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The role of Plk4 in DNA damage pathways and entry into mitosis.

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THE ROLE OF PLK4 IN DNA DAMAGE PATHWAYS AND ENTRY INTO
MITOSIS

by

Sepal Bonni

A Thesis
Submitted to the Faculty of Graduate Studies
Through Biological Sciences
In Partial Fulfillment of the Requirements for
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ABSTRACT

The roles that Plk4 plays in cell division and DNA damage pathways have not yet been clarified. Results presented provide further insight into this question. Plk4 protein levels were seen to decrease in response to UV damage specifically, but not ionizing radiation. Additionally, overexpression of Plk4 overrides the G1-S checkpoint arrest induced by DNA damage, leading to an accumulation of cells in the S-phase of the cell cycle. This suggests that Plk4 is inhibited by DNA damage, and suggests a possible role for Plk4 in S-phase entry.

Thus far only a few interacting partners and substrates are known for Plk4. My results show that Plk4 interacts with both Cdc25C and Cyclin B. Furthermore, Cdc25C was established to be a substrate of Plk4 through in-vitro kinase assays. The finding that Plk4 interacts with these key mitotic proteins provides further evidence that Plk4 may play a novel role in cell division.

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CHAPTER I

INTRODUCTION

Polo-Like Kinase Family

The Polo-Like kinases (Plks) are a family of serine-threonine kinases which play key roles in multiple stages of the cell cycle (Glover *et al.*, 1998). The founding member of this family was named Polo and was originally identified in *Drosophila melanogaster*. Subsequently, structural and functional homologs have been identified in all major model systems studied, indicating a conserved function and evolutionarily important role in ensuring species survival. Simple eukaryotes such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain one Plk family member, CDC5 and Plo1 respectively (Kitada *et al.*, 1993; Ohkura *et al.*, 1995), while more complex eukaryotes contain a higher number of Plk family members. *C. elegans* and *Xenopus laevis* have three Plk family members, whereas humans and mice contain four (Plk1, Plk2, Plk3 and Plk4 or Sak) (Barr *et al.*, 2003).

The Plks play important roles during multiple stages of the cell cycle including cytokinesis and mitosis as well as roles in DNA damage response pathways (Barr *et al.*, 2004). Furthermore, Plks are regulators of bipolar spindle formation, centrosome maturation, chromosome segregation and APC/C regulation (Barr *et al.*, 2004).

The Polo-Box Domain

The Plks share two highly homologous regions including an N-terminal protein kinase domain and one or two non-catalytic C-terminal polo-box domains (Figure 1).

Plks 1-3 contain two polo-box domains, whereas Plk4 only contains one. Each polo-box is ~80 amino acids in length separated by a ~20 amino acid linker region (Leung *et al.*, 2002; Cheng *et al.*, 2003). Cheng *et al.*, 2003 revealed that the two polo-boxes of Plk1 contain a six-stranded anti-parallel beta-sheet and an alpha-helix.

Lie *et al.*, 1998 suggested that the polo-box domain of Plk1 is necessary for localization. A single amino acid change of a conserved residue at position 414 in the polo-box from tryptophan to phenylalanine abrogates Plk1's localization to the spindle pole bodies. Furthermore, this mutation of the human Plk1 polo-box domain results in Plk1's inability to complement the temperature sensitive CDC5-1 mutant (Lee *et al.*, 1998). Complementation assays for the CDC5-1 mutant prove that W414 is the most crucial residue for the function of Plk1 polo-box domain (Lie *et al.*, 1998). Additionally, Hanisch *et al.*, 2006 reported that the catalytic domain of Plk1, lacking the polo-box domain is unable to localize to centrosomes and kinetochores. Hanisch *et al.*, 2006 also demonstrated that diminishing Plk1 or similarly overexpression of the polo-box domain of Plk1 resulted in mitotic arrest. Interestingly however, overexpression of the polo-box domain interfered with chromosome segregation whereas depletion of Plk1 impaired maturation and separation of centrosomes or bipolar spindle formation (Hanish *et al.*, 2006). However, on the contrary, it was also found that Plk1 protein in which both polo-box domains have been deleted still localizes to centrosomes as do the two polo-box domains of Plk1 expressed as GFP (green fluorescent protein) fusion proteins. This thus suggests that the polo-box domains are sufficient but not necessary for localization (Hudson unpublished data). The polo-box domain of Plk3 has also been implicated as a localization signal to centrosomes and spindle poles (Jiang *et al.*, 2006). Also, it has been

shown that both polo-box motifs are necessary for this localization to occur and that the polo-box domain is also sufficient for this localization. In addition, overexpression of the polo-box domain of Plk3 interferes with cell division and cytokinesis (Jiang *et al.*, 2006).

For Plk1-3, the polo-box domain including the region between them and a portion of the linker between the end of the kinase domain and the first polo-box domain, functions as a single phosphoserine/threonine-binding module (Elia *et al.*, 2003a). The crystal structure of the Plk1 polo-box domain showed that the phosphopeptide binding motif is located in the interface between the two polo-boxes (Cheng *et al.*, 2003; Elia *et al.*, 2003b). For Plk1, a molecular model has been proposed for the substrate binding of the kinase. The model proposes that initially the polo-box binds to a previously phosphorylated serine/threonine binding motif on the substrate which then positions the substrate such that the kinase domain of Plk1 can phosphorylate the same protein at a different site (Figure 2a) (Elia *et al.*, 2003a). The two residues of Plk1 that come in contact with the phosphate group are H538 and K540 and mutation of these residues results in loss of substrate binding and localization of centrosomes (Cheng *et al.*, 2003). If this model is accurate, Plk1 substrates should contain polo-box binding motifs and kinase phosphorylation motifs. This model thus proves accurate for Plk1 substrate Cdc25C. Cdc25C is phosphorylated on Thr-130 by Cdk1 and this site is involved in the interaction with the polo-box domain. Upon binding, Plk1 then phosphorylates Ser-198 of Cdc25C (Elia *et al.*, 2003a). Mutation of the putative Plk1 pThr-binding motif in Cdc25C abolishes the interaction of the enzyme with its substrate (Elia *et al.*, 2003a). Furthermore, mutation of the interaction sites within the polo-box domain of Plk1 not only abrogates phospho-substrate binding, but also eliminates localization of Plk1 to the

centrosomes (Elia *et al.*, 2003b). Also, quite interestingly, upon phospho-peptide binding of the polo-box domain of Plk1, Plk1 kinase activity is also stimulated (Elia *et al.*, 2003b). One model for this is that Plk1 activity is negatively regulated by the polo-box domain (Jang *et al.*, 2002). In the absence of a phosphorylated substrate, the polo-box domain interacts with the kinase domain and prevents substrate binding or activation. Upon binding of the polo-box domain with a phosphopeptide, the kinase domain is thus released and upon phosphorylation of the T-loop, the protein is converted to an active form (Elia *et al.*, 2003b).

An alternate model proposes that the polo-box domain phosphoserine / threonine substrates are essentially different from the substrates that the kinase domain phosphorylates. This model suggests that perhaps polo-box binding to phosphorylated docking proteins localizes the kinase domain to its substrates which may or may not be tightly associated with the docking protein (Figure 2b).

Trp-414, a key residue which is necessary for polo-box localization along with His-538 and Lys-540, residues which bind phosphorylated peptides, are conserved residues in Plk 1-3 however not in Plk4. Both Plk2 and Plk3 polo-box domains are found to function as phosphoserine/threonine binding motifs thus suggesting a similar mechanism of substrate interaction (Cheung *et al.*, 2003).

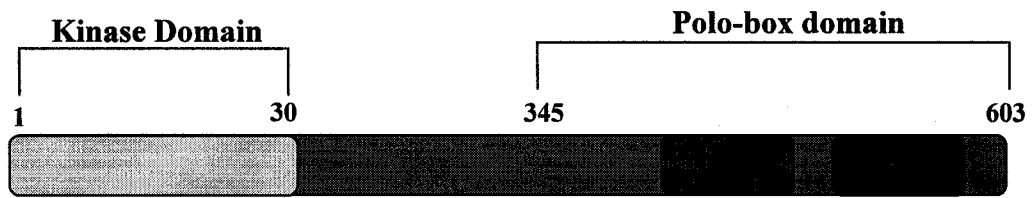


Figure 1. **Polo-like kinase structure.** Plks share a highly conserved protein kinase domain in the amino-terminal and common structural motifs called polo-boxes in the non-catalytic carboxy-terminal region. The kinase domain is represented by the yellow colour and the two polo-boxes are shown in red. The region prior to and including the two polo-boxes has been renamed the polo-box domain.

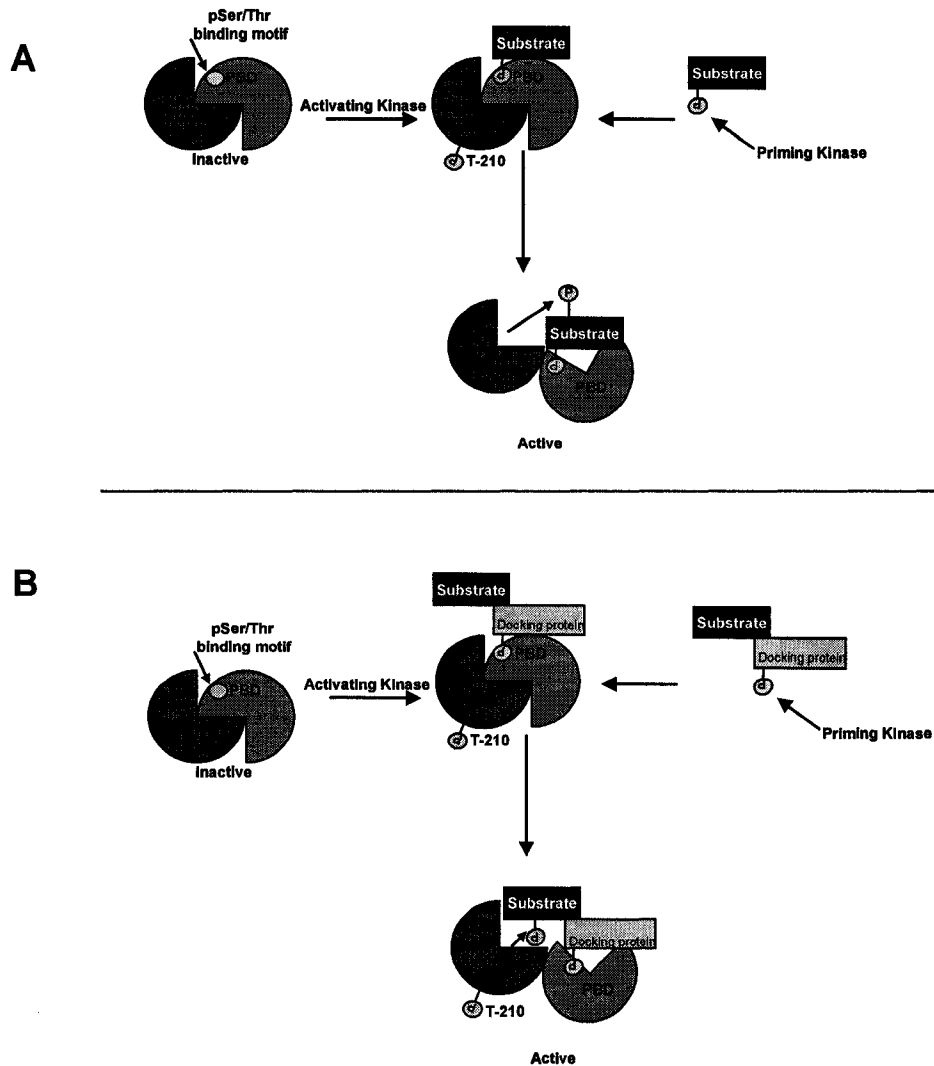


Figure. 2. **Two models for Plk1, Plk2 and Plk3 polo-box domain function.** Plks 1-3 are activated through phosphorylation of T-210 and binding of the polo-box domain to phosphorylated ligands. Which event occurs first remains to be elucidated. KD refers to the kinase domain and PBD refers to the polo-box domain. A. The polo-box domain binds to a site on a protein that has been previously phosphorylated by a priming kinase. This alleviates inhibition of the kinase domain and allows for phosphorylation of the same protein on a different site. B. In this model, the ligand to which the polo-box domain binds is different from the substrates which the kinase domain phosphorylates. Priming kinases phosphorylate sites on docking proteins and upon binding to the polo-box domain allows the catalytic domain of the Plk to phosphorylate the associated substrate.

Activation and Phosphorylation of Plks

Although it is widely known that the Plks require phosphorylation for activation, their upstream activating kinases have remained fairly elusive. Among the Plks, the mechanistic and structural detail of Plk1 activation has been the most extensively examined. The activation of other Plk family members is thought to occur through the same mechanism (Qian *et al.*, 1999).

A common mechanism for the activation of many protein kinases is phosphorylation in their respective T loop. Within the kinase domains of Plks, several conserved serine and threonine residues are present which could be potential sites for phosphorylation and activation of these kinases. T210 lies within the T-loop of the Plk kinase domain and mutation of this residue to Asp mimics phosphorylation and renders the kinase constitutively active. It is generally believed that phosphorylation at T210 is a requirement for Plk activation (Qian *et al.*, 1999).

Although much remains to be elucidated regarding the full activation of Plks, two candidates have been proposed for the phosphorylation of T210. These two candidates are human SLK and PKA along with *Xenopus* Plkk1 (Qian *et al.*, 1998).

Although mutation of another site in the kinase domain, S137 to Asp also renders the kinase active, there is no evidence for the direct phosphorylation of this residue. Furthermore, mutation of this serine (S137) to alanine did not prevent activation of Plk1/Plx1 suggesting that phosphorylation at this residue occurs, but it is not critical (Qian *et al.*, 1999). The possibility for additional post-translationally modified sites on Plks remains to be determined.

Plk Expression and Localization

The expression and localization patterns of Plks are dynamically regulated in a cell-cycle dependent manner. *Drosophila* Polo localizes to centrosomes at mitotic entry. Furthermore, during prometaphase, the protein associated with kinetochores and late in anaphase, prior to cytokinesis, it was seen to localize to the central spindle (Glover, 2005).

Yeast Plks have been shown to localize to spindle pole bodies but the timing of localization differs between CDC5 (budding yeast) and Plo1 (fission yeast). CDC5 localizes to the spindle pole bodies in G1 until the completion of mitosis (Song *et al.*, 2000). Conversely, Plo1 localizes to the spindle pole bodies only upon activation of Cdc2 and remains associated there until Cdc2 becomes inactive (Mulvihill *et al.*, 1999).

During G2, Plk1 localizes to the cytoplasm and the nucleus and is targeted to centrosomes (Golsteyn *et al.*, 1995). During prophase and metaphase Plk1 has been shown to localize to centrosomes and kinetochores where it persists until it is degraded. A portion of Plk1 relocates to the equatorial spindle midzone at anaphase (Lee *et al.*, 1998). Generally, Plk1 expression and activity is low in G0, G1 and S, begins to increase in G2 and peaks in M phase. Finally, Plk1 is degraded through the ubiquitin - proteasome pathway upon completion of mitosis (Lee *et al.*, 1995).

Plk2 and Plk3 most likely have overlapping roles with Plk1 as they localize to similar intracellular structures. However, Plk2 functions predominantly as a regulator of G1 progression in mammalian cells (Ma *et al.*, 2003a). Plk2 and Plk3, similar to Plk1, have both been shown to localize to the centrosomes (Wang *et al.*, 2002). In contrast to what is found for Plk1 and Plk2, the expression of Plk3 protein and mRNA is not cell

cycle regulated. Although Plk3 mRNA expression is only seen in G1, Plk3 protein level is seen at all stages of the cell cycle given Plk3s stability (Alberts and Winkles, 2004). Furthermore, Plk3 protein is rapidly degraded in the nucleus via the ubiquitin-proteasome pathway however, in order to sustain a long half-life, plk3 is most likely sequestered in the cytoplasm in a catalytically inactive form.

The multiple sites of the Plks localization patterns and differential expression during normal cell cycle progression reflect the many functions that these enzymes perform during the cell cycle.

Plk Overexpression and Mutant Phenotypes

Mutations in *Drosophila melanogaster* Polo, the founding member of the Plk family, resulted in several phenotypic defects (Sunkel and Glover, 1988). Although homozygous mutants of the gene successfully developed to adulthood, numerous mitotic defects were observed during development (Glover, 2005). Additionally, monopolar spindles were observed in the mutant cells with centrosomes which failed to separate (Llamazares *et al.*, 1991). In the absence of Polo function, several proteins fail to be recruited to the spindle pole and abnormal spindle poles form, reflecting the name of this protein (Llamazares *et al.*, 1991).

Both the budding yeast and the fission yeast contain Polo homologs with significant functional similarities to vertebrates Plks suggesting that the role of Plks is largely preserved throughout evolution (Glover *et al.*, 1998). A temperature-sensitive CDC5 mutant (the Plk homolog in budding yeast) arrests with a bipolar spindle late in mitosis (Byers *et al.*, 1974). Furthermore, in the first division of meiosis, it displays

abnormal behaviour of spindle pole bodies, and in the second division there is a failure of the spindles to elongate (Schlid *et al.*, 1980). Interestingly, studies by Lee *et al.*, 1997 showed that complementation of the temperature sensitive CDC5-1 mutant could be accomplished by Plk1. Additionally, Ouyang *et al.*, 1997 showed that Plk3 could also rescue this mutant, indicating that Plk1 and Plk3 have a conserved function in regulating mitotic/meiotic progression. Thus, this demonstrates that Plk1 and Plk3 both have conserved some CDC5 functions. In contrast, Plk2 and Plk4 do not rescue this mutant suggesting divergence of these Plk family members. Furthermore, this suggests that Plk2 and Plk4 have a non-redundant role in the cell cycle (Swallow *et al.*, 2005).

In fission yeast overexpression, or mutation of Plo1 resulted in the failure of the spindle pole bodies to complete either their duplication or separation resulting in monopolar spindles thus indicating the protein is necessary for bipolar spindle formation (Ohkura *et al.*, 1995). Also, Plo1 mutants failed to form the actin ring and septum, both of which are prerequisites for cytokinesis. Another consequence of overexpression of Plo1 was multiple septa being formed in cells at any stage of the cell cycle (Ohkura *et al.*, 1995).

Vertebrate Plk family member's knockdown and overexpression phenotypes have also been extensively examined. With respect to the Plk1 knockdown phenotype, prominent mitotic arrest was observed. Although Plk1 was dispensable for normal mitotic entry in human cells, as the majority of cells entered mitosis, Plk1 depleted cells failed to exit mitosis (Van Vugt *et al.*, 2004). Furthermore, upon entry into mitosis, Plk1 deficient cells were unsuccessful at forming a bipolar spindle and were incapable of aligning their chromosomes. The inability for the cells to align their chromosomes is

most likely caused by a defect in centrosome maturation, consistent with the observation that the Plks play key roles in the centrosome cycle (Seong *et al.*, 2002; Llamazares *et al.*, 1991). Indeed, Li *et al.*, 2002 did report that cells depleted of Plk1 by RNAi displayed an inhibition in centrosome amplification. Additionally, downregulation of Plk1 expression or activity in tumour cells induced apoptosis and inhibited tumour cell proliferation (Gray *et al.*, 2004). On the other hand, overexpression of Plk1 leads to multinucleation and enables tumour cells to override mitotic checkpoints leading to genomic instability and oncogenic transformation (Gray *et al.*, 2004).

In contrast to Plk1, overexpression of Plk3 leads to apoptosis and suppresses cellular proliferation and inhibits colony formation (Conn *et al.*, 2000). The Plk2 knockdown phenotype has been examined as well, and downregulation of Plk2 by RNAi has shown that Plk2 is required for centriole duplication (Warnke *et al.*, 2004). Furthermore, Plk2 knockout mice are viable and fertile, however they appear smaller than their wildtype littermates. Plk2^{-/-} mice reveal a role for Plk2 in embryonic development, as the null embryos display a slight delay in development and exhibit a diminished weight of the placentas (Ma *et al.*, 2003).

Plks and Centrosome Amplification

Localization and mutant phenotypes of polo-like kinases reveal a common characteristic role for Plks in bipolar spindle formation and centrosome duplication.

Drosophila Polo mutants fail to recruit key proteins to the centrosomes during centrosomal maturation. A complex of γ -TuRC and CP190 shows failure of recruitment to centrosomes in Polo mutants. This complex is responsible for capping the minus ends

of microtubules on centrosomes a necessary step for their nucleation (Barbosa *et al.*, 2000). Furthermore, Donaldson *et al.*, 2001 show that RNAi directed towards Polo results in failure of γ -tubulin recruitment to centrosomes. Another protein, Asp (a microtubule associated protein) has been found to be a substrate of Polo. Asp is necessary for microtubule-nucleating activity. Although Asp is able to localize in the absence of Polo, Polo is necessary to activate Asp at the microtubules (Avides *et al.*, 2001).

Fission Yeast Plk family member Plo1 associates with spindle poles and Fin1 has a role in this association. Fin1 is necessary for the regulation of spindle formation. Furthermore, increasing the levels of Fin1 results in recruitment of Plo1 to the spindle pole bodies in wild-type cells (Grallert *et al.*, 2002). One hypothesis is that Fin1 may phosphorylate proteins at the spindle pole bodies providing docking sites for Plo1 (Grallert *et al.*, 2002).

Xenopus Plx1 immunodepletion resulted in monopolar spindles with altered patterns of α -tubulin around the pole (Qian *et al.*, 1998). The mechanism through which this occurs is not completely understood, however it is thought that Plx1 affects bipolar spindle formation through the regulation of centrosome maturation and separation.

Plk1 has been shown to be necessary for the localization of several proteins to the centrosomes during the centrosome maturation process. Lane *et al.*, 1996 proved that injection of Plk1 antibodies into cells resulted in a decrease in the accumulation of γ -tubulin at the centrosome suggesting that Plk1 is necessary for γ -tubulin localization. Furthermore, the cells had monoastral microtubule arrays and duplicated centrosomes which were not separated (Lane *et al.*, 1996). Plk1 has also been shown to interact with

and phosphorylate α and β -tubulins as well (Feng *et al.*, 2002). Another centrosomal protein which Plk1 has been shown to interact with is Nip (ninein-like protein) (Casenghi *et al.*, 2003). This protein interacts with γ -tubulin and contributes to the organization of microtubules. Plk1 has been shown to phosphorylate Nip at the commencement of mitosis resulting in the proteins disengagement from centrosomes. Mutation of the Plk1 phosphorylation sites in Nip hindered spindle formation (Casenghi *et al.*, 2003). Thus, it is evident that Plk1 is necessary for bipolar spindle formation and centrosome maturation and separation.

It is therefore evident, that the polo-like kinase family plays a key role in bipolar spindle formation and centrosome (spindle pole body) maturation and separation.

Plks and Cytokinesis

Another common role for Plks is their involvement in cytokinesis. Yeast Plk Plo1 activity is necessary for formation of a division septum whereas overexpression of this kinase leads to ectopic septum formation (Ohkura *et al.*, 1995). Furthermore, Bahler *et al.*, 1998 show that Plo1 interacts with Mid1/Dmf1. Mid1/Dmf1 is a protein required for positioning of the cell division site. Plo1 localizes Mid1/Dmf1 to filamentous actin during mitosis from the nucleus (Sohrmann *et al.*, 1996). Two main components of septation are equatorial actin ring formation and equatorial microtubule organizing center (EMTOC) formation which is similar to the central spindle that forms in animal cells (Heitz *et al.*, 2001). Plo1 is necessary for EMTOC assembly and thus, Plo1 seems to be involved in several pathways which initiate septation (Heitz *et al.*, 2001).

Weak loss-of-function alleles of *Drosophila* polo kinase Polo display defects in cytokinesis (Carmena *et al.*, 1998). Polo associates with a motor protein Pavarotti (Adams *et al.*, 1998). Pavarotti (Pav) mutations result in a defective spindle mid-zone region and prevent cytokinesis from occurring (Carmena *et al.*, 1998). Additionally, Pav and Polo are dependent on one another for their correct localization. Mutation of either protein results in failure of the other to localize to spindle poles and spindle mid-zone (Adams *et al.*, 1998).

Xenopus Plx1 is localized to the midbody in late mitosis (Qian *et al.*, 1999) providing a role for this kinase in cytokinesis. Qian *et al.*, 1998 showed that injection of mRNA encoding a kinase active form of Plx1 into *Xenopus* embryos resulted in cleavage arrest. Embryos injected with wild-type Plx1 however divided normally. Therefore, the inactivation of Plx1 may be required for completion of cytokinesis in *Xenopus* embryos.

Mammalian Plks have also been implicated in cytokinesis. Initial support for the idea that Plk1 was involved in cytokinesis came from the discovery of the Plk1 substrate CHO1/MKLP-1 (mitotic kinesin-like protein) which induces microtubule bundling (Lee *et al.*, 1995). Additionally, overexpression of a dominant negative form of Plk1 causes cytokinesis failure, similar to the effect which is seen with the depletion of Plk1 in cells (Van Vugt *et al.*, 2004). Numerous other cytokinesis proteins were found to be phosphorylated by Plk1. Phosphorylation of NudC (nuclear distribution gene C) by Plk1 is essential for the execution of cytokinesis (Zhou *et al.*, 2003). Another protein which Plk1 phosphorylates is MKLP2. When MKLP2 is phosphorylated, it binds to the polo-box domain of Plk1. Depletion of the protein results in failure of cytokinesis and furthermore, disrupts Plk1 localization.

Thus, the polo-like kinase family of mitotic regulators are important regulators of cytokinesis.

Plk4 (Sak) Structure

Plk4 (Sak) was the last member of the human Plk family to be identified. Human and murine Plk4 contain a highly conserved kinase domain and a less conserved polo-box domain (Figure 3). Although little is known about the kinase activity of this protein, a Plk4 T170D mutation in the T-loop increases kinase activity, whereas a Plk4 K41M mutation in the ATP-binding domain eliminates activity (Swallow *et al.*, 2005).

Although Plks 1-3 are quite similar with respect to structure, Plk4 differs in that it harbours only a single polo-box motif at its extreme C-terminus (Leung *et al.*, 2002). The Plk4 polo-box forms intermolecular homodimers and consists in total of two alpha helices and two six-stranded antiparallel beta sheets. Each beta sheet contains four strands from one polo-box and two strands coming from the other polo-box. Although there are several similarities between the polo-box domain of Plk1 and Plk4 numerous differences also exist. Though each of the Plk1 polo-box domains consists of one six-stranded beta sheet and an alpha helix, the organization of the strands between Plk1 and Plk4 are strikingly different (Leung *et al.*, 2002; Elia *et al.*, 2003b). The two polo-box domains of Plk1 form an intramolecular heterodimer and in contrast to Plk4, the Plk1 beta-sheet consisted of six-self contained strands, and no strand exchange was observed. Although the polo-box region of Plk4 forms intermolecular homodimers, there is no evidence suggesting that the Plk4 polo-box binds phosphorylated peptides which is seen in the other Plk family members. Furthermore, the Plk4 polo-box domain is most likely

not involved in substrate binding, as the residues which are most likely involved in phosphopeptide binding by Plk1 are not conserved in Plk4 (Elia *et al.*, 2003b). However, Swallow *et al.*, 2005 report that the Plk4 polo-box domain is necessary but not sufficient for Plk4 binding to its substrate p53, suggesting that the polo-box region is a requirement for the binding of the protein to its substrate and interactions between subdomains of Plk4 are important for binding. However, results published by Habedanck *et al.*, 2005 and Leung *et al.*, 2002, support the idea that in contrast to the other Plk family members, loss of the polo-box domain of Plk4 does not abolish localization. Subcellular localization loss was only seen if a second self-association domain denoted the 'cryptic polo-box' was also deleted (Habedanck *et al.*, 2005).

The C-terminus of Plk4 contains three PEST sequences. PEST sequences are associated with decreased protein stability and the short half-life of proteins. Indeed, Plk4 does display a short half-life and the protein is destroyed by the anaphase-promoting complex (APC/C) following mitosis. Stability of this kinase is increased when the PEST sequences are deleted. Protection from PEST-dependent proteolysis and enhanced phosphorylation of the Plk4 kinase domain is observed upon Tec tyrosine kinase binding to the cryptic polo-box of Plk4. Subsequently, Plk4 is tyrosine phosphorylated by Tec (Yamashita *et al.*, 2001). Tec is the only known kinase that phosphorylates Plk4.

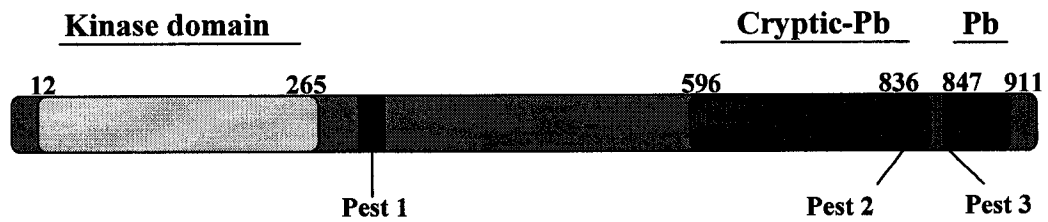


Figure 3. **Murine Plk4 structure.** Plk4 contains a kinase domain at the N-terminus, one polo-box domain at the C-terminus and 3 PEST sequences. Upstream of the polo-box domain is another self-association domain designated the cryptic-polo-box.

Plk4 Expression and Localization

Immunofluorescence staining has shown that endogenous Plk4 co-localizes with centrosomes throughout the cell cycle (Habedanck *et al.*, 2005). Both the protein and mRNA levels of Plk4 are regulated in a cell cycle dependent fashion, quite similar to the other Plk family members. Plk4 expression increases gradually from G1 to early mitosis after which it is destroyed by the anaphase promoting complex (Fode *et al.*, 1996). In mice there are two splice variants produced due to alternative splicing which results in the retention of an intron, followed by early termination of transcription in the Plk4-*b* transcript. Specifically, Plk4-*a* and Plk4-*b* transcripts both have exons 1-5 present however at which point the transcripts differ. Exons 6-15 complete Plk-*a*, whereas 147 base pairs adjacent to exon 5 completes Plk-*b* (Hudson *et al.*, 2000). In contrast, human Plk4 has only one transcript which has an insertion of 34 amino acids immediately contiguous to exon 5 relative to the murine Plk4-*a* protein sequence. Interestingly, this 34 amino acid insertion in human Plk4 is highly similar to the intron sequence that is retained in murine Plk-*b* (Hudson *et al.*, 2000). All of the work in the present study will focus on human Plk4 constructs.

Plk4 Null Phenotype

Plk4 was the first polo-like kinase family member to be mutated in vertebrates. The murine Plk4 null phenotype was found to be embryonic lethal with embryos arresting at E7.5, shortly after gastrulation. Staining for the mitotic markers phosphorylated histone H3 and Cyclin B1 showed that a large percentage of the cells were blocked in mitosis. Phosphorylated histone H3 is rapidly dephosphorylated upon

completion of mitosis. The Plk4 null embryos stained positive for phosphorylated histone H3 at a much higher frequency than Plk4 wildtype embryos indicating a block or delay in exit from mitosis. Also non-degradable forms of Cyclin B1 were seen in anaphase and telophase inhibiting exit from mitosis in Plk4 null embryos but not in Plk4 wildtype embryos (Hudson *et al.*, 2001). Proteolysis of cyclin B1 begins as soon as the last chromosome aligns on the metaphase plate and is complete prior to the onset of anaphase. Cyclin B1 degradation is required for the onset of anaphase and thus non-degradable forms blocks the exit of cells from mitosis (Clute *et al.*, 1999). Therefore, Plk4 deficiency leads to an arrest or delay of cell cycle progression in mitosis (Hudson *et al.*, 2001). Plk4 null embryo explants displayed a similar phenotype. Blastocyst outgrowths stained positive for phosphorylated histone H3 and Cyclin B1 and displayed an increase in dumbbell-shaped cells blocked in anaphase or telophase (Hudson *et al.*, 2001). These results suggest that Plk4 is necessary for exit from mitosis and more importantly, that Plk4 is essential for cell viability.

Plk4 and Cancer

Plk4 heterozygous mice embryonic fibroblasts (MEFs) also have a unique phenotype when compared to wild-type MEFs. Plk4^{+/-} MEFs have a much slower growth rate, an increased number of centrosomes, abnormal spindles, as well as misaligned and misdirected chromosomes. Thus haploid levels of Plk4 are not sufficient to sustain wildtype Plk4 phenotype and lead to chromosome instability (Hudson *et al.*, 2001; Ko *et al.*, 2005).

The phenotype of Plk4 heterozygous mice was also examined. Despite the fact that Plk4 heterozygous mice have no evident abnormalities at a young age, with increased age, the heterozygous mice develop obvious tumours at a rate of 50% compared to only 3% of their wildtype litter mates (Hudson *et al.*, 2001; Ko *et al.*, 2005). Primary heptaocellular carcinoma was the predominant category of tumour formation. The second most common lesions were papillary adenocarcinomas in the lungs. Additionally, several mice also developed axilla and upper chest wall tumours (Ko *et al.*, 2005). Human PLK4 is found on chromosome 4q28. Intriguingly, loss or rearrangement of human chromosome 4q28 is common in hepatomas, the same region where PLK4 is located (Ko *et al.*, 2005).

Furthermore, a two-thirds partial hepatectomy (PH) model was utilized to examine liver regeneration in Plk4 heterozygous and wildtype mice. Interestingly, at 44 hours the Plk4 heterozygous hepatocytes displayed enlarged cellular and nuclear area compared to the wildtype hepatocytes (Ko *et al.*, 2005). This is indicative of cells which experience a delay in the cell cycle. Furthermore, at 7 days post-PH the Plk4 +/- hepatocytes had atypical morphology compared to the wildtype, with the liver architecture distorted. Also, the first cell cycle following PH in Plk4 heterozygous hepatocytes resulted in a delay of cell cycle progression and in particular a loss in the correct levels of Cyclin B, D and E. At 9-12 months, all of the Plk4^{+/-} mice had abnormal liver histology and 4 of 11 mice had grossly apparent liver tumours. The tumours from Plk4^{+/-} mice retained their heterozygosity at four polymorphic markers suggesting that the tumours were a result of Plk4 haploinsufficiency. These results all suggest that Plk4

heterozygosity may be linked to hepatoma development and that deregulation of this kinase can contribute to carcinogenesis (Ko *et al.*, 2005).

Plk4 and Centrosome Duplication

Dating back to 1914, Theodor Boveri proposed the idea that there was a relationship between the loss of tissue architecture typical of human tumors, aneuploidy and centrosomal abnormalities (Boveri *et al.*, 1914). One mechanism in which Plk4 may lead to tumour progression is through centriole overduplication. Endogenous Plk4 localizes to centrosomes throughout the cell cycle. Interestingly, centriole amplification occurs in Plk4 heterozygous MEF's and also with the overexpression of Plk4 (Hudson *et al.*, 2001; Ko *et al.*, 2005; Habedanck *et al.*, 2005).

There are two mechanisms in which centrosome overduplication can occur. These two mechanisms include legitimate centriole overduplication in S phase or through failure of cell division (Nigg *et al.*, 2002). Interestingly, overexpression of Plk4 caused centrosome overduplication in the presence or absence of aphidicolin which blocks the cells in the S phase of the cell cycle. This indicates that Plk4 indeed caused legitimate centriole overduplication in S phase, rather than through failure of cell division (Habedanck *et al.*, 2005). Furthermore, centrosome localization of Plk4 required regions of the protein that were located upstream of the polo-box domain, in the C-terminal non-catalytic domain and centriole amplification could only occur with the kinase domain present. Mutants which lacked the kinase domain or the C-terminal region could not cause centriole amplification (Habedanck *et al.*, 2005). Additionally, overexpression of Plk4 only caused centrosome duplication in the presence of Cdk2. To that end, in the

absence of Plk4, Cdk2 could not cause centrosome overduplication either. Thus, these two proteins must cooperate to cause the centrosome over duplication phenotype which is observed with the overexpression of Plk4 (Habedanck *et al.*, 2005).

Loss of function experiments determined that indeed Plk4 is essential for centriole amplification. When endogenous Plk4 is depleted in cells, the centrosome amplification phenotype is suppressed. Also, cells undergo a reduction in centrosome number with each passage through the cell cycle with a predominant phenotype of monopolar spindles with a single centriole in the center 48 hours post Plk4 siRNA (Habedanck *et al.*, 2005). Thus, with continued passage through the cell cycle, cells depleted of Plk4 undergo a reduction of centrioles indicating that Plk4 is indispensable for centriole duplication (Habedanck *et al.*, 2005).

Plk4 heterozygous MEF's also display a similar phenotype such that centrosome overduplication is observed. One possible explanation for this is believed to be that Plk4 heterozygous MEF's fail to restrict Cyclin E and Cdk2, which results in overduplication of centrosomes. This could possibly be an outcome of the decreased p53 and p21 levels seen in Plk4 heterozygous MEF's (Ko *et al.*, 2005). Another explanation for this phenotype could be that reduced Plk4 activity causes cell division failure and thus centrosomes are overduplicated or vice versa such that cell division failure results as a consequence of irregular centrosome duplication (Habedanck *et al.*, 2005). Increased centrosome number leads to polyploidy and aneuploidy which in turn can contribute to the increased frequency of tumours observed in Plk4^{+/-} mice.

Thus, Plk4 plays a crucial role in centrosome duplication however it is not the sole regulator of this phenomenon. Plk4 and Cdk2 presumably cooperate in the centrosome duplication pathway.

The Plks and DNA Damage Pathways

One of the most important functions of Polo-like kinases is their involvement in DNA damage checkpoint pathways. Cell cycle checkpoints ensure that one stage of the cell cycle is completed at high fidelity prior to the initiation of the following stage. A loss of checkpoint function can result in genetic instability and/or transformation (Xie *et al.*, 2005). Plk 1, 2 and 3 are key proteins that are important to cell cycle regulation and have been implicated in DNA damage checkpoint pathways.

Plk1 has been shown to physically bind to tumour suppressor p53 and inhibit its transcriptional activity. However, upon DNA damage to the cells, critical DNA damage sensor proteins are activated such as ataxia telangiectasia mutated protein (ATM) and ATM- and Rad3-related protein (ATR). In turn, these kinases phosphorylate downstream targets including the polo-like kinases. Plk1 has been shown to be inhibited by ATM and ATR and thus is inhibited by DNA damage (Smits *et al.*, 2000). This inhibition of Plk1 by ATM and ATR abolishes Plk1's inhibition of p53 and as a result p53 levels can increase leading to cell cycle arrest or apoptosis. Although these observations implicate Plk1 as a negative regulator of DNA damaged cell cycle arrest, Plk1 may also play a positive role in DNA damage checkpoint activation. Plk1 has been shown to phosphorylate and possibly activate Chk2 (Tsvetkov *et al.*, 2003) which ultimately leads to the inactivation of Cdc25C and thus entry into mitosis can no longer occur.

Other mammalian Plks have also been implicated in the DNA damage checkpoint activation pathway. Plk2 mRNA expression is increased in response to cellular stresses such as UV damage and this response is dependent on p53 expression. Additionally, p53 binds to the Plk2 promoter and induces transcription (Burns *et al.*, 2003).

In sharp contrast to Plk1, Plk3 acts as a positive regulator of DNA damage pathway activation. Plk3 has been shown to interact and activate p53 and the degree of this interaction is increased in response to DNA damage. Also in response to DNA damage, Plk3 is activated in an ATM-dependent manner (Xie *et al.*, 2001). Additionally, Plk3 has been shown to phosphorylate and activate Chk2 in concert with ATM (Bahassi *et al.*, 2002). Thus Plks 1, 2 and 3 all play significant roles in DNA damage response pathways (Figure 4).

Plk4 has also recently been implicated in DNA damage pathways. The protein has been shown to co-immunoprecipitate with p53 (Swallow *et al.*, 2005). Furthermore, through chip profiling Li *et al.*, 2005 have shown that p53 repressed Plk4, through an indirect mechanism as direct binding of p53 to the Plk4 promoter region was not seen. Plk4 expression was repressed in a p53-dependent manner such that when cells were exposed to etoposide to activate p53, a dramatic decrease in Plk4 expression was seen in p53 wildtype cells however not in p53 null cells. This indicates a direct relationship between p53 status and downregulation of Plk4 expression. Lastly, Li *et al.*, 2005 showed that Plk4 RNAi induced apoptosis, whereas overexpression of the protein attenuated p53-mediated apoptosis indicating that Plk4 repression likely contributes to p53-induced apoptosis. Thus, the role of Plk4 in DNA damage has yet to be clearly defined and the role this kinase plays in response pathways remains to be elucidated.

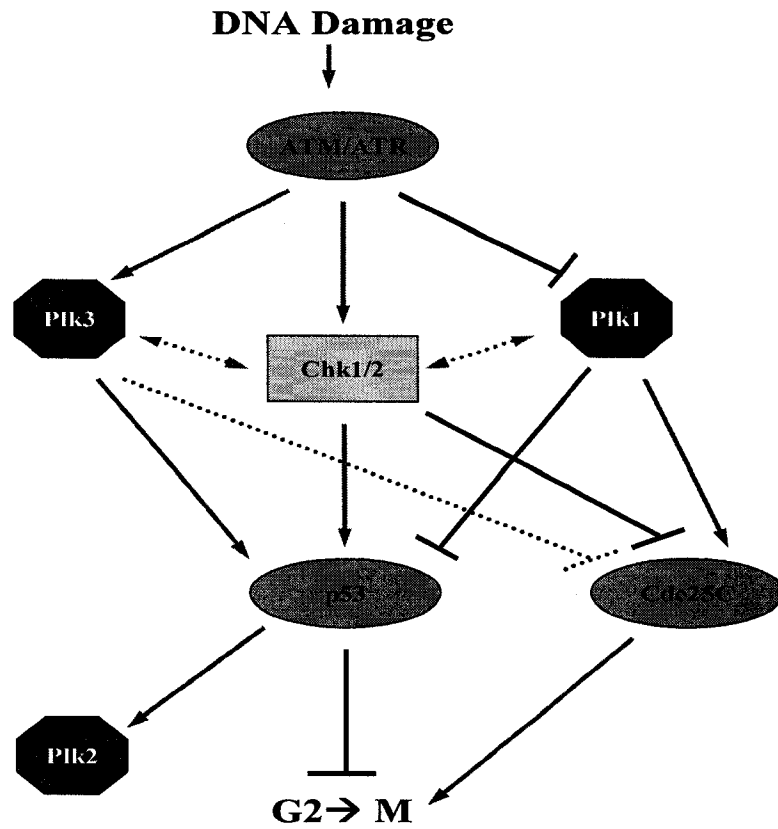


Figure 4. Major DNA damage pathways where Plks have been implicated. Solid red arrows indicate positive regulation whereas solid black lines indicate negative regulation. Dotted lines indicate proposed regulatory role however the precise role remains to be elucidated.

The Plks and Entry into Mitosis

Mitotic entry requires the activation of the Cdk1/Cyclin B complex in eukaryotic cells. The kinase activity of Cdk1/Cyclin B is controlled by phosphorylation and dephosphorylation events on Cdk1 and the accumulation of Cyclin B protein. Myt1 and Wee1 protein kinases phosphorylate Thr14 and Tyr15 respectively on Cdk1 (Russell and Nurse, 1987; McGowan and Russell, 1993; Mueller *et al.*, 1995). Phosphorylation on these residues is inhibitory due to the blocking of ATP binding sites (Artherton-Fessler *et al.*, 1993). Upon completion of G2, dephosphorylation of inhibitory sites on Cdk1 by Cdc25C triggers the activation of the Cdc2/Cyclin B complex (Nishida *et al.*, 2002). Furthermore, phosphorylation of Cdk1 is also needed to activate the kinase. In addition to the accumulation of Cyclin B and activation of Cdc2, nuclear localization of Cdc2/Cyclin B and Cdc25C are thought to be essential for the induction and coordination of M-phase events (Li *et al.*, 1997). During interphase, Cdc25C is mainly cytoplasmic due to the phosphorylation of S216 by checkpoint kinases Chk1/2 and CTAK-1 which creates a binding site for 14-3-3 protein (Peng *et al.*, 1997). Similarly to Cdc25C, Cyclin B is also cytoplasmic during interphase due to the existence of a cytoplasmic retention sequence and a nuclear exclusion motif (Hagting *et al.*, 1999). During mitosis, Cdc25C and Cyclin B are both localized to the nucleus. Activation of these proteins and localization to the nucleus is done primarily through phosphorylation of key residues on the protein (Li *et al.*, 1997). Although it is thought that one of the enzymes which activates Cdc25C at G2/M is Cdk1 (thus creating a positive feedback loop), other activating kinases of Cdc25C exist (Peng *et al.*, 1997). Cyclin B1 is thought to be phosphorylated in the nuclear exclusion motif at four conserved residues which promotes

nuclear import. Mutation of these four residues (Ser 126, 128, 133 and 147) to alanine abolishes the nuclear import of the protein (Hagting *et al.*, 1999). Furthermore, although initial activation of Cdk1/Cyclin B occurs at the centrosomes, many of the kinases responsible for this activation remain to be elucidated (Hagting *et al.*, 1999). The polo-like kinases are one group of kinases that have been implicated in the phosphorylation and localization of Cdc25C and Cyclin B.

Plx, the *Xenopus* polo-like kinase family member, was the first to be implicated in the interaction with Cdc25C and Cyclin B (Abrieu *et al.*, 1998). Abrieu *et al.*, 1998 showed through immunodepletion studies that Plx is indeed indispensable for activation of Cdk1/Cyclin B. Furthermore, the study showed that the Cdk1/Cyclin B complex promotes activation of Plx which in turn leads to Cdc25C activation. As mentioned, Cdc25C activation leads to the dephosphorylation and activation of Cdk1/Cyclin B thus creating a positive feedback loop (Abrieu *et al.*, 1998).

Plk4 family members Plk1 and Plk3 both have significant roles in the entry into mitosis. Roshak *et al.*, 2000 showed that Plk1 phosphorylates Cdc25C; however which sites Plk1 phosphorylates was not determined. Phosphorylation of Cdc25C by Plk1 resulted in the activation of this phosphatase as was assessed by dephosphorylation of Cdk1/Cyclin B (Roshak *et al.*, 2000). Additionally, Toyoshima-Morimoto *et al.*, 2002 showed that Plk1 phosphorylates S198 of Cdc25C which is located in the nuclear export signal of the phosphatase, leading to nuclear localization of the protein. One possibility for the nuclear accumulation of Cdc25C is that phosphorylation of S198 of Plk1 inhibits 14-3-3 binding. Furthermore, an interaction between Plk1 and Cyclin B has also been determined. Plk1 phosphorylates Cyclin B1 on a serine residue (S147) in the nuclear

export signal, and induces its nuclear entry during prophase (Toyoshima-Morimoto *et al.*, 2001).

Bahassi *et al.*, 2004 have shown that Plk3 co-fractionates and co-immunoprecipitates with Cdc25C. Furthermore, they have shown that Plk3 phosphorylates Cdc25C on S191, and to a lesser extent on S198, sites located within the proteins nuclear exclusion motif. Mutation of S191 to alanine abolishes the localization (Bahassi *et al.*, 2004). Furthermore, overexpression of wildtype Plk3 induces nuclear accumulation of Cdc25C however this accumulation is however not seen with overexpression of kinase-dead Plk3. Nuclear accumulation of Cdc25C is abrogated when Plk3 is inhibited with siRNA further supporting Plk3's involvement in Cdc25C phosphorylation and nuclear localization (Bahassi *et al.*, 2004).

Evidence suggests that Plk4 kinase regulates both Cdk1 and APC/C and thus suggests a possible role for Plk4 in the entry and perhaps exit from mitosis. Plk4 heterozygous hepatocytes also have a loss of acuity of Cyclin B1 levels suggesting a further role of Plk4 in entry into mitosis. Plk4 is primarily found to localize to centrosomes, the site at which activation of Cdk1/Cyclin B initially occurs (Jackman *et al.*, 2003). These observations along with Plk4 family member's interactions with Cdc25C and Cyclin B1 suggest a possible role for Plk4 in entry into mitosis.

Therefore, given that Plk4 family members play key roles in DNA damage checkpoint responses and mitotic entry, the purpose of the present study is to elucidate a role for Plk4 in DNA damage pathways and entry into mitosis.

CHAPTER II

MATERIALS AND METHODS

Production of Competent *Escherichia coli* (*E. coli*) cells

In order to obtain competent *E. coli* for subsequent transformation the procedure outlined below was followed. 10 mLs of TYM broth in a 50 mL flask was inoculated with *E. coli* cells from glycerol stocks and grown for 16 hours at 37°C. 1 mL of the resultant culture was added to 100 mL prewarmed TYM broth in a 500 mL flask and grown at 37°C until an OD₆₀₀ of 0.5 was reached. Cultures were then cooled on ice with gentle swirling for five minutes and then transferred to a sterile, round-bottom centrifuge tube. The cells were centrifuged for 10 minutes at 4000 x g at 4°C, the supernatant decanted and the pellet resuspended gently in 30 mL cold TFB1. The cells were then collected by centrifugation for 10 minutes at 4000 x g at 4°C with the supernatant carefully discarded. Bacterial cells were kept on ice throughout the procedure. The cells were then resuspended in 4 mL ice-cold TFB2 buffer and 100 uL aliquots were placed in sterile microcentrifuge tubes. The aliquots were frozen in liquid nitrogen and stored at -80°C.

Transformation and DNA purification

In order to obtain plasmid DNA needed for subsequent experimental procedures, competent *E. coli* cells (Top10 F') were transformed as follows: Approximately 10-100 ng of plasmid DNA was added to the competent cells and incubated on ice for 30 minutes. Following this, the cells were heat shocked at 42°C for 40 seconds and then placed immediately back on ice. 500 uL of Luria-Bertani (LB) broth was then added and

the cells were incubated at 37°C for 45 minutes. 100 uL of cells were subsequently plated on LB agar plates supplemented with 100 ug/mL ampicillin and incubated overnight at 37°C. The following day, a single colony was inoculated with 100 mL LB supplemented with 100 ug/mL ampicillin and grown for 16 hours at 37°C. The DNA was purified using a QIAGEN Plasmid Maxi Prep Kit (Qiagen Inc.) in compliance with the manufacturer's specifications.

Cell Culture

HEK-293 and NIH-3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Sigma). In order to aid in the prevention of contamination the media was also enriched with penicillin (100 U/mL) / streptomycin (100 ug/mL) (Gibco), amphotericin B (2.5 ug/mL) (Mediatech) and gentamycin (50 ug/uL) (Hyclone). Cell cultures were maintained at 37°C with 5% CO₂.

Transfection and Cell Lysis

Twenty-four hours prior to transfection, cells were seeded onto 100 mm tissue culture dishes at a density of 1×10^6 cells / dish (~20-40% confluence). Cells were routinely transiently transfected with approximately 6ug of the FLAG vector (Invitrogen) using Effectene transfection reagent (Qiagen Inc.) in compliance with the manufacturer's specifications. All other FLAG-tagged constructs were transfected using equimolar amounts relative to the vector control. At the indicated times post transfection, cells were washed three times with ice cold PBS and then lysed on ice for 20 minutes with 1 mL lysis buffer. Cells were then scraped off the plate and lysates were clarified by

centrifugation for 20 minutes at 13,000 x g at 4°C.

UV and IR damage

In order to induce DNA damage, cells were exposed to ultra-violet radiation at the indicated doses using a Stratalink UV crosslinker (Stratagene), or similarly, cells were exposed to the indicated doses of ionizing radiation (Faxitron X-ray Corp., R650). Cells were then incubated at 37°C with 5% CO₂ for the indicated times (6 or 16 hours) and lysed as previously described. Following lysis, immunoprecipitation, Western blot analysis or flow cytometry analysis was performed.

Antibodies

For immunoprecipitation reactions, 1 ug of anti-Cdc25C (Santa-Cruz), anti-FLAG (Sigma) or anti-Cyclin B1 (Sigma) were incubated with 1 mg of lysate. For Western blot analyses, 1 ug of the specified primary antibody was incubated in 10 mL of TBST and a dilution of 1:60000 was used for the appropriate anti-mouse or anti-rabbit secondary antibodies which were conjugated to horseradish peroxidase (HRP) (Amersham). This was dependent on the species of the primary antibody. Alternatively, protein A HRP (Amersham) was used as secondary antibody at a dilution of 1:10000.

Immunoprecipitation

Following cell lysis and centrifugation, immunoprecipitation of FLAG-tagged expression plasmids and endogenous Cdc25C and Cyclin B1 was performed by incubating approximately 1 mg of lysate with 1 ug of the indicated antibody for 1 hour at 4°C on a

vertical rotator. Immunocomplexes were then precipitated with 60 uL of a 20% protein G-sepharose slurry (GE lifesciences) at 4°C for one hour. Following the one hour incubation with the protein G-sepharose beads, the immunocomplexes were pelleted at 13,000 x g at 4°C for 1 minute. The precipitates were then washed three times at 4°C with 500 uL of TNT buffer. Following this, the immunoprecipitates were resuspended in 15 uL of 2X SDS sample buffer and boiled for 5 minutes. The proteins were then separated by SDS-polyacrylamide gel electrophoresis and Western blot analysis was performed.

Western Blotting

Cell lysates and immunocomplexes were resuspended in 20 uL of sodium dodecyl sulphate (SDS) sample buffer and incubated at 100°C for five minutes. Proteins were then resolved by SDS-polyacrylamide gel electrophoresis. Gels were subsequently incubated in transfer buffer for 15 minutes at room temperature on a shaker. In the meantime, polyvinylidene fluoride (PVDF) membrane was activated with methanol for 20 seconds and then equilibrated for 10 minutes in transfer buffer. Transfer of the proteins from the gel to the membrane was done using a semi-dry transfer apparatus (Biorad). Three whatman filters were immersed in transfer buffer and then placed on the transfer apparatus. PVDF membrane was then placed on top of the three filters and the gel was placed on top of the membrane. Three additional whatman filters were immersed in transfer buffer and then placed on top of the gel. The filters were then hand rolled to ensure no air bubbles were trapped in any layer. The gel was transferred to the membrane at a suitable voltage and time depending on the number of membranes

transferred. Following transfer of the proteins to the PVDF membrane, the membrane was blocked using TBST for one hour at room temperature in order to reduce non-specific binding sites on the membrane. Following blocking, the membrane was incubated with primary antibody for one hour. The membrane was then washed three times for ten minutes and subsequently incubated with the appropriate secondary antibody which was conjugated to horseradish-peroxidase for 45 minutes. After an additional three 10 minute washes with TBST, the proteins of interest were visualized using enhanced chemiluminescent substrate (Pierce).

Flow Cytometry

For cell cycle analysis purposes, a flow cytometer (Beckman Coulter, FC500) was utilized. Cells were prepared as follows: At the indicated times post transfection, NIH-3T3 cells were collected and pelleted at 500 x g for 5 minutes. Cells were then washed with 10 mL room-temperature PBS and pelleted again at 500 x g for 5 minutes. Supernatant was carefully removed and cells were fixed by resuspending in 1 mL 3.7% paraformaldehyde and kept on ice for 1 hour. Cells were then centrifuged at 500 x g for 5 minutes and washed in 10 mL cold PBS. Supernatant was removed and cells were resuspended in 1 mL cold 70% ethanol and kept on ice for 1 hour. Cells were then pelleted at 500 x g for 5 minutes, resuspended in 1 mL PBS and DNA was stained with 10 μ L of propidium iodide (5 mg/mL stock) for 1 hour at room temperature.

GST-Cdc25C Protein Purification

To maximize expression of recombinant proteins *E. coli* BL21 cells were utilized which allow for optimal expression of recombinant protein. *E. coli* BL21 backgrounds are deficient in a number of proteases. This allows them to accumulate recombinant proteins at a high rate and help reduce the degradation of some proteins during purification. Therefore the pGEX2T-Cdc25C plasmid was introduced into this cell type by transformation (see above). A single colony was inoculated overnight in 50 mL of LB supplemented with 100 ug/mL of AMP. The following day 1 L of LB was inoculated with 10 mL of overnight culture. Cultures were grown at 37°C to an A₆₀₀ of 0.6 and IPTG (Fisher) was added to a final concentration of 0.5 mM. The temperature was then dropped to 25°C to improve solubility of the protein, and the cultures were induced for another 16 hours. Cells were then pelleted at 5000 x g for 15 minutes at 4°C, the supernatant discarded and the cells were resuspended in 20 mL cell lysis buffer. The cells were then sonicated on ice, centrifuged at 10 000 x g for 30 minutes at 4°C, and the supernatant was added to a column containing GST-agarose beads. The column was then placed on a vertical rotator for four hours to allow the protein to bind to the beads. The supernatant was then allowed to flow through the column and the beads were washed two times with column wash buffer. 3 mL's of elution buffer were added to the column and incubated at 4°C overnight. The following day, the protein was eluted. SDS-PAGE and Western blot analyses were performed to visualize the purified protein.

Kinase Assay

In order to detect Plk4 kinase activity, HEK-293 cells were transiently transfected with FLAG-Plk4, FLAG-K41M and FLAG-T170D expression plasmids. 16 hours post transfection cells were lysed and the cell lysate was incubated with 1 ug anti-FLAG antibody at 4°C for 45 minutes and precipitated with 60 uL of a 20% protein G-sepharose slurry (GE lifesciences) at 4°C for 45 minutes. The immunoprecipitates were washed three times in TNT buffer and once in kinase buffer. The immunocomplexes were resuspended in 20 uL of kinase buffer and the reaction was started by incubation with 10 uCi [γ -³²P] ATP (Amersham Biosciences) and 5 ug of GST-Cdc25C. After incubation for 45 minutes at 30°C, 20 uL of 2X SDS sample buffer was added to stop the reaction. Samples were boiled for 5 minutes and then analyzed by SDS-polyacrylamide gel electrophoresis. The gel was then transferred to a membrane and a phosphoimager was utilized to visualize the bands. Following this, the membrane was then probed with an anti-FLAG antibody in order to detect the immunoprecipitated protein. The same blot was subsequently probed with an anti-Cdc25C antibody to confirm equal loading of the protein.

CHAPTER III

RESULTS

Plk4 and DNA Damage pathways

UV Radiation Inhibits Plk4 Protein Expression

On the basis of the role of Plk1, 2 and 3 in DNA damage pathways and the interaction of Plk4 with checkpoint effector p53, the effects of DNA damage on Plk4 protein expression was investigated. To this end, NIH-3T3 cells were transiently transfected with an expression plasmid for FLAG-Plk4. 16 hours post transfection, cells were exposed to varying doses of ultra-violet (UV) and ionizing radiation (IR). Six hours post irradiation, cells were lysed and immunoprecipitation was done using an anti-FLAG antibody. As can be seen in Figure 5, with increasing doses of UV radiation, Plk4 protein expression decreases. At a dose of 70mJ/cm² Plk4 protein was no longer present.

In order to determine if the decrease in protein levels found with increasing doses of UV radiation also occurred with other types of DNA damage, Plk4 protein levels were examined in response to ionizing radiation. NIH-3T3 cells were transiently transfected with FLAG-Plk4 and exposed to increasing doses of ionizing radiation. Six hours post irradiation, cells were lysed and immunoprecipitation using an anti-FLAG antibody was performed to examine Plk4 protein levels. Surprisingly, Plk4 protein levels remained constant with increasing doses of ionizing radiation (Figure 6).

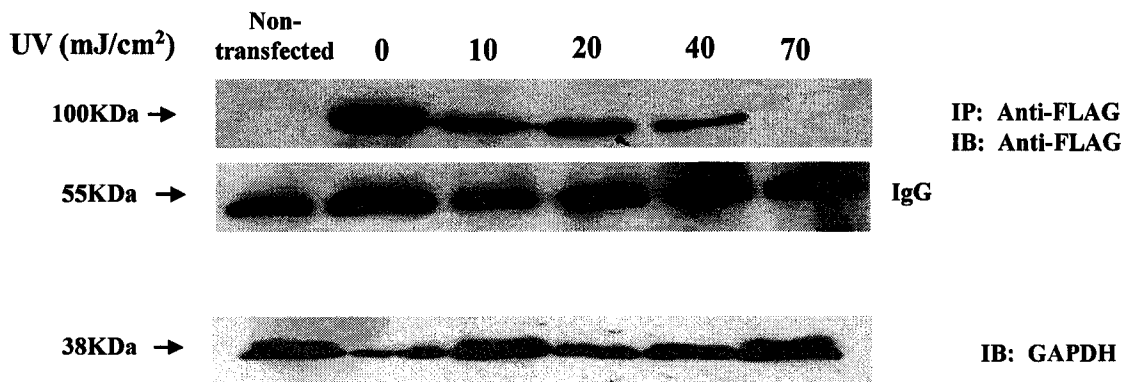


Figure 5. Plk4 protein levels decrease with increasing doses of UV radiation. NIH-3T3 cells were transiently transfected with an expression plasmid for Flag-Plk4. 16 hours post transfection cells were exposed to increasing doses of UV radiation. Cells were incubated for another six hours and then lysed. Immunoprecipitation and Western blot analysis were performed using an Anti-Flag antibody. Lysates were probed with an anti-GAPDH antibody as a loading control.

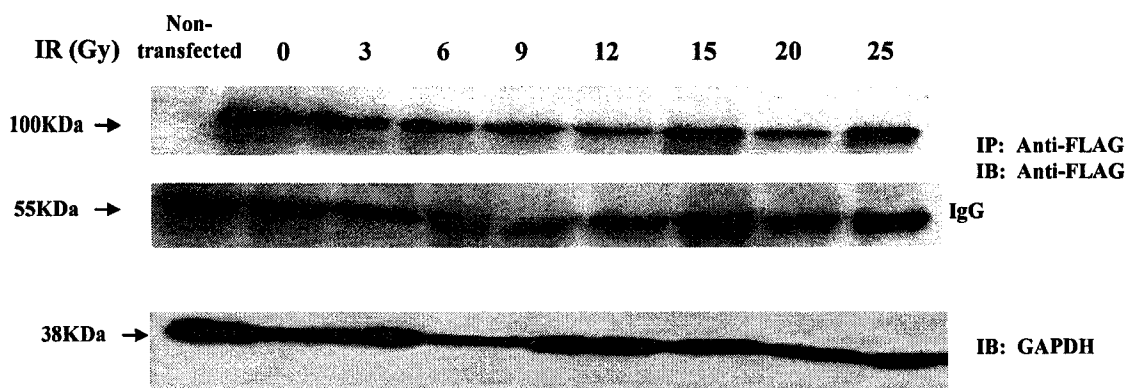
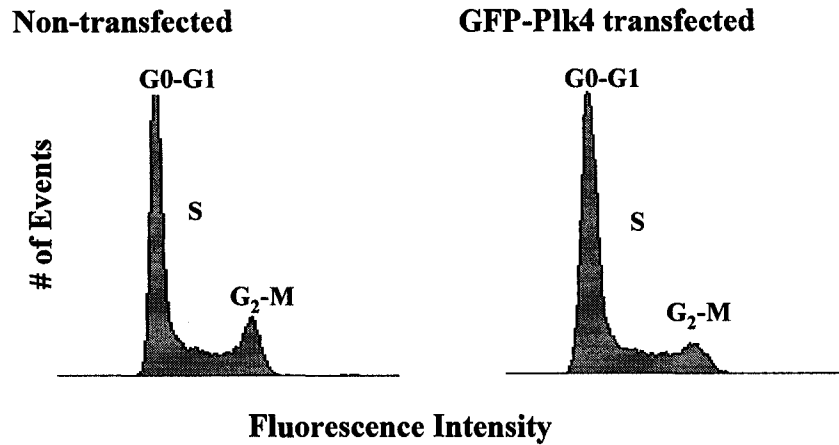


Figure 6. **Plk4 protein levels remain constant with increasing doses of IR.** NIH-3T3 cells were transiently transfected with Flag-Plk4. 16 hours post transfection cells were exposed to increasing doses of IR. Cells were incubated for another six hours and then lysed. Immunoprecipitation and Western blot analysis were performed using an anti-Flag antibody. Cell lysates were probed using an anti-GAPDH as a loading control.

Overexpression of Plk4 Causes Recovery from DNA Damage Induced Cell Cycle Arrest

To further investigate the function of Plk4 in DNA damage response pathways, the role of Plk4 in cell cycle arrest after DNA damage was investigated. To explore this, NIH-3T3 cells were transfected with an expression plasmid for GFP-Plk4. Untransfected cells were used as a control. 10 hours post transfection cells were exposed to 20 mJ/cm² of UV radiation or left untreated and 16 hours post irradiation cell cycle profiles were analyzed using a flow cytometer. A dose of 20 mJ/cm² was chosen since the cells did not undergo apoptosis 16 hours post UV radiation at this dose. Furthermore, examining the previous experiment, Plk4 protein still persists at this dose. Examining Figure 7A, non-transfected, non-treated control cells had a similar cell cycle profile to non-treated cells over-expressing Plk4. This thus suggests that Plk4 overexpression in the absence of DNA damage does not disturb the cell cycle. In the non-transfected control cells, 16 hours post irradiation approximately sixty percent of the cells remained arrested in G0/G1 and forty percent in G2/M thus exhibiting cell cycle arrest as would be expected with DNA damage (Figure 8). In contrast, in cells that overexpress Plk4 protein, a strikingly different cell cycle profile was obtained upon exposure to UV damage. Interestingly, when cells overexpressing Plk4 were subjected to radiation, rescue of the cell cycle block imposed by DNA damage was observed (Figure 7B). Cells that were overexpressing Plk4 pushed the cell cycle through the G1-S cell cycle block and lead to an accumulation of cells in the S phase of the cell cycle however almost no cells were seen in G2/M (Figure 7B).

A No UV



B 16 hours Post UV

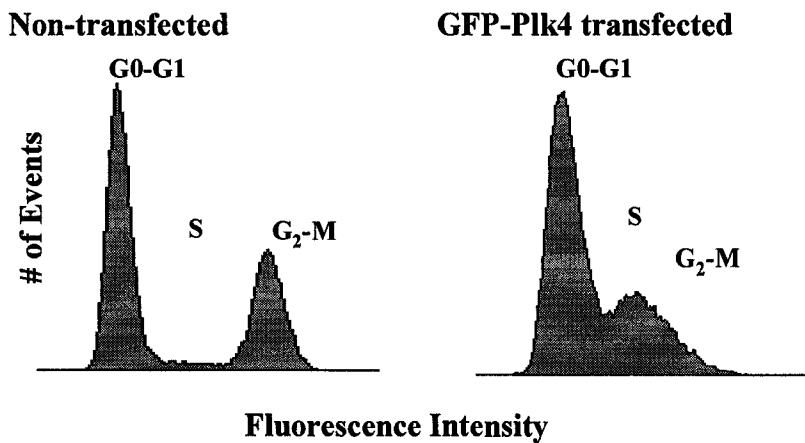


Figure 7. Overexpression of Plk4 causes recovery from DNA damage induced cell cycle arrest. The experiment was repeated three times and shown here is representative data from one trial. A. NIH-3T3 cells were transiently transfected with an expression plasmid for GFP-Plk4 or left untransfected. Cell cycle analysis was done in the absence of DNA damage. B. Cells were transiently transfected with GFP-Plk4 or left untransfected. 10 hours post transfection cells were exposed to 20mJ/cm² of UV or left untreated. The cells were then incubated for another 16 hours following which cell cycle analysis was performed using flow cytometry.

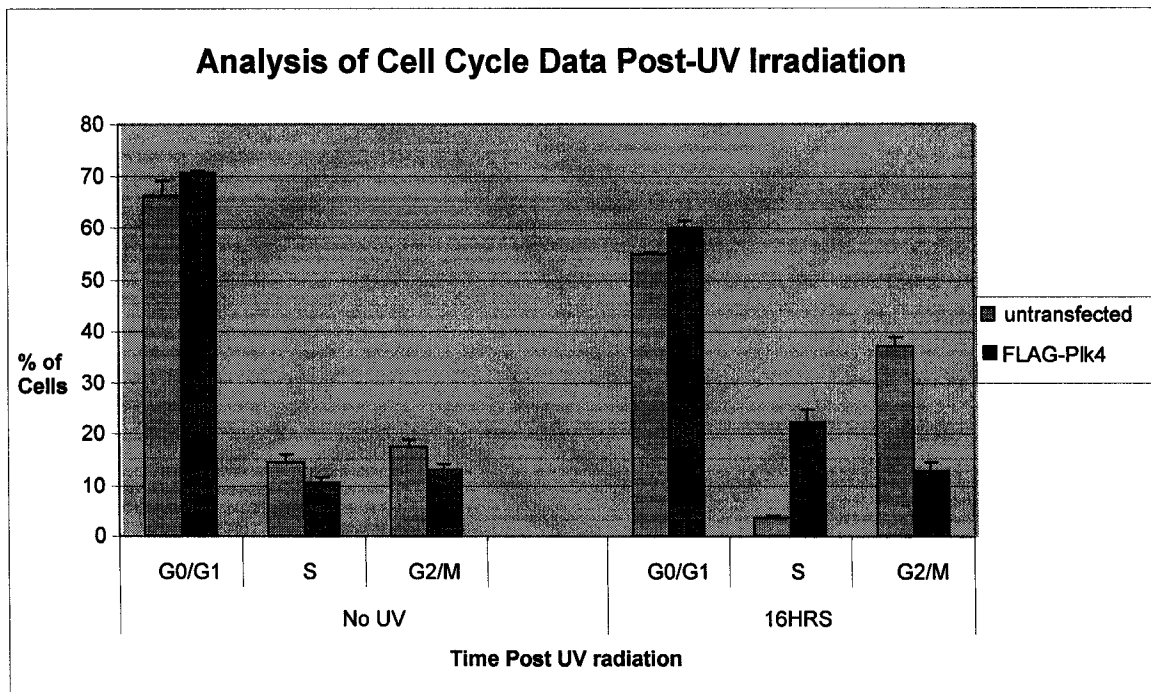


Figure 8. Analysis of Cell Cycle Data Post-UV Irradiation. With no irradiation, both transfected and non-transfected cells exhibited normal cell cycle profiles. However, 16 hours post irradiation, non-transfected cells were blocked in the cell cycle, whereas GFP-PIk4 transfected cells did not exhibit DNA damage induced cell cycle arrest. The experiment was repeated three times.

Plk4 and Entry into Mitosis

Interaction of Plk4 with Cyclin B1

Plk1 has been shown to phosphorylate S147 in the nuclear export signal of Cyclin B1 and induce the proteins nuclear translocation (Toyoshima-Morimoto *et al.*, 2001). This observation of Plk1 prompted us to investigate a possible interaction between Plk4 and Cyclin B1. To further investigate this possibility, HEK-293 cells were transiently transfected with expression plasmids for FLAG-tagged Plk4 domain specific constructs (Figure 9). The expression of the FLAG constructs was confirmed in cell lysate by immunoblot analysis with an antibody against the FLAG epitope followed by probing with an anti-GAPDH antibody to confirm equal protein loading (Figure 10A). Whole cell lysates prepared from transfected cells were immunoprecipitated with an anti-Cyclin B1 antibody and Western blot analysis was performed using an anti-FLAG antibody. As shown in Figure 10B, FLAG-Plk4, FLAG-154N, FLAG- Δ Pb, FLAG-K41M and FLAG-T170D were co-immunoprecipitated with the endogenous Cyclin B1. However, co-immunoprecipitation of deletion constructs FLAG-R1 or FLAG-Pb was not seen with Cyclin B1 (Figure 10B). FLAG-YVHI was used as a negative control to ensure that the interaction which was observed was not due to the FLAG-tag. After probing with an anti-FLAG antibody to show co-immunoprecipitation with cyclin B1, the membrane was stripped and probed with an anti-Cyclin B1 antibody to ensure an equal amount of protein was immunoprecipitated. These results clearly demonstrate that Plk4 interacts with Cyclin B1 however the polo-box domain is not necessary for this interaction to occur. Furthermore, the cryptic polo-box or the polo-box domain are not sufficient for this

interaction to occur, suggesting that perhaps the interactions of sub-domains of Plk4 are necessary for this interaction to occur.

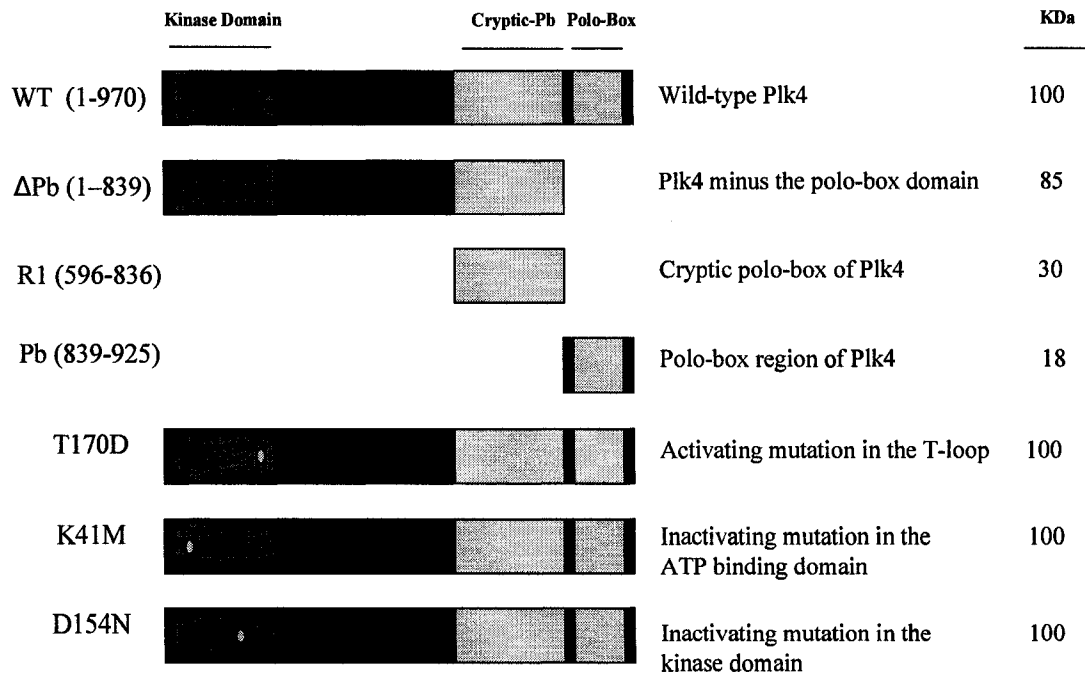


Figure 9. Plk4 domain specific constructs. A schematic representation of Plk4 deletion mutants and Plk4 kinase dead and kinase active mutants.

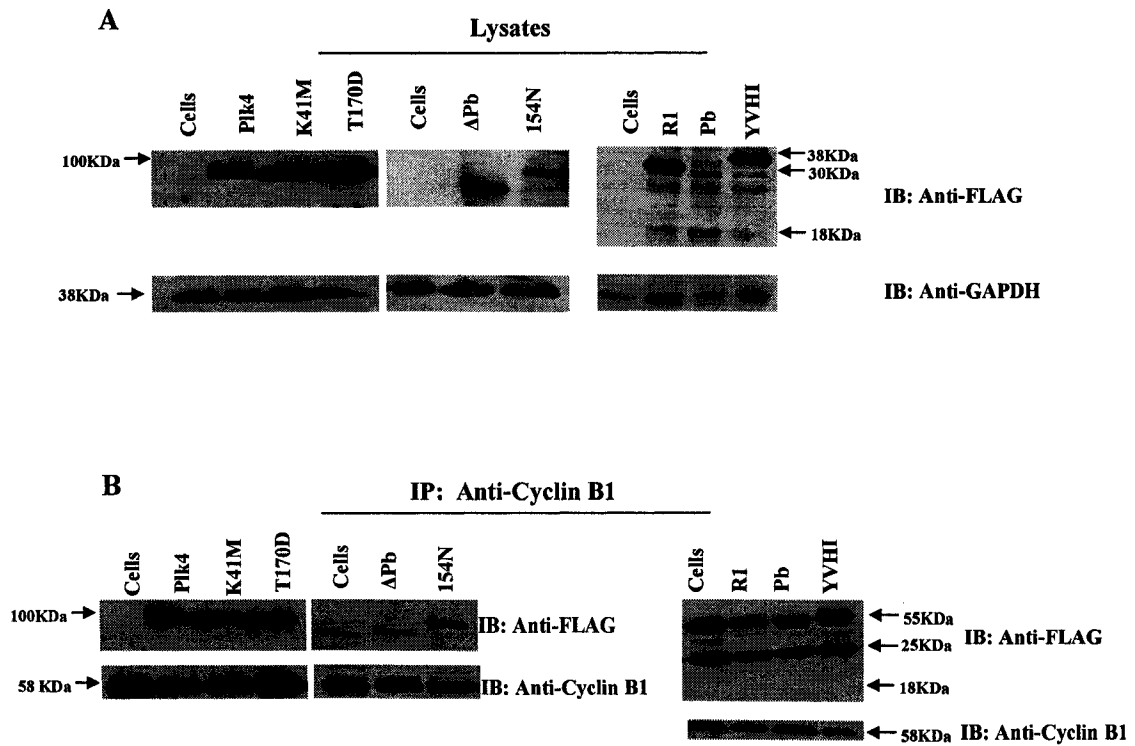


Figure 10. Plk4 co-immunoprecipitates with endogenous Cyclin B1. HEK-293 cells were transiently transfected with expression plasmids for FLAG-Plk4 proteins. 16 hours post transfection cells were lysed. (A) Lysates of transfected proteins were immunoblotted with an anti-FLAG antibody to determine the transfection efficiency and then probed with anti-GAPDH to ensure equal protein loading.

(B) Immunoprecipitation was performed using an anti-Cyclin B1 antibody. The immunocomplexes were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody. Full length Plk4, kinase dead Plk4 (K41M), kinase active Plk4 (T170D), Δ Pb-Plk4 (deletion of polo-box domain of Plk4) and an alternate kinase dead Plk4 (154N) all co-immunoprecipitated with endogenous Cyclin B1. Co-immunoprecipitation of the deletion constructs, cryptic-polo-box domain (R1) and the polo-box domain (Pb) was not observed. Whole cell lysates of non-transfected cells and the transfected construct encoding FLAG-YVHI protein served as negative controls. After immunoblotting with an anti-FLAG antibody to show the interaction, the membrane was stripped and reprobed with an anti-Cyclin B1 antibody.

Interaction of Plk4 with Cdc25C

Nuclear localization of Cdc25C plays an essential role in maintaining the activity of Cdc2/Cyclin B in the nucleus during mitosis by counteracting inhibitory phosphorylation (Toyoshima-Morimoto *et al.*, 2002). Plk1 and Plk3 have both been shown to phosphorylate phosphatase Cdc25C. These observations prompted us to investigate possible interactions between Plk4 and Cdc25C. For this purpose, HEK-293 cells were transiently transfected with expression plasmids for FLAG-tagged Plk4 domain specific constructs. Cell lysates were initially probed with an anti-FLAG antibody to ensure expression of the plasmids followed by probing with a GAPDH antibody to ensure equal protein loading (Figure 11A). Whole cell lysates prepared from the transfected cells were immunoprecipitated with an anti-Cdc25C antibody and Western blot analysis was performed using an anti-FLAG antibody. Similar to the results observed with the Cyclin B1 co-IP, FLAG-Plk4, FLAG-154N, FLAG- Δ Pb, FLAG-K41M and FLAG-T170D were co-immunoprecipitated with endogenous Cdc25C. Non-transfected cell lysate and FLAG-YVHI were used as negative controls (Figure 11B). Co-immunoprecipitation of the deletion constructs FLAG-R1 and FLAG-Pb was not observed (Figure 11B). After the initial Western blot analysis using the anti-FLAG antibody was performed to show the interaction between these two proteins, the membrane was stripped and probed with anti-Cdc25C to ensure equal amounts of protein was immunoprecipitated (Figure 11B). These results confirm that Plk4 interacts with Cdc25C while in the case of both the polo-box domain and the cryptic-polo box no interaction was detected.

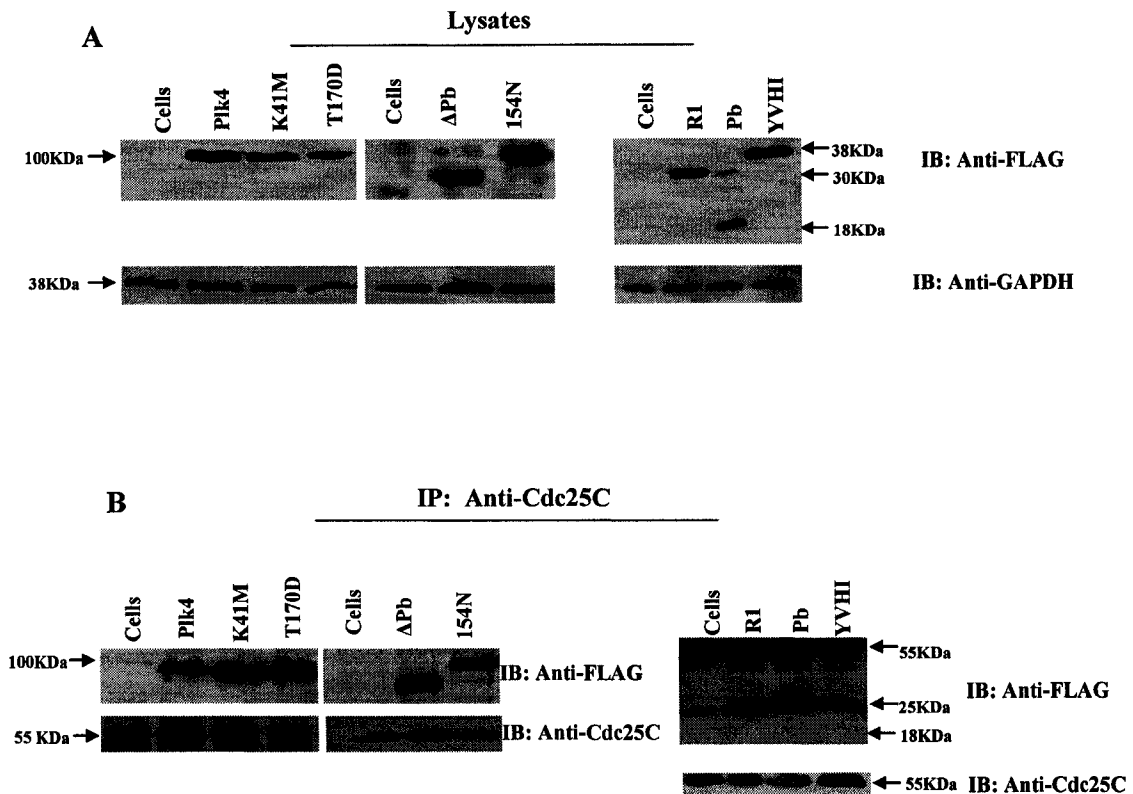


Figure 11. Plk4 co-immunoprecipitates with endogenous Cdc25C. HEK-293 cells were transiently transfected with expression plasmids encoding FLAG-Plk4 protein. 16 hours post transfection cells were lysed. (A) Whole cell lysates were immunoblotted with an anti-FLAG antibody to show the levels of FLAG-tagged proteins, followed by probing with an anti-GAPDH antibody to confirm equal protein loading. (B) Immunoprecipitation was performed using an anti-Cdc25C antibody on prepared lysates and western blotting was performed using an anti-FLAG antibody. Full length Plk4, kinase dead Plk4 (K41M), kinase active Plk4 (T170D), Δ Pb-Plk4 (deletion of polo-box domain of Plk4) and an alternate kinase dead Plk4 (154N) all co-immunoprecipitated with endogenous Cdc25C. Co-immunoprecipitation of the deletion constructs, cryptic-polo-box domain (R1) and the polo-box domain (Pb) was not observed. Whole cell lysates of non-transfected cells and a transfected construct encoding FLAG-YVHI protein served as negative controls. The membrane was probed with an anti-Cdc25C antibody to ensure an equal amount of immunoprecipitated protein.

Plk4 Phosphorylates Cdc25C In-Vitro

The interaction of Cdc25C and Plk4 suggests the possibility that Cdc25C may be a substrate of Plk4. To test this hypothesis, firstly the GST-Cdc25C fusion protein was purified for use as substrate. To confirm the purity of the protein, SDS-PAGE was performed and the gel was Coomassie stained (Figure 12A). Immunoblotting was performed using an anti-Cdc25C antibody to further confirm the purity of the GST-Cdc25C protein. Purified GST-Cdc25C protein yielded primarily a protein product of 83kDa and a 55 kDa proteolytically cleaved product, corresponding to the size of the cleaved Cdc25C protein (Figure 12B). FLAG-Plk4, FLAG-T170D and FLAG-K41M were transiently transfected into HEK-293 cells and immunoprecipitation was performed using an anti-FLAG antibody. The bacterially produced GST-Cdc25C was incubated with the respective FLAG-Plk4 immunoprecipitated constructs in the presence of [γ -³²P] ATP and subjected to SDS-PAGE. Figure 12C shows that GST-Cdc25C is phosphorylated by FLAG-Plk4 and FLAG-T170D however not by the kinase dead Plk4 mutant, FLAG-K41M. In addition to phosphorylation of the full-length GST-Cdc25C and GST-Cdc25C cleavage products, Plk4 autophosphorylation was observed as evident by the strong band at 100kDa (Figure 12C). The membrane was probed with an anti-FLAG antibody to confirm the expression of FLAG-Plk4, then stripped and re-probed with an anti-Cdc25C antibody to ensure equal protein loading (Figure 12D). These results show that Cdc25C is indeed a substrate of Plk4. Further experimentation is required to determine if the site of phosphorylation is similar to that of Plk1 or Plk3.

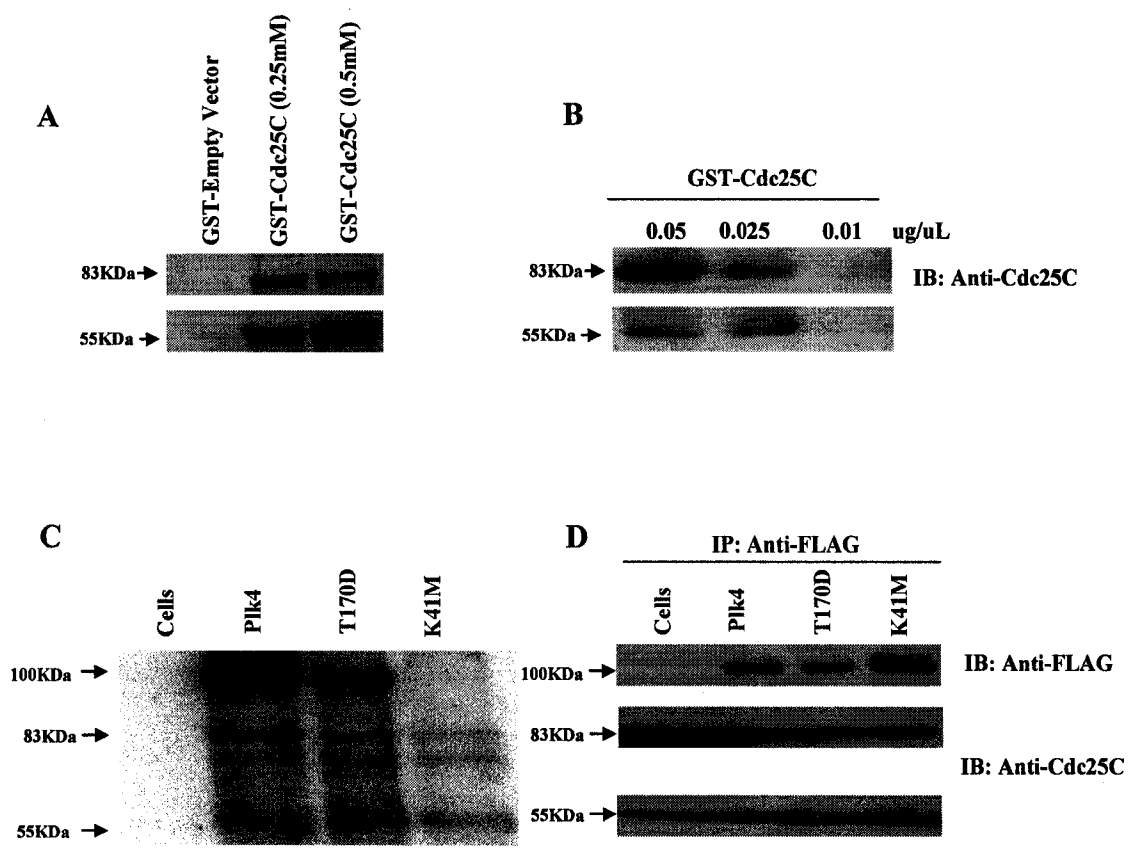


Figure 12. Plk4 phosphorylates GST-Cdc25C. (A) GST-Cdc25C was expressed in and purified from bacteria using varying concentrations of IPTG. The fusion protein was purified using a GST column and SDS-PAGE was performed. Purification resulted primarily in an 83kDa band corresponding to GST-Cdc25C and a band at 55kDa corresponding to cleaved Cdc25C. (B) Western blot analysis was performed with the fusion protein to ensure that the observed bands were in fact GST-Cdc25C. Varying dilutions of the protein were loaded onto a gel and SDS-PAGE was performed. GST-Cdc25C was detected using an anti-Cdc25C antibody. (C) Bacterially produced GST-Cdc25C was incubated with or without FLAG-Plk4 in the presence of ATP, subjected to SDS-PAGE, transferred to a membrane and then analyzed using a phosphoimager (Alpha-Imager). (D) The membrane was then subjected to Western blot analysis with an anti-FLAG antibody to ensure transfection efficiency, and then stripped and re-probed with anti-Cdc25C to ensure equal protein loading.

CHAPTER IV

DISCUSSION

One of the main roles of the polo-like kinase family is their involvement in DNA damage pathways. In response to DNA damage, Plk1 activity is inhibited, whereas Plk3 is activated (Xie *et al.*, 2001; Ando *et al.*, 2004). Since Plk1 has been shown to phosphorylate and inactivate the tumour suppressor p53, the inhibition of Plk1 activity in response to DNA damage consequently leads to the activation of p53 (Xie *et al.*, 2001). Furthermore, Plk3 has been shown to phosphorylate and activate p53 and thus, activation of Plk3 in response to DNA damage leads to further p53 stabilization (Ando *et al.*, 2004). Plk4 has also recently been implicated in DNA damage response pathways. An association between Plk4 and p53 protein has been established and the observation that p53 is a substrate of Plk4 has been previously documented (Swallow *et al.*, 2005). Plk4 has also been shown to be transcriptionally repressed by p53 and Plk4 mRNA levels decrease in response to DNA damage in a p53-dependent manner (Li *et al.*, 2005). Although Plk4 mRNA levels in response to DNA damage have been previously examined, protein levels in response to damaged DNA have not been determined. To this end, it was of interest to determine if the protein level of Plk4 also decreased in response to DNA damage. Unfortunately, endogenous Plk4 protein levels could not be examined due to the unavailability of a suitable Plk4 antibody. Thus, Plk4 protein was overexpressed in NIH-3T3 cells and overexpressed protein levels were examined in response to DNA damage. Through Western blot analysis, it was determined that overexpressed Plk4 protein levels do indeed decrease with increasing doses of ultra-violet

radiation, however protein levels remain constant in response to ionizing radiation. Although Li *et al.*, 2005 observed a decrease in Plk4 mRNA levels in response to DNA damage in a p53-dependent manner, the decrease in protein levels observed with UV radiation cannot be explained by a decrease in the amount of translated protein since the NIH-3T3 cell line utilized is inactive in p53. Hence, this decrease in Plk4 protein level seen with UV radiation must occur through a p53 independent mechanism. One hypothesis for this could be that Plk4 protein is subjected to post-translational modification upon exposure to ultra-violet radiation and targeted for ubiquitin-mediated degradation. Also, it is interesting to note that the decrease in Plk4 protein was only seen with UV radiation however was not seen with ionizing radiation. An explanation for this observation could include that UV light and x-rays, which respectively induce pyrimidine dimers and DNA double strand breaks, use different signaling factors (Lakin *et al.*, 1999). For example, in response to ionizing radiation, ATM is activated; however, UV-damaged DNA activates ATR protein kinase (Lakin *et al.*, 1999; Canmann *et al.*, 1998; Tibbetts *et al.*, 1999). Furthermore, in contrast to ionizing radiation, a role for p53 in response to UV radiation has not been clarified. UV radiation induces a G1 arrest in cells with wild-type p53. However surprisingly, this same G1 arrest is also seen in cells which p53 is inactivated (Chang *et al.*, 1999). Thus, the decrease in Plk4 protein level must have occurred through a p53 independent mechanism and Plk4 conceivably acts through p53 dependent and p53 independent pathways.

Upon exposure of cells to DNA damage reagents, the genomic stability of cells is threatened. In order to effectively repair damaged DNA, and hence protect genomic integrity, cells undergo transient cell cycle arrests (Lindahl *et al.*, 1993). The two major

DNA damage cellular checkpoints occur at the G1-S transition and at the G2-M transition (Kaufmann *et al.*, 1996). These arrests allow cells time to repair the damaged DNA prior to replicating DNA or commencing mitosis. Cells which are exposed to the DNA damaging agents UV and ionizing radiation undergo G1 and G2 phase cell cycle blocks (Maki *et al.*, 1997). These responses are under the control of a variety of genes which belong to different overlapping pathways. The Plk family has been implicated in the involvement of cell cycle arrest upon DNA damage. As previously stated, Plk1 is inhibited in response to DNA damage (Ando *et al.*, 2004). Furthermore, overexpression of Plk1 was shown to overcome DNA damage cell cycle arrest (Smits *et al.*, 2000). Since Plk4 has also been implicated in DNA damage response pathways, it was interesting to determine if Plk4 overexpression also pushes the cells through the cell cycle when exposed to DNA damage. Upon exposing untransfected cells to UV and ionizing radiation, an obvious G1 and G2/M cell cycle block was observed. Quite interestingly, overexpression of Plk4 rescued the cells from DNA damage induced cell cycle arrest. More specifically, these results indicate that overexpression of Plk4 appears to drive cells past the G1-S DNA damage checkpoint and leads to the accumulation of cells in the S phase. Additionally, as is apparent by the absence of cells in G2/M, entry into G2/M is halted, however entry into G1 can still occur. One explanation for these observations could be that DNA damage inhibits Plk4, and overexpression of the protein overcomes this inhibition and drives cells past the G1-S cell cycle checkpoint, suggesting a role for Plk4 in the entry into S phase. Moreover, this hypothesis is somewhat supported by the fact that overexpression of Plk4 leads to multiple centrosomes since centrosome duplication occurs in S phase (Habedanck *et al.*, 2005; Hudson *et al.*, 2001).

Quite interestingly, Plk4 expression is increased in colorectal tumours (Macmillan *et al.*, 2001). One mechanism in which an increased expression of Plk4 could lead to tumour formation is if overexpression of Plk4 pushes cells past the G1-S checkpoint in the presence of damaged DNA, this could ultimately lead to genomic instability. A second mechanism in which Plk4 overexpression could lead to cancer may perhaps be the previously mentioned overduplication of centrosomes in the S phase which thus leads to aneuploidy. Consequently, the observation that overexpression of Plk4 drives cells past the G1-S checkpoint has vast implications such that Plk4 overexpression could lead to a novel means by which chromosomal instability and eventually cancer occur.

Cdc25C is a key protein involved in mitotic entry. Moreover, this mitotic regulator serves as a target of checkpoint pathways delaying entry into mitosis in response to DNA damage or stalled replication. Under normal (non-stressful) conditions dephosphorylation and activation of the Cdk1/Cyclin B complex by phosphatase Cdc25C ultimately leads to mitotic entry (Dalal *et al.*, 1999). Cdc25C localization and activation is dependent on phosphorylation and dephosphorylation events and many of the enzymes responsible for these events remain to be elucidated.

In interphase, Cdc25C is phosphorylated on S216 by CTAK-1 which allows for the binding of 14-3-3 protein and maintains cytoplasmic localization of this protein (Peng *et al.*, 1997). Also in response to DNA damage, Cdc25C is phosphorylated by checkpoint kinases Chk1/2 on S216 which leads to the cytoplasmic localization and ultimately inactivation of this protein (Peng *et al.*, 1997). However, upon completion of G2 Cdc25C is activated and localized to the nucleus. Phosphorylation of this protein is a key event leading to its nuclear accumulation (Dalel *et al.*, 1999). Quite similar to

Cdc25C, Cyclin B1 nuclear accumulation is also necessary for mitotic entry to occur. Interestingly, evidence indicates that the initial activation of the Cdk1/Cyclin B complex occurs at the centrosome (Jackman *et al.*, 2003), the main site of Plk4 localization. To this end, it was of interest to determine if Plk4 interacted with these mitotic regulators and DNA damage checkpoint targets. Quite interestingly, full length Plk4 was found to co-immunoprecipitate with both Cdc25C and Cyclin B1. Although the polo-box region of other Plk family members has been implicated as a phosphopeptide substrate binding motif (Hanisch *et al.*, 2006), the polo-box region of Plk4 was not necessary or sufficient for this interaction to occur. A potential problem could arise when the reciprocal co-immunoprecipitations would be performed. Considering the molecular weight of Cdc25C is 55kDa, the signal corresponding to this protein would be masked by the IgG heavy chain. Attempts using protein-A as a secondary antibody in place of anti-rabbit proved unsuccessful and a prominent band masking the Cdc25C signal was detected in the non-transfected control lane (data not shown).

The association of Plk4 with these proteins indicates that Plk4 could regulate these mitotic proteins. Furthermore, it sheds light on a novel role for Plk4 in the entry into mitosis.

The interaction of Plk4 with Cdc25C suggests that Cdc25C may be a substrate of Plk4. Indeed, Cdc25C was found to be a substrate of Plk4 through in-vitro kinase assays. Transport of proteins which contain a nuclear export signal is often times modulated by phosphorylation of amino acids within this motif, leading to nuclear import (Schwindling *et al.*, 2004). Phosphorylation of sites within the nuclear export signal of Cdc25C can abolish 14-3-3 binding and may ultimately lead to the nuclear accumulation of Cdc25C.

As previously discussed, S216 phosphorylation of Cdc25C occurs in interphase and phosphorylation of this residue leads to binding with 14-3-3 protein and thus cytoplasmic localization (Peng *et al.*, 1997). Quite interestingly, S191 and S198, residues found in the nuclear export signal of Cdc25C, are phosphorylated by Plk1 and Plk3 and lead to the nuclear accumulation of the protein (Toyoshima-Morimoto *et al.*, 2002; Bahassi *et al.*, 2003).

Although it has not been currently determined which sites Plk4 phosphorylates on Cdc25C, it was serendipitously determined that Plk4 phosphorylates a Cdc25C-derived peptide (*Cell Signalling Technology*, datasheet). Figure 13 shows the alignment of this peptide with a portion of the human Cdc25C protein. Intriguingly, this peptide corresponds to a region of the nuclear export signal of Cdc25C, which is the main motif responsible for Cdc25C nuclear import and export. Of the two serine residues found within the nuclear export signal of Cdc25C, S191 but not S198, is conserved in the peptide which was shown to be phosphorylated by Plk4 (Figure 13). These results suggest that Plk4, similar to its family members Plk1 and Plk3 phosphorylate Cdc25C and furthermore, this phosphorylation event could perchance occur at S191. Moreover, if Plk4 phosphorylates Cdc25C in its nuclear exclusion motif it could further promote nuclear accumulation of this protein.

Although the synthesis and destruction of regulatory proteins such as cyclins is important for cell cycle regulation, localization of these proteins to the right place at the right time is a fundamental step in ensuring proper cell cycle controls. Often times, this localization is accomplished through phosphorylation and dephosphorylation events.

Thus, one hypothesis could be that Plk4 could act as an important coordinator for intracellular localization of Cdc25C to activate Cdc2/Cyclin B at the centrosomes.

Although further experimentation is necessary, a hypothesis for the observations observed could be that DNA damage may prevent activation of Cdc25C through inhibition of Plk4, if in fact Plk4 is an activating kinase for Cdc25C as is its family member Plk1 (Toyoshima-Morimoto *et al.*, 2002). Perhaps the inhibition of Plk4 has a critical role in establishing the cell cycle block seen after DNA damage. All of these results suggest that Plk4 inhibition is an important event in DNA damage-induced cell cycle arrest. On the basis of these findings, a possible model in which activation of Cdc25C is prevented in response to DNA damage through interference with Plk4 activation is proposed (Figure 14).

Furthermore, the implications of these results are that Plk4 could thus, similar to its family members Plk1 and Plk3, lead to the activation or inactivation of key mitotic regulators. The elucidation of proteins leading to the activation or inactivation of these mitotic proteins leads to a further understanding of the events which lead to cellular division. Uncontrolled cellular proliferation is a hallmark on the journey towards tumour development. Therefore further characterization of the key proteins involved in regulating cell proliferation will provide critical insight into identifying potential therapeutic targets for intervention.



Figure 13. **Alignment of Cdc25C-derived peptide and human Cdc25C protein.** The top panel (blue) depicts a partial sequence of the human Cdc25C protein. The nuclear export signal is blocked in grey. The bottom sequence depicts the Cdc25C-derived peptide implicated as a substrate of Plk4 (*Cell Signalling Technology*, datasheet). Asterisks (*) indicate Plk1 and Plk3 phosphorylation sites.

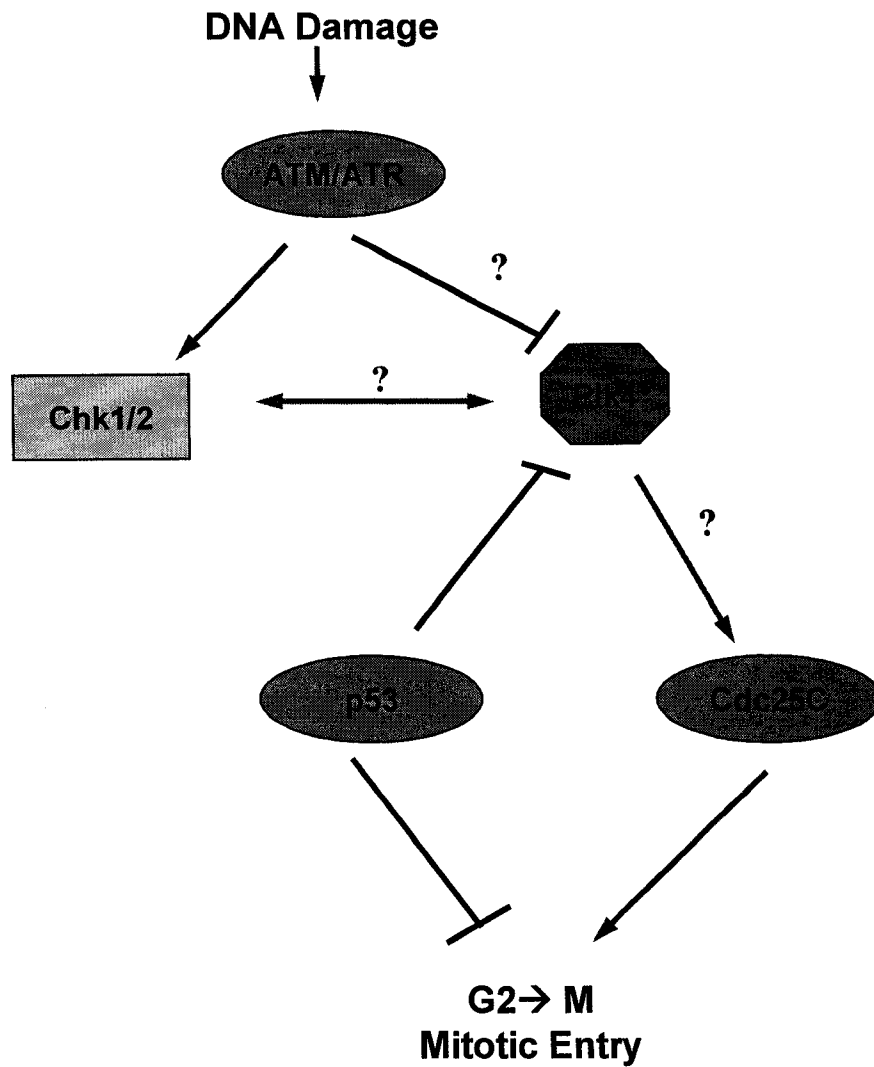


Figure 14. A proposed pathway through which Plk4 acts.

Red lines indicate proposed positive regulation whereas black lines indicate proposed negative regulation. In this proposed model Plk4 is involved in the activation of Cdc25C which leads to mitotic entry. Thus, in response to DNA damage, activation of Cdc25C is prevented through inhibition of Plk4 via ATM/ATR checkpoint kinases and mitotic entry is halted.

CHAPTER V

FUTURE DIRECTIONS

A reduction in overexpressed Plk4 protein level was observed in response to UV damage. Ideally, this experiment would be conducted looking at endogenous Plk4 protein levels; however a suitable Plk4 antibody has yet to be optimized. Given that a reduction in overexpressed Plk4 protein level was seen in response to UV radiation and not ionizing radiation suggests a role for Plk4 in UV DNA damage pathways. Several unanswered questions remain and it would thus be interesting to explore if this decrease in protein level seen was in fact due to ubiquitination. In order to determine this, after transfection cells would be subjected to UV radiation at increasing doses. Six hours post radiation, cells would be lysed and immunoprecipitation would be performed using anti-FLAG antibody, followed by western blotting using an anti-ubiquitin antibody. It can also be further determined if this decrease in protein level was dependent on the proteasome, by using a proteasome inhibitor such as MG-132. If in fact this decrease in Plk4 protein was dependent on ubiquitin-mediated proteasomal degradation, the decrease in protein level would not be seen in the presence of this inhibitor. Ideally, this experiment would be performed using endogenous Plk4 protein, however at this time this is not an option due to the unavailability of a suitable Plk4 antibody. Additionally, seeing as this experiment was performed in NIH-3T3 cells, one can assume that the decrease in Plk4 protein level is occurring in a p53-independent manner since these cells contain inactive p53. Supporting this, a role for p53 has not been clarified in UV damage response pathways (Chang *et al.*, 1999). However, Lie *et al.*, 2005 determined that p53

represses Plk4, thus implying Plk4 in p53-dependent and p53-independent response pathways. It would thus be interesting to test this experiment in a p53-null cell line such as Saos-2 and a p53 wildtype cell line such as U-2OS, to see if this response is entirely p53 independent. Furthermore, Li *et al.*, 2005 found that Plk4 mRNA levels decrease in cancer cell lines in response to the DNA damaging agent etoposide. To this end, it would be appealing to determine if UV and ionizing radiation in turn cause a decrease in Plk4 mRNA level and a decrease in the kinase activity of the protein.

The observation that overexpression of Plk4 pushes cells through the G1-S DNA damage checkpoint in the presence of damaged DNA suggests a possible role for Plk4 in the entrance into the S-phase of the cell cycle or possibly that Plk4 is inhibited in response to DNA damage. It would thus be of interest to determine if Plk4 phosphorylates and activates (or inactivates) Cdk2 or Cyclin E leading to entry into S-phase. In order to determine this, co-immunoprecipitation assays would initially be done to show that Plk4 interacts with these proteins. Following this, *in-vitro* kinase assays could be performed to establish if Cdk2 and Cyclin E are in fact substrates of Plk4. The observation that the overexpression of Plk4 causes centrosome overduplication in the presence of Cdk2 (Habedanck *et al.*, 2005) further supports this hypothesis.

One possible mechanism by which Plk4 is involved in mitotic entry is through interaction with key mitotic proteins Cdc25C and Cyclin B. These proteins are in turn targets of DNA damage pathways. Co-immunoprecipitations were initially done to determine that Cdc25C and Cyclin B interact with Plk4. Furthermore, it was determined that the polo-box domain was not necessary for these interactions to occur. It would however be interesting to determine if the 'cryptic' polo-box region of Plk4 is necessary

for these interactions to occur. Additionally, whether Cdk1 is an interacting partner of Plk4 and if Cyclin B is a substrate of Plk4 remain to be elucidated through co-immunoprecipitation experiments and in-vitro kinase assays.

Co-immunoprecipitation assays determined that Cdc25C is indeed an interacting partner of Plk4. Interestingly through in-vitro kinase assays, Cdc25C was found to be a substrate of Plk4. Therefore, it is of interest to establish which sites Plk4 phosphorylates on Cdc25C. This can be done by site directed mutagenesis of potential sites or truncation mutants of the phosphatase. Upon determining which sites Plk4 is responsible for phosphorylating the next question which needs to be answered is what effect this phosphorylation has on Plk4. For example, this site may lead to the activation of Cdc25C in turn leading to mitotic entry. On the contrary, Plk4 may inactivate Cdc25C (similar to Chk1) and prevent mitotic entry. If the site which is being phosphorylated is within Cdc25C's nuclear exclusion motif, phosphorylation of this site may change the localization pattern of this protein. Perhaps this phosphorylation event leads to the accumulation of Cdc25C in the nucleus, similar to Plk4 family members Plk1 and Plk3 (Toyoshima-Morimoto *et al.*, 2002; Bahassi *et al.*, 2004). Phosphorylation of Cdc25C by Plk4, and interaction of Plk4 with Cyclin B, may suggest that Plk4 is important for mitotic entry and DNA damage response pathways.

APPENDIX A: Solutions

LB Media (1L)

10 g tryptone
5 g yeast extract
10 g NaCl
Adjust pH to 7.0 and autoclave.

LB-AMP Plates (1L)

10 g tryptone
5 g yeast extract
10 g NaCl
15g Agar
Autoclave and add AMP to a concentration of 100 ug/mL after cooling.

Lysis Buffer

50 mM Tris-HCl pH 7.4
150 mM NaCl
1 mM EDTA
1% Triton X-100
1 protease inhibitor tablet (Roche) per 10 mL buffer

PBS

pH 7.4
137 mM NaCl
2.7 mM KCl
4.3 mM Na₂HPO₄
1.4 mM KH₂PO₄

TNT

0.1% Triton X-100
50 mM Tris-HCl pH 7.4
150 mM NaCl

Running Buffer

25 mM Tris
250 mM Glycine
0.1% SDS

Transfer Buffer (1 L)

3.03 g tris base
14.4 g glycine
200 mL methanol

2 X SDS-PAGE sample buffer (10 mL)

125 mM Tris pH 6.8
2.0 mL 100% glycerol
4.0 mL 10% (w/v) SDS
0.5 mL 0.1 % bromophenol blue

1 X TBST (Tris buffered saline and tween)

100 mM Tris-Cl pH 7.5
150 mM NaCl
0.1% Tween

Kinase Buffer

60 mM HEPES pH 7.5
3 mM MgCl₂
3 mM MnCl₂
50 mM NaF
1.2 mM DTT
1 protease inhibitor tablet (Roche) per 10 mL buffer

GST-Protein Purification Buffers

Cell Lysis Buffer

100 mM NaCl
50 mM Tris pH 7.5
0.1 mM EDTA
0.1% Triton X-100
2 mM DTT
1 protease inhibitor tablet (Roche) per 10 mL buffer

Column Wash Buffer

100 mM NaCl
50 mM Tris pH 7.5
1 mM DTT

Elution Buffer

100 mM NaCl
50 mM Tris pH 7.5
10 mM Glutathione

Competent Cell Solutions

TFB1

30 mM KOAc
50 mM MnCl₂

100 mM KCl
10 mM CaCl₂
7% glycerol

TFB2

10 mM MOPS pH 7.0
75 mM CaCl₂
10 mM KCl
7% glycerol

TYM broth

2% bactotryptone
0.5% yeast extract
0.1 M NaCl
10 mM MgSO₄

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