined. WT HCT116 cells have significantly decreased Cdc25A mRNA levels after 24 hours of hypoxia whereas Cdc25B mRNA levels remained unchanged again demonstrating that the loss of Cdc25A is a specific process and not part of the global repression of protein translation and gene expression in response to environmental stress (210). It is also interesting to note that despite the dependence on p21 for Cdc25A repression, p21 itself did not appear to be increased by hypoxia. In response to DNA damage by agents such as SN38 and doxorubicin, p21 protein levels are elevated and Cdc25A mRNA levels are decreased (80). The findings in this study are in agreement with the findings of others that p21 protein levels are not increased in response to hypoxia (149). It remains to be determined if repression of Cdc25A by p21 requires and increase in the protein levels of the CdkI as well as how hypoxic exposure leads to the p21-dependent decrease in Cdc25A. One potential explanation for a hypoxia-mediated change in p21 activity in the absence of detectable protein upregulation is that changes in the redox state of cells can result in changes in p21 activity. Indeed, p21 has been shown to be *S*-nitrosylated by nitric oxide resulting in increased p21 activity and downstream signaling (211, 212). The redox-mediated post transcriptional regulation of p21 levels has also been described (213). It is

therefore possible that under hypoxic conditions, which is paradoxically associated with elevated levels of reactive oxygen species and oxidative stress (214), p21 activity might be increased in the absence of increased p21 transcription. The involvement of p21 in the regulation of the cell cycle is discussed further in Chapter 5. My study represents the first report of p21 and miR-21 acting to decrease Cdc25A mRNA levels under hypoxic conditions. To date the regulation of Cdc25A in response to extracellular stimuli and potential genotoxic stress has focused primarily on increased protein degradation as a consequence of checkpoint activation. The kinetics Cdc25A protein loss were consistent with suppression of mRNA levels, which would require substantially more time than the polyubiquitination and proteasomal degradation of Cdc25A in response to checkpoint activation given the need for upregulation and processing of miR-21 and a reduction in Cdc25A transcription by p21. The regulation of Cdc25A by miR-21 represents a novel level of regulation in response to extracellular stimuli that may allow for cell cycle regulation. Although the existence of a relationship between p21 and miR-21 remains to be established, it is possible that these mediators of Cdc25A gene expression may function in concord to reduce levels of Cdc25A at the onset of hypoxia and subsequently maintain suppression of the phosphatase until normoxic conditions are restored. Indeed, it has been proposed that whereas p21 is not required for the induction of cell cycle arrest in response to hypoxia, it may be responsible for the maintenance of cell cycle arrest and for preventing premature cell cycle resumption (149). By reducing Cdc25A, hypoxic cells may be protected from the potential genetic instability that would arise if DNA replications were allowed to proceed under hypoxic conditions increasing the likelihood of replication fork stalling (215).

The adaptation of cells to changes in their surroundings is an important feature in the maintenance of genomic stability and ultimately survival of the organism. But the regulation of Cdc25A by hypoxia represents a double-edged sword: the sustained repression of Cdc25A under hypoxic conditions may protect cancer cells from the insult of chemotherapeutic and radiation therapy by preventing cells from actively cycling and thus decreasing their susceptibility to DNA damaging agents. Chapter 5 will explore the functional consequences of Cdc25A suppression on cell cycle progression to gain further perspective on the regulation of the cell cycle by hypoxia.

4 IDENTIFICATION OF THE ROLE OF HIF-1α IN REGULATION OF CDC25A BY HYPOXIA

4.1 INTRODUCTION

Cells undergo a variety of cellular responses when placed under hypoxic conditions. These responses include the activation of signaling pathways that regulate cell proliferation, angiogenesis and cell death. Cancers cells have harnessed these pathways allowing them to survive under hypoxic conditions. HIF-1α is an important mediator of the cellular response to hypoxia, which has been hypothesized to regulate Cdc25A in human cancer cell lines (82). A possible role for HIF-1α is supported by evidence that HIF-1α can actually disrupt expression of c-Myc driven genes and also is required for cell cycle arrest (141, 172). Thus, Cdc25A may represent a key piece of the HIF-1-α-mediated response to hypoxia in cancer cells. The following study examined the requirement for HIF-1α in the repression of Cdc25A and the induction of hypoxia-mediated cell cycle arrest in the colon cancer cell line HCT116.

4.2 RESULTS

4.2.1 THE ROLE OF HIF-1α IN HYPOXIA-MEDIATED DOWNREGULATION OF CDC25A

Figures 7 and 17 demonstrate that HIF-1α was rapidly and robustly stabilized in response to hypoxia in two independent colon cancer cell lines (HCT116 and DLD1). An isogenic HCT116 cell line lacking HIF-1α (HCT116 HIF-1α -/-) was used to determine if the presence of HIF-1α was required for the reduction in Cdc25A phosphatase levels.

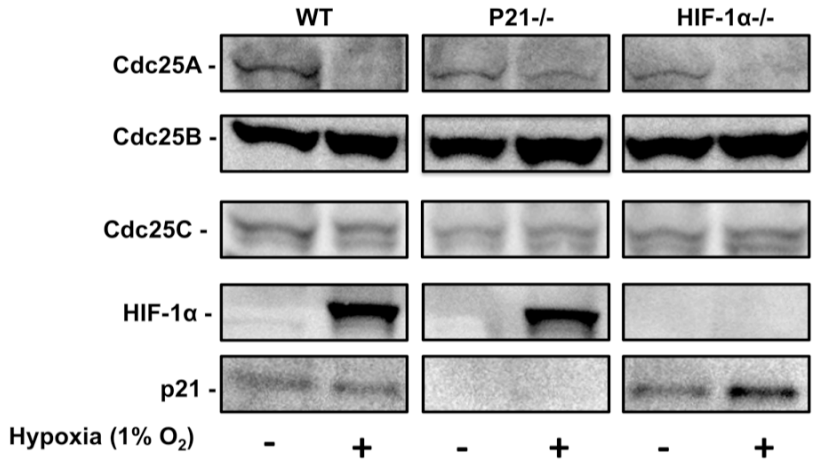

Figure 21: p21 but not HIF-1α is required for hypoxia-mediated loss of Cdc25A.

p21-/- HCT116, HIF-1α -/- HCT116 and WT HCT116 cells were cultured under normoxic or hypoxic conditions for 24 hours after which Cdc25A, Cdc25B, Cdc25C, HIF-1α, p21 and β-tubulin levels were determined by Western blotting. Western blots are representative of three independent experiments (N=3).

These cells were exposed to hypoxic conditions for 24 hours followed by analysis of Cdc25A levels by Western blotting. As can be seen in Figure 21, the absence of HIF-1α expression did not abrogate the hypoxia-mediated loss of Cdc25A suggesting that HIF-1α is not required for suppression of Cdc25A by hypoxia. Induction of HIF-1α in HCT116 cells by the hypoxia-mimetic cobalt chloride (Figure 22) resulted in an increase in HIF-1α levels but did not decrease Cdc25A indicating that HIF-1α induction alone was insufficient to recapitulate the phenotype observed in hypoxic cells.

Interestingly, hypoxia caused an increase in p21 levels in HIF-1α -/- HCT116 cells. This was particularly interesting given that it has been reported that HIF-1α can actually increase p21 expression (172).

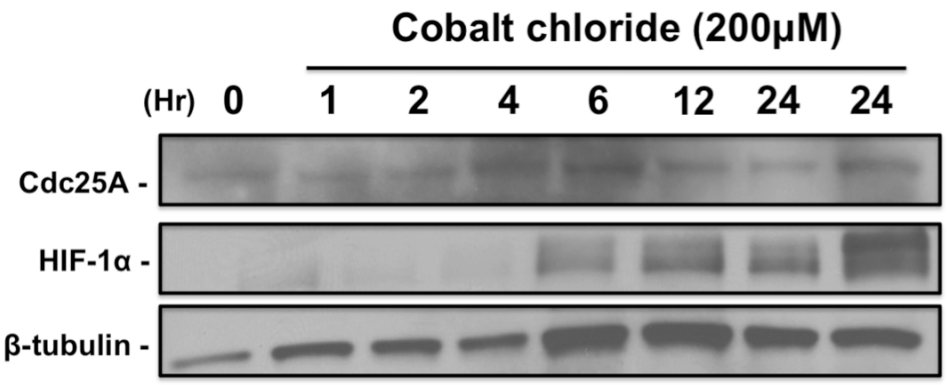

Figure 22: Cobalt chloride treatment does not suppress Cdc25A protein.

HCT116 cells were treated with 200 μM cobalt chloride for 0 to 24 hours followed by harvesting of whole-cell lysates. Cdc25A and HIF-1α protein levels were determined by Western blotting. β-tubulin levels were used as a loading control. Western blots are representative of three independent experiments (N=3).

4.3 DISCUSSION

HIF-1α is a critical mediator of the cellular response and the adaptation of cells to low oxygen conditions. HIF-1α levels are increased in a number of human cancers and elevated HIF-1α levels are associated with disease progression (216, 217). Intriguingly, these studies have also correlated high levels of HIF-1α with increased cell proliferation in a number of human cancers including colon cancer (217). It was therefore conceivable that the repression of Cdc25A observed in hypoxic cells may be dependent on HIF-1.

In this study I confirmed that HIF-1α was not required for the loss of Cdc25A. This study has also shown that Cdc25A was not affected by hypoxia-mimetics, such as cobalt chloride, which act to mimic the stabilizing effect of hypoxia on HIF-1α. In other words cells must be placed under *bone fide* hypoxic conditions in order to repress Cdc25A. The findings in this study suggest that the regulation of Cdc25A under hypoxic conditions is triggered by a hypoxia sensing mechanism that is independent of HIF-1α. It is now recognized that a number of mechanisms with different sensitivities may effectively act as oxygen sensors and coexist in the same cell (218). These mechanisms can broadly classified into those dependent on perturbations in mitochondrial function and those relying on the synthesis or degradation of mediators. In the model used in this study it is more likely that the latter sensors are involved given the notion that mitochondrial function is not affected by moderate hypoxia (1% oxygen). The redox state of the cell as well as the production of reactive oxygen species have been described to function as oxygen sensors (218). A recent study has described the regulation of Cdc25A by nitrosative stress has demonstrated that these entities may be able to play a role in the

regulation of Cdc25A under hypoxic conditions. It remains to be determined if moderate hypoxia used in this study elicits changes in the redox state of cells and if reactive oxygen species are generated and ultimately if these sensors are linked to p21 and miR-21 to drive repression of Cdc25A. Another possible explanation for the HIF-1α-independent regulation of Cdc25A under hypoxic conditions is through inhibition of oxygen-sensitive prolyl hydroxylases. Enzymatic hydroxylation of residues within proteins is a novel mechanism of signal transduction and given the dependence of these enzymes on oxygen, hydroxylation, or the absence of, could modulate the activity of signaling pathways under hypoxic conditions. Although prolyl hydroxylases and their functions in HIF-1α stabilization have been extensively studied (219-221), they also have functions in other signaling pathways that remain to be fully characterized. For example, hydroxylases are involved in the regulation of IκB, an important mediator of the NF-κB pathway, and in the modulation of RNA polymerase II (222). It therefore seems likely that protein hydroxylation may play a role in HIF-1α independent responses to hypoxia and may be involved in the repression of Cdc25A by p21 and miR-21. Further studies are needed to determine the identity of the oxygen sensing mechanism responsible for increasing miR-21 and driving miR-21 and p21 mediated repression of Cdc25A under hypoxic conditions.

5 HYPOXIA-MEDIATED CELL CYCLE ARREST

5.1 INTRODUCTION

Cdc25A is a critical regulator of the mammalian cell cycle and is required for cell cycle progression and mitosis. Hypoxia is an important aspect of the tumor microenvironment and elicits a plethora of changes in cellular behavior affecting amongst other things cellular metabolism, proliferation and apoptosis. The present study has established that Cdc25A mRNA levels are decreased in a p21 and miR-21 dependent fashion in response to hypoxia. The induction of cell cycle arrest in response to loss of Cdc25A is well established (45, 71, 223). The aim of this study was to test the hypothesis that the observed hypoxia-mediated repression of Cdc25A and respective actions of p21 and miR-21 were also required for hypoxia-mediated cell cycle arrest.

5.2 RESULTS

5.2.1 INDUCTION OF CELL CYCLE ARREST BY HYPOXIA

Extended periods of hypoxia (24-48 hours) were shown to inhibit proliferation of HCT116 cells without induction of cell death (Figure 23). Visual inspection of cells and staining with Trypan blue did not indicate an increase in non-viable cells between normoxic and hypoxic cultures. The inhibition of cell proliferation and repression of Cdc25A are temporally similar, occurring within about 24 hours after exposure to hypoxia and warranting an analysis of the cell cycle distribution in these cell populations. Incubation of HCT116 cells under hypoxic conditions for 24 and 48 hours resulted in a significant decrease in the late G_2/early M phase population (PH3-positive cells) (Figure 24A). Examination of the DNA content of these cells reveled an increase in the S- phase portion of the population along with decreased mitotic cells suggesting that cell cycle arrest is induced in response to hypoxia (Figure 24B).

I performed mitotic trapping studies to determine if HCT116 cells incubated under hypoxic conditions for 24 hours and then treated with nocodazole for an additional 24 hours were able to reach mitosis or if they are becoming arrested at an earlier phase of the cell cycle. These studies confirm that cell cycle arrest is being induced rather than a delay in cell cycle progression. A delay in cell cycle progression, in particular S-phase, can occur under conditions limiting the availability of deoxyribonucleotides or by DNA lesions hindering replication fork progression (224). Hypoxia has been shown cause both decreased deoxyribonucleotide supplies and replication fork stalling. Normoxic cells

were trapped in the G_2/M phase (Figure 25, white bars), hypoxic cells on the other hand, did not accumulate in early mitosis, indicative that these cells are arresting at an earlier point in the cell cycle either in G_1, S or early G_2 phase (Figure 25, black bars). These findings indicate that hypoxic cells are arresting prior to the addition of nocodazole and because of this, no accumulation of cells in G_2/M is observed. Normoxic cells are still actively cycling at 24 hours (24 hours of normoxia followed by 24 hours in the presence of nocodazole) and therefore accumulate in the G_2/M phase in the presence of the mitotic inhibitor nocodazole.

These data demonstrate that under hypoxic conditions, HCT116 cells undergo cell cycle arrest with similar kinetics as the observed loss of Cdc25A protein and in agreement with the consequence of Cdc25A suppression.

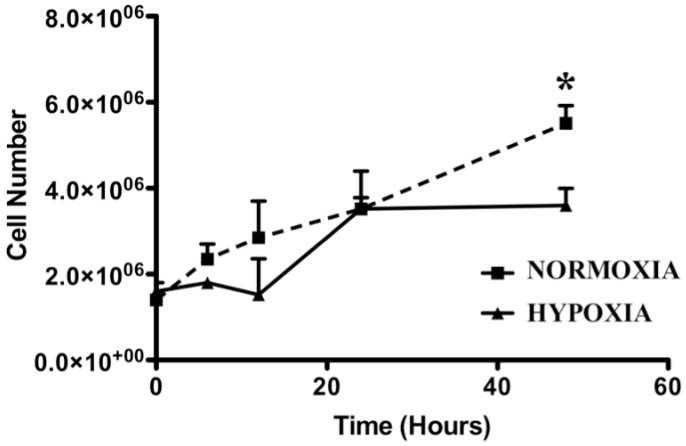

Figure 23: Cell proliferation is inhibited by hypoxia.

Time course profile of HCT116 cell growth under hypoxic and normoxic conditions. HCT116 cells were counted after indicated periods of time under normoxic (■) or hypoxic conditions (▲). Data is presented as the mean ± SEM (n=3). * = $P < 0.05$.

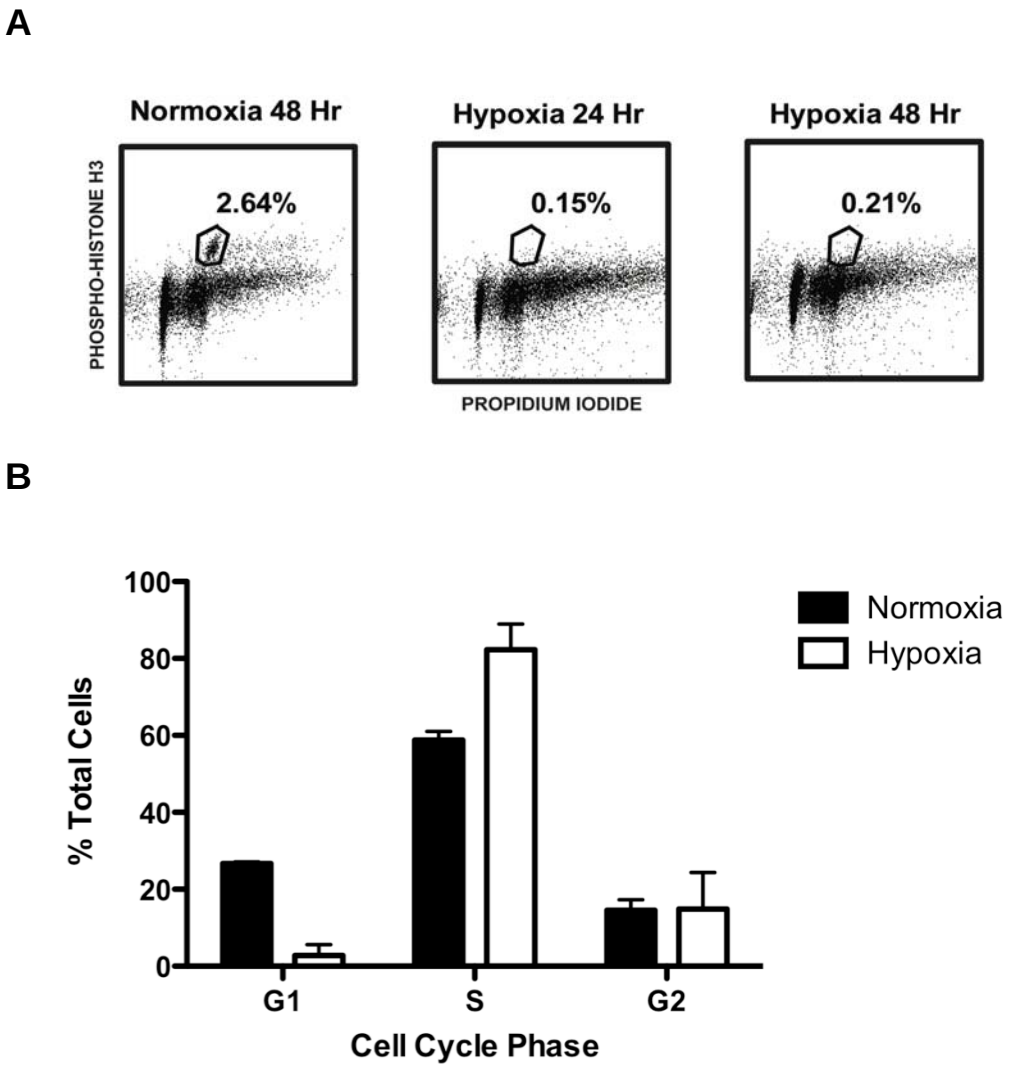

Figure 24: Loss of the mitotic fraction of cells and S-phase arrest with hypoxic exposure.

HCT116 Cells were cultured under hypoxic conditions for 24 and 48 hours and stained with phospho-histone H3 and PI. A) Dot plots indicating DNA content and percentage of mitotic cells (phospho-histone H3 positive cells in hexagon). B) Cell cycle distribution under normoxic and hypoxic conditions after 48 hours based on DNA content. Cell cycle distribution was determined using ModFit software. Data are presented as mean ± SEM bars (N=3).

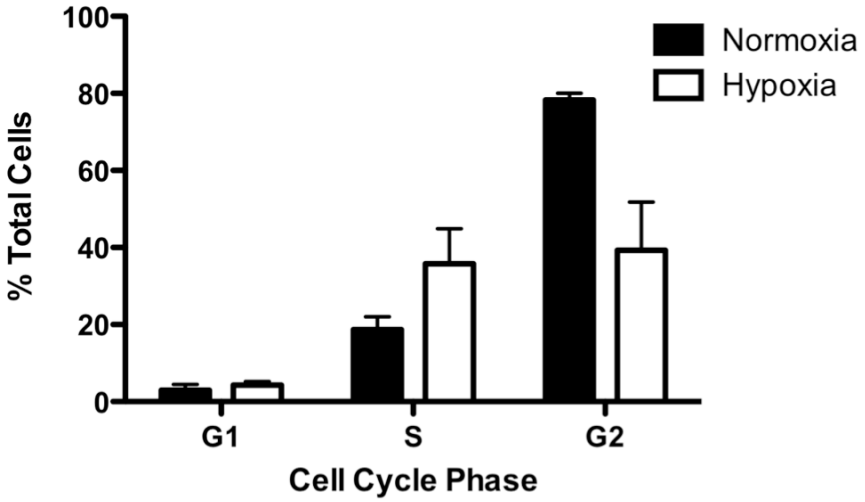

Figure 25: Induction of S-phase arrest in hypoxic cells.

HCT116 cells were subjected to mitotic trapping. Cells were exposed to hypoxia for 24 hours followed by an additional 24 hours in the presence or absence of 100 ng/mL nocodazole. Cells were harvested and stained with a phospho-histone H3 antibody and PI followed by analysis by flow cytometry. Data are presented as mean ± SEM bars. Cell cycle distribution was determined using ModFit software (N=3).

Unsynchronized HCT116 cells were incubated under hypoxic conditions for 48 hours and then stained with bromodeoxyuridine (BrdU) and 7-actinomycin D (7-AAD) to establish if hypoxic cells are arresting in S-phase. BrdU is taken up by cells actively undergoing DNA synthesis, and therefore in S-phase, and 7-AAD stains DNA (Figure 27). Normoxic cell populations were found to have about 50% of cells actively going through S-phase, which under hypoxic conditions was reduced by approximately 40% indicative of cells undergoing S-phase arrest.

p53 is a modulator of the cellular response to stress and DNA damage. The absence of p53 in HCT116 cells did not affect hypoxia-mediated repression of Cdc25A and as can

be seen in Figure 26, p53 -/- HCT116 cells exhibited a similar phenotype to the isogenic WT HCT116 cells (Figure 24A) indicative that p53 was also not required in the induction of cell cycle arrest in response to hypoxia. HCT116 cells were treated with hydroxyurea, an agent that causes S-phase arrest via the depletion of deoxyribonucleotides and induction of double strand breaks, to confirm that HCT116 cells are in fact capable of undergoing S-phase arrest. As expected an almost complete loss BrdU positive cells was observed indicating that these cells can undergo S-phase arrest (Figure 27). Similar experiments were performed in p21 -/- HCT116 cells revealing that like WT cells, hydroxyurea was capable of inducing S-phase arrest (Figure 27).

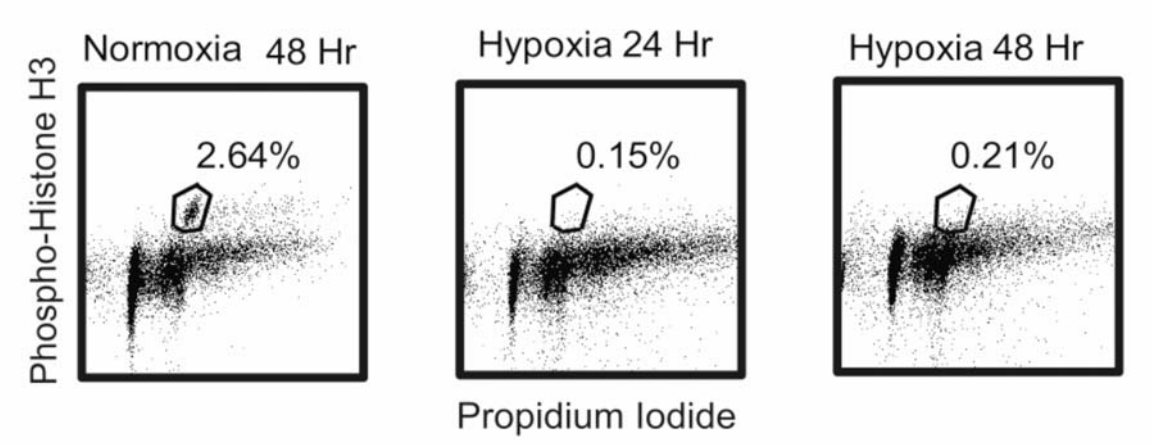

Figure 26: p53 is not required for hypoxia-mediated cell cycle arrest.

p53 -/- HCT116 cells were exposed to hypoxia for 24 and 48 hours or maintained under normoxic conditions for 48 hours and stained with an anti-phospho-histone H3 antibody and propidium iodide for the detection and evaluation of mitotic cells (contained in the hexagon) and DNA content respectively. (N=3).

However, hypoxia did not elicit a significant reduction in S-phase cells after 48 hours in p21-/- HCT116 cells indicating that p21 was required for down regulation of Cdc25A and also for induction of cell cycle arrest. It must be noted that p21-/- cells have a proliferative defect that slows down the cell cycle leading to a reduced population size of cells undergoing S-phase (225).

Taken together these data indicated that moderate hypoxia induced p21-dependent cell cycle arrest and that this arrest appeared to block cells in S-phase as would be expected from Cdc25A suppression.

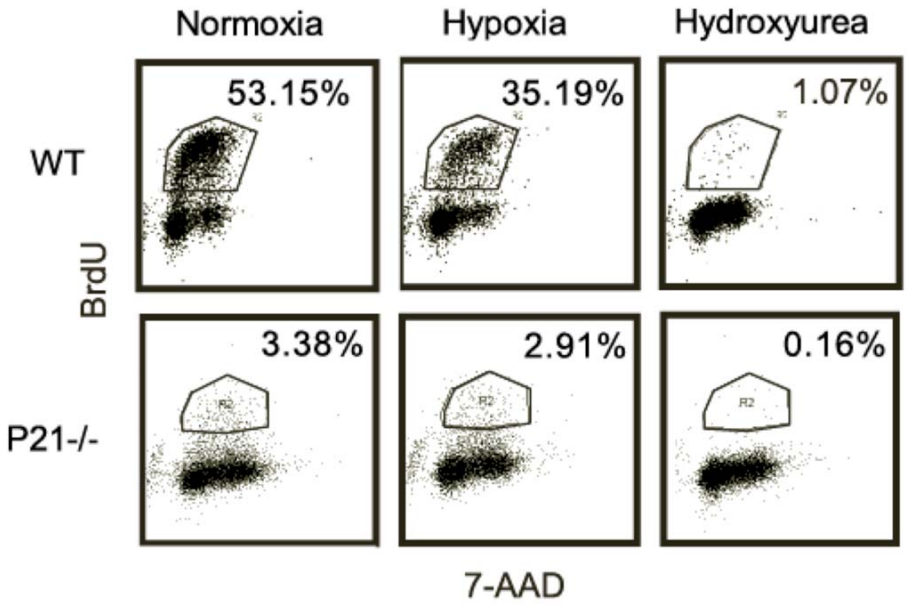

Figure 27: p21-dependent induction of S-phase arrest.

BrdU staining of WT and p21-/- HCT116 cells exposed to 48 hours hypoxia or 1 mM hydroxyurea as a control for induction of S-phase arrest. BrdU positive cells are indicated in the polygons. Percentages indicate the proportion of cells in S-phase (BrdU positive). The dot plots are representative of three separate experiments (N=3).

5.2.2 REQUIREMENT OF HIF-α FOR HYPOXIA-MEDIATED CELL CYCLE ARREST

The induction of cell cycle arrest in response to hypoxia has been discussed in the Chapter 1. One study concluded that HIF-1α is required for cell cycle arrest (141). However, this study was conducted in non-transformed cells and the same dependence on HIF-1α may not exist in cancer cells. WT and HIF-1α -/- cells were exposed to hypoxia for 48 hours and then stained with an anti-phospho-histone H3 (PH3) antibody to detect mitotic cells and propidium iodide (PI) to determine DNA content providing an indication of whether cells are in the G_1 or G_2 phase of the cell cycle. As can be seen in Figure 28, HIF-1α -/- cells did not arrest under hypoxic conditions as indicated by the presence of mitotic cells in the hypoxic population as well as no apparent change in the distribution of cells based on DNA content compared with WT cells. These findings demonstrate that HIF-1α was required for cell cycle arrest under hypoxic conditions in colon cancer cells.

5.2.3 MIR-21 AS A REGULATOR OF HYPOXIA MEDIATED CELL CYCLE ARREST

mir-21 has been established as a regulator of Cdc25A under hypoxic conditions and has been shown to be upregulated in hypoxic HCT116 cells (Figure 19). The role of miR-21 dependent Cdc25A suppression on the cell cycle was determined using DLD1 WT and

DLD1 miR-21 KO cells that were exposed to hypoxia for 48 hours and then analyzed for BrdU incorporation and DNA content (7-AAD staining) (Figure 29). WT cells exhibited a hypoxia-dependent reduction in BrdU incorporation indicative of S-phase arrest (approximately 56%). This effect was comparable to that observed in HCT116 cells exposed to hypoxia for 48 hours (Figure 27).

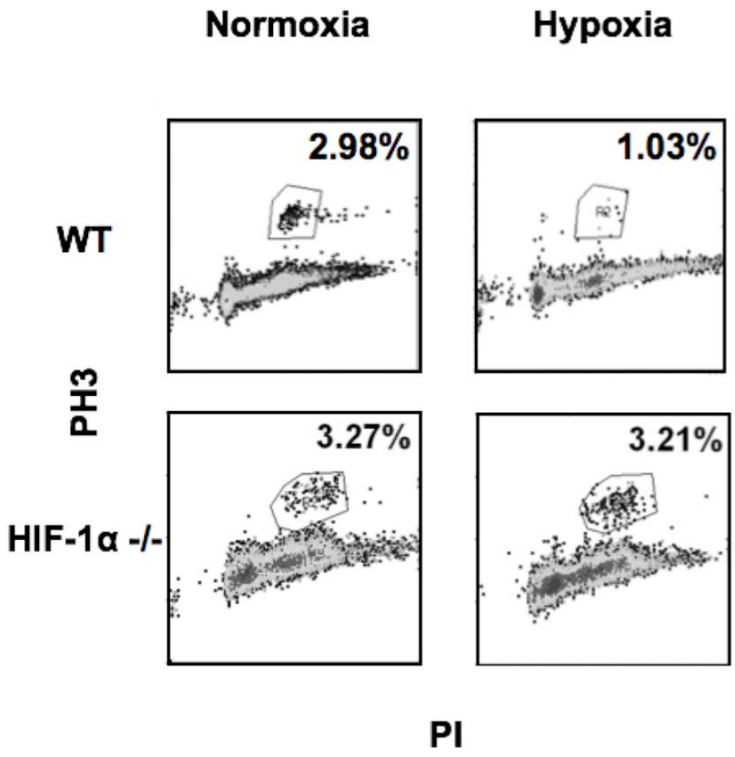

Figure 28: HIF-1α -/- cells do not undergo hypoxia-induced cell cycle arrest.

WT and HIF-1α -/- HCT116 cells were exposed to hypoxia for 48 hours or maintained under normoxic conditions for 48 hours prior to staining with anti-phospho-histone H3 (PH3) and propidium iodide (PI). 20,000 cells were counted for each condition by flow cytometry. Percentages indicate the proportion of cells contained in the polygon gates representing cells in mitosis (PH3 positive) contained in the polygons. The experiment shown is representative of three independent experiments (N=3).

The miR-21 KO cells on the contrary did not exhibit a significant change in BrdU incorporation following hypoxic exposure (approximately 8% decrease in S-phase cells). These data indicated that miR-21 was not only required for repression of Cdc25A but also for induction of S-phase arrest in hypoxic cells.

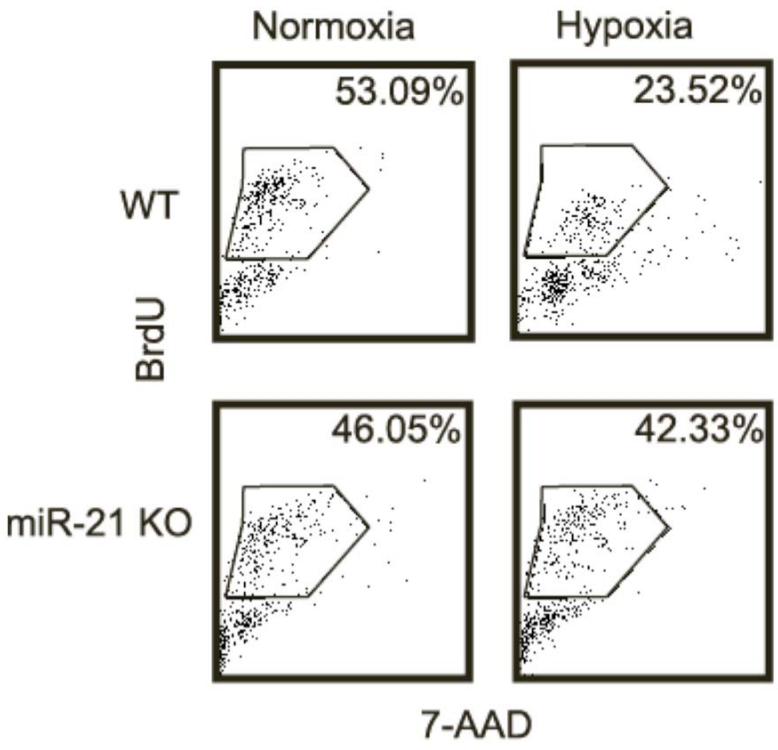

Figure 29: Hypoxia induces miR-21 dependent S-phase arrest.

DLD1 WT and miR-21 KO cells were cultured under hypoxic conditions for 48 hours and then stained with BrdU and 7-AAD and analyzed by flow cytometry. BrdU positive cells are indicated in the polygons. Percentages indicate the proportion of cells in S-phase (BrdU positive). The dot plots are representative of three separate experiments (N=3).

5.3 DISCUSSION

Loss of Cdc25A in response to DNA damage results in S-phase cell cycle arrest. In fact, Cdc25A has been shown to be rate limiting for entry into S-phase by activation of CdK2 (45, 223). In the present study, it has been shown that repression of Cdc25A mRNA levels by hypoxia elicited a p21- and miR-21-dependent cell S-phase cell cycle arrest. Hypoxia has for some time been known to cause cell cycle arrest but the mechanisms by which cells sense hypoxia and subsequently relay signals to undergo cell cycle arrest are poorly understood. Severe hypoxia causes rapid S-phase arrest as a result of checkpoint activation leading to the engagement of checkpoint kinases (143, 148, 149, 165). The role of Cdc25A in cell cycle arrest induced by severe or moderate hypoxic conditions has not been reported. The data shown herein demonstrated that Cdc25A could be modulated at the level of gene expression and mRNA stability by p21 and miR-21 respectively and that suppression of Cdc25A resulted in S-phase arrest in response to moderate hypoxia.

This study provides a possible mechanism for the induction of S-phase cell cycle arrest through the repression of Cdc25A, resulting in inactivation of Cdk/Cyclin complexes independently of checkpoint activation. The role of Cdc25A in modulating the cell cycle has been extensively studied (71, 75, 226-228) with the regulation of Cdc25A in response to DNA damage and other cellular stresses being mediated by increased Cdc25A degradation following phosphorylation of the phosphatase and through decreased gene expression via transcriptional repression (80, 81). The degradation of Cdc25A protein occurring very rapidly and the gene repression being a more delayed sustained response.

The dependence of hypoxia-induced S-phase arrest on p21 may be due to transcriptional repression of Cdc25A or due to the action of p21 as inhibitor of Cdk2/Cyclin E. Repression of Cdc25A mRNA levels by p21 has been described in response to DNA damage and has been shown to occur by direct binding to the *Cdc25A* promoter (80, 81). The latter study has described this mechanism to be dependent on p53 in contrast to the present study where p53 -/- HCT116 cells arrested in a similar fashion to the WT HCT116 cells. The induction of cell cycle arrest by direct inhibition of Cdk2/Cyclin E is less likely given that p21 levels were not increased in HCT116 cells following induction of hypoxia for 24 hours. Previous studies have shown that p21 levels are increased in response to DNA damaging agents by p53-dependent and -independent mechanisms leading to inhibition of cell cycle progression by Cdk2 inhibition and transcriptional repression (80). In the present study, no detectable increases in p21 protein levels were observed under hypoxic conditions, consistent with the findings of others (149). This raises the question as to whether the transcriptional repression capability of p21 requires an increase in protein levels or if basal p21 levels under hypoxic conditions can induce transcriptional repression? It has been suggested that p21 may not be required for the initial induction of cell cycle arrest but instead for the maintenance of arrest and prevention of cell cycle resumption (149). Testing this hypothesis will yield important information into the mechanisms of hypoxia-mediated cell cycle arrest.

HIF-1α regulates the expression of a large number of hypoxia responsive genes but is also able to repress the expression of genes involved in regulation of the cell cycle such as p21 (172). Under hypoxic conditions, HIF-1α can counteract c-Myc repression of p21, thus increasing levels of the CdkI and inducing G_1/S cell cycle arrest. In contrast to the

previous findings, the present study has shown that HIF-1α was required for induction of cell cycle arrest without a detectable increase in p21(Figure 21). The absence of an increase in p21 may be due to the fact that p21 levels were examined after 24 hours and a hypoxia-mediated increase in p21 may occur earlier. Another explanation is that the HCT116 cells used in this study are not completely isogenic and the p21 pathway may have been altered. This notion might also explain why cells lacking HIF-1α had increased p21 under hypoxic conditions, contrary to what would be expected if HIF-1α is responsible for increasing p21. It has also been suggested that alterations in the cell lines used in this study may preclude a robust induction of p21. Indeed HCT116 cells have an altered p21 response even upon induction of DNA damage (229). Further investigation into the non-transcription effects of HIF-1α and its relationship with p21 are warranted. Interestingly, the role of HIF-1α in the present study initially appears to be contrary to notion that HIF-1α promotes tumor growth.

Hypoxia-inducible genes are known to regulate multiple biological pathways including cell proliferation, angiogenesis, cellular metabolism, apoptosis, immortalization and migration (125). Many known oncogenic pathways overlap with hypoxia-induced pathways and cancer cells may take advantage of these pathways during tumorigenesis. Whereas a number of the pathways activated by HIF-1α promote tumor growth by triggering cell proliferation and resistance to apoptosis, the response to hypoxia observed in the present study does not appear to directly promote tumorigenesis although the inhibition of cell cycle progression under hypoxic conditions may represent a protective mechanism for cells in order to avoid death and resist DNA damage-inducing agents. More research is needed to reconcile the seemingly paradoxical roles of HIF-1α in

cancer. One possibility is that in some of the studies mentioned above, HIF-1α overexpression was an important factor in the establishment of tumorigenic phenotypes whereas in the model used in this study, HIF-1α levels were transiently increased by hypoxia but the transcription factor was not overexpressed by ectopic expression as has been the case in a number of studies. The correlation between HIF-1α levels and tumorigenicity raises the question as to whether HIF-1α leads to more aggressive tumors or if aggressive tumors generate higher levels of HIF-1α?

The ability to survive under hypoxic conditions distinguishes tumor cells from normal cells. Both normal and transformed cells display a wide array of hypoxic responses dependent and independent of HIF-1α but the exact pathways involved in each case remain to be determined. The regulation of Cdc25A under hypoxic conditions does not require HIF-1α but the subsequent cell cycle arrest is abrogated in cells lacking of HIF-1α. These findings suggest that HIF-1α is contributing to hypoxia-mediated cell cycle arrest by a mechanism that may compensate for the loss of Cdc25A such as increased expression of the kinases involved in inhibitory phosphorylations of Cdks. In addition, it is possible that HIF-1α is causing the inhibition of Cdk2 independently of Cdc25A resulting in a similar phenotype of cell cycle arrest. This data reinforces the importance of HIF-1α in the cellular response to hypoxia and is in agreement with the concept that inhibiting HIF-1α in tumor cells and forcing cells to cycle in the presence of DNA damaging agents may represent an attractive target for cancer therapy (125, 230).

The recent discovery of a novel method of Cdc25A regulation at the level of translation in response to nitrosative stress (231) indicates that proteasome-mediated degradation of Cdc25A and decreased gene expression are not the sole mechanisms of Cdc25A

regulation in response to cellular stresses. This study presents evidence for yet another novel mechanism of regulation of Cdc25A by miR-21 in response to hypoxic stress. It is proposed that miR-21 is required not only for decreased Cdc25A mRNA and protein levels but also for S-phase replication arrest. miR-21 is overexpressed in numerous cancer types including colon cancer and its role in the regulation of the cell cycle through repression of Cdc25A may shed light on the phenomenon of increased therapeutic resistance of hypoxic tumors. The resistance of hypoxic tumors chemotherapeutic drugs and radiation therapy is based on the poor accessibility of drugs to the tumor sight due to aberrant angiogenesis but also due to the absence of molecular oxygen required for radiation and chemotherapy induced DNA damage. For effective induction of DNA damage and subsequent apoptosis, cells must be actively cycling. The fact that hypoxia also induces cell cycle arrest could diminish the effectiveness of DNA damaging agents thus protecting cells from sustaining DNA damage and undergoing apoptosis. mir-21 is increased in hypoxic cells and is required for both repression of Cdc25A and also for hypoxia-mediated cell cycle arrest. It is conceivable that under hypoxic conditions, upregulation of miR-21 resulting in suppression of Cdc25A represents a protective mechanism allowing cells to survive and remain viable under hypoxic conditions. Although miR-21 is increased in hypoxic cells, its mechanism of regulation remains to be discovered. The independence of Cdc25A suppression from HIF-1α, suggests that increases in miR-21 in response to hypoxia may also be independent of the hypoxia-responsive transcription factor. The role of miR-21 in tumorigenesis has been largely associated with the promotion of tumor growth, invasion and metastasis, increased cell proliferation and inhibition of apoptosis (175, 232, 233) and the targets of miR-21

leading to these phenotypes are primarily the products of tumor suppressor genes (232). In the present study, miR-21 appears to be negatively regulating the levels of Cdc25A, a proto-oncogene. This effect may have come about evolutionarily to protect cells from hypoxia and is conserved in cancer and possibly promoted by increased miR-21 expression levels.

The findings in this study along with the frequent overexpression of miR-21 in human cancer make this miR an attractive target for cancer therapy and its inhibition may represent a new tool in overcoming the resistant phenotype of hypoxic tumors. The data presented in this study appears to conflict with the finding that miR-21 is overexpressed in human cancers and so is Cdc25A. If miR-21 negatively regulates Cdc25A, then it would be expected that Cdc25A would not be overexpressed. It is however possible that tumors found to overexpress miR-21 do not overexpress Cdc25A and vice versa. Another important aspect is that although hypoxia represents an important feature of the tumor microenvironment, it is typically transient and therefore the function of miR-21 in the regulation of Cdc25A may differ from it's role in tumorigenesis in the absence of hypoxia. In summary, the data shown herein describes a novel, hypoxia-mediated, mechanism for the regulation of the cell cycle. At the center of this pathway is Cdc25A, which upon downregulation can induce cell cycle arrest. The downregulation of Cdc25A appears to be controlled by two mediators: mir-21, which is upregulated in response to hypoxia, and p21, which has also been shown to be increased by hypoxia and is known to repress Cdc25A gene expression. Both of these mediators are needed for repression of Cdc25A and induction of cell cycle arrest. Whether there is a link between p21 and miR-

21 remains to be determined as does the mechanism of miR-21 upregulation under hypoxic conditions.

6 CONCLUSION

The role of the tumor microenvironment in tumorigenesis is becoming clear and modulation of the cell cycle represents an important adaptation conferring a survival advantage to cancer cells. Tumor hypoxia has been shown to promote tumorigenesis and is proposed to be a major factor in the resistance of solid tumors to chemo- and radiation therapy. The changes in the physiology associated with tumor growth that lead to hypoxic regions has been well studied, however, the molecular pathways involved in the cellular response to hypoxia leading to changes in cell cycle progression and cell proliferation remain poorly described. In the design of this study, it was hypothesized that Cdc25A is an essential mediator in the process of hypoxia-mediated cell cycle arrest. The data in this study has revealed a novel mechanism for the regulation of Cdc25A and it is proposed that via Cdc25A-dependent modulation of the cell cycle, hypoxic cells undergo S-phase arrest, an event that may confer a survival advantage to cancer cells under sustained hypoxic conditions. This study provides evidence that under hypoxic conditions, colon cancer cells downregulate the levels of the phosphatase Cdc25A through a mechanism of decreased mRNA levels involving miR-21 and p21. This represents a previously unknown mechanism of regulation of Cdc25A and cell cycle progression. The role of miR-21 in the regulation of Cdc25A observed in this study is

substantiated by the findings that miR-21 is upregulated in response to hypoxia and that Cdc25A is a putative target for miR-21. A novel method of Cdc25A regulation at the level of translation in response to nitrosative stress has recently been described (231, 234) indicating that proteasome-mediated degradation of Cdc25A and decreased gene expression are not the sole mechanisms of Cdc25A regulation in response to cellular stresses. The implications of this new pathway may extend beyond the boundaries of cancer and the discovery of a novel mode of cell cycle regulation under hypoxic conditions may also contribute to our understanding of the pathophysiologies of several other diseases including cardiac hypertrophy, acute and chronic vascular disease, pulmonary disease, stroke, and Alzheimer's disease where hypoxia may play an important role in disease progression and Cdc25A overexpression has been reported.

APPENDIX A

ANTIBODIES FOR WESTERN BLOTTING

Antigen	Antibody species	Dilution	Vendor
Cdc25A (F-6)	Mouse	1:100	Santa Cruz Biotechnology
Cdc25B	Mouse	1:2500	BD Pharmingen
Cdc25C	Rabbit	1:1000	Santa Cruz Biotechnology
Chk1	Rabbit	1:5000	Santa Cruz Biotechnology
Chk2	Mouse	1:200	Santa Cruz Biotechnology
HA	Mouse	1:1000	Covance Research Products, Inc.
HIF-1α	Mouse	1:250	BD Pharmingen
p21	Rabbit	1:500	Calbiochem
Phospho-Chk1 (Ser245)	Rabbit	1:1000	Cell Signaling Technology
Phospho-Chk2 (Thr68)	Rabbit	1:1000	Cell Signaling Technology
β-Tubulin	Mouse	1:20,000	Cederlane Laboratories

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