INDUCTION OF CDC25B FOLLOWING DNA DAMAGE: IMPLICATIONS FOR CELL CYCLE RESUMPTION AND TUMORIGENESIS

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INDUCTION OF CDC25B FOLLOWING DNA DAMAGE: IMPLICATIONS FOR CELL CYCLE RESUMPTION AND TUMORIGENESIS

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The overall hypothesis of this dissertation was that Cdc25B is an important regulator of the cellular response to DNA damage and defection from the normal response could promote tumorigenesis by enhancing genomic instability. Conventionally, DNA damage is generally thought to inhibit Cdc25 functionality to induce cell cycle arrest. However, recently a crucial role of Cdc25B in the cell cycle resumption after DNA damage was identified. To understand the precise regulation of Cdc25B following DNA damage, I examined the effect of mechanistically distinct DNA damaging agents on Cdc25B. Secondly, experiments were performed to elucidate how Cdc25B participates in the recovery from the checkpoints induced cell cycle arrest. Finally, the mechanism by which Cdc25B contributes to *anti*-BPDE induced tumorigenesis was investigated. The results of our studies revealed that Cdc25B was rapidly induced following DNA damage and levels of Cdc25B regulated the number of cells existing G2 into mitosis. Increased expression of Cdc25B did not affect the G2/M checkpoint engagement immediately following DNA damage; however, increased Cdc25B reduced the time required for cell cycle resumption. Using UV irradiation as the prototypic damaging agent, the increase in Cdc25B levels was found to be regulated by ATR/Chk1 via post-transcriptional mechanism, potentially by affecting Cdc25B protein stability. Furthermore, Cdc25B was found to be essential for *anti*-BPDE-induced neoplastic transformation. Additionally, Cdc25B facilitated resumption in the presence of DNA damage following *anti*-BPDE thus indicating that Cdc25B contributes to

tumorigenesis by regulating premature recovery from checkpoints without completion of DNA repair. Finally, increased Cdc25B activated checkpoints in the absence of overt DNA damage suggesting that Cdc25B enables genomic instability by promoting selection of cells with deregulated checkpoint signaling.

To conclude, studies presented in this dissertation identified a novel role of Cdc25B following DNA damage and elucidated the molecular mechanisms by which Cdc25B regulates *anti*-BPDE induced tumorigenesis.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

The following chapter has been modified from the original manuscript to provide greater detail and a consistent format throughout this dissertation.

Chapter 3: Bansal P and Lazo JS. Induction of Cdc25B regulates cell cycle resumption after genotoxic stress. Cancer Research (In Press)

FOREWARD

In dreams and in life nothing is impossible.

-Fortune cookie at Fuel and Fuddle

I am not known for my patience. Patience is a polite quality and often appropriate, but it rarely gets things done. Impatience, however, is hunger for results and intolerance for excuses and delays.

- Lance Armstrong

Dedicated to my family.

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1.0 INTRODUCTION

1.1 MAMMALIAN CELL CYCLE

Regulation of cell cycle is crucial for all eukaryotes including unicellular and multi-cellular organisms. In eukaryotes cell cycle is divided into four distinct phases where progression through each phase is regulated by cyclin dependent kinases (Cdk) and their cognate cyclins. During two of these four phases a cell carries out two basic events necessary for productive cell division: generation of a single and faithful copy of its genetic material (the synthetic or S phase) and division of the cellular component into two identical daughter cells (mitosis or M phase). The other two phases of the cell cycle represent gap periods, G1 and G2. G1 precedes S phase and G2 M phase, respectively. In these periods a cell prepares itself for more active S and M phase (1). When cells stop proliferating, most cells in an adult organism, they enter a quiescent state known as G0. Most quiescent cells have the capacity to re-enter cell cycle, except terminally differentiated cells, where re-entry is regulated by signaling pathways activated by different mitogens (1).

1.2 MOLECULAR PLAYERS OF CELL CYCLE

Since their discovery over 20 years ago, Cdks have been shown to play a crucial role in the central enzymatic process required for cell cycle progression. The first member of the Cdk family (Cdc2) was identified in genetic screens for *Schizosaccharomyces pombe (S. pombe)* and *Saccharomyces cerevisiae (S .cerevisiae)* mutants with defects in the cell division cycle (2). Following this several members have been identified in human and mouse cells. According to the latest versions of the human and mouse genomes, there are 11 genes encoding cdks and 9 other genes encoding Cdk-like proteins with conserved primary structure. Among these, only 5 members have been implicated in the cell cycle regulation: Cdk1, Cdk2, Cdk3, Cdk4, and Cdk6 (3).

Cdks, a group of serine/theronine kinases, form the catalytic subunit of a heterodimeric complex comprising cyclins as the regulatory subunit. At the beginning of G1, Cdk4/6 associate with new synthesized D-type cyclins enabling the complex to phosphorylate members of retinoblastoma (Rb) family (4, 5). Phosphorylation at some of the sites on Rb releases the repressive effect of Rb on E2F leading to the expression of genes required for completion of G1 and entry into S phase (6-8). One of the crucial transcriptional targets of Rb-E2F complexes are E-type cyclins. As illustrated in Fig.1, cyclin E associates with Cdk2 in mid to late G1 resulting in the activation of the complex. Activated cyclinE/Cdk2 further phosphorylate Rb leading to irreversible inactivation of Rb (8, 9). This process is believed to render cells independent of mitogenic signals and corresponds to the "restriction point" (1). There is some evidence suggesting that Cdk3 also participates in the inactivation of Rb during G0 exit, thus contributing to the cells progression beyond the restriction point (10). Once beyond the restriction point, Cdk2 activity is thought to be essential for initiating DNA replication by facilitating loading of the

MCM chromosome maintenance proteins onto origins of replication (3). Once cells enter S phase, inhibition of cyclin E/Cdk2 is at least in part culminated by the degradation of cyclin E by the SCF-Fbxw7 ubiquitin ligase to prevent re-replication of the DNA (11). In the mid to late S phase cyclin A begins to accumulate and with the degradation of cyclin E, Cdk2 associates with the abundant cyclin A. Cyclin A/Cdk2 complex then phosphorylates several proteins required for the proper S phase completion and exit (3). At the end of the S phase cyclin A associates with cdk1. During G2, cyclin A is rapidly degraded on the contrary cyclin B is actively synthesized. As a result, Cdk1 binds to B- type cyclins – an association shown to be essential for initiating mitosis. Finally, inactivation of cyclin B/Cdk1 is required for exiting mitosis (12, 13).

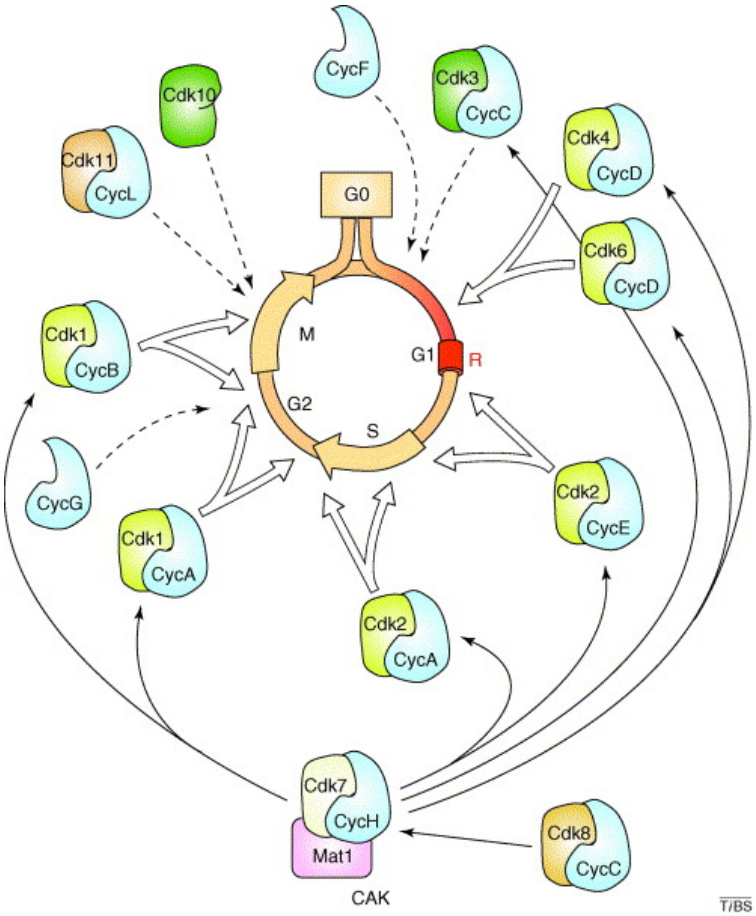


Figure 1. Regulation of cell cycle by cdks

Adapted from Malumbres and Barbacid, 2005. Mammalian cyclin-dependent kinases. Trends in Biochemical Sciences, 11: 630-641.

1.3 REGULATION OF CDK ACTIVITY

Because Cdk activity is paramount to cell cycle progression, it is regulated by several processes to ensure precise timing of its activity. The processes include: a) association with cognate cyclin; b) binding to negative regulators, Cdk inhibitors (CDKI); and c) activating and inhibitory phosphorylation. Temporal expression of cyclins restricts the activity of Cdk to specific phases of cell cycle. With in a specific phase of the cell cycle – two families of CDKI: INKK4 family (p16 ^{INKK4a}, p15 ^{INKK4b}, p18 ^{INKK4c}, and p19 ^{INKK4d}) and the Cip and Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) further regulate Cdk activity (14). It has been shown previously that INK4 proteins specifically bind to and inhibit monomeric Cdk4 and Cdk6 (15). Cip and Kip, on the other hand, promiscuously inhibit cyclin-Cdk complexes. Furthermore, Cdks are also subjected to activating and inhibitory phosphorylation. Activating phosphorylation at the threonine residue (Thr 161 in Cdk1 and Thr 160 in Cdk2) is carried out by the complex of proteins comprising cyclin H/Cdk7/Mat1, also known as Cdk activating kinase (CAK) (3, 16). On the contrary, phosphorylation at Thr 14 and Tyr 15 in the ATP binding domain prevents phosphorylation of its substrates. These phosphorylations are carried out by Wee1 and Myt1 kinases. Wee1 is nuclear tyrosine specific kinase whereas Myt1 is dual specific kinase localized to ER and Golgi complex (17, 18). To achieve full catalytic activation of Cdks, the dual specific phosphatase Cdc25 dephosphorylates triple phosphorylated Cdks at Thr 14 and Tyr 15 (Fig. 2)

(19).

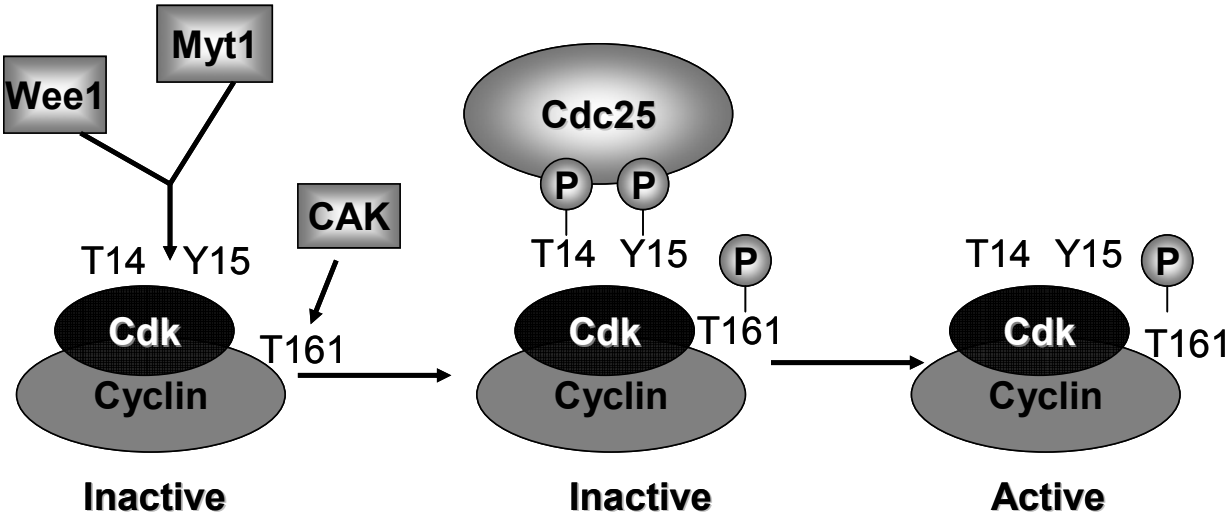


Figure 2. Activation of Cdks by Cdc25

Cdc25s activate Cdks by removing inhibitory phosphate groups on Thr 14 and Tyr 15. These sites are initially phosphorylated by Wee1 and Myt1 in the ATP binding domain of the kinase. Phosphorylation at Thr 161 is activating phosphorylation carried out by CAK.

1.4 CDC25 DUAL SPECIFIC PHOSPHATASE

Cdc25 was initially identified in *S. pombe* as a 25th gene to influence the cell cycle. The Cdc25 mutant failed to divide resulting in an enlarged phenotype but could be reversed by the deletion of Wee 1. These results provided phenotypic evidence that Cdc25 is functionally antagonist to Wee1 and it might function as a phosphatase (20) . Subsequently, three mammalian genes were identified, namely Cdc25A, B and C, which complemented a cdc25^{ts} temperature sensitive yeast strain (21-23). At the protein level, the three isoforms are highly homologous in their carboxy terminus. The carboxy terminus constitutes 30% of the protein and is marked by a

conserved Leu-Ile-Gly-Asp motif (Fig. 3). It also harbors the catalytic site of the enzyme comprising the canonical Protein Tyrosine Phosphatase (PTPase) active site motif His-Cys-X5-Arg. Mutation in the active Cys abolishes the catalytic activity of the enzyme (24). Like other PTPases, Cdc25 also employs a two step process to hydrolyze the phosphate ester bond on Thr and Tyr residue located in the substrate-Cdks (25). Briefly, Cdc25 catalyzes the formation of a transient phosphoenzyme intermediate by transferring the phosphate to a catalytic Cys residue. The dephosphorylated substrate is expelled from the active site using an acidic amino acid residue to protonate a tyrosine phenolic oxygen. Subsequently, active Cdc25 is generated when an amino acid activates a water molecule thus allowing hydrolysis of the phosphoenzyme intermediate and generation of inorganic phosphate (25, 26). Compared to other PTPases, Cdc25 has a shallower active site, which allows Cdc25 to function as dual specific phosphatase by accommodating less extended phosphoserine and phosphothreonine residues (27). Finally, there is little homology in the amino termini (the regulatory domain) of Cdc25 isoforms, which may contribute to the diverse nature of their biological activities (24).

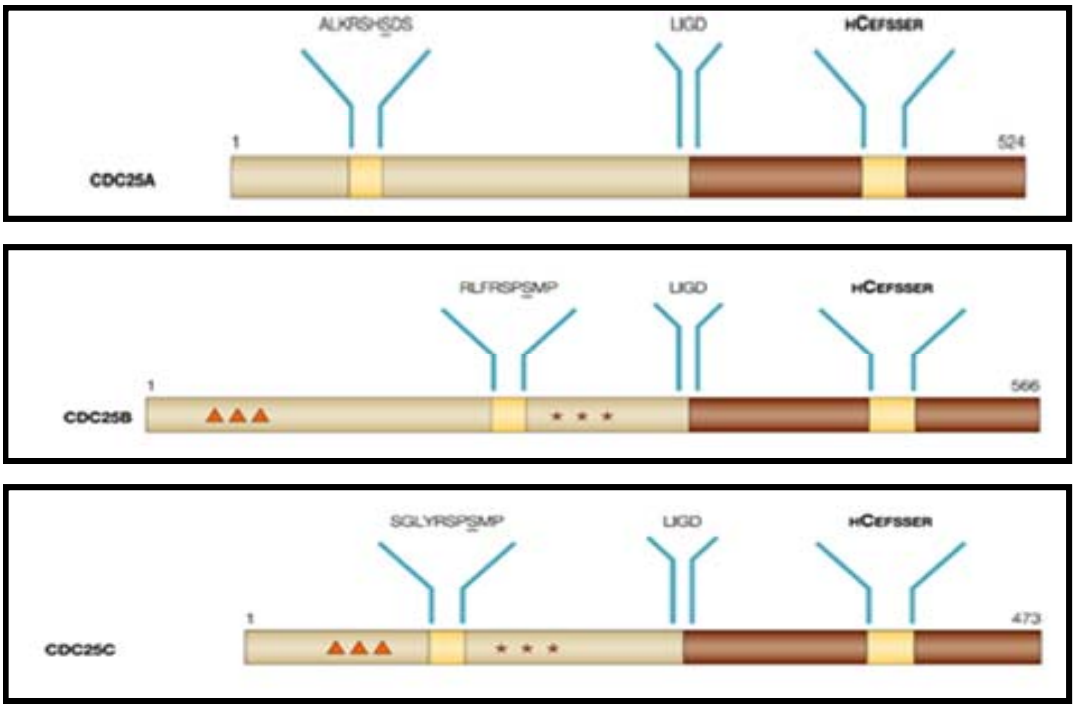


Figure 3.Three isoforms of Cdc25

The three isoforms of Cdc25 are encoded by different genes. At protein level, their amino terminal represents the regulatory domain which is subjected to several regulatory phosphorylations. The carboxy terminal, marked by Leu-Ile-Gly-Asp, harbors the catalytic activity of these enzymes containing protein tyrosine phosphatase (PTPase) active site motif His-Cys-X5-Arg where X represents any amino acids. Identified nuclear-localization sequences are indicated with asterisks and known nuclear-export sequences are indicated with triangles. Adapted from Lyon *et al.,* 2002. Dual-specificity phosphatases as targets for antineoplastic agents. Nat Rev Drug Discov, 1:961-76.

1.4.1 Regulation of Cell Cycle by Cdc25

In the past decade, the role of Cdc25s in cell cycle progression has been extensively characterized. In the traditional model, Cdc25A regulated the transition from G1 to S by

activating cyclin E/Cdk2 and cyclin A/Cdk2 complexes. Furthermore, Cdc25B and C were though to control the transition from G2 to M by activating cyclin A/Cdk1 and cyclin B/Cdk1 complexes (Fig.4) (28). This model was based on the evidence that microinjection of Cdc25A antibodies in the cell caused G1 arrest and Cdc25B or Cdc25C blocked entry into mitosis (29, 30). However, recent evidence from several groups suggests that all three Cdc25s may contribute to G1-S and G2-M transitions, and in mitosis the three isoforms may exhibit specific activity towards cyclinB/cdk1 (Fig.5). It was shown that siRNA and/or antisense molecules to Cdc25B or Cdc25C inhibit S phase progression in human cells, although, the specific targets of Cdc25B and Cdc25C are not known (31, 32). Similarly, ectopic Cdc25A expression induced unscheduled mitosis while Cdc25A siRNA led to a decrease in the percentage of mitotic cells (33, 34). Interestingly, Bartek and colleagues showed that in mitosis cyclin B/Cdk1 stabilizes Cdc25A by phosphorylating Cdc25A at Ser17 and 115 and recent report suggested that Cdc25A might regulate chromosome condensation during mitosis (35, 36). In contrast, Cdc25B appears to function as a “mitotic inducer” by activating cyclin B/Cdk1 at the centrosome where Cdc25B undergoes Aurora A (serine/threonine kinase) dependent phosphorylation (37). A more recent study has shown that initial activation of cyclin B/Cdk1 by Cdc25B is required for centrosome separation before the onset of mitosis (35).

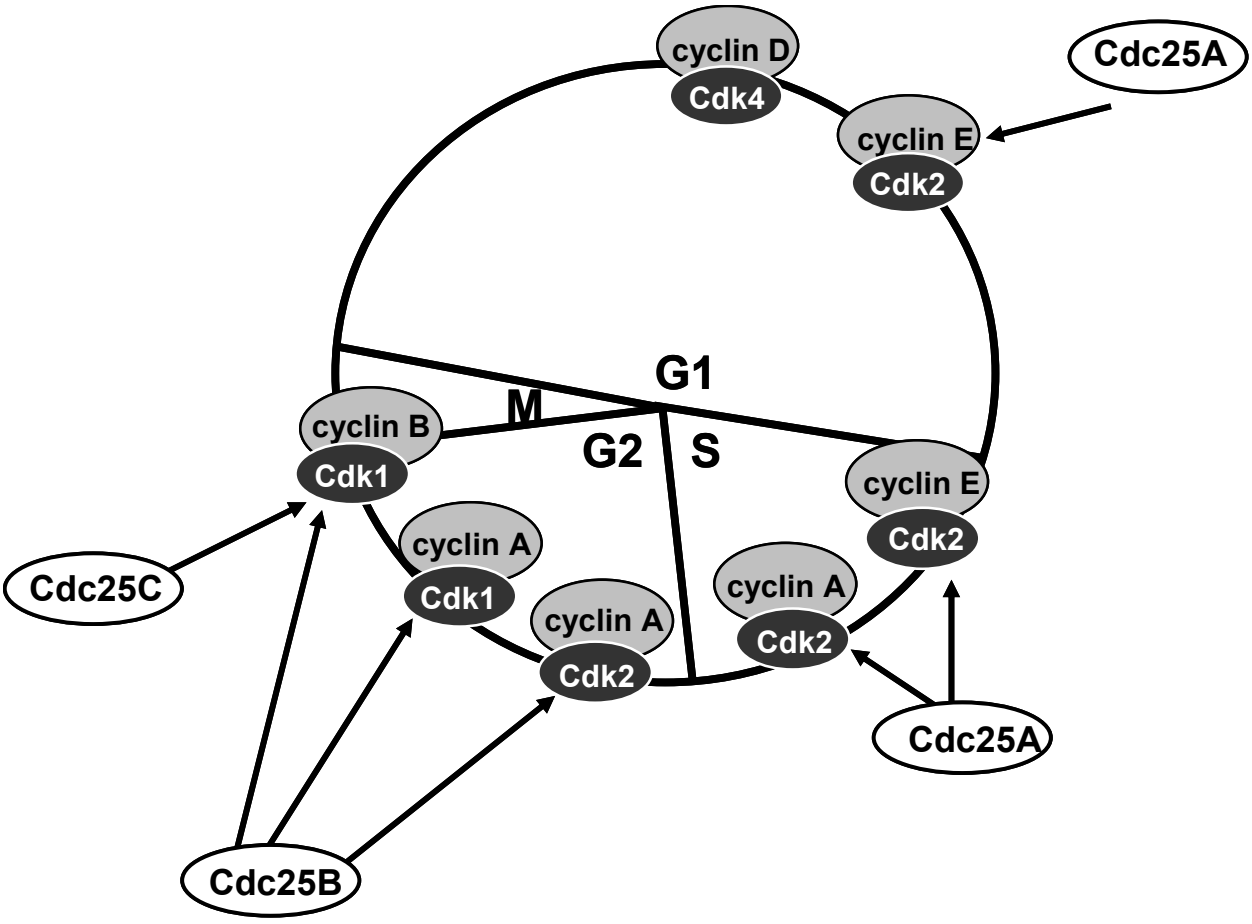


Figure 4. Figure 4. Regulation of Mammalian cell cycle by Cdc25s

Schematic representing activities of Cdc25 isoforms in different phases of cell cycle. Adapted from Lyon *et al.,* 2002. Dual-specificity phosphatases as targets for antineoplastic agents. Nat Rev Drug Discov, 1:961-76.

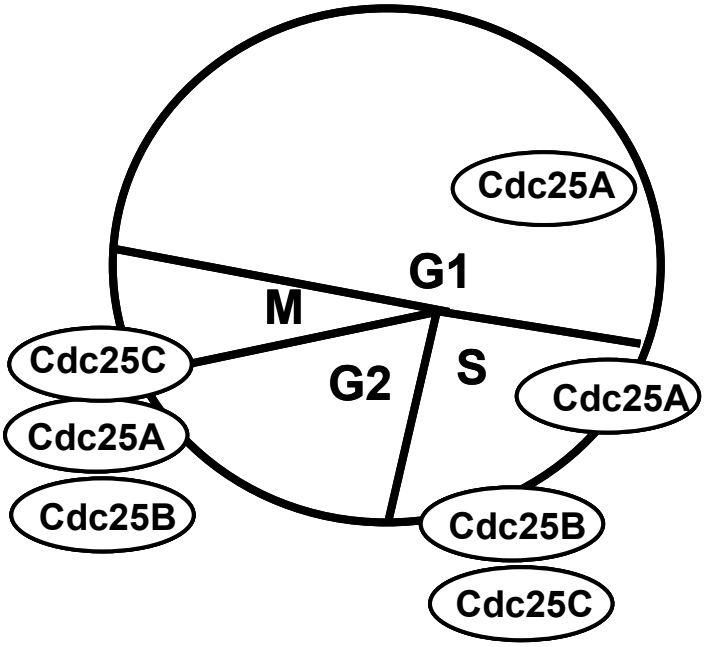


Figure 5. New model representing Cdc25s activities during cell cycle

Figure representing temporal and combinatorial contribution of all three Cdc25 phosphatases to achieve precise control over cell cycle progression.

Collectively, these findings support a model where a temporal and combinatorial contribution of all three Cdc25 phosphatases is required to achieve precise control over cell cycle progression. Nonetheless, recent finding from Piwnica-Worms and colleagues indicate that Cdc25B and Cdc25C are not required for mouse development and mitotic entry (38). The cell cycle regulation of Cdc25A and response to DNA damage was found to be normal in cells lacking both Cdc25B and Cdc25C. These results suggest that Cdc25A or an as yet unidentified phosphatase could functionally compensate for the loss of Cdc25B and Cdc25C in mice. Nevertheless, these findings should not come as complete surprise because similar redundancies have been shown for Cdks. In particular, Cdk4, Cdk6 and Cdk2 are dispensable for mitotic cell cycle; however, they are essential for the proliferation of some endocrine and hematopoietic cells

and Cdk2 is necessary for meiotic division of male and female germ cells (3, 39-41). Similarly, Cdc25B is required for resumption of meiosis during oocyte maturation (42). These findings suggest that cell cycle regulators have evolved from common ancestors to accomplish specialized functions but have retained the ability to complement most of the functions of other family members.

1.4.2 Regulation of Cdc25 Activity

As a key controller of cell cycle progression, activities of Cdc25 phosphatases are tightly regulated by multiple mechanisms including abundance in the cell, splice variants, subcellular localization and phosphorylation status. Among the three isoforms Cdc25C is expressed throughout the cell cycle whereas Cdc25A and Cdc25B are labile proteins (19). Cdc25A is rapidly turned over by two different ubiquitin ligases (APC/C and SCF complex). While APC/C is involved in regulating Cdc25A protein levels at the exit of mitosis, SCF regulates protein levels during S and G2 phases and in response to DNA damage (36, 43). Similarly, a recent study showed that a non-phosphorylated motif in Cdc25B is recognized by the F box/β-TRCP component of the SCF complex, which could regulate Cdc25B degradation (44). However, it is unclear whether or not the SCF-mediated degradation of Cdc25B is functionally important.

In the human Cdc25 family, each isoforms also have multiple splice variants; 2 for Cdc25A, 5 for Cdc25B and 5 for Cdc25C (45). Although data are lacking, it seems reasonable to speculate that the Cdc25 splice variants could have different roles in cells or may differ in their cell cycle distribution. In addition, alternative splicing may eliminate specific consensus

phosphorylation sites, which could affect their regulation or biological functions. Thus, further studies are required to understand the significance of these splice variants.

It has been shown previously that activation of Cdk1 in the centrosome is required for the commitment of cells into mitosis (46). Therefore, regulation of Cdc25 localization with in cells is critical for maintaining the proper order of cell cycle transition. In agreement with this concept, the localization of Cdc25 during interphase and mitosis has been extensively studied. Cdc25 localization has been shown to be regulated by binding to 14-3-3 and nuclear export signals (NES) and nuclear localization signals (NLS) within the protein (47-52). Specifically, Cdc25B is thought to localize in the cytoplasm during interphase. Cytoplasmic localization of Cdc25B is regulated by binding to 14-3-3 through Ser309 phosphorylation. Furthermore, this interaction enables 14-3-3 to negatively regulate Cdc25B by preventing it to interact with its substrates or nuclear accumulation. Interestingly, Bulavin *et al.* showed that phosphorylation at Ser307 during mitosis prevents phosphorylation at Ser309 and promotes nuclear accumulation of Cdc25B (53). In addition, several groups have reported centrosomal localization of Cdc25B where it provides first stimulus for Cdk1 activity prior to the onset of mitosis (54-56). Therefore, Cdc25B appears to have a unique role in initiating mitosis that warrants further investigation.

1.4.3 Role of Cdc25B in Cancer

De-regulation of cell cycle is one of the hallmarks of cancer. In cancer cells de-regulation is perpetuated by alterations in the signal transduction pathways that lead to proliferation in response to external signals. The high frequency of mutations in the growth factors, their receptors, and their downstream effectors in cancer cells is testament of their significance in the oncogenic process. Cdc25s were first shown to be involved in the oncogenic transformation

when co-expression of Cdc25A or Cdc25B but not Cdc25C with oncogenic mutants H-ras lead to formation of transformed foci in normal mouse embryo fibroblasts (57). Subsequently, several studies in a number of model systems confirmed an oncogenic role for the Cdc25 phosphatases. Interestingly, a gain-of-function allele of *cdc-25.1* gene in *C. elegans* caused excess proliferation of intestinal cells and depletion of Cdc25 by RNAi lead to reduced proliferation (58). Similarly, targeted over-expression of Cdc25B in the mammary glands of transgenic mouse cell lines enhanced the proliferation of mammary epithelial cells resulting in precocious alveolar hyperplasia (59). However, using a similar strategy another study saw no changes in mammary cells or other cell types. Interestingly, the mice had an increased susceptibility to carcinogen-induced mammary tumors (60). Furthermore, we found that benzo[a]pyrene diol epoxide, the ultimate carcinogen derived from benzo[a]pyrene in cigarette smoke, increases Cdc25B expression in lung cancer cells indicating that Cdc25B could contribute to benzo[a]pyrene diol epoxide induced lung carcinogenesis (61). Cdc25B has also been shown to be over-expressed in different human cancers including prostate cancer, esophageal squamous cell carcinoma, breast cancer, colorectal carcinoma, pancreatic ductal adenocarcinoma, head and neck cancer, gastric carcinoma nonsmall cell lung cancer, thyroid neoplasm, non-Hodgkin's carcinoma, neuroblastoma and endometrial cancer (62-68). Moreover, Cdc25B2 (one of the Cdc25B splice variants) can function as a co-activator for the steroid receptors. Surprisingly, the effect of Cdc25B2 on steroid receptor is independent of its phosphatase activity (69). This suggests that Cdc25B can regulate breast and prostate cancer progression by potential activation of steroid receptors.

Taken together, these findings suggest that Cdc25B might play a significant role in cancer development. Nevertheless, the exact mechanism by which Cdc25B contributes to cancer

development is still not elucidated. One of the hypotheses is by driving cell proliferation Cdc25B might regulate cancer development; however, no correlation has been observed in the majority of cancer cells between Cdc25B over-expression and rate of proliferation (66). Alternatively, over-expression of Cdc25B might promote premature entry in to G2/M in the presence of DNA damage leading to inappropriate distribution of chromosomes and aneuploidy (70).

1.5 DNA DAMAGE AND CELL CYCLE CHECKPOINTS

During its life a cell encounters DNA damaging agents either through endogenous or exogenous sources; these include ionizing or ultraviolet radiation (IR or UV, respectively), various chemicals or drugs, and reactive cellular metabolites. These DNA damaging agents can either directly damage one of the three billion bases or break the phosphodiester backbone on which the bases reside. Therefore, continuous surveillance of the genetic material and rapid action to repair any DNA damage or eliminate genetically unstable cells are essential to prevent the evolution of diseases such as cancer. To execute these processes, all eukaryotes have evolved an extensive network to detect unreplicated or aberrant DNA structures, to spread the signal, and finally to coordinate the DNA repair process with cell cycle checkpoint pathways (71). The term "checkpoint" refers to biochemical pathways that cells employ to actively delay progression through the cell cycle in response to DNA damage. Depending on the phase of the cell cycle that is being inhibited by DNA damage, the checkpoints are coined as G1/S, intra-S, and G2/M checkpoints (72).

1.5.1 Molecular Components of Cell Cycle Checkpoints

In a canonical checkpoint response, proteins are grouped into four categories depending on their primary function. These groups include sensors, mediators, transducers, and effectors (Fig.6). The phosphoinositide 3-kinase-like kinase (PIKK) family members, ATM (ataxia telangiectasia mutated) and ATR (AMT-and Rad3-related) are sensors which are activated instantaneously following DNA damage (73). ATM primarily responds to double-strand breaks whereas ATR responds to all cellular stress responses that share inhibition of replication-fork progression as a common mechanism. ATR has also been shown to be engaged in cellular responses to double stand breaks, possibly compensating for ATM (72, 73). In order to activate their substrates, ATM and ATR depend on the group of proteins called mediators. These proteins participate in the activation either by recruiting ATM to the site of the damage or by facilitating the interaction of ATM and ATR with their substrates. Among several substrates, Chk1 and Chk2 (serine/threonine kinase) play a crucial role in propagating the signal to coordinate a pan-cellular response to DNA damage by targeting effector proteins from various cellular processes. To induce cell cycle delay or arrest, these kinases act on Cdc25 phosphatases (71-74).

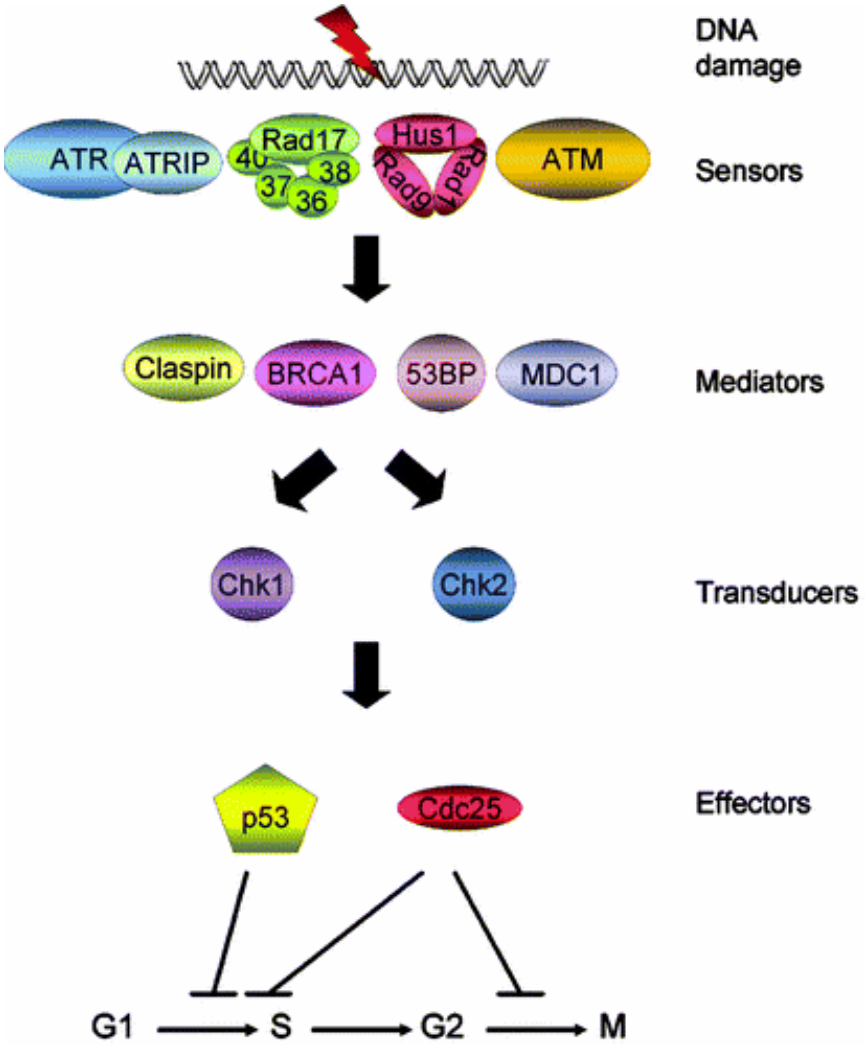


Figure 6. Components of mammalian checkpoint

DNA damage in a cell is sense by sensors which in turn transduce the signal to transducers with the aid of mediators. Transducers then relay the signal to effectors. These effectors inhibit cell cycle progression. Adapted from Sancar *et al.* 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Review Biochem, 73:39-85.

1.5.2 Cdc25s as Targets of Cell Cycle Checkpoints

Cdc25 was first demonstrated to be a key target for Chk1 and Cds1 (the homologue of Chk2) in fission yeast (75). Following DNA damage, phosphorylation of Cdc25 by Chk1 and Cds1 provides a binding site(s) for the 14–3-3 proteins, Rad24 and Rad25, which are required for checkpoint arrest, and cause Cdc25 to be excluded from the nucleus. Similarly, in human cells Chk1 and Chk2 phosphorylate the N-terminal region of Cdc25C on Ser216. Subsequent binding to 14–3-3 leads to an increased cytoplasmic localization of Cdc25C and blocks interaction of Cdc25C with its substrate (76). Cdc25B has also been reported to be affected by at least some forms of genotoxic stress. Thus, in response to UV, the p38/MAPKAPK2 signaling pathway is activated and Cdc25B is phosphorylated at Ser309. This leads to 14-3-3 binding, which is thought to restrict Cdc25B to the cytoplasm (77, 78). As illustrated in Fig.7, Chk1 and Chk2 employ a distinct mode for regulating Cdc25A following DNA damage. Chk1 and Chk2 regulate rapid degradation of Cdc25A protein levels leading to sustained inhibitory phosphorylation of Cdk2, causing a G1 arrest and a block in S phase entry (33, 43, 79-82).

In the unperturbed cells, Chk1 promotes basal Cdc25A protein turnover by direct phosphorylation on multiple Cdc25A residues (Ser75, Ser123, Ser178, Ser278, and Ser292). While Chk2 also appears to phosphorylate all of these serine residues except Ser278, Chk2 is only implicated in Cdc25A turnover following genotoxic stress (43, 80, 83). It has been proposed that accelerated proteolysis of Cdc25A following DNA damage could be due to the increase kinetics of Cdc25A phosphorylation mediated by cooperative action of Chk1 and Chk2. Phosphorylated Cdc25A is recognized by β-TRCP (F box) that targets Cdc25A for proteasomal degradation by the Skp1/Cul1/F box protein complex (43, 80). Abrogation in this pathway leads

to radioresistant DNA synthesis in response to DNA damage - a phenotype indicative of a defect in the intra-S-phase checkpoint that leads to genomic instability (83).

Recently, several groups have reported findings that challenge the simplified model described above. For example, Cdc25C is shown to be degraded in response to arsenite and is down-regulated by p53 following doxorubicin (topoisomerase II poison) (84, 85). Similarly, p21 causes transcriptional repression of Cdc25A following DNA damage (86). Paradoxically, our group and Kang *et al.* reported increased expression of Cdc25B protein levels following DNA damaging agents (61, 87). These findings indicate that Cdc25B regulation by DNA damaging agents might be antithetical to other family members and could play an important role in a yet to be recognized process. Interestingly, checkpoint response was found to be normal in cells lacking Cdc25B and C suggesting that Cdc25A might compensate for loss of both phosphatases to regulate normal checkpoint response (38). In contrast, stabilization of Cdc25A by mutation of Ser75 to alanine or Ser75 and Ser123 to alanines was not enough to overcome the UV or IR induced S phase checkpoint therefore challenging the hypothesis that Cdc25A is the master phosphatase regulating checkpoints (81, 88).

Thus, these studies suggest that regulation of Cdc25s following DNA damage might be far more complex than initially anticipated. The final outcome of Cdc25 levels might depend on several factors including amount and context of DNA damage, relative expression and localization of Cdc25s at the time of DNA damage and finally the phase of cell cycle progression.

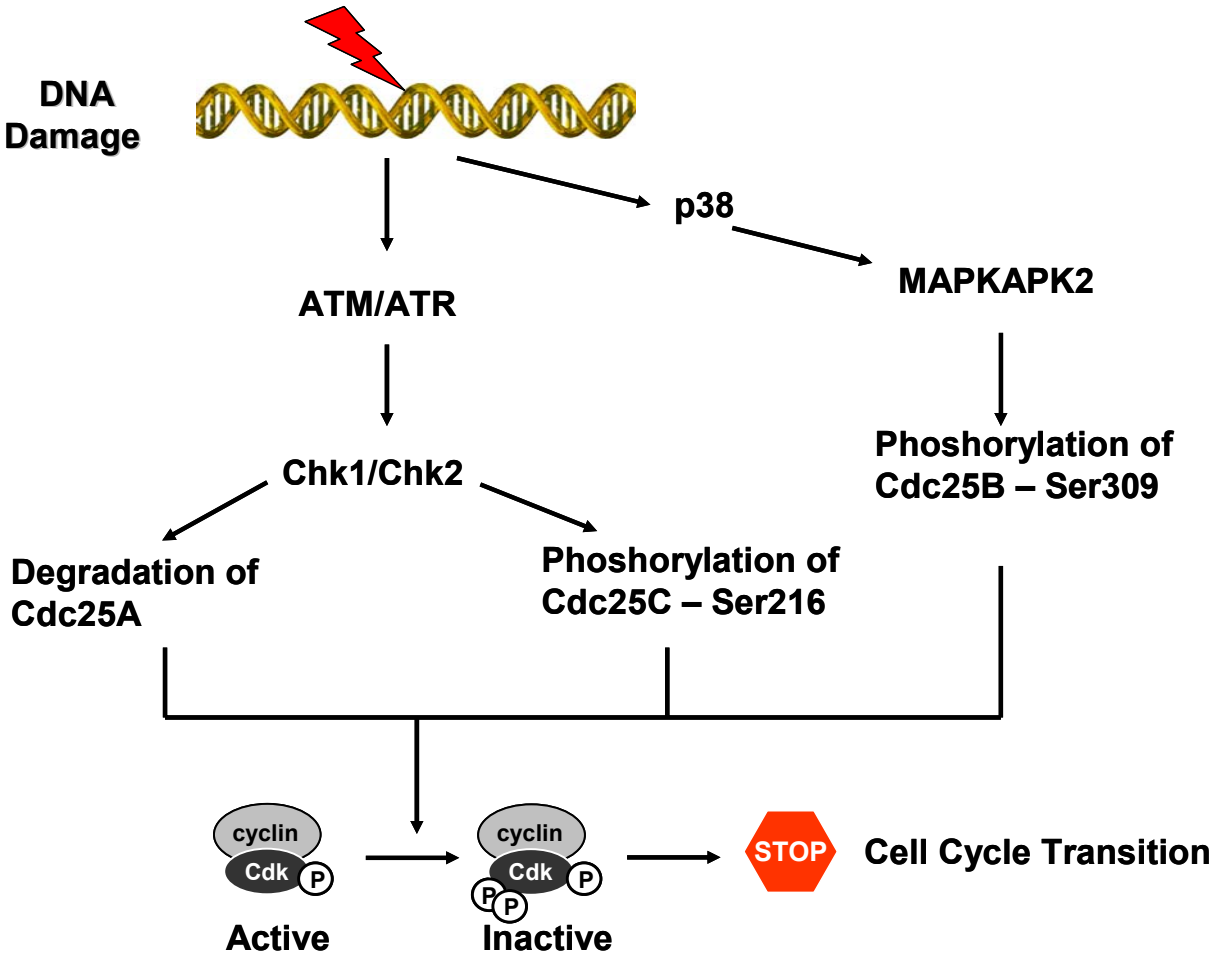


Figure 7. Checkpoint inactivate Cdc25s to induce cell cycle arrest

Schematic representing the molecular mechanisms by which cells inhibit activity of Cdc25s to induce cell cycle arrest.

1.6 CELL CYCLE RESUMPTION AFTER A DNA DAMAGE-INDUCED CHECKPOINT ARREST

Although DNA damage clearly can cause cell cycle arrest, the factors that control cell cycle resumption or "recovery" have only recently become the subject of attention. Recovery

was first examined in the yeast cells and based on the results from yeast it is clear that recovery is a more complex process than simply the removal of the DNA lesions that would attract the attention of DNA damage-sensing proteins (89). In *S. cerevisiae*, recovery depends upon on the Polo-like kinase Cdc5, casein kinase II, phosphatases Ptc2 and Ptc3, and the helicase Srs2 and is accompanied by loss of Rad53p kinase activity and Chk1 phosphorylation (90-93) .

Checkpoint inactivation or recovery was first studied in mammalian cells by Medema *et al.* (94). They investigated the requirement for a cell cycle restart in G2 DNA damage–arrested cells by inhibiting ATM and ATR with caffeine. Inhibition of ATM/ATR allowed the cell cycle to restart by abrogating the DNA damage–induced arrest. Using this method, they found that both Plk1 and Cdc25B are essential for mitotic entry when cells recover from a DNA damage checkpoint–induced arrest. In contrast, both Plk1 and Cdc25B were dispensable for mitotic entry in the absence of DNA damage. Interestingly, the requirement for Plk1 during recovery was lost in Wee1-depleted cells, suggesting that Plk1 in human cells might function as an upstream inhibitor of Wee1. Plk1 has been shown to promote proteosomal degradation of Wee1 by phosphorylating Wee1. In addition, p53 induced oncogenic PPM1D (Serine/Theronine phosphatase) was shown to dephosphorylate ATM/ATR targeted phosphorylation sites on Chk1 and p53 following DNA damage. Dephosphorylation of these proteins by PPMID correlated with reduced intra-S and G2/M checkpoint following DNA damage. These results suggest that primary function of PPM1D is to reverse cell cycle checkpoint responses and regulate return of the cells to a homeostatic state following completion of DNA repair (95). Similarly, Chk1 and claspin, a regulator of Chk1 activation, are targeted for degradation after replication stress to facilitate cell cycle resumption following genotoxic stress (96-98). Overall, above studies highlight the significance of cell cycle resumption and indicate that several components of the

eukaryotic checkpoint signaling pathway are essential for resumption of cell cycle progression following genotoxic stress. However, one important aspect that is currently poorly understood is how seemingly opposite processes of cell cycle arrest and resumption are coordinated within a cell after DNA damage without compromising genomic integrity. To better understand this, a comprehensive study examining the chronology of events after DNA damage is essential.

1.7 HYPOTHESIS AND SPECIFIC AIMS

As mentioned previously, Cdc25s are rapidly inactivated following DNA damage to induce cell cycle arrest. However, the DNA damaging carcinogen (7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (*anti*-BPDE) caused a marked increase in the expression of Cdc25B mRNA and protein levels in terminal squamous differentiated human bronchial epithelial cells and in lung cancer cells, but not in undifferentiated bronchial cells (61). In addition, Cdc25B was highly expressed in radiation-resistant and tumorigenic clones of NIH3T3 (87). These results are dissimilar to previously published reports describing Cdc25B inhibition in response to UV by p38/MAPKAPK2 pathway (77, 78). One potential explanation for the discrepancy could be differential regulation of Cdc25B following mechanistically distinct DNA damaging agents. However, it is still surprising that a positive regulator of mitotic entry is upregulated in response to some DNA damaging agents. Moreover, in light of the recent findings demonstrating a crucial role of Cdc25B in cell cycle resumption following DNA damage it is imperative to understand the precise temporal regulation of Cdc25B following DNA damage. Secondly, Cdc25B is frequently overexpressed in several tumors and it may promote genomic instability by regulating premature recovery from checkpoints without completion of DNA repair

as opposed to inadequate activation of DNA damage checkpoints as previously suggested (89). Therefore, subsequent studies are required to distinguish between premature checkpoint recovery and inadequate checkpoint activation for the better understanding of role of Cdc25B in tumorigenesis. Therefore, the underlying hypothesis of this dissertation is that Cdc25B is an important regulator of the cellular response to DNA damage and defection from the normal response could promote tumorigenesis by enhancing genomic instability. Studies in this dissertation were first designed to delineate the effects of mechanistically distinct DNA damaging agents on Cdc25B expression. Secondly, experiments were performed to understand how checkpoints regulate Cdc25B to induce cell cycle arrest and how Cdc25B participates in the recovery from the checkpoints induced cell cycle arrest. Finally, the work described in this dissertation will help to shed light on the molecular mechanisms by which Cdc25B regulates *anti*-BPDE induced tumorigenesis.

1.7.1 Induction of Cdc25B Regulates Cell Cycle Resumption after Genotoxic Stress

To elucidate the role of Cdc25B following DNA damage, I examined the effects of various DNA damaging agents on Cdc25B protein expression. Using UV as prototypic damaging agent, I characterized the mechanism (s) of increased Cdc25B expression and subcellular localization of Cdc25B. The functional significance of the increased Cdc25B protein levels was determined by employing two antithetical approaches (1) Conditional overexpression of Cdc25B and (2) Down-regulation of Cdc25B using shRNAi. Using these approaches, the effects of Cdc25B on cellular progression after DNA damage was investigated by trapping cells into mitosis. Finally, the effect of increased Cdc25B levels on G2/M checkpoint was analyzed to determine whether Cdc25B regulates exit from G2/M checkpoint.

1.7.2 Cdc25B Phosphatase is Essential for Benzo[*a*]pyrene-7, 8-diol-9, 10-epoxide Induced Neoplastic Transformation

In chapter 4, experiments were performed to determine whether Cdc25B is essential for *anti*-BPDE induced tumorigenesis and to understand how Cdc25B might regulate tumorigenesis. For testing the essentiality of Cdc25B, Cdc25B WT and KO MEFs were chronically treated with *anti*-BPDE and chronically treated cells were injected into nude mice. To delineate the significance of upregulation of Cdc25B, cells over-expressing Cdc25B were treated with *anti*-BPDE and cell cycle re-entry was examined.

2.0 EXPERIMENTAL METHODS

2.1 REAGENTS

Etoposide, cisplatin, and bleomycin were obtained from Sigma-Aldrich Co (St. Louis, MO). Caffeine, nocodazole, MG132, Crystal Violet and tetracycline were obtained from Calbiochem (La Jolla, CA). BP and its metabolites were obtained from the National Cancer Institute, Chemical Carcinogen Reference Standard Repository (Frederick, MD). UCN-01 was generously provided by Dr. Baskaran (University of Pittsburgh) provided by For UVC exposure UVC Cross linker from Stratagene (La Jolla, California) was used. For ionizing radiation, Gammacell 1000 irradiator from Atomic Energy of Canada Ltd. (Mississauga, ON) with a ^{137}Cs was used. PI/RNase was obtained from BD Biosciences (Franklin Lakes, NJ). The antibody against Cdc25B (#610528), which has been extensively validated by us, was from BD Transduction Laboratories (Franklin Lakes, NJ). Other primary antibodies used were: Cdc25A, and Cdc25C from Santa Cruz Biotechnology (Santa Cruz, CA); Histone H3 (Ser10) from Upstate Biotechnology (Lake Placid, NY); Cdk1 (Tyr15) Chk1 (Ser317), Chk1 (Ser345), p53 (Ser15) p53, γ-H2AX and PARP, from Cell Signaling Technology (Danvers, MA); HA from Covance (Berkeley, CA) and ß-tubulin from Cedarlane Laboratories (Hornby, Ontario, Canada). Primary antibodies dilutions and species is provided in the Appendix A Horseradish Peroxidase (HRP) conjugated goat anti-mouse antibody or HRP conjugated goat anti-rabbit

antibody were obtained from Jackson ImmunoResearch (West Grove, PA). Enhanced chemiluminescence reagent was obtained from Amersham Pharmacia (Piscataway, NJ).

2.2 CELL CULTURE

A549 cells (ATTC, Manassas, VA) were maintained in Basal Medium Eagle supplemented with 1% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin–streptomycin; p53+/+ and p53-/- , Chk2-/- HCT116 cells (a gift from Prof. Bert Vogelstein, Johns Hopkins University) in McCoy's medium with 10% FBS and 1% penicillin–streptomycin and Hela (ATTC) in Dulbecco's Minimum Essential Medium (DMEM) with 10% FBS, 1% penicillin–streptomycin. Wildtype and knockout Cdc25B mouse embryonic fibroblasts (MEFs) (a gift from Dr. Peter Donovan, Johns Hopkins University) were maintained in DMEM with 20% FBS, 1% L-glutamine and 1% penicillin–streptomycin. U2OS expressing HA-Cdc25B3 under tetracycline-regulated promoter (a gift from Prof. Bernard Ducommun, Université Paul Sabatier) were maintained in DMEM supplemented with 10% FBS (tetracycline screened), G418 (100 μg/ml), 1% penicillin–streptomycin and 2 μg/ml tetracycline. CCL202, human lung fibroblasts were obtained from ATCC (Manassas, VA). CCL202 cells were maninted in Eagle's Minimum essential Medium, 10% FBS, 2mM L-glutamine.

Cells were plated and incubated for 24 hours to yield 25-30% density prior to treatment with compounds or UV or IR exposure. Stock solutions of Etoposide and Cisplatin were prepared in dimethyl sulfoxide (DMSO) and Bleomycin in distilled water. Cells were exposed to the drugs for the indicated time periods. For UV exposure cells were washed twice with pre-warmed (37°C) phosphate buffer saline (PBS) and were irradiated in the absence of

medium. Following irradiation cell culture medium was added to the cells and cells were incubated for indicated time periods at 37°C. For IR cells were treated in the presence of the medium. The CCL202 cells were exposed to the desired concentration of *anti*-BPDE for the indicated time periods. Stock solution of *anti*-BPDE was prepared in DMSO, and an equal volume of DMSO was added to the controls. Induction of DNA damage in the presence of elevated levels of Cdc25B was examined by culturing cells in the absence of tetracycline for the indicated time periods.

2.3 WESTERN BLOTTING AND SUBCELLULAR FRACTIONATION

Cell were harvested and lysed in modified RIPA buffer (50 mM Tris pH 7.6, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 12 mM ß-glycerol phosphate, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µg/ml AEBSF, 10 µg/ml soybean trypsin inhibitor and 1 mM PMSF), incubated on ice for 30 minutes with brief vortex mixing every 10 minutes, and centrifuged at 13,000 x *g* for 15 minutes to clear the lysates. Western blotting was done as described previously (61) with the exception that proteins were transferred overnight to a nitrocellulose membrane at 30 V to allow maximum protein transfer. Cell fractionation was performed as previously described (99). Briefly, cells were lysed in sucrose buffer (320 mM sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, and 0.5% NP-40) and incubated on ice for 2 min or until they were determined to be lysed completely by examination under phase contrast microscope.

2.4 RECOMBINANT FULL LENGTH CDC25B

Epitope-tagged $His_6Cdc25B_2$, was expressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid (His_6) as described previously (100).

2.5 RNA INTERFERENCE AND RNA MEASUREMENTS

The vector for creating Cdc25B knockdown HCT116 cells was obtained from Ambion (Austin TX); pSilencer 4.1 containing a gene for puromycin resistance. Oligonucleotides encoding short hairpin RNA interference (shRNAi) targeting Cdc25B (target sequence for Cdc25B4 vector, AAAGGCGGCTACAAGGAGTTC or Cdc25B3 vector GTTCAGCAACATCGTGGATAA) were ligated into the vector according to the manufacturer's instructions. Vector expressing interference RNA with limited homology to any known sequence (scramble vector) was provided by the manufacturer and used as a negative control. Briefly, pSilencer plasmid was transfected in HCT116 using Lipofectamine-PLUS (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were replated and allowed to attach overnight. Thereafter, clones were selected with 0.5 μg/ml puromycin. Clones were picked 2 weeks later, expanded by culturing in the presence of 0.5 μg/ml puromycin, and screened for Cdc25B knockdown using Western blotting. For Cdc25B smartpool small interfering RNA (siRNA) (Dharmacon, Inc. Lafayette, CO) transfection, HCT116 cells were plated and incubated for 24 hours to yield 50-55% density. Cells were transfected according to the manufacturer's recommendation (Invitogen). Briefly, 100 nM of Cdc25B smartpool siRNA

was transfected using Lipofectamine 2000. Twenty four hours after transfection, cells were split to yield 30-35% density the next day. Cells were harvested as described elsewhere.

2.6 RT-PCR

Total RNA was extracted from cells using RNeasy (Qiagen, CA). cDNA was synthesized from 2 µg of RNA using random hexamer (Amersham, Buckinghamshire, UK) with Superscript RNase H-reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The reverse-transcribed cDNA from each sample was subjected to PCR amplification using Taq polymerase (Promega, Madison, WI) and primers. The sequence of the primers used was as described previously (61). The PCR conditions for the amplification of *Cdc25B* and *β-actin* genes used consisted of 24 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by a final incubation at 72°C for 7 minutes. Amplified products were separated by 2% agarose gel electrophoresis and bands were visualized by staining with ethidium bromide.

2.7 G2 CHECKPOINT RECOVERY ASSAY AND FLOW CYTOMETRY

For G2 checkpoint recovery assay, U2OS cells were plated for 24 hours and Cdc25B was induced by washing cells with PBS twice then incubating in the presence of culture medium for 15 minutes at 37°C and finally culturing in the absence of tetracycline for additional 16 hours. Control cells were cultured in the presence of tetracycline to suppress Cdc25B expression. At the

end of the 16 hour induction, cells were mock or UV treated (as indicated) and cells were either harvested at the indicated time points (asynchronous cells) or were trapped in mitosis with 23 hour nocodazole (1 μM) incubation. For the analysis of mitotic cells, both floated and attached cells were harvested or collected. For BPDE experiments cells were either treated with DMSO or 100nM BPDE and mitotic re-entry of asynchronous cell populations was examined by harvesting cells at the indicated time points. For HCT116 Scr and Cdc25B4#2 cells, cells were plated for 24 hours and were either mock or UV treated (as indicated). Cycling cells were then trapped in mitosis with 18 hour nocodazole (1 μM) incubation.

Identification of mitotic cells was carried out by simultaneously staining cells with PI and phospho-Histone H3 (Ser10) as described previously (101). Briefly, cells were fixed overnight in 70% ethanol and permeabilized with PBS, 0.25% Triton X100 for 8 minutes on ice. Following washing in PBS-1% BSA, cells were incubated with 1 μg of anti-phospho-Histone H3 for 2 hours. Cells were washed one more time before incubating with anti-rabbit IgG –Alexa 488 (1:75 for U20S and 1:100 for HCT116 cells) for 40 minutes in the dark. Finally, cells were washed and resuspended in 500 μl PI for 15 minutes prior to FACS analysis. For the FACS, 20,000 cells were analyzed and the data analysis was performed using WinMDI to determine phospho-Histone H3 positive cells.

2.8 VIABILITY STAIN

HCT116 and U20S cells were plated for 24 hours. Next day, Scr and Cdc25B4#2 cells were treated with mock or UV ($30J/m^2$) and cells were fixed 48 hours later with para formaldehyde as described previously (102). Briefly, cells were fixed with freshly prepared 3.7%

paraformaldehyde for 15 minutes, washed and then stored in PBS. Cells were visualized by staining the cells with crystal violet for 5 minutes. Cells were then washed with distilled water to remove excess stain. U20S cells were processed similarly except Cdc25B was induced by removing tetracycline or culturing in the presence of 10ng/ml of tetracycline just after mock or UV ($15J/m^2$) treatment.

3.0 INDUCTION OF CDC25B REGULATES CELL CYCLE RESUMPTION AFTER GENOTOXIC STRESS

Cdc25 phosphatases propel cell cycle progression by activating cyclin-dependent kinases (Cdk). DNA damage is generally thought to inhibit Cdc25 functionality by inducing proteasomal degradation of Cdc25A and phosphorylation-mediated sequestration of Cdc25B and Cdc25C to the cytoplasm. Recently, a critical role for Cdc25B in the resumption of cell cycle progression through mitosis after DNA damage has been identified. In this study, the fate of Cdc25B following mechanistically distinct DNA damaging agents (etoposide, cisplatin, bleomycin, ionizing irradiation or UV irradiation) was examined and surprisingly a rapid increase in cellular Cdc25B levels was observed following DNA damage. Using UV irradiation as the prototypic damaging agent, we found that the increase in Cdc25B levels was regulated by Chk1 and was independent of p53. Interestingly, Cdc25B levels controlled the number of cells progressing into mitosis following UV but they did not affect G2/M checkpoint engagement immediately following DNA damage. Increased Cdc25B was found to reduce the time required for cell cycle resumption. Furthermore, induced Cdc25B was found to be functionally positioned to activate cytoplasmic Cdk1 to regulate resumption. Collectively, these data support a model in which Cdc25B accumulation after DNA damage is an important anticipatory event for cell cycle resumption and might function as one of the arbiter for cell survival.

3.1 INTRODUCTION

The Cdc25 subfamily of the protein tyrosine phosphatases are important regulators of mammalian cell cycle checkpoints, which control proliferation and genomic integrity (19). The three mammalian homologues, Cdc25A, Cdc25B and Cdc25C, positively regulate cell cycle progression by activating cyclin-dependent kinase (19, 24, 28). Cdc25B has been proposed to have a unique role in initiating mitosis by activating Cdk1 at centrosomes (37).

Eukaryotic cells have evolved surveillance mechanisms to guard their DNA from genotoxic stress. One critical mechanism is inhibition of cell cycle progression, thus allowing cells to repair damaged DNA and preventing replication or transmission of altered DNA to the next generation. Cdc25s are rapidly inhibited to induce cell cycle arrest as described in Chapter 1. However, the molecular mechanisms employed by checkpoints to inactivate Cdc25B and the exact role of Cdc25B in checkpoint response are far from being completely understood. For example, it is unclear why the site targeted by p38/MAPKAPK2 to inhibit Cdc25B in response to UV is also basally phosphorylated in unperturbed cells (77). In the absence of stress, the same site (Ser309) is phosphorylated by Chk1 and pEG3 (a member of the KIN1/PAR-1/MARK family) (55, 103). Therefore, it is unresolved how phosphorylation at Ser 309 could possibly cause cell cycle arrest. One potential explanation could be spatio-temporal regulation of Cdc25B activity as suggested by a recent study in which Cdc25B was selectively inhibited in the centrosomes following DNA damage (56).

In contrast some groups believe that Cdc25B is crucial for cell cycle resumption following DNA damage induced cell cycle arrest. In these studies, cell cycle resumption was analyzed by measuring the percentage of cells exiting from G2 by trapping in cells mitosis with paclitaxel or nocadozole for 18 or 23 h (94, 104). In some studies, resumption was recapitulated

by inhibiting checkpoint signaling using caffeine. However, one of the drawbacks of this experimental design is that it does not truly study resumption from "cell cycle arrest" following DNA damage rather it induces checkpoint abrogation. Secondly, it also does not explain the mechanism by which DNA damage activated signaling pathways might regulate proteins involved in resumption. It only provides limited insight onto which protein might participate in resumption. To gain a deeper insight into cell cycle arrest and resumption, I analyzed the effect of DNA damaging agents on Cdc25B expression and cell cycle progression at different time points following DNA damage.

Thus, in this study I re-evaluated the effects of different types of DNA damaging agents on Cdc25B. The objective of the study was to delineate how different types of DNA damaging agents regulate Cdc25B. For this I first optimized conditions for analyzing protein levels of endogenous Cdc25B. I found that mechanistically distinct DNA damaging agents rapidly induced Cdc25B expression and that levels of Cdc25B determined the kinetics of cell cycle resumption following DNA damage induced cell cycle arrest.

3.2 RESULTS

3.2.1 Cdc25B Induction by DNA Damage

After an extensive investigation of commercially available Cdc25B antibodies to detect endogenous Cdc25B, I determined that an anti-Cdc25B monoclonal antibody from BD Transduction Laboratories (#610528) was most specific. As shown in the Fig. 8.A, this Cdc25B monoclonal antibody detected only two bands in the lysates of wildtype Cdc25B MEFs in a

region of the predicted molecular mass (~ 65 KDa) of Cdc25B whereas in the Cdc25B null MEF lysates only the lower band was detected even after loading more lysate (Fig. 8.A, lane 3). These results confirm that the upper band was Cdc25B. Other commercially available antibodies to Cdc25B consistently cross-reacted with many additional bands and were unaffected in the MEFs lacking Cdc25B or after shRNAi (data not shown). Thus, I believe the other currently available antibodies are not fully validated. With the BD Transduction antibody, the Cdc25B band ran closer to the recombinant His-tagged-human Cdc25B2 protein, further supporting that the upper band was indeed Cdc25B. This was further corroborated by a decreased expression of Cdc25B in the clones picked after Cdc25B specific shRNAi treatment (Fig. 8.B). Similar results were obtained when I used Cdc25B smartpool (Dharmacon) in transient transfection assays (Fig. 8.C). Notably, Cdc25B specific shRNAi/siRNA did not decrease the levels of the non-specific lower band. Thus, for all subsequent experiments I used the BD Transduction monoclonal antibody to detect endogenous Cdc25B in cells.

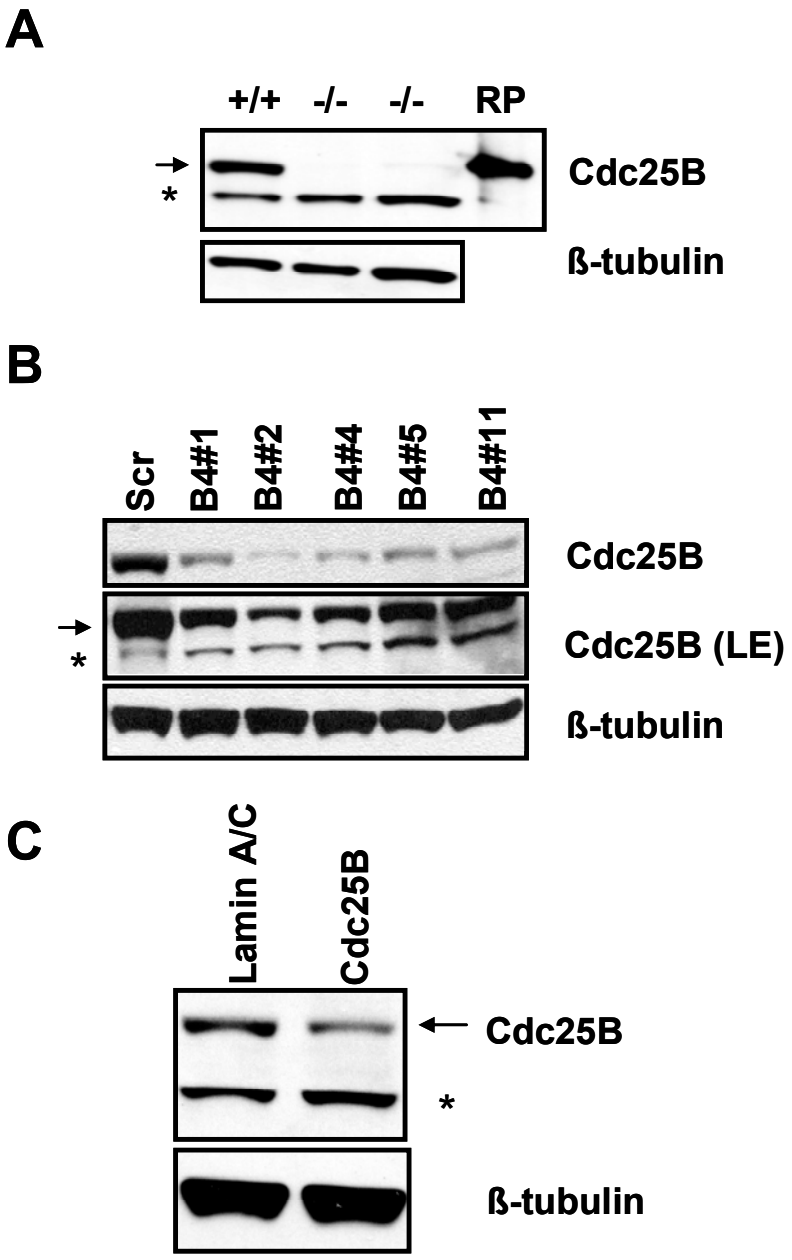


Figure 8. Detection of endogenous Cdc25B by western blotting

(A) Early passage of Cdc25B wildtype (+/+) and null (-/-) MEFs were harvested to analyze the Cdc25B expression. RP represents recombinant full-length purified human His-tagged Cdc25B2. (B) Cdc25B expression was also measured in cells selected for stable knock down of Cdc25B. (C) HCT116 cells transfected with smartpool siRNA against Cdc25B were harvested 48 hours later to analyzed Cdc25B expression. Cells transfected with siRNA against

Lamin A/C served as control. An arrow indicates Cdc25B and an asterisk is a non-specific band. LE is long exposure.

3.2.2 Agents causing different forms of DNA damage induced endogenous Cdc25B protein levels

To investigate the effects of DNA damaging agents on endogenous Cdc25B expression, I treated asynchronous A549 cells with different clinically used anticancer drugs: the topoisomerase–II poison etoposide (30 μM), the DNA cross-linker cisplatin (25 μM) and the radiomimetic bleomycin (25 μM) for 0, 1, 4 and 24 hours (Fig. 9). These drug concentrations were based on previous published findings revealing significant DNA damage (105, 106). Unexpectedly, all of these agents increased Cdc25B protein levels in cells within 1 hour (Fig. 9). With etoposide treatment, Cdc25B levels were elevated for at least 24 hours whereas with cisplatin and bleomycin treatment the increase in Cdc25B was more transient and basal levels were seen at 24 hours. Treatment with anticancer drugs did not affect the levels of the lower band (data not shown). As expected, treatment with these drugs decreased Cdc25A and transcriptionally increased p53 levels, although the kinetics for p53 induction was slower than that for Cdc25B. Additionally, Cdc25B levels increased within 1 hour after treatment with 10 Gy of ionizing irradiation, a prototypical DNA damaging agent, with levels remaining high for at least 4 hours (Fig. 9). The kinetics of Cdc25B increase after ionizing irradiation was similar to that of p53. Therefore, Cdc25B induction was a promiscuous response shared by mechanistically distinct DNA damaging agents and this observation supports our previous finding that the DNA damaging carcinogen, benzo[a]pyrene diol epoxide, increases Cdc25B expression in these lung cancer cells (61).

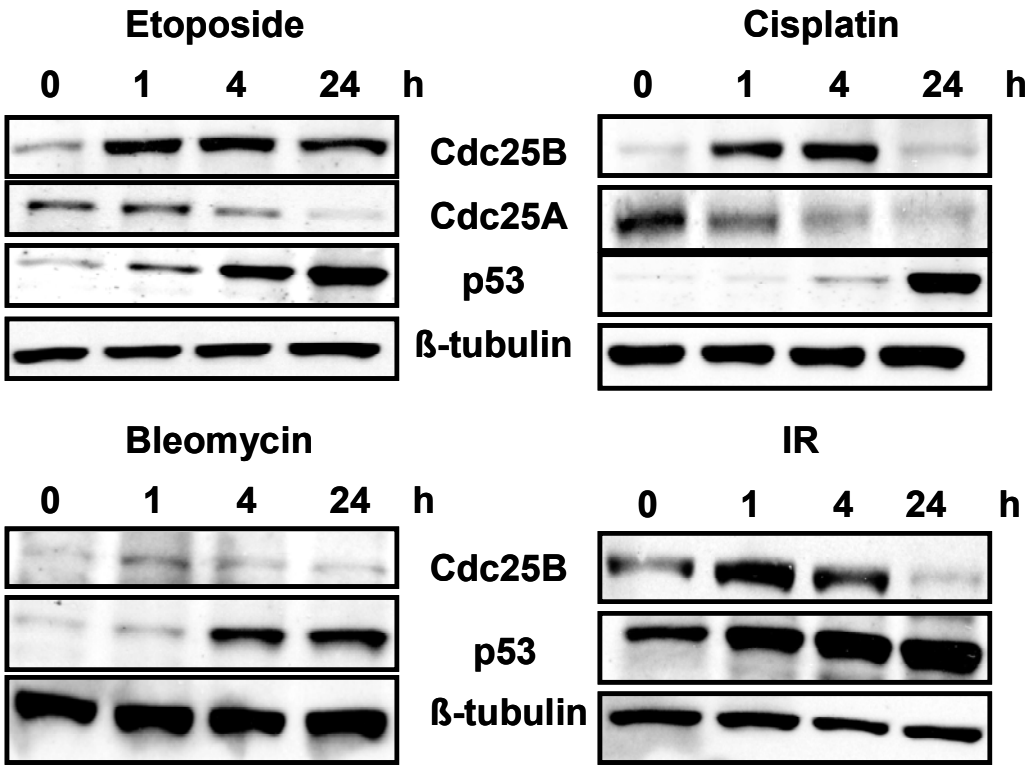


Figure 9. Agents causing different forms of DNA damage induced endogenous Cdc25B protein levels

A549 cells were treated with DMSO or etoposide (30 μM), cisplatin (25 μM), bleomycin (25 μM) and harvested at different time points. HCT116 cells were treated with IR 10 Gy and harvested at different time points. Levels of indicated proteins were examined by Western blotting. Each panel is representative of n= 4-6.

3.2.3 p53 Independence of Cdc25B Induction by UV

I next examined the effect of DNA damage by UV irradiation on Cdc25B expression. As shown in Fig. 10, A549 cells treated with UV (60 J/m^2) had increased Cdc25B protein levels within 30 minutes after irradiation and the levels were persistently increased for 24 hours post-treatment. Because one previous report showed that Cdc25B levels were unaffected in Hela cells

exposed to UV (77), I examined the effect of UV irradiation on endogenous Cdc25B expression in Hela cells to exclude cell type specific effects. I observed increased Cdc25B levels within 30 minutes after UV exposure and they remained elevated for at least 24 hours, similar to A549 cells (Fig. 10). Furthermore, immediate induction of Cdc25B was independent of p53. Thus, increased Cdc25B was observed in UV exposed isogenic HCT116 cells that expressed or lacked p53 (Fig. 10).However, the increase in Cdc25B expression at 4 h and 24 h was attenuated in p53 -/- cells, indicating that p53 might play a role in maintaining increased expression of Cdc25B following UV irradiation .

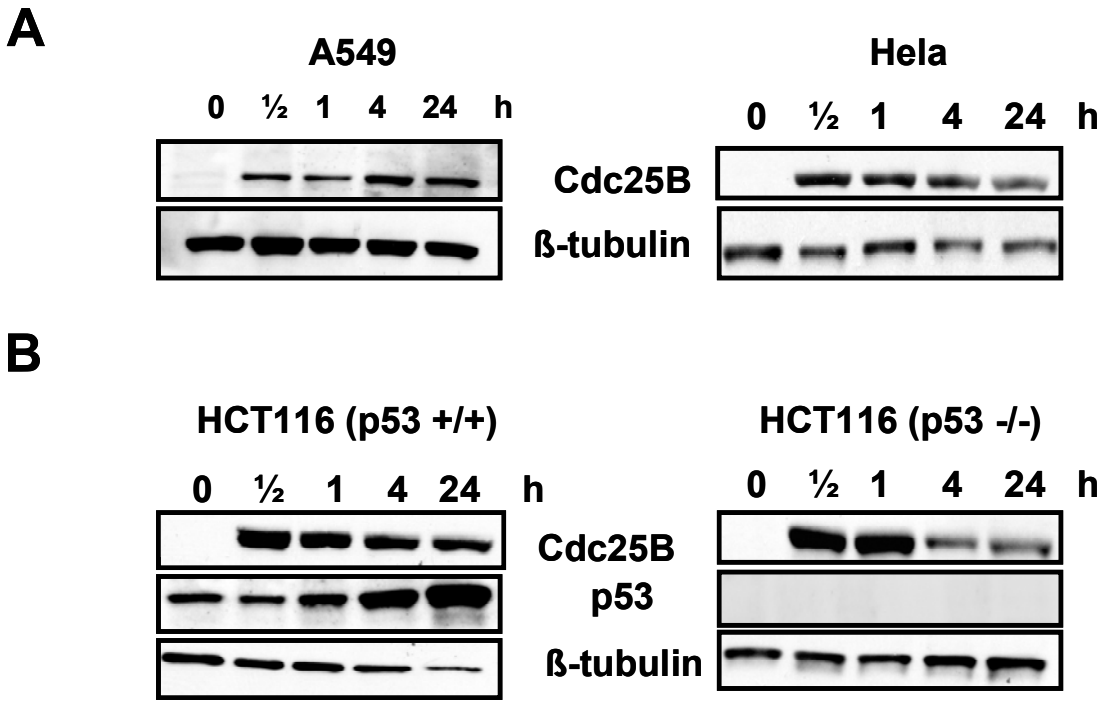


Figure 10. Cdc25B induction by UV is independent of p53 levels

A) A549 and Hela cells were washed with PBS twice and then irradiated with 60 J/m^2 of UV in the absence of medium. Following exposure, cell culture medium was added and cells were harvested at different time points for determination of endogenous Cdc25B expression by

western blotting. (B) HCT116 (p53 +/+) and HCT116 (p53-/-) cells were treated as described for panel A.

I also studied the effect of UV on Cdc25B wildtype and null MEFs. Cdc25B expression was increased in the Cdc25B wildtype MEFs whereas no Cdc25B was detected in Cdc25B null cells as expected (Fig. 11). Furthermore, Cdc25B induction was attenuated in cells stably expressing Cdc25B shRNAi (Cdc25B4#2) compared with cells with scrambled shRNAi (Fig. 11). Finally, to study the effect of UV irradiation on induced Cdc25B, I treated U20S cells expressing a tetracycline (tet-off) regulated HA-tagged Cdc25B. After removal of tetracycline from the medium, HA-Cdc25B expression was induced and cells were either mock or UV treated. Cells treated with UV expressed more HA-Cdc25B at 24 hours and 48 hours compared to the mock treated cells. Induction of exogenous Cdc25B was confirmed using an antibody to the HA-epitope tag (Fig. 11).

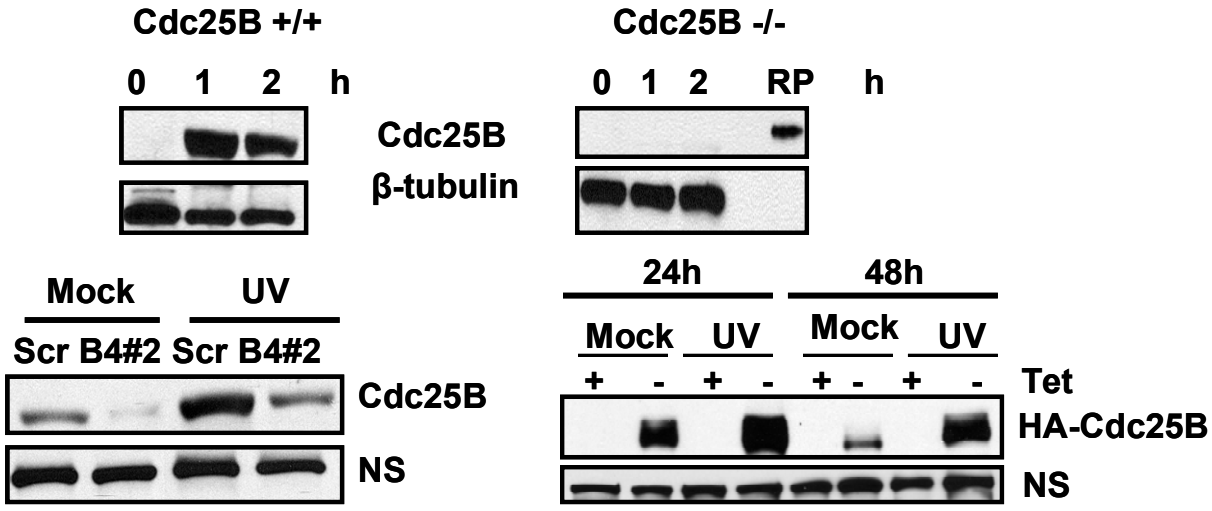


Figure 11. Validation of Cdc25B induction following UV irradiation

Cdc25B +/+ and -/- MEFs were treated as described under Figure 10. HCT116 Scr and B4#2 cells were treated with either mock or UV (60 J/m^2) for 16 hours as indicated. NS is non-specific band. U2OS cells were cultured either in the presence or absence of tetracycline to induce HA-Cdc25B3 and the effect of either mock or UV (15 J/m^2) irradiation on exogenous Cdc25B was analyzed at the indicated time points.

I next determined the minimum dose of UV required to increase endogenous Cdc25B expression. In A549 cells, doses as low as 15 J/m^2 were sufficient to induce Cdc25B levels within 1 hour (Fig. 12). To examine the effect of UV treatment on endogenous Cdc25B expression in a long-term assay, I treated HCT116 (p53+/+) cells with UV doses ranging from 5-60 J/m^2 and harvested clones 8 days later. In this assay, doses ≥ 5 J/m^2 induced significant levels of Cdc25B (Fig. 12). The effect of doses higher than 15 J/m^2 could not be evaluated due to toxicity and the lack of sufficient colonies for analysis. These results support previous findings demonstrating an increase in Cdc25B levels following chronic exposure to ionizing radiation (87).

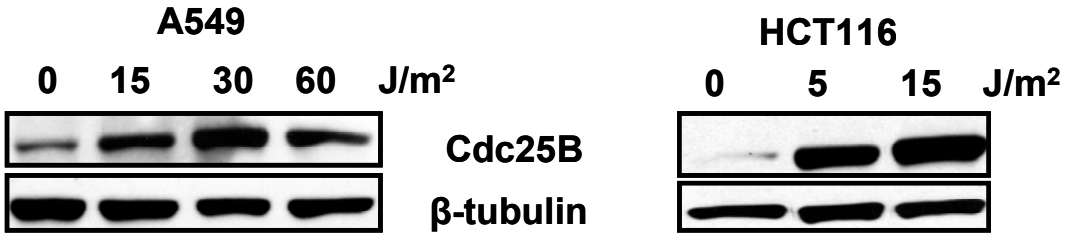


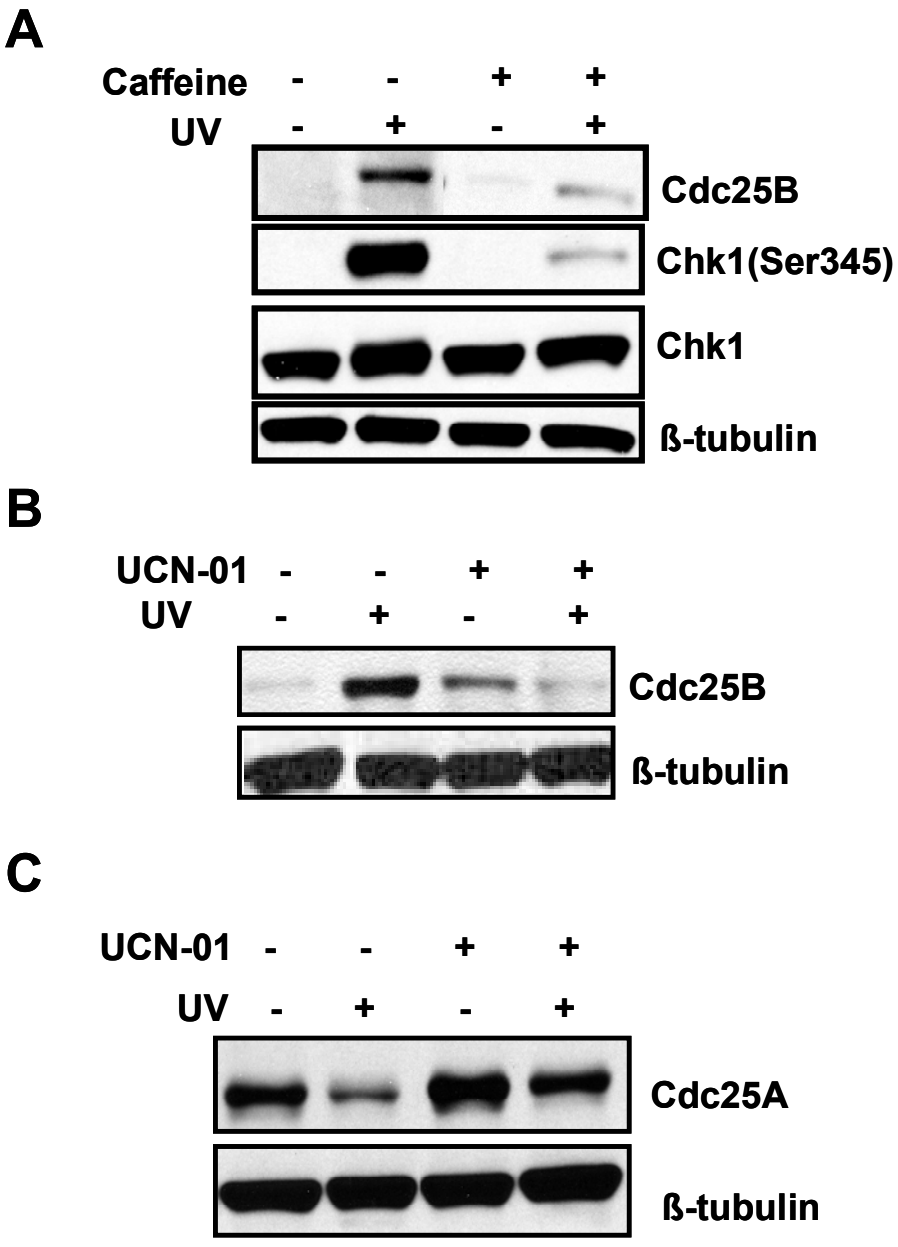
Figure 12. Cdc25B induction following UV irradiation is dose dependent

A549 cells were treated with different doses of UV and 1 hour later cells were harvested. We plated 800 HCT116 (p53 +/+) cells in 6 cm dish and 24 hours later cells were treated with different doses of UV. The surviving clones were harvested 8 days post-treatment for Cdc25B

expression. Levels of indicated proteins were examined by Western blotting. Each figure is representative of n= 4-6.

3.2.4 Checkpoint Regulates Cdc25B Induction

Checkpoint activation in response to DNA damage results in the Chk1 dependent phosphorylation of Cdc25A and Cdc25C. Because Cdc25B has canonical Chk1/Chk2 phosphorylation sites and Chk1 has been shown to phosphorylate Cdc25B, it was reasonable to hypothesize that Cdc25B induction might be checkpoint regulated (75, 107). Indeed, Chk1 was rapidly phosphorylated and activated in HCT116 cells following UV treatment (Fig. 13.A). To investigate the role of ATR/Chk1 pathway, I pretreated cells with caffeine (5 mM) to block ATR/ATM activation for 30 minutes before mock or UV (60 J/m^2) irradiation. As illustrated in Fig. 13.A, pretreatment with caffeine blocked the increase in Cdc25B seen 1 hour after UV exposure. Similar results were also observed in A549 cells (data not shown). To determine the role of Chk1 in Cdc25B upregulation, I pretreated cells with UCN-01 (300 nM) for 30 minutes and measured Cdc25B levels 1 hour after mock or UV treatment (60 J/m^2). UCN-01 blocked the increase in Cdc25B expression with UV (Fig.13.B). UCN-01 prevented loss of Cdc25A following UV (Fig. 13.C) confirming that the efficacy of UCN-01 in inhibiting Chk1 activity. These results also highlighted the mechanistically distinct regulation of Cdc25A and B isoforms by checkpoint signaling following DNA damage. I also examined Cdc25B induction in HCT116 Chk2-/- cells. Cells were either mock or UV (60 J/m^2) treated and harvested 1 hour later. As shown in the Fig. 13.D, the increase in Cdc25B level was independent of Chk2.


A
Caffeine - - + +
UV - + - +
Cdc25B
Chk1(Ser345)
Chk1
ß-tubulin
B
UCN-01 - - + +
UV - + - +
Cdc25B
ß-tubulin
C
UCN-01 - - + +
UV - + - +
Cdc25A
ß-tubulin

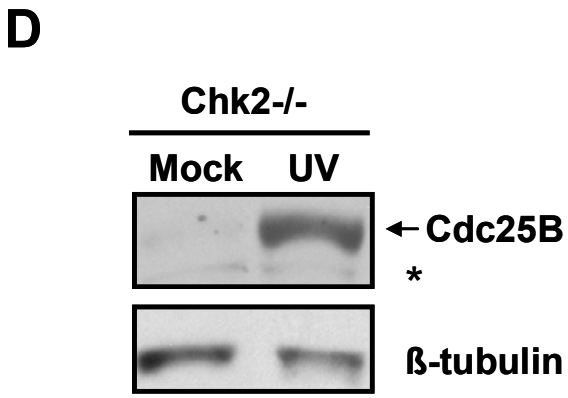


Figure 13. Cdc25B induction following UV irradiation is regulated by ATR/Chk1

(A) HCT116 cells were pretreated with vehicle or caffeine (5 mM) for 30 minutes before UV (60 J/m^2) or mock irradiation for 1 hour. (B and C) HCT116 cells were pretreated with UCN-01 (300 nM) for 30 minutes or vehicle before UV (60 J/m^2) or mock irradiation for 1 hour. Panel (B) represents Cdc25B levels and Panel (C) represents Cdc25A levels.(D) HCT116 Chk2-/- were treated either with mock or UV (60 J/m^2) and harvested 1 hour later. * Indicates a non-specific band. Each figure is representative of n=4.

I also noted similar Cdc25B mRNA levels 4 hours after UV treatment using semi-quantitative RT-PCR (Fig. 14.A). The labile nature of Cdc25B was confirmed by MG132-mediated proteasomal inhibition, which revealed increased Cdc25B levels by 24 hours (Fig. 14.B). These results support previous findings showing proteasome mediated degradation of Cdc25B (108). Collectively; my results are consistent with a model in which the increase in Cdc25B protein levels after UV is regulated by ATR/Chk1 pathway via post-transcriptional mechanism, potentially by affecting Cdc25B protein stability.

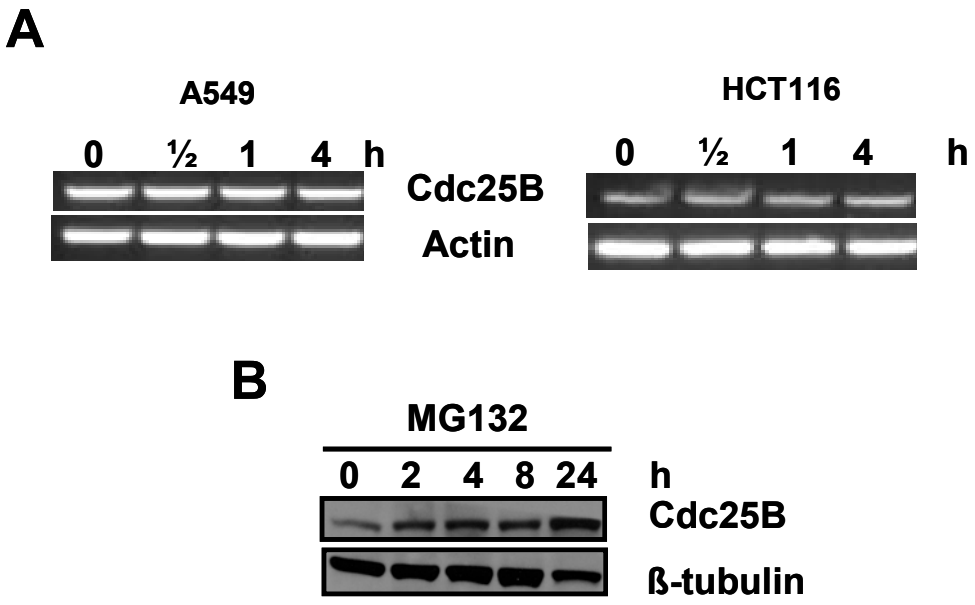


Figure 14. Regulation of Cdc25B expression by UV irradiation

(A) RT-PCR for Cdc25B expression in A549 and HCT116 cells after UV (60 J/m^2) irradiation. Cells were irradiated with UV as described in Figure 10. The conditions for RT-PCR were optimized to ensure linearity of the signal. (B) HCT116 cells were treated with MG132 (10 μM) for different times and cells were harvested to analyze expression of Cdc25B using western blotting.

3.2.5 Cdc25B Regulates Mitotic Entry Following DNA Damage

Recent evidence suggests that Cdc25B, but not Cdc25A or Cdc25C regulates cell cycle re-entry following DNA damage produced by doxorubicin (94). Stimulated by our observation that Cdc25B expression was increased within 1 hour in cells following exposure to mechanistically distinct DNA damaging agents, I hypothesized that levels of Cdc25B might be crucial in regulating the rate of cell cycle resumption following DNA damage-induced cell cycle

arrest. Thus, asynchronous cells were treated with mock or UV irradiation and cells exiting G2 were trapped in mitosis with nocodazole (1 μM) treatment for 18 hours (HCT116) or 23 hours (U2OS). Mitotic arrest was detected either by probing lysates with a phospho-Histone H3 (Ser10) antibody or by staining cells with PI and phospho-Histone H3 (Ser10) and analyzing cells with a flow cytometer. To examine the effect of Cdc25B in this assay, I reduced intracellular Cdc25B levels by >75% using shRNAi (Fig. 15.A). In the absence of DNA damage, 69.2 $\pm$ 2.8% of control cells (Scr) (n=3) and 59.4 $\pm$ 4.0% of Cdc25B depleted cells (N=3) were trapped in mitosis (Fig.15.B and C) consistent with previous studies suggesting Cdc25B is not required for normal cell cycle progression (38). After 30 J/m^2 UV, I observed a slight decrease in the percentage of cells trapped in mitosis by nocodazole for control cells (60.3 ± 0.5%; n=4) whereas only 40.5 ± 1.1% of Cdc25B depleted cells (n=4) were arrested in mitosis. The difference in mitotic trapping was even more pronounced at a higher dose of UV (60 J/m^2) where 25.0 ± 1.1% of control cells (n=5) and 14.7 ± 0.6% of Cdc25B depleted cells (n=5) were trapped in mitosis. At both doses of UV the difference between Scr and Cdc25B depleted cells was statistically significant (Fig. 15.D). Interestingly, with all of the conditions, about 80% of the cell population was in G2/M (Fig. 15.D) suggesting that even after DNA damage a majority of cells could progress from G1 and S phase into G2 phase but entry into mitosis was dependent on levels of Cdc25B and was directly correlated to the intensity of DNA damage. These results were confirmed using a different shRNAi against Cdc25B (Fig. 16). In these cells, depletion of cdc25B decreased the % of cells trapped in mitosis after UV 60J/m^2 treatment compared to Scr cells suggesting this was not due simply to an off-target effect of a single shRNAi.

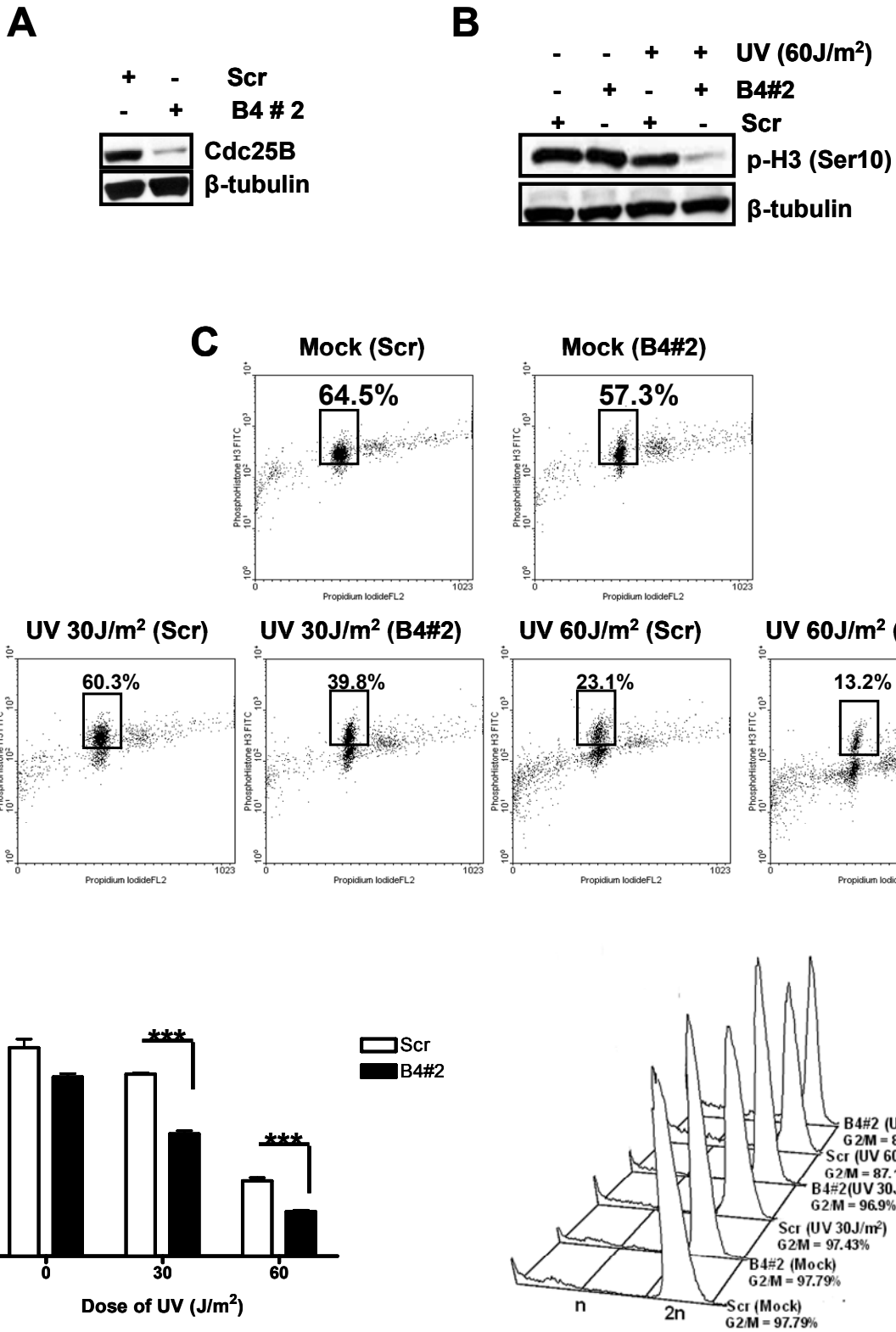

A
+ - Scr
- + B4 # 2
Cdc25B
β-tubulin
B
- - + + UV (60J/m2)
- + - + B4#2
+ - + - Scr
p-H3 (Ser10)
β-tubulin
C
Mock (Scr)
64.5%
Mock (B4#2)
57.3%
UV 30J/m2 (Scr)
60.3%
UV 30J/m2 (B4#2)
39.8%
UV 60J/m2 (Scr)
23.1%
UV 60J/m2 (B4#2)
13.2%
Phosphohistone H3 FITC
Propidium IodideFL2
D
% of cells in mitosis
Dose of UV (J/m2)
Scr
B4#2
***
***
B4#2 (UV 60J/m2)
G2/M = 80.13%
Scr (UV 60J/m2)
G2/M = 87.12%
B4#2(UV 30J/m2)
G2/M = 96.9%
Scr (UV 30J/m2)
G2/M = 97.43%
B4#2 (Mock)
G2/M = 97.79%
Scr (Mock)
G2/M = 97.79%
n
2n

Figure 15. Depletion of Cdc25B decreases the entry into mitosis following DNA damage

(A) HCT116 cells were transfected with scramble (Scr) and Cdc25B (B4# 2) shRNAi. Clones were selected to enhance Cdc25B depletion. Asynchronous cells were harvested to analyze Cd25B levels. (B) Western blot analysis of phospho-Histone (Ser10) (mitosis marker) in Scr (controls cells) and Cdc25B shRNAi cells after mock or UV (60 J/m^2) irradiation and subsequent treatment with 1 μM nocodazole for 18 hours to trap cells in mitosis. For the analysis both attached and floating cells were collected. (C) Cells treated as described in panel (B) were fixed at the end of 18 hours of trapping and phospho–Histone H3 positive cells were determined by flow-cytometer. (D) Bar graph and histogram representation of the data in (C). Results are mean $\pm$ SE (n=3-5). Statistical significance was determined by two tail unpaired t – test. ***, P<0.0001. For histogram the percentage of cells in G2/M represents mean.

To understand how increased levels of Cdc25B affected cellular progression after DNA damage, I exploited a previously described tetracycline regulated U2OS cell system (104). Ectopic HA-Cdc25B expression was induced prior to treatment with either mock or UV irradiation (Fig.17.A). In the absence of DNA damage, 73.5 $\pm$ 1.4% of control cells were in mitosis, which was similar to Cdc25B overexpressing cells (69.4 $\pm$ 1.1%) (Fig. 3.10.B and C). In the presence of DNA damage (UV 30 J/m^2), however, 4.1 ± 0.3% of UV exposed control cells and 8.1 ± 0.5% of UV exposed Cdc25B overexpressing cells were trapped in mitosis by nocodazole treatment (Fig.17.B, C and D). In my experiments, I have observed that U20S cells are more sensitive to UV compared to HCT116 cells (Fig.15 and 16). Also, HCT116 cells are deficient in mismatch repair protein hMLH1, which impairs their ability to arrest in G2/M for prolonged period following DNA damage (109). As a result, UV treated U20S cells were more

resistant to nocodazole trapping. Hence, in the mock treated cells about 80% of cells were in G2/M following nocodazole trapping whereas in UV (30 J/m^2) treated cells only 18.5% of cells were in G2/M for control and 21.5% of cells for Cdc25B overexpressing cells.

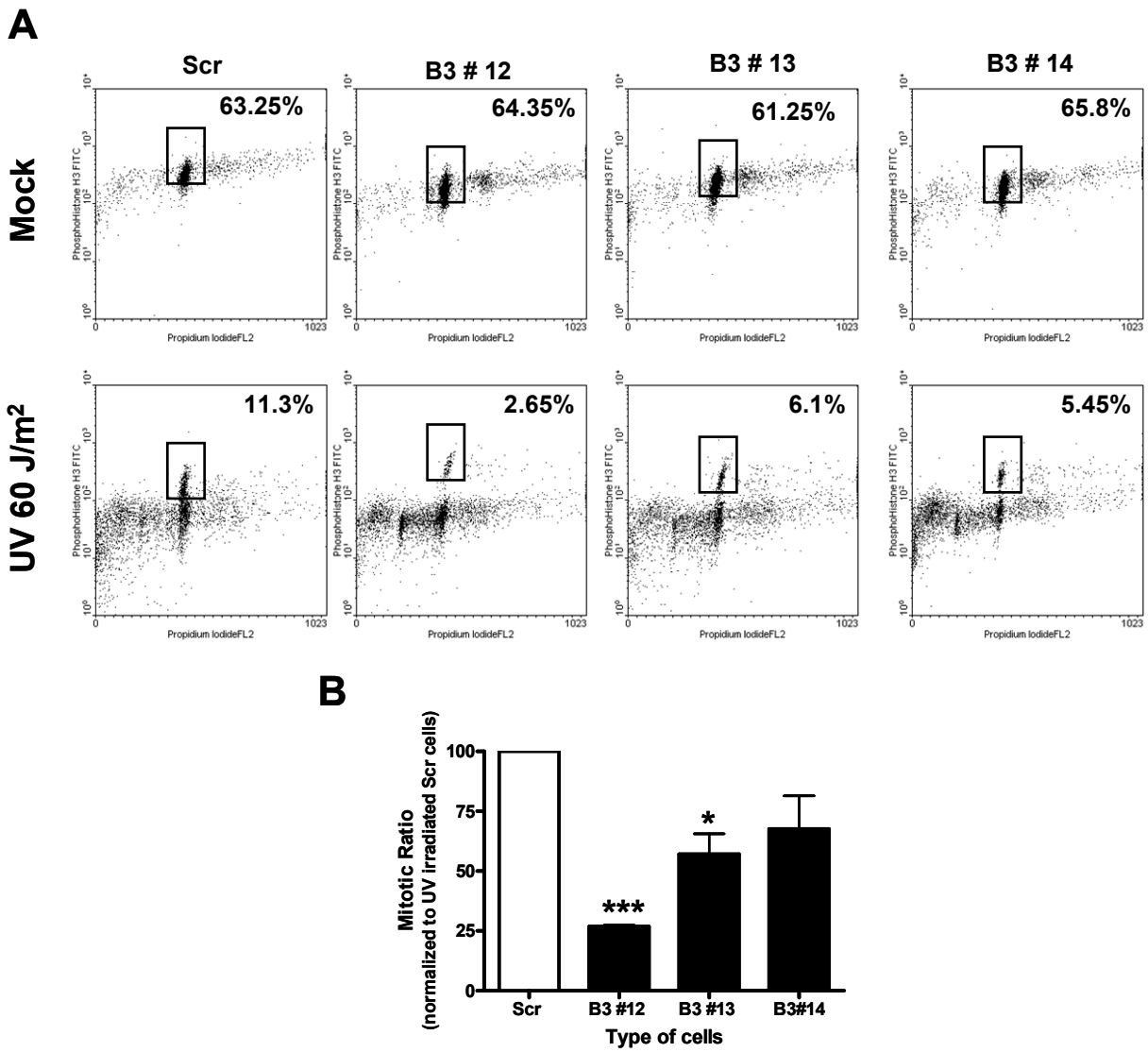


Figure 16. Depletion of Cdc25B with different Cdc25B shRNAi also decreases the entry into mitosis following DNA damage

(A) HCT116 cells were transfected with scramble (Scr) and Cdc25B3 shRNAi. Clones were selected to enhance Cdc25B depletion and B3#12, B3#13, and B3#14 were used for the analysis. Asynchronous cells (Scr and B3#12, B3#13, and B3#14) were treated and analyzed as

described under Figure 15. (B) Each bar represents % of mitotic cells following UV irradiation (60 J/m^2) normalized to UV (60 J/m^2) treated Scr cells. Results are mean ± SE (n=3-4). Statistical significance was determined by two tail unpaired t – test. ***, P<0.0001 and * P< 0.05.

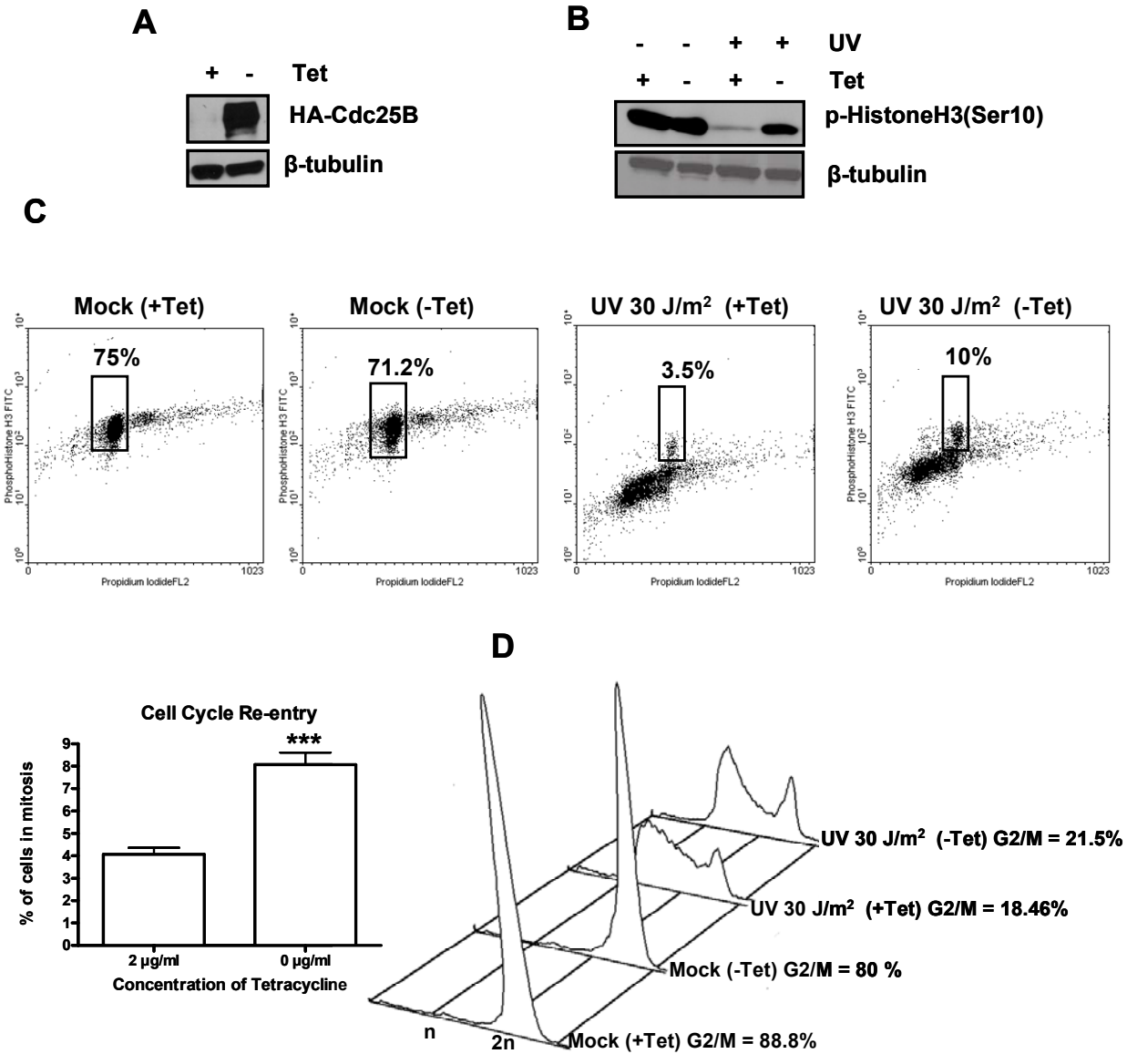
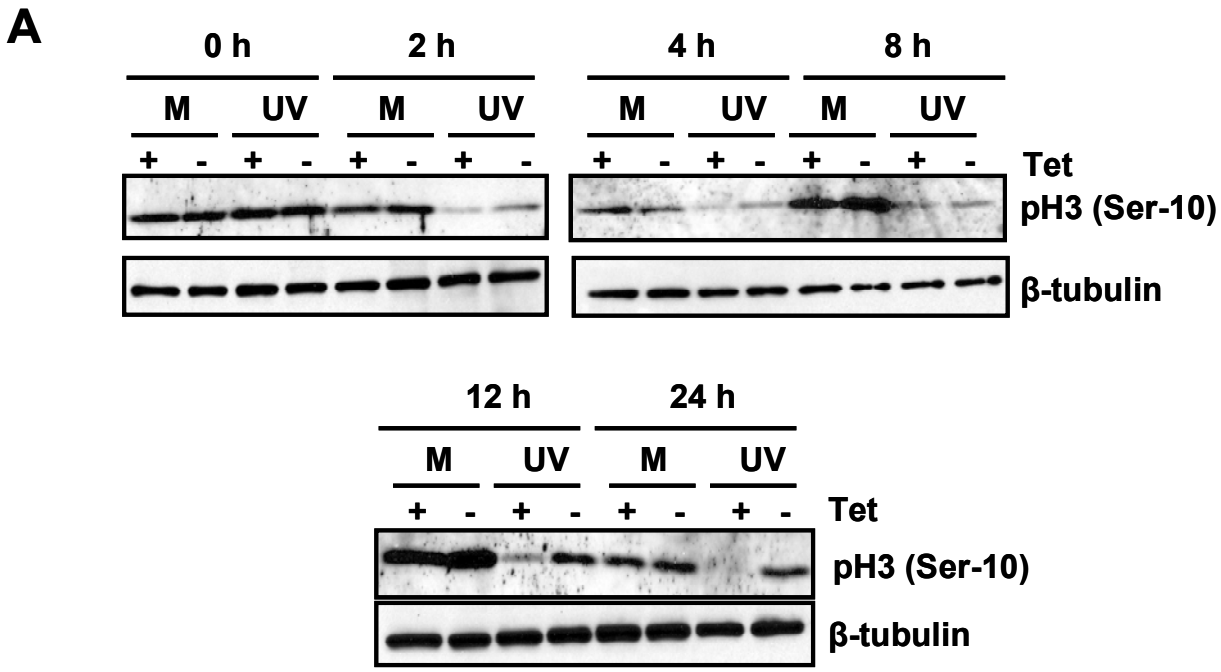


Figure 17. Over-expression of Cdc25B increases the entry into mitosis following DNA damage

(A) Expression of HA-tagged Cdc25B in U2OS cells expressing Cdc25B under the control of tetracycline–regulated promoter for 16 hours. Cdc25B was detected using a HA antibody. (B) U2OS cells expressing Cdc25B for 16 hours (-Tet) or controls cells (Tet) were treated as described in (Figure 15.B) except the dose of the UV was 30 J/m^2 and cells were trapped for 23 hours with nocodazole. (C) Cells were fixed at the end of 23 hours of trapping and phospho-Histone H3 levels were determined. (D) Bar graph and histogram representation of the data in (C). Results are mean $\pm$ SE (n=6). Statistical significance was determined by 2 tail unpaired t – test. ***, P<0.0001. For histogram the percentage of cells in G2/M represents mean.

Finally, if Cdc25B was important in the regulation of cell cycle resumption, then cells expressing more Cdc25B should exit the G2/M checkpoint earlier than control cells. To test this hypothesis, I induced Cdc25B for 16 hours by removing tetracycline and treated asynchronous cells with either mock or UV irradiation (15 J/m^2). At 2, 4, and 8 hours post-treatment, phospho-Histone H3 (Ser10) staining decreased indicative of a loss of the mitotic population and the engagement of a G2/M checkpoint, which was independent of Cdc25B levels (Fig. 18.A). In contrast, at 12 and 24 hours, Cdc25B overexpressing cells recovered from G2/M checkpoint whereas in control cells the G2/M checkpoint was still enforced. Similar results were observed by examining phospho-Histone H3 (Ser 10) using flow cytometry (Fig. 18.B). Four hours after UV treatment, control and Cdc25B overexpressing cells had fewer mitotic cells compared to the corresponding mock treated cells consistent with activation of the G2/M checkpoint. When normalized to unirradiated controls, 0.36 ± 0.01% of control cells and 0.34 ±

0.02% of Cdc25B overexpressing cells were in mitosis (Fig.18.B and C). At 12 hours post-treatment, the G2/M checkpoint was still enforced in control cells (0.22 ± 0.01%) whereas Cdc25B overexpressing cells resumed cell cycle followed by an increase in the percentage of the population in mitosis (0.86 ± 0.06%) (Fig.18.B and C). Collectively, these results demonstrate that Cdc25B had a fundamental role in cells progression into mitosis following DNA damage and checkpoint exit.



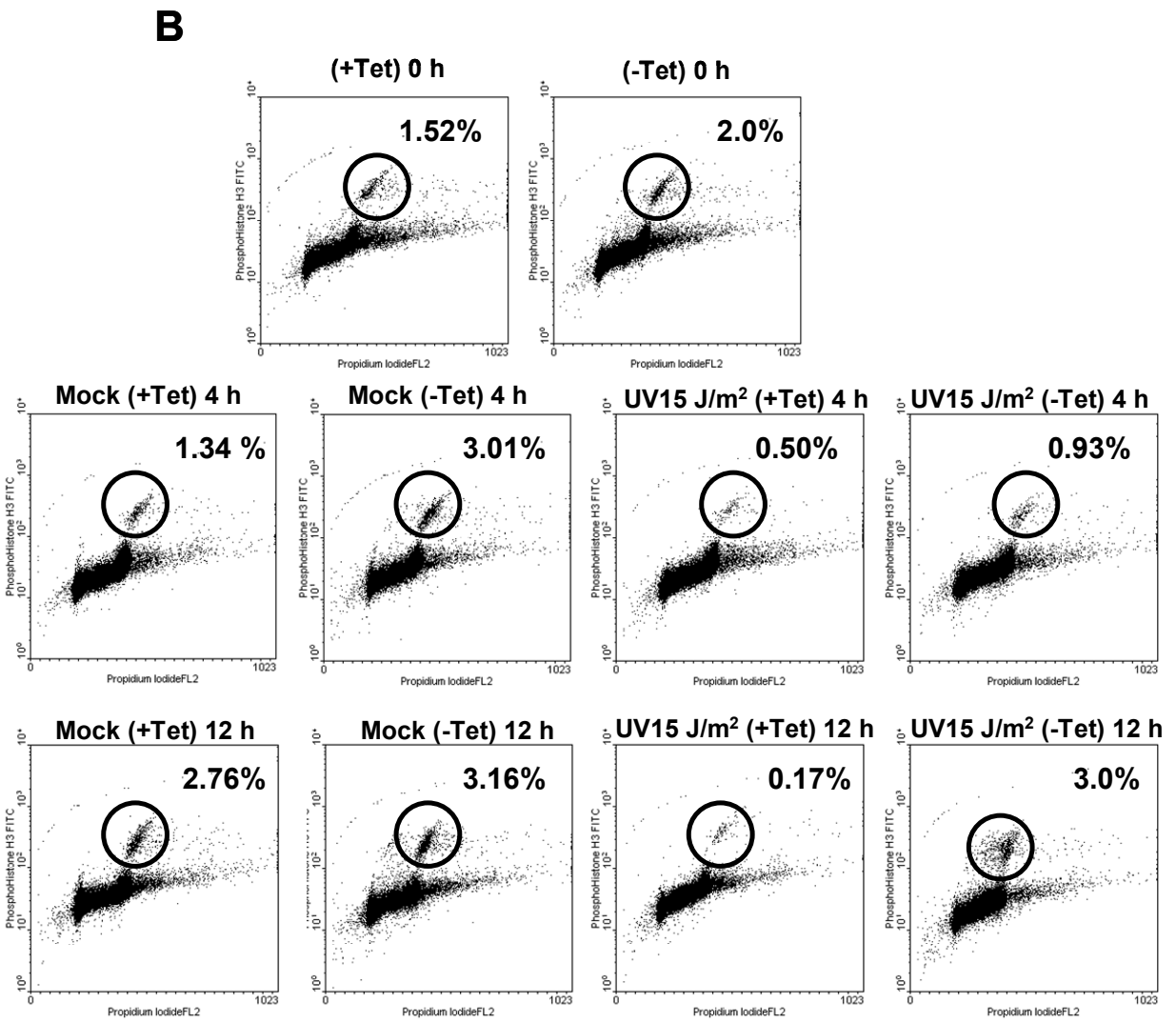

B
(+Tet) 0 h
1.52%
(-Tet) 0 h
2.0%
Mock (+Tet) 4 h
1.34 %
Mock (-Tet) 4 h
3.01%
UV15 J/m2 (+Tet) 4 h
0.50%
UV15 J/m2 (-Tet) 4 h
0.93%
Mock (+Tet) 12 h
2.76%
Mock (-Tet) 12 h
3.16%
UV15 J/m2 (+Tet) 12 h
0.17%
UV15 J/m2 (-Tet) 12 h
3.0%
PhosphoHistone H3 FITC
Propidium IodideFL2
C
Mitotic Ratio
(% of unirradiated control)
1.00
0.75
0.50
0.25
0.00
***
Tet
- Tet
0
4
12
Time (h)

Figure 18.Over-expression of Cdc25B accelerates resumption of cell cycle

(A) Cdc25B was induced for 16 hours before either mock or UV (15 J/m^2) irradiation. Floating and attached cells were harvested at the indicated time points to detect phospho-Histone H3 (mitotic marker) by western blotting (B) Cells were also fixed at the indicated time points and phospho-Histone H3 levels were determined by flow-cytometer. (C) Bar graph representation of the data in (B). Each bar represents % of mitotic cells following UV irradiation normalized to unirradiated control. Results are mean $\pm$ SE (n=4-5). The number indicates the concentration of the tetracycline for the experimental conditions. Statistical significance was determined by two tail unpaired t – test. ***, P<0.0001.

3.2.6 Cdc25B regulates Wee1 degradation

Recently, Van Vugt *et al.* showed that in cells depleted with Wee1, Plk1 and Cdc25B are not required for cell cycle resumption suggesting these proteins may act upstream of Wee1 to regulate recovery. It was further shown that Plk1 controls recovery by regulating Wee1 degradation. In this study the effect of Cdc25B on Wee1 degradation was not addressed (94). However, previous studies have shown that Cdk1, a Wee1 substrate, regulates Wee1 degradation by phosphorlyating Wee1 at Ser123 before the onset of M –phase (110, 111). Since Cdc25B activates Cdk1 to regulate entry into mitosis, in the absence of DNA damage, I hypothesized that Cdc25B regulates cell cycle recovery by regulating Cdk1 dependent Wee1 degradation. To test this hypothesis, I examined Wee1 levels in U20S cells treated with mock or UV (30 J/m^2) irradiation. In this experiment, I over-expressed Cdc25B by removing tetracycline for 16 hrs followed by mock or UV treatment. Cells exiting G2 were trapped in mitosis as described earlier. In the absence of DNA damage when a majority of cells are in mitosis (Fig. 17.B, C) the levels

of Wee1 were down regulated irrespective of Cdc25B levels (Fig. 19). However, in the presence of UV, control cells had higher levels of Wee1 compared to Cdc25B over expressing cells (lane 3 and lane 4). These results correlate with our flowcytometry data (Fig.19) where I showed that over-expression of Cdc25B increases the number of cells trapped in mitosis following UV. Based on these preliminary findings I propose that Cdc25B regulates recovery from DNA damage induced cell cycle arrest by regulating Wee1 degradation, potentially by activating Cdk1.

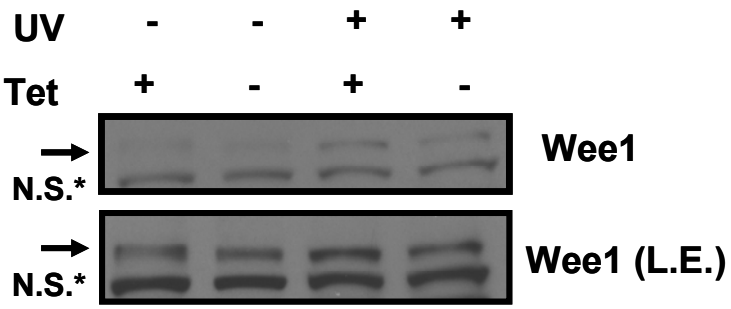


Figure 19. Cdc25B regulates Wee1 degradation

Asynchronous U20S cells expressing Cdc25B for 16 hours (-Tet) or control cells (+Tet) were mock or UV (30 J/m^2) treated. Cells were subsequently trapped in mitosis for 23 hours with nocodazole. At the end of 23 hours incubation, floating and attached cells were harvested to analyze Wee1 levels by western blotting. Non specific (NS) band was used to normalize Wee1 expression. Figure is representative of n=3. LE stands for long exposure.

3.2.7 Localization of Cdc25B Following DNA Damage

As an important regulator of cell cycle, specifically onset of mitosis, Cd25B activity is tightly regulated. One of the mechanisms by which cells regulate Cdc25B activity is through

subcellular localization facilitated by interactions with 14-3-3. Cdc25B Ser309 phosphorylation enables binding to 14-3-3β. Mutation of Ser309 abolishes cytoplasmic localization of Cdc25B during the interphase (47-49). This site is also subjected to checkpoint-dependent regulation (77, 78). To gain insight into how increase Cdc25B might regulate cell cycle re-entry, I examined Cdc25B localization in cell treated with leptomycin-B (LMB), a nuclear export inhibitor, and UV. In untreated asynchronous HCT116 cells, Cdc25B was primarily located in the cytoplasm (Fig. 20.A). To determine the effect of nuclear export inhibitor, LeptomycinB (LMB) and UV on subcellular distribution of Cdc25B, I subjected asynchronous HCT116 cells to an *in situ* fractionation protocol at various times points after LMB and UV exposure. Treatment with the LMB did not significantly affect subcellular distribution of Cdc25B. Cdc25C was cytoplasmic in the presence or absence of LMB. In contrast, modest nuclear Cdc25A redistribution was seen with LMB treatment after 6 h. These results suggest that among the three isoforms, Cdc25A most actively shuttles between nucleus and cytoplasm in the interphase. These results support previous findings that Cdc25B and C are cytoplasmic and move in the nucleus only prior to mitosis (112, 113). Alternatively, Cdc25B and Cdc25C may use different nuclear export pathway which is insensitive to LMB. Further studies examining Cdc25B and Cdc25C localization will help elucidate their localization under normal and stress conditions.

To examine the effects of UV on the subcellular localization of Cdc25A, B and C, I mock or UV irradiated asynchronous cells and harvested them 0.5 or 4 h later. As shown in the Fig.20.B, Cdc25A levels decreased significantly within 30 min, consistent with previous reports documenting significant loss of Cdc25A in the whole cell extracts 30 min after UV treatment (79). The increased Cdc25B was found localized in the cytoplasmic compartment at both 0.5 and 4 h after UV irradiation (Fig. 20.B). Cdc25C levels remained constant in the cytoplasm

supporting model that Cdc25C is retained in the cytoplasm in the presence of DNA damage to prevent premature activation of Cdk1 in the nucleus (76).

To understand the functionality of the increased Cdc25B levels, I determine the localization of Cdc25B substrate Cyclin B1/Cdk1 in asynchronous cells following UV treatment. Cyclin B1/Cdk1 were found in both the compartments (cytoplasmic and nuclear), although with slightly higher nuclear distribution (Fig. 20.C). Interestingly, with UV treatment levels of cyclin B1/Cdk1 were decreased in the nucleus in a time dependent manner. This supports previous findings that 14-3-3σ sequesters cyclin B1/Cdk1 in the cytoplasm to prevent nuclear accumulation of cyclin B1/Cdk1 following DNA damage (114). These results show that Cdc25B and its substrate are localized in the same compartment following DNA damage.

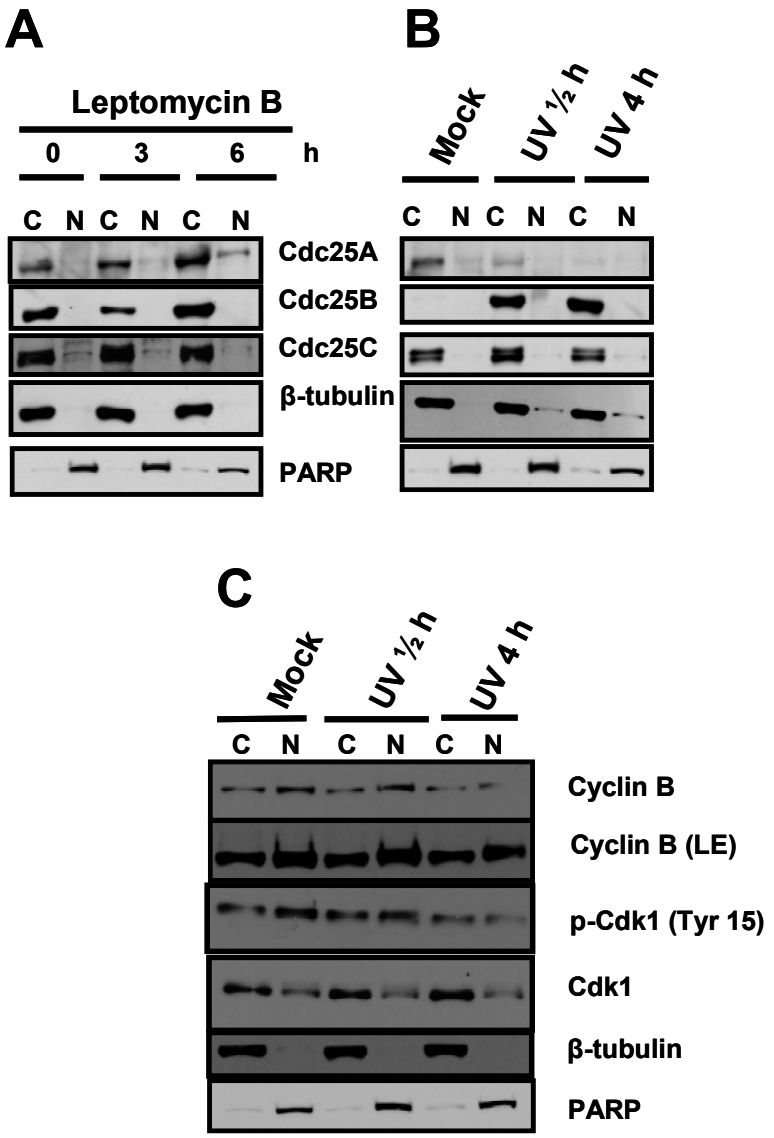


Figure 20. Localization of Cdc25B following DNA damage

(A) HCT116 cells were treated with leptomycin B (10 ng/ml) for indicated time points and were sequentially extracted to yield cytoplasmic (C) and nuclear (N) fractions. (B and C) Cells were treated with 60 J/m^2 of UV and processed to yield cytoplasmic and nuclear fractions. Subcellular fractions were immunoblotted with the indicated antibodies. Each figure is representative of two independent experiments. LE stands for long exposure.

3.2.8 Enhancement of UV Induced Cytotoxicity by Cdc25B Depletion

The induction of endogenous Cdc25B by mechanistically distinct DNA damaging agents suggests the increase expression could have functional consequences with respect to cell survival following DNA damage. Therefore, I examined cell survival under conditions where endogenous Cdc25B levels were reduced by RNAi. In Cdc25B depleted cells, fewer cells survived after 2 days of UV (30 J/m^2) compared to control cells (Fig. 21.A and B). Similar results were obtained with a different clone of Cdc25B depleted cells (data not shown). These results suggest that levels of Cdc25B are critical determinant of cell survival following DNA damage.

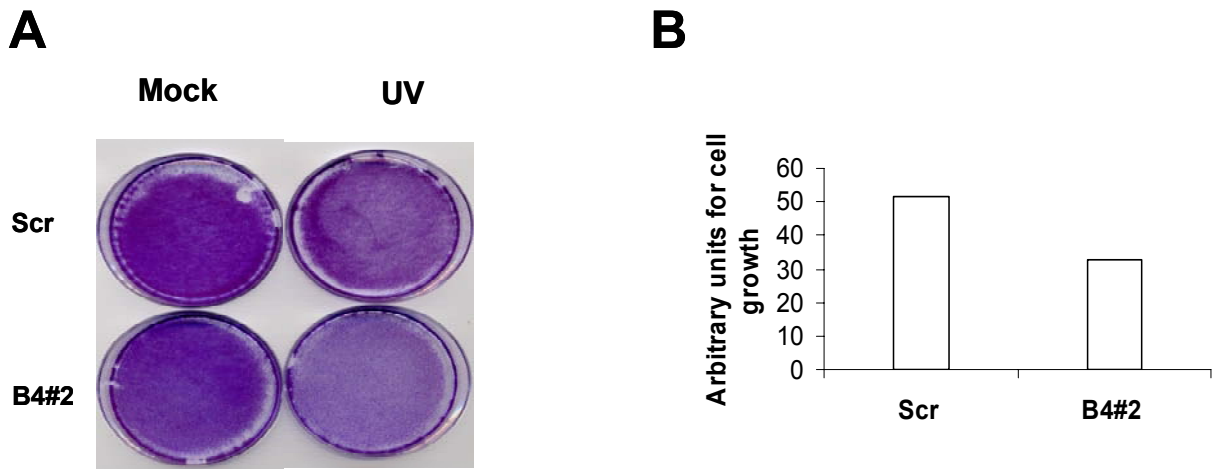


Figure 21.Survival of Cdc25B depleted cells following UV irradiation

A) HCT116 Scr (control) and B4#2 cells treated with mock or UV (30 J/m^2). Cells were stained with crystal violet 48 h later. (B) Quantification of number of cells survived after 48 h. Cells were counted in 3 fields per plate. Percentage of survival was calculated by normalizing to mock.

Next, I examined whether the decreased survival seen with Cdc25B depletion could be rescued by providing different levels of exogenous Cdc25B. For this U2OS cells were exposed to UV (15 J/m^2) and one population was grown in the presence of 2 μg/ml tetracycline to inhibit overexpression of Cdc25B, a second population was grown in 10 ng/ml of tetracycline to produce moderate overexpression expression of Cdc25B and the third population was grown with highly overexpressed levels of Cdc25B (Fig. 22.A). The decrease in cell survival after UV treatment was found to be inversely proportional to Cdc25B levels (Fig.22.B and C). Initially, these results were surprising because I anticipated that increased levels of exogenous Cdc25B would assist in resumption leading to increased cell survival. However, it is possible that the Cdc25B overexpression accelerated the recovery without the completion of DNA repair resulting in cell death. This is supported by our finding that the cell survival was inversely proportional to Cdc25B levels. Taken together these results indicate that manipulating the expression of Cdc25B could regulate survival following DNA damage potentially by affecting rate of cell cycle re-entry.

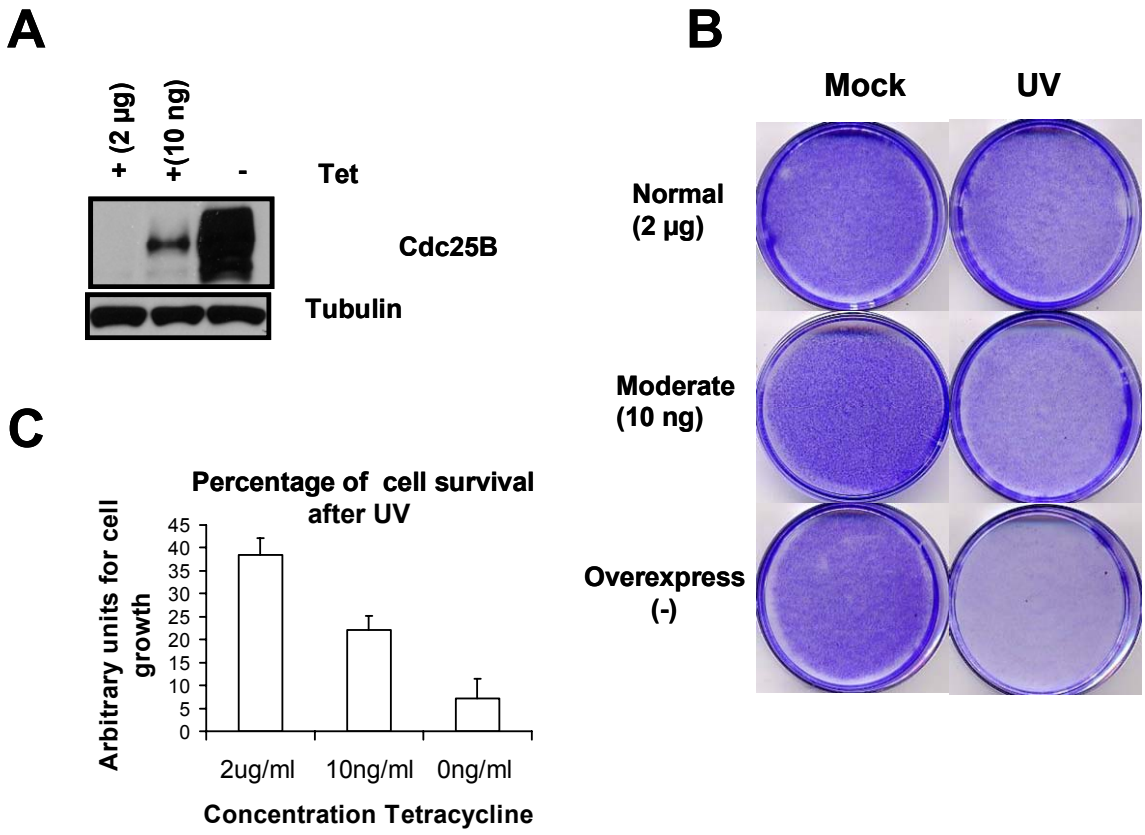


Figure 22.Enhancement of UV induced cytotoxicity in Cdc25B overexpressing cells

(A) Expression of HA-Cdc25B in U2OS cells. U2OS cells were treated with mock or UV (15 J/m^2) and Cdc25B was induced or not induced by incubating cells in the presence of tetracycline (2 µg/ml), tetracycline (10 ng/ml) or absence of tetracycline. Cells were stained with crystal violet 48 h later. (B) Quantification of number of cells survived after 48 h. Cells were counted in 6 fields per plate. (C) Percentage of survival was calculated as described under Figure 21.

3.3 DISCUSSION

It is well documented that Cdc25s are targeted in response to DNA damage but the exact contributions of the three mammalian Cdc25 homologues toward the resumption of cell cycle progression are not completely understood. Interestingly, Cdc25A stabilization does not appear to be sufficient to overcome ionizing or UV irradiation induced S phase checkpoints (88). In addition, Cdc25C knockout cells have normal G2/M checkpoint response (115). These findings indicate additional regulators cooperate to regulate cell cycle progression after DNA damage. In this chapter, I described a previously unrecognized elevation in endogenous Cdc25B following DNA damage generated by diverse types of genotoxic insults. I proposed that elevation in Cdc25B was an anticipatory response to resume cell cycle after DNA damage-induced cell cycle arrest.

My results are in contrast with one report in which no increase in Cdc25B levels were observed 1-2 hours following UV (20 J/m^2) irradiation (77). One potential explanation for the differences in the experimental results could be the reagents used to detect endogenous Cdc25B. The nature and specificity of the antibody used in those experiments, however, are not clear. At the time of the previous publication, Cdc25B null cells and Cdc25B shRNAi were unavailable. I have observed that several commercially available antibodies were useful when Cdc25B was overexpressed but failed to detect endogenous Cdc25B (data not shown). Other possible explanation could be the difference in the response of splice variants to DNA damage. There are five splice variants of Cdc25B and it is possible that Bulavin *et al.* were detecting a splice variant, which was not regulated in response to DNA damage (77). This seem unlikely, however, as the monoclonal antibody used in our assay should detect all the splice variants. Nonetheless, this warrants further analysis.

The biochemical factors that regulate cell cycle resumption following DNA damage are still being defined. Interestingly, at least some factors that participate in the canonical checkpoint response seem to also regulate cell cycle resumption. Thus, Plk1, which is a well characterized checkpoint target, is inhibited in response to DNA damage by ATM/ATR to induce cell cycle arrest. Plk1 also regulates cell cycle resumption following DNA damage-induced cell cycle arrest (94). I demonstrated that Cdc25B, which can be inhibited by p38/MAPKAPK2 phosphorylation at Ser309 following DNA damage, was rapidly induced following DNA damage and proposed this helps regulate cell cycle resumption. One could envisage that Cdc25B phosphorylation at Ser309 is operative only in the G2 cell population to prevent premature entry into mitosis in the presence of DNA damage. The decrease in mitotic cells at 4 hours following UV, which was independent of Cdc25B expression level (Fig. 18), could be due to the lack of dephosphorylation of Ser309, which is important for progression through mitosis (53). A key issue that needs to be resolved, however, is how opposing functions are orchestrated in an orderly manner to allow cells to recover from the effects of DNA damaging agents without compromising genomic integrity.

One possible explanation is that in the period immediately following DNA damage, checkpoints regulating cell cycle arrest are more active thus allowing time for DNA repair. This would be in agreement with our data showing that cells overexpressing Cdc25B arrested normally after DNA damage. Nonetheless, they resumed cell cycle progression significantly earlier than control cells (Fig. 18). As described previously, degradation of Cdc25A is a key event in arresting cells in G2 after DNA damage whereas our data suggests that accumulation of Cdc25B regulates cell cycle resumption. Based on this I proposed that initial cell cycle arrest following DNA damage might be more dependent on the loss of Cdc25A whereas accumulation

of Cdc25B regulates cell cycle resumption. This could explain the absence of checkpoint defects in Cdc25BC double knockouts immediately after exposure to ionizing radiation (38). Interestingly, cell cycle re-entry after UV irradiation was not formally studied in this background. Although a rapid increase in Cdc25B expression would seem premature for its role in cell cycle resumption, temporally the Chk1 activity begins to decline 1 hour after UV, which I have confirmed by examining Chk1 (Ser 345) phosphorylation (activating phosphorylation) following UV (data not shown). These findings suggest a testable hypothesis that increased Cdc25B is a cellular priming factor for cell cycle resumption once DNA damage repair is complete and the ATR/Chk1 pathway prepares cells for cell cycle resumption before inactivation of checkpoint signaling. Alternatively, gradual accumulation of Cdc25B following DNA damage may also be required to increase Cdk1 activity to a level that would be sufficient to overcome the inhibition accumulated during the DNA damage induced cell cycle arrest. The dependence on increased Cdc25B levels as a limiting factor only after DNA damage was supported by our result that over-expression of Cdc25B did not significantly increase the number of cells in mitosis in the absence of DNA damage (Fig. 17).

Van Vugt *et al.* previously found Cdc25B and Plk1 were not required for recovery from DNA damage in Wee1 depleted cells. Cdk1 has been shown to regulate Wee1 degradation at the onset of mitosis (94). Therefore, I examined the levels of Wee1 in Cdc25B overexpressing cells exiting G2 and found to be lower than control cells (Fig. 19). These results support the hypothesis that Cdc25B might regulate cell cycle re-entry following DNA damage by promoting Wee1 degradation.

Both basal and UV-induced Cdc25B were found in the cytoplasm (Fig. 20). Interestingly, cyclin B1/Cdk1 was also found to redistribute to cytoplasm in these cells following

UV treatment. Thus, the induced Cdc25B is functionally positioned to activate cytoplasmic Cdk1 and to promote cell cycle progression once the DNA damage was repaired. Furthermore, Cdc25B might regulate Cdk1 activity in the centrosome following DNA damage considering the central role of the centrosome in coordinating mitotic entry (46).

Based on our results and previous studies (89, 104), I propose that Cdc25B activity is tightly controlled following DNA damage and is one of the crucial factor in regulating survival (Fig. 21 and 22). In some cellular conditions it can act to accelerate cell cycle progression by activating Cdk1 once the repair is complete. Activation of Cdk1 results in degradation of Wee1, a negative regulator of Cdk1, through Plk1 to ensure further activation of Cdk1. In other cellular conditions, for example when damage is not repairable, the same self-amplification process results in mitotic catastrophe due to premature activation of Cdk1. The crucial role Cdc25B plays in regulating cell cycle resumption could also help explain the oncogenic properties of this phosphatase. It is possible that overexpression of Cdc25B in tumor cells overwhelms the process of cell cycle resumption and survival resulting in division of cells with damaged DNA, thereby contributing to genomic instability observed in cancer cells. This could explain the increased susceptibility to breast cancer induction by 9, 10-dimethyl-1, 2 benzanthracene in mice that ectopically overexpressed Cdc25B in mammary glands (60). Finally, our results reinforce the rationale for Cdc25B inhibition as an adjuvant approach for anticancer therapy because inhibition of Cdc25B by small molecule inhibitor might impair checkpoint recovery and increase the efficacy of DNA damaging agents.

4.0 CDC25B PHOSPHATASE IS ESSENTIAL FOR BENZO[*A*]PYRENE-7, 8-DIOL-9,10-EPOXIDE INDUCED NEOPLASTIC TRANSFORMATION

Cdc25B phosphatase controls entry into mitosis and regulates recovery from G2/M checkpoint-induced arrest. In the present study we show that exposure of mouse embryonic fibroblasts (MEFs) to the ultimate carcinogen *anti*-benzo[*a*]pyrene-7, 8-diol-9, 10-epoxide (*anti*-BPDE) resulted in a concentration and time-dependent increase in Cdc25B protein levels. Chronic exposure of wild type ($Cdc25B^{+/+}$) MEFs to *anti*-BPDE (0.1 μM) caused neoplastic transformation characterized by colony formation in culture and tumor production in nude mice. In contrast, the Cdc25B null MEFs were resistant to *anti*-BPDE-induced transformation. Furthermore, a carcinogenic dose of the parent hydrocarbon (benzo[*a*]pyrene) increased Cdc25B protein levels in the target organ, lung. The biological importance of elevated Cdc25B levels was documented by premature recovery of Cdc25B overexpressing cells in the presence of DNA damage. Furthermore, over-expression of Cdc25B, in the absence of *anti*-BPDE, also caused DNA damage and activation of checkpoints hence inducing stressful environment ideal for selection of cells with compromised checkpoints. This was supported by decreased protein levels of p53 and p21 in the transformed WT MEFs. To summarize, Cdc25B has an essential role in *anti*-BPDE-induced neoplastic transformation, by regulating cell cycle resumption without completion of DNA repair and selecting cells with compromised checkpoints, thus leading to genetic instability.

4.1 INTRODUCTION

4.1.1 Background

Benzo[*a*]pyrene (BP) is the prototype of the polycyclic aromatic hydrocarbon family of environmental pollutants that are tumorigenic in experimental animals and suspected human carcinogens (116). The tumorigenic activity of BP has been attributed to its metabolite benzo[*a*]pyrene-7,8-diol-9,10-epoxide (*anti*-BPDE), which is formed *via* epoxidation and hydration reactions catalyzed by cytochrome P450-dependent monooxygenases and epoxide hydrolase, respectively (117-120). *Anti*-BPDE is highly reactive toward cellular nucleophiles including DNA and glutathione (GSH) (121, 122). Covalent modification of the epoxide functional group of *anti*-BPDE with exocyclic amino groups of the purine bases in DNA is considered a critical reaction in the initiation of *anti*-BPDE-induced cancers (122).

We showed previously that *anti*-BPDE exposure of lung cancer cells and terminal squamous differentiated human bronchial epithelial cells, but not undifferentiated bronchial epithelial cells, resulted in induction of Cdc25B protein expression (61). In addition, the growth rate of lung cancer cells increased significantly in comparison with untreated cells following chronic exposure to *anti*-BPDE. In the present study in collaboration with Professor Shivendra V. Singh (University of Pittsburgh), we extend these findings by investigating whether Cdc25B is essential for *anti*-BPDE-induced neoplastic transformation using previously described (123) mouse embryonic fibroblasts (MEFs) from wild type ($Cdc25B^{+/+}$) and Cdc25B null mice ($Cdc25B^{-/-}$). We also delineate the mechanisms by which Cdc25B could regulate *anti*-BPDE induced tumorigenesis in MEFs.

4.1.2 *anti*-BPDE Increases Cdc25B Protein Levels

To determine whether *anti*-BPDE altered Cdc25B levels in MEFs, we treated wild type MEFs with various concentrations of *anti*-BPDE. As illustrated in Fig. 23.A, we noted a concentration-dependent induction of Cdc25B. In time course studies using 10 μM *anti*-BPDE (Fig. 23.B), the induction of Cdc25B protein level was evident as early as 4 hours after treatment and persisted for the duration of the experiment (24 hours post-treatment). Consistent with the known function of Cdc25B in catalyzing dephosphorylation of Thr14 and Tyr15 of Cdk1, *anti*-BPDE mediated induction of Cdc25B protein was accompanied by a sharp decline in the level of Tyr15 Cdk1 (Fig. 23.B) similar to terminal squamous differentiated bronchial epithelial cells and lung cancer cells. The Cdc25B mRNA levels were either unaltered or only modestly increased in wild type MEFs after *anti*-BPDE treatment (data not shown).

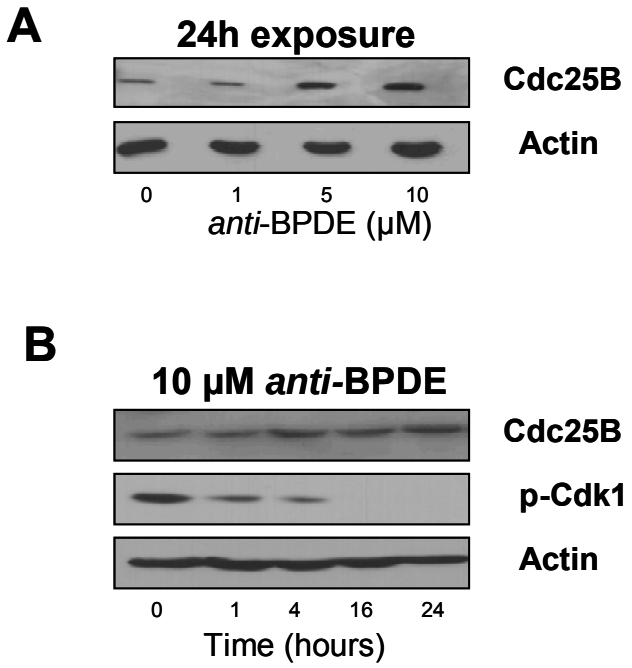


Figure 23.*Anti*-BPDE increases Cdc25B protein levels in MEFs

(A) Immunoblotting for Cdc25B protein using lysates from wild type MEFs treated for 24 hours with the indicated concentrations of *anti*-BPDE. The membrane was stripped and reprobed with anti-actin antibody to ensure equal protein loading. Data shown are from a representative experiment that was repeated three times using independently prepared lysates with similar results. (B) Immunoblotting for Cdc25B and ^{Tyr15}Cdk1 using lysates from wild type MEFs treated with 10 μM *anti*-BPDE for the indicated time periods. The membrane was stripped and reprobed with anti-actin antibody to ensure equal protein loading. Data are from a representative experiment that was repeated twice with similar results.

Next, we addressed whether induction of Cdc25B protein was unique to *anti*-BPDE by determining the effects of parent hydrocarbon (BP), benzo[*a*]pyrene-7,8-diol (precursor of *anti*-BPDE), and benzo[*a*]pyrene-7,8,9,10-tetrol (hydrolysis product of *anti*-BPDE) on Cdc25B protein level by immunoblotting (Fig. 24). Unlike *anti*-BPDE, the level of Cdc25B protein was unaffected by treatment of MEFs with either BP or benzo[*a*]pyrene-7,8-diol or benzo[*a*]pyrene-7,8,9,10-tetrol (Fig. 24). These results indicated that the epoxide functional group of *anti*-BPDE was required for its inductive effects on Cdc25B protein level.

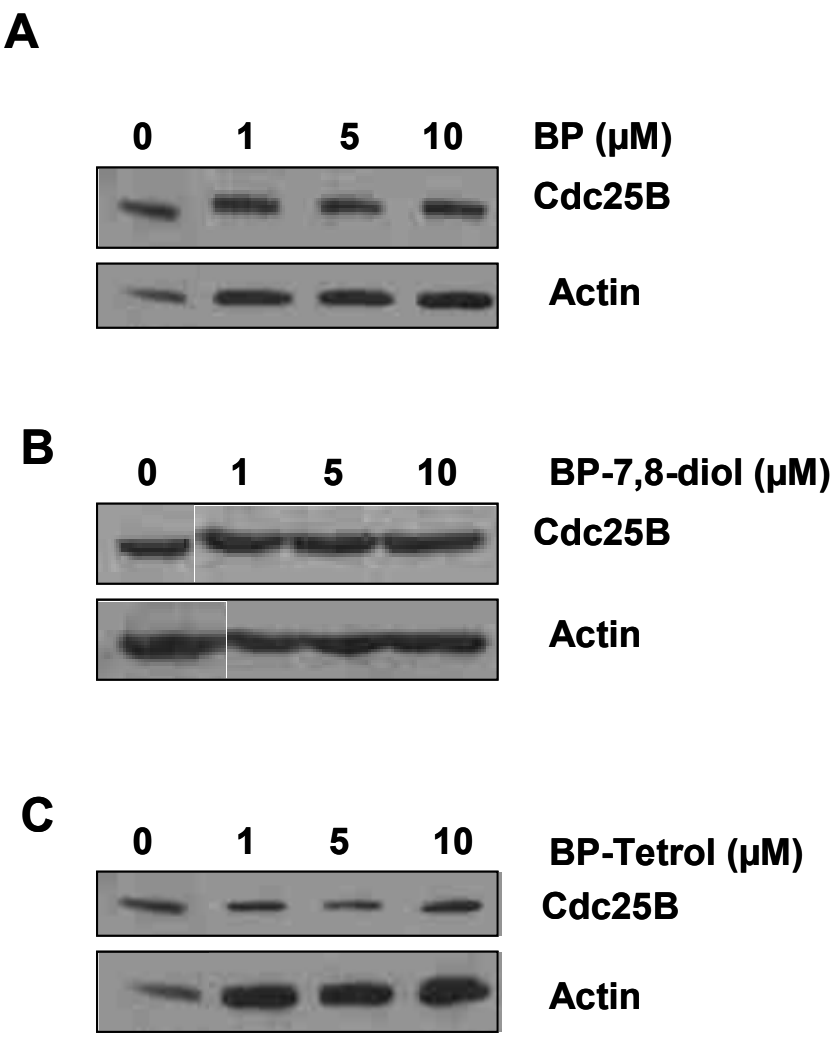


Figure 24.Increase in Cdc25B levels is dependent on *anti*-BPDE

Immunoblotting for Cdc25B protein using lysates from wild type MEFs treated for 24 hours with the indicated concentrations of (A) benzo[*a*]pyrene, (B) benzo[*a*]pyrene-7,8-diol, or (C) benzo[*a*]pyrene-7,8,9,10-tetrol. The blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Immunoblotting was performed at least twice using independently prepared lysates, and the results were comparable.

4.1.3 Cdc25B is Essential for *anti*-BPDE Induced Tumorigenesis

We next determined the potential functional significance of Cdc25B induction after *anti*-BPDE treatment. We postulated that if Cdc25B induction was important in *anti*-BPDE-induced neoplasia it might affect *anti*-BPDE-induced neoplastic transformation. Using previously described MEFs from wild type and Cdc25B null mice, we observed the wild type MEFs began to form colonies of rounded cells after repeated exposures to 0.1 μM *anti*-BPDE over a period of 4 weeks (Fig. 25.A, panel II). Similar results were seen in three separate cultures of the wild type MEFs that were exposed to *anti*-BPDE. In contrast, *anti*-BPDE treatment of Cdc25B null MEFs did not cause a visible change in their morphology (Fig. 25.A, panel IV). The fibroblast-like morphology was also maintained when either the wild type or the Cdc25B null MEFs were chronically exposed to DMSO (Fig.25.A, compare panels I and III).

To confirm the transformed phenotype, we subcutaneously injected athymic mice with the *anti*-BPDE exposed wild type MEFs. As seen in Fig.25.B (panel II), the *anti*-BPDE exposed wild type MEFs produced tumors after subcutaneous injection in nude mice. Tumors were observed in each of the four mice that were injected with *anti*-BPDE exposed wild type MEFs. Fig.25.C depicts the growth curve for tumors resulting from subcutaneous injection of *anti*-BPDE exposed wild type MEFs. In contrast, the *anti*-BPDE exposed Cdc25B null MEFs failed to produce tumors in nude mice (compare panel IV of Fig.25.B for a representative mouse of this group). The DMSO exposed MEFs of both types (wild type and Cdc25B null) were also non-tumorigenic in nude mice (Fig.4.3.B, panels I and III, respectively).

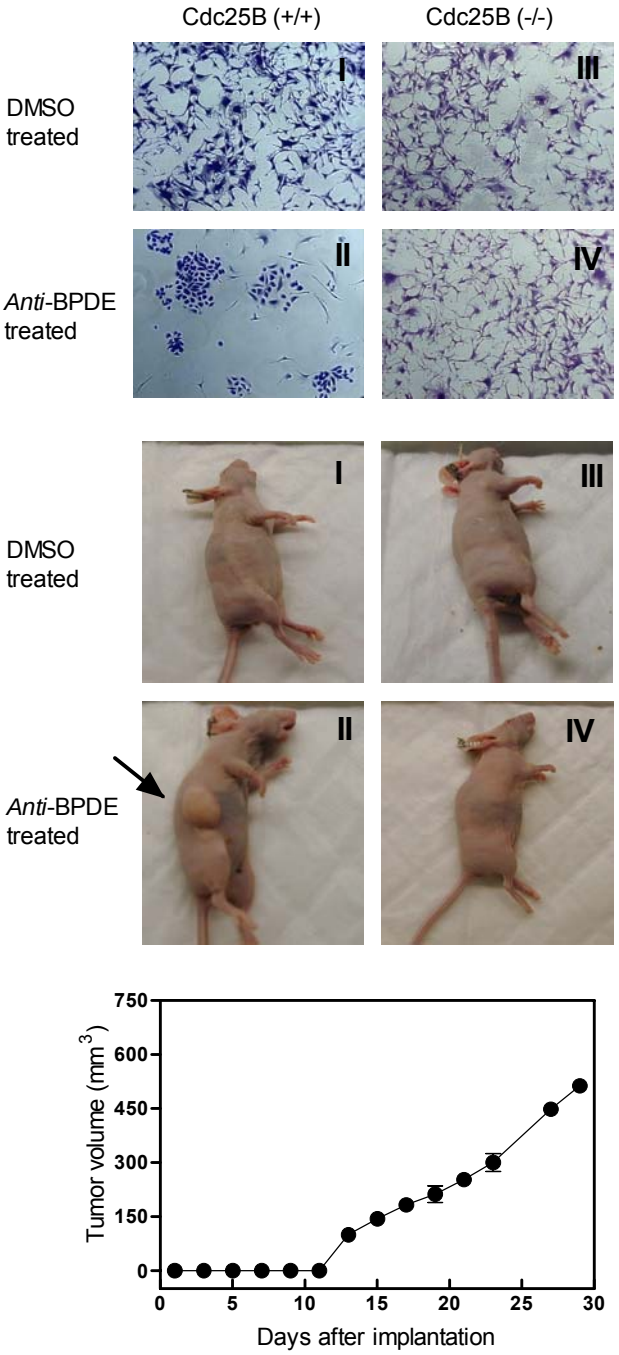


Figure 25.Cdc25B is essential for *anti*-BPDE induced tumorigenesis

(A) Light microscopy of crystal violet stained cultures of DMSO exposed (panels I and III) or *anti*-BPDE exposed (panels II and IV) MEFs derived from wild type mice (panels I and II) or Cdc25B null mice (panels III and IV). Note that only *anti*-BPDE exposed wild type MEFs

(panel II) acquired the ability to form colonies. (B) Tumorigenic potential of *anti*-BPDE exposed wild type MEFs in female athymic mice. Compare panel II of Figure 3b for a representative mouse of this group. (C) Growth curve for tumors upon subcutaneous injection of 10^6 *anti*-BPDE exposed wild type MEFs. Tumor volume data are means ± SE (n=4).

4.1.4 BP Administration Increases Pulmonary Cdc25B *in vivo*

To further test the hypothesis that the *anti*-BPDE induction of Cdc25B protein was biologically relevant, we examined the *in vivo* effect of BP administration to mice on Cdc25B protein content in the lung, which is a target organ for BP-induced cancer in mice (124). Female A/J mice, a strain sensitive to BP-induced pulmonary tumorigenesis, were orally treated with a carcinogenic dose of BP or an equal volume of the corn oil (vehicle for BP). Four mice from both groups were sacrificed at different time intervals, and the lungs and livers were collected and processed for Cdc25B immunoblotting (Fig.26). Densitometric scanning of the Cdc25B immunoreactive bands followed by normalization to loading control (actin) revealed that 24 hour after BP administration there was a significant increase in pulmonary Cdc25B (Fig.26) In contrast, hepatic Cdc25B protein level was unaffected by BP administration (data not shown), as would be predicted by the lack of BP-induced hepatocarcinogenesis in this mouse model.

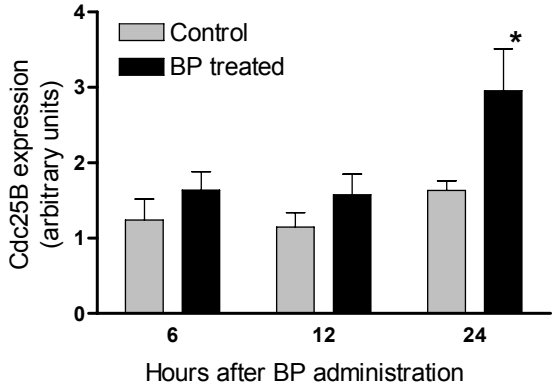


Figure 26.BP administration increases pulmonary Cdc25B protein level *in vivo*

Effect of BP administration on pulmonary Cdc25B protein level *in vivo* in female A/J mice. (A)The effect of BP treatment on pulmonary Cdc25B protein level was determined by immunoblotting using 14,000 x g supernatant fractions from the lungs of control (corn oil treated) and BP treated mice. Data are mean ± SE (n=4). **P* < 0.05, control *versus* treated groups by Student's *t*-test.

4.1.5 Decreased expression of p53 and p21 in chronically treated wildtype MEFs

To gain further insight into the mechanism by which Cdc25B protein induction contributes to *anti*-BPDE induced transformation, we examined the expression of p53 and p21 in the transformed wild type MEFs exposed to *anti*-BPDE. It has been shown previously that p53 plays a key role in G1 checkpoint, and p53 mutation occurs in many lung tumors and even in the normal bronchial epithelium of smokers (125-129). It is note worthy that chronic treatment of wildtype MEFs with *anti*-BPDE resulted in the loss of p53 and p21. This loss appeared to at least

partially require the presence of Cdc25B as loss of p53 and p21 was attenuated in the Cdc25B null MEFs (Fig.27A and B).Thus, these results suggest that p53 and p21 may act downstream of Cdc25B in *anti*-BPDE induced transformation or decrease expression of p53 and p21 could be the consequence of the transformed phenotype.

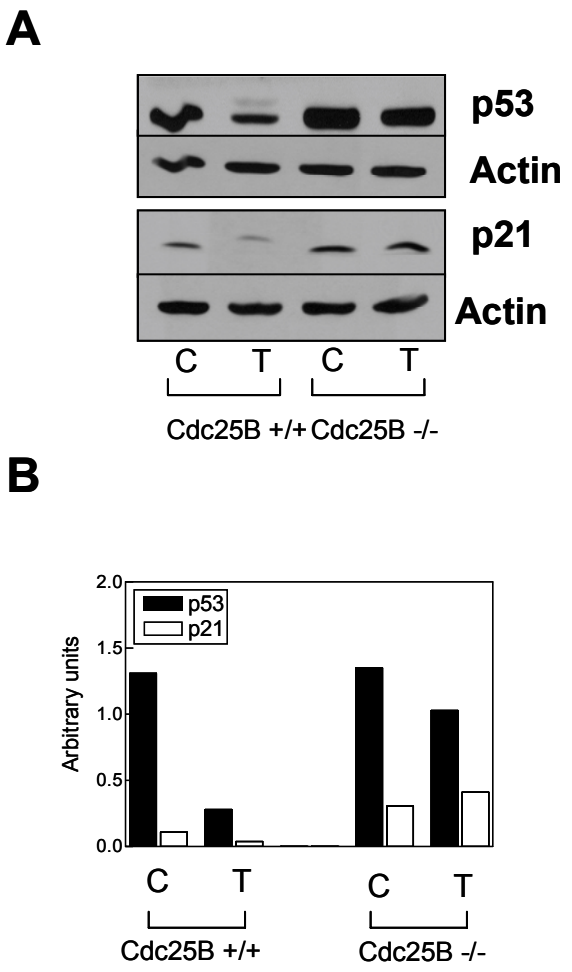


Figure 27.Decreased expression of p53 and p21 in chronically treated wildtype MEFs

(A) Immunoblotting for p53 and p21 proteins using lysates from wild type ($Cdc25B^{+/+}$) and Cdc25B null ($Cdc25B^{-/-}$) MEFs chronically exposed to 0.1 μM *anti*-BPDE (denoted as T) or DMSO (denoted as C) over a period of 4 weeks. The experiment was repeated twice using

independently prepared lysates, and the results were similar. (B) Densitometric scanning data for p53 and p21 immunoblots after normalization to actin loading control**.**

To summarize findings from this section, *anti*-BPDE increased Cdc25B expression in MEFs and Cdc25B was found to be essential for *anti*-BPDE induced neoplastic transformation. Furthermore, BP administration in a Female A/J increased expression of Cdc25B in the lung where it was metabolized to the *anti*-BPDE. These results suggest that BP inhalation through cigarette smoke could predispose to lung cancer by increasing expression of an oncogene Cdc25B phosphatase.

4.2 RESULTS

4.2.1 *anti*-BPDE increases Cdc25B expression in human lung fibroblast

To validate the hypothesis that BP inhalation through cigarette smoke could predispose to lung cancer by increasing expression of Cdc25B, I investigated the effect of *anti*-BPDE treatment in human lung fibroblast (CCL-202). For this, CCL-202 cells were treated with freshly prepared 10 μM *anti*-BPDE and cells were harvested at the indicated time points. As illustrated in Fig.28, exposure to *anti*-BPDE increased Cdc25B protein levels with 30 minutes and the levels remained high until 24 hour post-treatment.

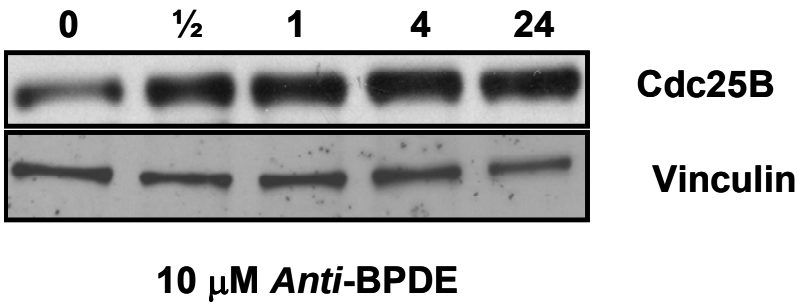


Figure 28.anti-BPDE increases Cdc25B expression in human lung fibroblast cell line

CCL-202 cells were plated 24h before exposure to *anti*-BPDE. Cells were treated with *anti*-BPDE and harvested at the indicated time point. Immunoblotting was performed as indicated. Figure represents two independent experiments done in duplicate.

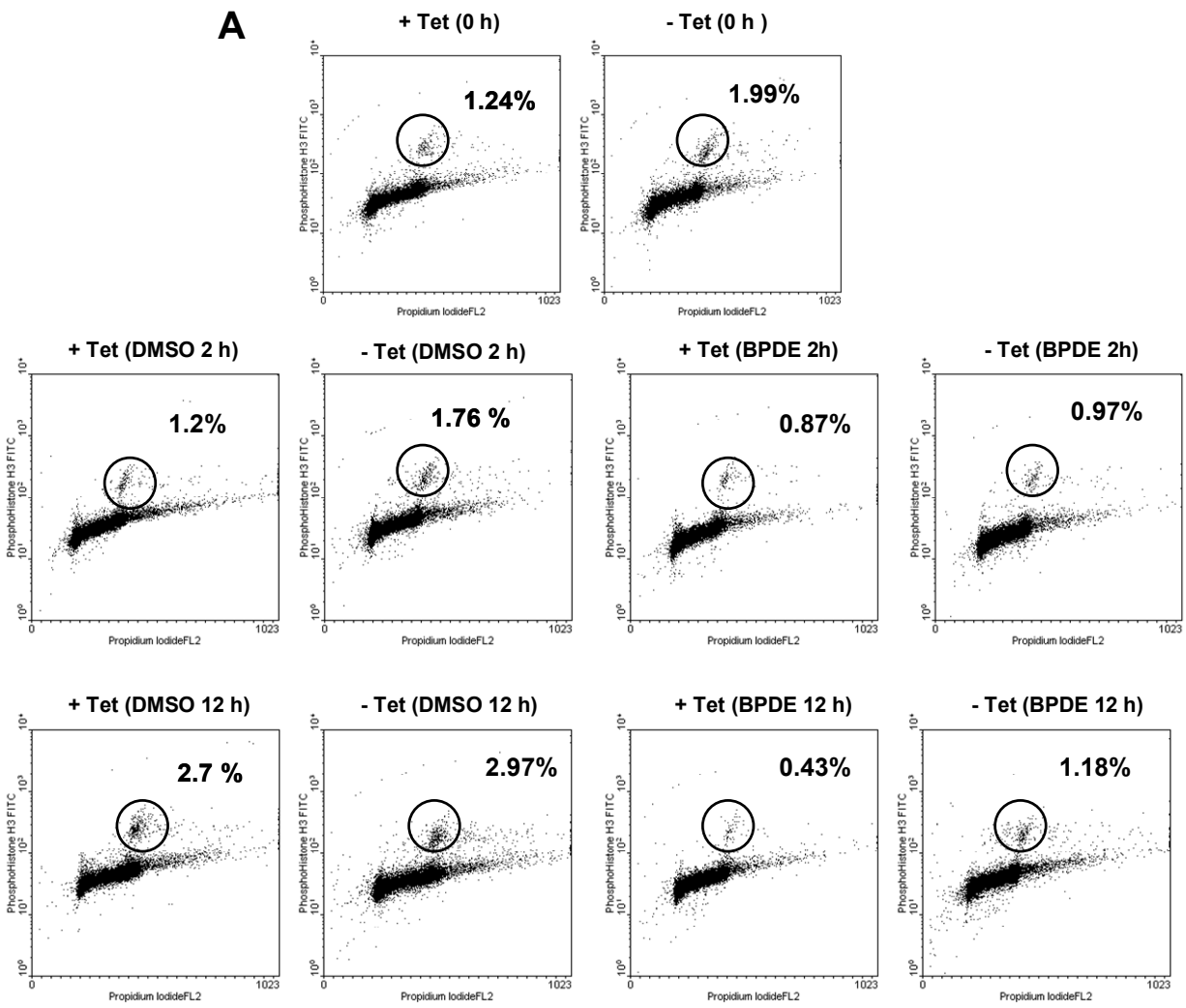
4.2.2 Cdc25B Regulates Recovery in the Presence of DNA Damage

Cdc25B has recently been identified to have a crucial role in the cell cycle resumption following DNA damage (94, 104) and this process might enable cells to survive genotoxic stress. A disruption in the presumptive protective pathway could contribute to tumor development by permitting cell division without completion of DNA repair. I, therefore, postulated that BPDE-induced transformation might be linked to Cdc25B induction and the subsequent promotion of cell cycle resumption in the presence of DNA damage. To test this hypothesis, I first examined mitotic re-entry in asynchronous cell populations with differing levels of Cdc25B after exposure to *anti*-BPDE (100 nM). Tetracycline was removed from U2OS cells for 16 hours to induce Cdc25B expression before *anti*-BPDE treatment. Using Histone H3 Ser10 phosphorylation as a marker of mitotic re-entry, I found that both control and Cdc25B overexpressing U2OS cells

initially activate a G2/M checkpoint as shown by the reduction in the percentage of mitotic cells at 2 hours post-treatment (Fig.29.A). The relative mitotic ratio of *anti*-BPDE treated control cells was 0.77 ± 0.06 %, which was similar to the *anti*-BPDE treated Cdc25B overexpressing cells (0.71 ± 0.11%) (Fig. 29.B). In contrast, 12 hours after *anti*-BPDE treatment, there was a pronounced inhibition of mitotic re-entry in cells with suppressed ectoptic Cdc25B (+tet) (0.19 ± 0.01%) whereas Cdc25B overexpressing U2SO cells (-tet) had a higher mitotic ratio (0.46 ± 0.08%) (Fig.29.A and B). Twenty four hours post-treatment, Cdc25B overexpressing cells (-tet) were fully recovered as indicated by percentage of mitotic cells of 1.16 ± 0.06% while control cells populations (+tet) recovered only partially (0.53 ± 0.04%). Furthermore, based on the DNA profile of cells at 12 hours compared to 0 and 2 hours, DMSO treated cell progressed through cell cycle as indicated by increase in the number of cells in G2. Cell cycle progression of *anti*-BPDE treated cells was delayed with a majority of cells in S phase (Fig. 29.C). Additionally, 24 hours after *anti*-BPDE treatment 47.9 ± 3.6 % of control cells and 40.5 ± 1.8 % of Cdc25B overexpressing cells were in G2/M phase indicating cells progression from S phase at 12 hours. In contrast, DMSO treated cells potentially divided between 12 and 24 hours as indicated by increased % of cells in G1 (29.6 ± 2.0 % of control cells and 19.7 ± 0.7% of Cdc25B overexpressing cells). These results suggest that *anti*-BPDE treated cells were not arrested at 24 hour post-treatment; however, these cells were delayed in cell cycle compared to DMSO treated cells. In summary, these findings highlight that it is imperative to investigate the effect of DNA damaging agent on cell cycle distribution at different time points following treatment to avoid misinterpretation of the data.

As shown in Fig.29.D, treatment with *anti*-BPDE caused DNA damage within 2 hours post-treatment indicated by γH2AX phosphorylation (marker for double strand breaks). The

intensity of γH2AX phosphorylation increased with time (4 and 8 hours) in BPDE treated samples. Cell populations that maintained high ectopic Cdc25B levels displayed prolonged γH2AX irrespective of exposure to *anti*-BPDE. It is noteworthy that Cdc25B overexpressing cells treated with *anti*-BPDE resumed cell cycle progression despite this apparent DNA damage.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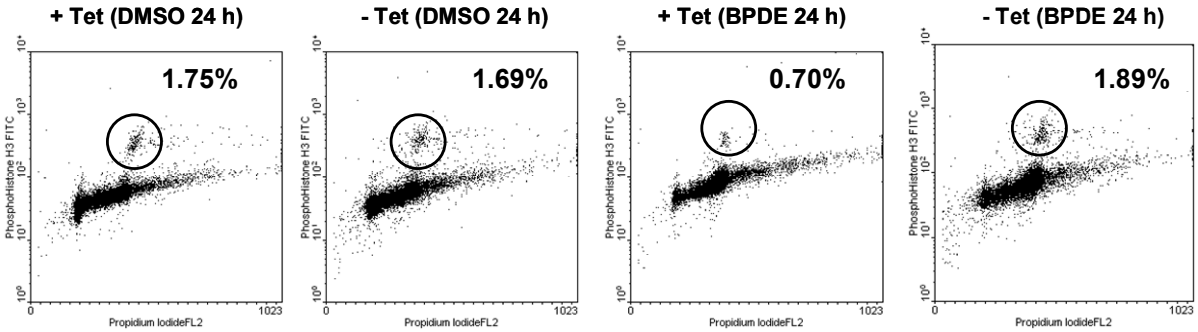
+ Tet (DMSO 24 h)
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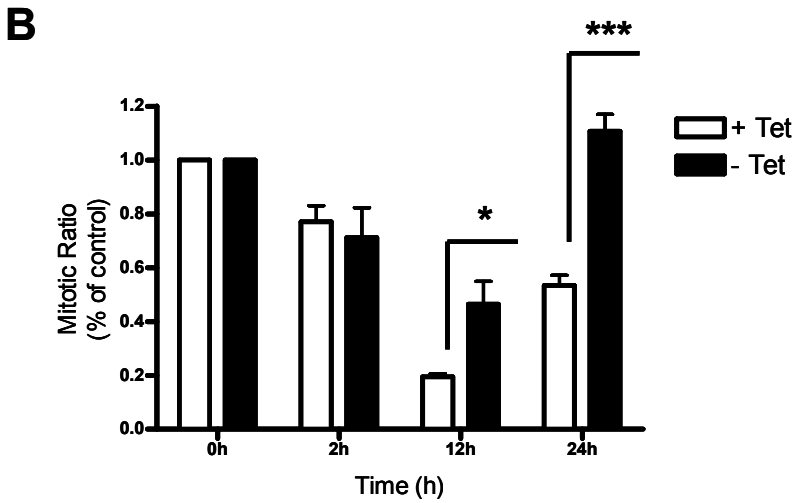
B
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*
+ Tet
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Mitotic Ratio
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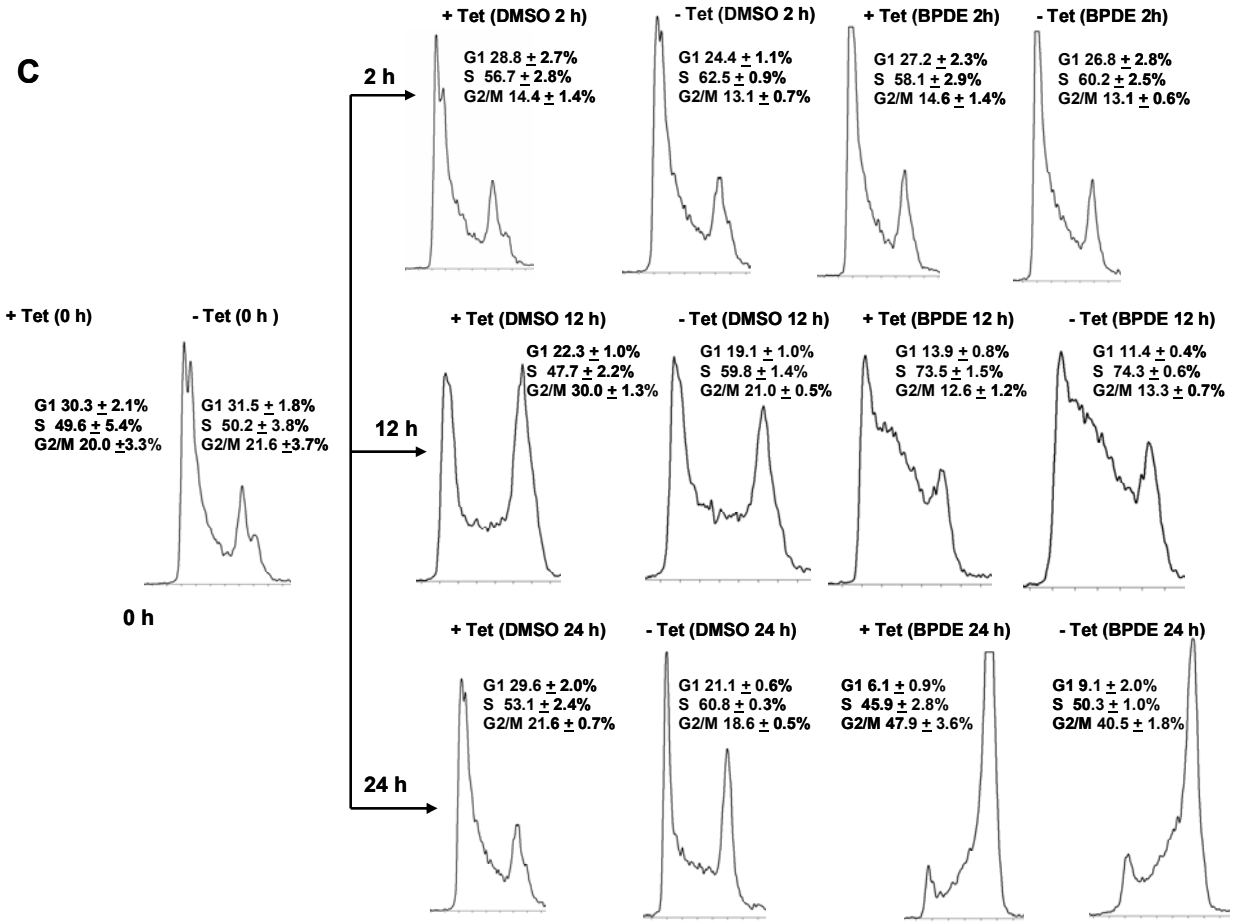

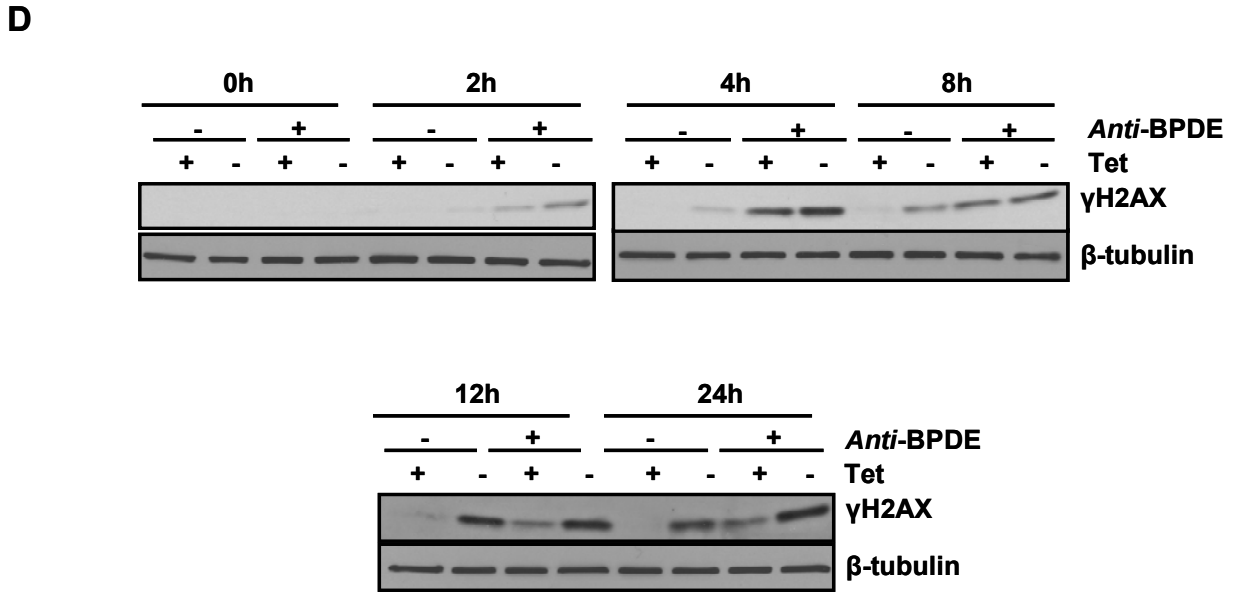

Figure 29.Cdc25B regulates recovery in the presence of DNA damage

(A) U2OS cells were cultured for 16 hours in the presence (wildtype Cdc25B) or absence of of tet (elevated Cdc25B). The two populations were then treated with either DMSO or *anti*-BPDE (100 nM). Cells were fixed at the indicated time points and stained for phospho-Histone H3. (B)Each bar represents % of mitotic cells following *anti*-BPDE treatment normalized to DMSO control. Results are mean $\pm$ SE (n=4). Statistical significance was determined by two tail unpaired t – test. * , $P < 0.05$ and ***, $P<0.0001$.(C) The DNA profile of the samples in (A). (D) Immunoblotting of cell lysates for γH2AX from Cdc25B induced or wildtype U2OS cells treated with DMSO or *anti*-BPDE. Cells were harvested at different time points as indicated. The experiment was repeated twice using independently prepared lysates, and the results were similar.

4.2.3 Cdc25B Induction Deregulates Checkpoints

Recent studies indicate that cells over-expressing oncogenes such as Cyclin E, Cdc25A or E2F1, activate DNA damage response including phosphorylated ATM, Chk2, phosphorylated γH2AX and p53 (130, 131). Similarly, I also observed phosphorylated γH2AX in Cdc25B over-expressing cells (Fig. 29.D). Since de-regulation of checkpoint response could enhance the likelihood of genomic instability, I further characterized the effect of Cdc25B over-expression on checkpoint activation. For this, I examined the phosphorylation status of Ser15 on p53 and Ser317 on Chk1 (markers of activated checkpoint). As illustrated in Fig.30, hyperphosphorylation of these DNA damage sensing proteins was sustained for at least five days after elevation of etcopic Cdc25B and for most markers the degree of phosphorylation was comparable to that seen with UV irradiation. These results imply that acute elevation of Cdc25B levels generated a stressful environment ideal for selection of cells with compromised

checkpoints. Interestingly, chronic treatment of wildtype MEF with *anti*-BPDE resulted in the loss of p53 and p21 indicating compromised checkpoints (Fig.27). These results imply that p53 and p21 could be downstream of Cdc25B in *anti*-BPDE induced neoplastic transformation.

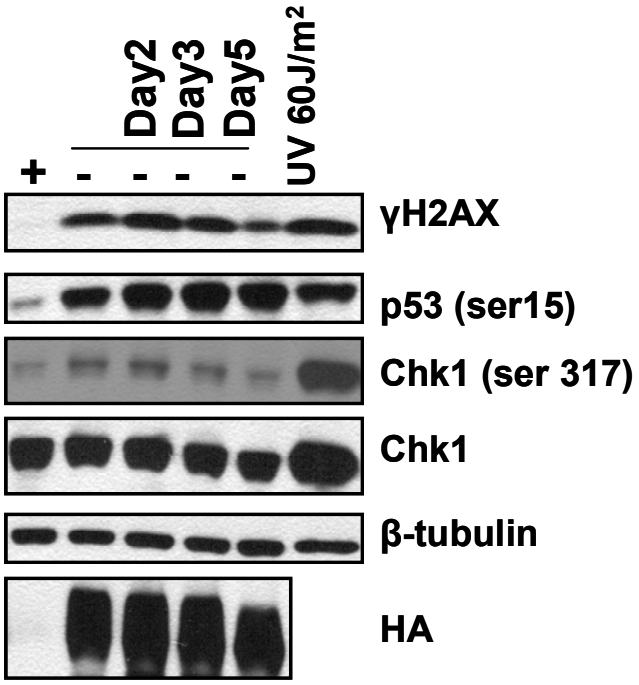

Figure 30.Cdc25B induction could deregulate checkpoint

Overexpression of Cdc25B induces DNA damage. U2OS cells harvested 1-5 days after tet removal and DNA damage assessed by immunoblotting for phosphorylated p53 or Chk1 or expression of γH2AX. The Figure represents the results of two independent experiments performed in duplicate.

4.3 DISCUSSION

The Cdc25 phosphatases are vital controllers of cell entry and progression into mitosis (19). Our understanding of the relative importance of each of the family members in the

regulation of Cdk-cyclin complexes after DNA damage induced cell cycle arrest continues to evolve. Although initial studies using mice lacking functional Cdc25B suggested a relatively normal phenotype (123), more recent data from several groups indicate that Cdc25B is required for an efficient exit from G2 arrest (94). Protein such as Plk-1, which controls the half-life of the Cdc25-inactivating kinase Wee1, is also required for this process (94). These observations have lead to the hypothesis that an increase in endogenous Cdc25B could be part of the normal response to recover from DNA damage. The data presented herein indicate that induction of Cdc25B protein could be an important event in *anti*-BPDE-induced neoplasia. Consistent with previous studies, treatment of wild type MEFs with *anti*-BPDE resulted in a transformed phenotype that was characterized by colony formation in culture and tumor production in nude mice upon subcutaneous injection. In contrast, exposure to MEFs derived from Cdc25B null mice failed to form colonies or to produce tumors in athymic mice (Fig. 25).

The induction of a Cdc25 family member after carcinogen-mediated DNA damage would seem at first rather surprising as this could facilitate Cdk activation and cell cycle progression rather than checkpoint activation. Indeed, with most DNA damaging agents Cdc25A is phosphorylated and rapidly degraded (79, 80, 83, 88). Recent studies indicate *anti*-BPDE can induce Chk1 dependent degradation of Cdc25A (132). Our results demonstrating that cells activate G2/M checkpoint immediately following *anti*-BPDE irrespective of Cdc25B levels (Fig. 29) are consistent with the model that initial cell cycle arrest following DNA damage might be more dependent on the loss of Cdc25A. If, however, there is an increase in basal level of Cdc25B due to chronic exposure to DNA damaging *anti*-BPDE then resumption could happen in the presence of DNA damage thus increasing the risk for genetic instability (Fig. 29.D). This could explain why transgenic mice overexpressing Cdc25B in mammary epithelium have

increased susceptibility to 9,10-dimethyl-1,2-benzanthracene-induced mammary tumorigenesis (60). These results are analogous to a recent study showing that Chk1 and Plk1 regulate checkpoint adaptation/premature recovery in human cells following ionizing radiation (133). These findings suggest that human cells, like their yeast counterpart, can divide in the presence of DNA damage and can increase the risk for development of genetically unstable cells that may progress toward cancer. Interestingly, positive regulators (Plk1 and Cdc25B) of premature recovery/ checkpoint adaptation are overexpressed in many human tumors and negative regulator (Chk1) is mutated in several malignancies (74, 134-137). Taken together, these findings indicate that Cdc25B may promote genomic instability by regulating premature recovery from checkpoints without completion of DNA repair.

It is interesting to note that the increased expression of Cdc25B even in the absence of an overt DNA damaging agent also resulted in DNA damage and activation of checkpoints (Fig. 30). These results are consistent with previous reports describing activation of checkpoints in response to replication stress induced by over-expression of Cdc25A, Cyclin E and E2F (130, 131). Based on these results it was proposed that in precancerous lesions, oncogenic stress could regulate selection of cells with compromised checkpoint signaling thus leading to genetic instability and tumor progression. Hence, markedly decrease levels of p53 and p21 in the chronically *anti*-BPDE exposed wild type MEFs compared to Cdc25B null MEFs is consistent with a role for Cdc25B in checkpoint deregulation.

Finally, induction of Cdc25B may not be restricted to the carcinogen *anti*-BPDE. Recently, Cdc25B was found to be elevated in cells chronically exposed to ionizing radiation. Repeated exposure to ionizing radiation leads to tumorigenic phenotype in these cells (87). These results suggest that increase in Cdc25B levels could potentially play an important role in

tumorigenesis induced by a number of different DNA damaging agents. I propose that Cdc25B predisposes cells to genetic instability by regulating cell cycle resumption without completion of DNA repair and selecting cells with compromised checkpoints.

5.0 DISCUSSION

There is considerable evidence supporting the hypothesis that tumorigenesis is a multistep process and these steps represent genetic alterations driving the progressive transformation of normal human cells into malignant cells (138-140). Hanahan and Weinberg posited that these genetic alterations broadly affect six capabilities in cells, which collectively dictate malignant growth (141). These capabilities include: self sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Furthermore, it has been suggested that alterations in these capabilities are shared by all types of cancer albeit specific cancer could acquire these capabilities through different mechanistic strategies.

Acquisition of these capabilities requires sequence of mutations which could be an inefficient process considering each cell has a complex array of DNA damage monitoring and repair enzymes. These genome guardians strive to ensure that DNA sequence information remains pristine. Furthermore, checkpoints operate at critical times in the cell's life to maintain karyotypic order. Even then, cancer appears at substantial frequency in the human population indicating that genomes of tumor cells must acquire increased mutability in order for the process of tumor progression to reach completion (142). Hanahan and Weinberg (141) proposed that defects in "caretaker" systems such as germline *p53* and *CHEK2* mutations in Li Fraumeni syndrome, inactivating ATM mutations in ataxia telangiectasia, defective DNA damage repair

with *Brca1/2* and *Nbs1* mutations in familial breast cancer patients, and Nijmegen breakage syndrome patients (143-147), respectively could enable genomic instability, therefore, allowing precancerous cells to gain capabilities to become malignant.

Recently, two different groups reported activation of the ATR/ATM-regulated checkpoints in early neoplastic lesions (130, 131). Activation of checkpoints in these lesions was linked to oncogenic stress and preceded mutations in the p53. Based on this it was proposed that checkpoints function as a barrier against tumor progression and prevent genomic instability. However, few cells surmount this barrier and development of cancer in turn depends on the selection of these cells leading to cell proliferation, survival, increased genomic instability and tumor progression. These findings were the first to link oncogenic stress induced checkpoint activation with the evolution of cancer and also provided insight into the mechanisms by which cancer cells "break" caretaker systems.

The biological functions of Cdc25B have been enigmatic with confounding literature reports. Several groups have reported that Cdc25B has a unique role of initiating mitotic events (35, 37, 113). Cdc25B activates centrosomal pool of Cdk1 before the onset of mitosis. To precisely coordinate the transition from G2 to mitosis, Cdc25B activity and subcellular localization is tightly regulated by phosphorylation at several sites. This phosphorylation is apparently facilitated by different kinases including Chk1, Aurora A, pEG3, and (37, 54, 148, 149). Even though cells have developed this complex network to regulate Cdc25B activity, the Cdc25B knockout mice develop normally. Moreover, no defects in cell cycle was observed in Cdc25B kncokout MEFs ((42), Lazo unpublished observation). These observations challenged the importance of Cdc25B in the regulation of mitosis. Nonetheless, knockout animals have some severe limitations as an experimental model and absence of phenotype does not always

reflect the importance of the function of the deleted protein. For example, during the development of the genetically modified mouse, other functionally related genes could compensate for the lost gene by overactivation or overexpression. Results with human cells lacking Cdc25B due to siRNA support the conclusion that under normal conditions Cdc25B is redundant for controlling entry into mitosis (94).

Nevertheless, Cdc25B has been found to be overexpressed in several cancers (19). Furthermore, we found that *anti*-BPDE increases Cdc25B expression in lung cancer cells indicating that Cdc25B could contribute to *anti*-BPDE induced lung carcinogenesis (61). In addition, transgenic mice that overexpress Cdc25B in mammary epithelium exhibit increased susceptibility to 9, 10-dimethyl-1, 2-benzanthracene-induced mammary tumorigenesis (60). These studies emphasize the importance of Cdc25B in tumorigenesis. Interestingly, the exact mechanism by which Cdc25B contributes to tumorigenesis is not completely understood. Several hypotheses that have been posited to explain oncogenic properties of Cdc25B include: (a) Cdc25B regulates cancer development by driving unrestricted cell proliferation, (b) overexpression of Cdc25B leads to inadequate activation of checkpoints thus causing genomic instability, (c) overexpression of Cdc25B promotes genomic instability by regulating premature recovery from checkpoints without completion of DNA repair, and (d) overexpression of Cdc25B creates oncogenic stress in precancerous lesions leading to selection of cells with compromised checkpoint signaling.

In all of the above mentioned hypotheses, DNA damage and checkpoints seems to have an important role in Cdc25B regulated tumorignesis. Furthermore as described previously, checkpoints are intimately linked to cell cycle regulation and could function as enablers of genomic instability when deregulated. Therefore, it is important to fully understand the

molecular details of impact of checkpoints on Cdc25B. Studies presented in this dissertation were designed to delineate the effect of DNA damage on Cdc25B.

In this study, the fate of Cdc25B following mechanistically distinct DNA damaging agents (cisplatin, bleomycin, etoposide, IR, UV and *anti*-BPDE) was examined and surprisingly a rapid increase in cellular Cdc25B levels was observed following DNA damage. Using UV as a prototypic agent, the increase in Cdc25B protein level was found to be concentration- dependent, p53 independent, sustained for 8 days post-treatment and attenuated by shRNAi against Cdc25B. UV irradiation also increased levels of HA-tagged Cdc25B where Cdc25B was expressed through tet-regulated heterologous promoter (Chapter 3.2.2 and 3.2.3). By blocking the checkpoint signaling using pharmacological inhibitors or genetic mechanisms (Chk2-/- HCT116 cells), I discovered that the increase in Cdc25B protein levels after UV was regulated by the ATR/Chk1 pathway (Chapter 3.2.4). Furthermore, UCN-01 prevented loss of Cdc25A following UV in a similar experimental conditions, showing distinct mode of regulation of Cdc25A and B isoforms by checkpoint signaling following DNA damage.

Activation of Chk1 following UV results in hyperphosphorylation of Cdc25A leading to accelerated SCF-dependent degradation through F box protein, β-TRCP (43). Interestingly, Cdc25B has been shown to be a labile protein under non-stress conditions (108). Cdc25B stability is dependent on the phosphorylation by Cyclin A/Cdk1.Recently; UCN-01 was shown to be a direct inhibitor of MAPKAPK2 within cells (150). Therefore, it is possible that UCN-01 blocked increased in Cdc25B could be partially mediated by MAPKAPK2. Moreover, MPAKAPK2 has been shown to phosphorylate Cdc25B at some of the same sites as Chk1 (54). Therefore, it could be postulated that in the presence of DNA damage, Chk1 or MAPKAPK2 increase Cdc25B protein stability leading to a rapid increase in Cdc25B levels following DNA

damage. It is possible that Chk1 or MAPKAPK2 phosphorylates Cdc25B at certain sites that disrupts the basal turnover of Cdc25B. Validating this hypothesis would entail a) confirming whether both or one of the above mention kinase is involve in Cdc25B induction following DNA damage using siRNA, b) confirming increased protein stability following DNA damage, c) determining sites phosphorylated by Chk1/MAPKAPK2 following DNA damage, and d) evaluating the impact of phosphorylating events on protein stability by mutating the sites identified in c). Finally, results from these studies would provide further insight into the Cdc25B regulation following DNA damage.

Recently, Chk1 was shown to be targeted for degradation following replication stress to terminate checkpoint signaling and facilitate recovery (98). Proteolytic degradation of Chk1 was trigged by the phosphorylation at Ser345, a known target site for the upstream activating kinase ATR. In our experimental conditions, we did not observe decrease in total Chk1 levels following UV irradiation (data not shown). However, phosphorylation at Ser345 rapidly declined after 1 hour supporting previous finding that PPMID dephosphorylates Chk1 to terminate checkpoints (95). These results led us to the hypothesis that Cdc25B accumulation is a priming factor for cell cycle resumption and the ATR/Chk1 pathway prepares cells for cell cycle resumption before inactivation of checkpoint signaling. To test that levels of Cdc25B are crucial in regulating the rate of cell cycle resumption following DNA damage-induced cell cycle arrest, I developed a stable knowdown cell line expressing shRNAi against Cdc25B. I also used previously described tet regulated Cdc25B overexpressing cells(104). These two antithetical approaches showed that Cdc25B levels controlled the number of cells progressing into mitosis following UV but did not affect G2/M checkpoint engagement immediately following DNA damage. Increased Cdc25B was found to reduce the time required for cell cycle resumption. These results along with

previous findings support a model in which activation of checkpoint following DNA damage decreases Cdc25A levels to induce cell cycle arrest and increases Cdc25B levels to resume cell cycle before their inactivation. As illustrated in Fig.31, the model envisions that in the first two hours following DNA damage, signaling pathway regulating cell cycle arrest are more dominant, thus allowing cells to repair the damaged DNA. In the following time period, gradual accumulation of Cdc25B may increase Cdk1 activity to a level that would be sufficient to overcome the inhibition accumulated during the DNA damage induced cell cycle arrest. This in turn allows cells to resume cell cycle between 8 and 24 hours.

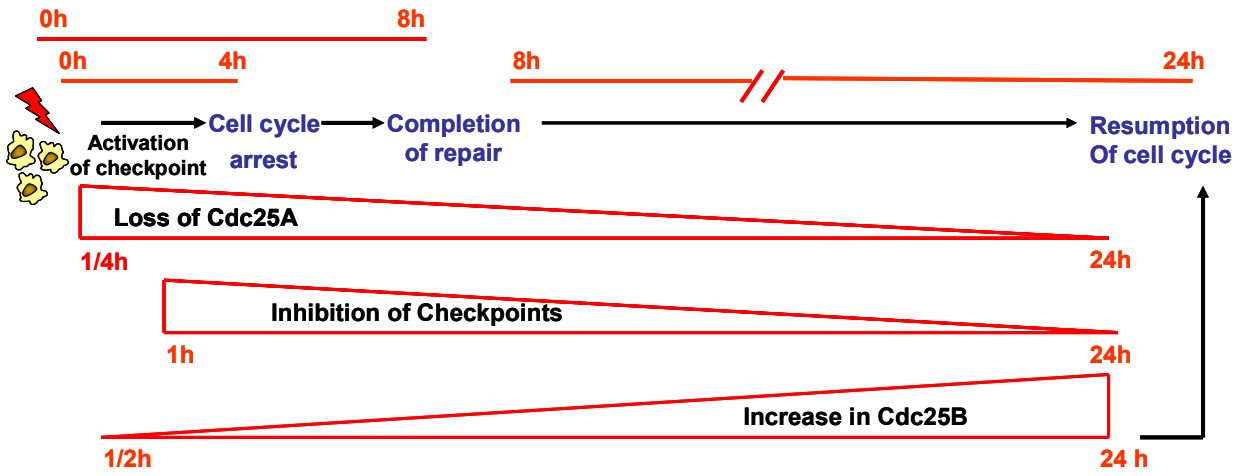

Figure 31. A schematic representing the sequence of events that take place after DNA damage to facilitate repair and regulate cell cycle resumption

Since there is a time lag between the rapid induction of Cdc25B and recovery it is not understood how cells restrained Cdc25B activity to orchestrate timely resumption without compromising genomic integrity. Interestingly, in a recent study ATM was shown to regulate recovery by phosphorylating Artemis (94, 151), which is a phospho-protein important for V(D)J

recombination, nonhomologous end-joining of double-strand breaks, and regulation of the DNA damage-induced G2/M cell cycle checkpoint (152-155). ATM phosphorylates Artemis at four different serine (516, 534, 538 and 645) residues within 30 minutes following IR. Among these four sites, Ser534 and 538 are rapidly dephosphorylated whereas phosphorylation on Ser516 and 645 is maintained for 24 hours post-irradiation. Mutation of Ser516 and 645 result in the defective recovery from the G2/M cell cycle checkpoint. This defective recovery is due to promotion of an enhanced interaction between unphosphorylated Cyclin B and Cdk1 by mutant Artemis, which in turn promotes inhibitory phosphorylation of Cdk1 by the Wee1 kinase. Based on these results it has been proposed that ATM regulates checkpoint recovery through modifications of Artemis that occur shortly after DNA damage, thus setting a molecular switch that acts upon completion of DNA repair. It could be envisioned that dephosphorylation of the other two residues (Ser534 and 538) might act as a signal to Artemis to regulate recovery upon completion of DNA repair. These results suggest that a similar molecular switch controlling Cdc25B activity might also exist for Cdc25B and further studies are required to validate this hypothesis.

In the latter section of the dissertation, I investigated the mechanism by which Cdc25B contributes to *anti*-BPDE induced tumorigenesis (Chapter 4). For this, we first confirmed that Cdc25B is essential for *anti*-BPDE-induced neoplastic transformation using previously described (123) mouse embryonic fibroblasts (MEFs) from wild type ($Cdc25B^{+/+}$) and Cdc25B null mice ($Cdc25B^{-/-}$) (Chapter 4.1.3). I further showed that increase in Cdc25B facilitated cell cycle resumption following *anti*-BPDE-induced cell cycle arrest and this resumption may occur in the presence of persistent DNA damage (Chapter 4.2.2). These results suggest that chronic exposure to *anti*-BPDE, e.g., through cigarette smoking, could increase basal Cdc25B levels in lung

epithelial cells and may enhance genomic instability by regulating premature recovery from checkpoints; hence, reinforcing the hypothesis that Cdc25B contributes to tumorigenesis by regulating premature recovery from checkpoints without completion of DNA repair.

Increased Cdc25B activated checkpoints in the absence of overt DNA damage indicating that increase in Cdc25B levels could induce oncogenic stress observed in precancerous lesions that activates checkpoints and facilitate selection of cells with compromised checkpoint signaling. These observations support a model where Cdc25B, similar to Cdc25A, enables genomic instability by promoting selection of cells with deregulated checkpoint signaling. This model was further supported by the observed decreased expression of p53 and p21 in chronically treated WT MEFs (Chapter 4.1.5). Future studies examining Cdc25B expression, markers of checkpoints, and p53 mutations in clinical samples derived from smokers would be extremely beneficial to understand the significance of Cdc25B in smoking induced lung cancer and might help elucidate the use of Cdc25B as a diagnostic marker for detecting lung cancer.

Finally, to summarize the effect of increase Cdc25B levels following DNA damage on cell's overall ability to survive, I developed a model describing three different scenarios. As illustrated in the Fig. 32, in cellular conditions when the process of DNA repair is completed, increase in Cdc25B expression following DNA damage would promote cell proliferation by regulating cell cycle progression. In other cellular conditions, where damage is not repairable, cells might utilize the same pathway to remove genetically unstable cells by prematurely activating Cdk1 resulting in mitotic catastrophe. Finally, in cells chronically exposed to DNA damaging agents, the increase in Cdc25B levels could facilitate resumption without the completion of DNA repair, which may lead to tumor progression through genomic stability. The factors that help decide between the scenario 2 and 3 remain to be defined. It would be

interesting to explore the role of Cdc25B in cell cycle re-entry from the quiescent state G0 and in the terminally differentiated neurons.

To conclude, studies in this dissertation help elucidate an important role of Cdc25B in cell cycle resumption following DNA damage induced cell cycle arrest and provided insight into how defection from the normal response could contribute to tumorigenesis by using *anti*-BPDE induced lung carcinogenesis as a model.

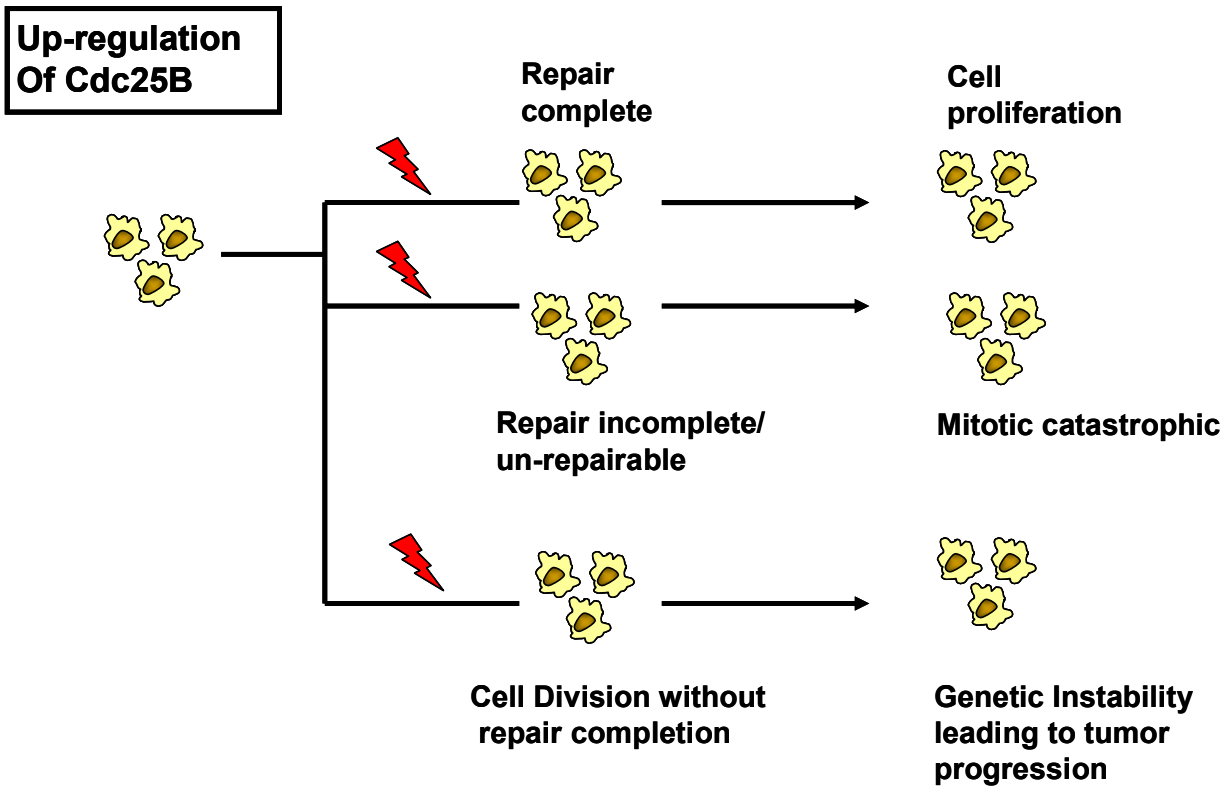

Figure 32. A model depicting the role of Cdc25B in cellular response to DNA damage.

APPENDIX A

Antibodies for Western blotting

Name of the protein	Vendor	Dilution for primary antibody	Dilution for secondary antibody
Cdc25A	Santa Cruz (F6)	1:100 (o/n)	1:1000 (1h)
Cdc25B	BD Biosciences	1:500 (o/n)	1:2000 (1h-2h)
Cdc25C	Santa Cruz (C-20)	1:500 (o/n)	1:2000 (1h)
ß - Tubulin	Cederlane	1:5000 (1h)	1:5000 (1h)
Vinculin	Santa Cruz	1:200 (o/n)	1:2000 (1h)
PARP	Cell Signaling	1:1000 (o/n)	1:2000 (1h)
p53	Cell Signaling	1:1000 (o/n)	1:3000 (2h)
p53 (Ser15)	Cell Signaling	1:1000 (o/n)	1:1000 (1h)
γ- H2AX	Cell Signaling	1:1000 (o/n)	1:1000 (1h)
Phospho-cdc2	Cell Signaling	1:1000 (o/n)	1:2000(1h)
Cdk1	Santa Cruz	1:1000 (o/n)	1:2000 (1h)
Chk1(Ser317)	Cell Signaling	1:1000 (o/n)	1:1000 (1h)
Chk1 (Ser345)	Cell Signaling	1:1000 (o/n)	1:1000 (1h)
Chk1	Santa Cruz (G4)	1:200 (o/n)	1:2000 (1h)
Histone H3 (Ser10)	Upstate Biotechnology	1:500 (o/n)	1:3000 (1h) or 1:1000 (1h)
HA	Covance	1:1000 (o/n)	1:5000 (1h)
Cyclin B1 (GSN1)	Santa cruz	1:200 (o/n)	1:2000

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