1-1-2007

Identification of Plk4 interacting partners and establishment of Plk4 stable cell lines.

Melissa Lauren Ganuelas
University of Windsor

Identification of Plk4 Interacting Partners and Establishment of Plk4 Stable Cell Lines

by

Melissa Lauren Ganuelas

A Thesis
Submitted to the Faculty of Graduate Studies
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

2007

Your file Votre référence
ISBN: 978-0-494-35009-6
Our file Notre référence
ISBN: 978-0-494-35009-6

ABSTRACT

Oncogenesis, or the development of cancer, can arise when an imbalance exists between cellular proliferation and differentiation, with a propensity for accelerated cell division. Critical enzymes are in place to ensure that cells progress through the cell cycle in a timely and regulated manner; the Polo-like kinases are one family of enzymes that exemplifies this regulatory function. Plk4 (Sak) is the most recently discovered mammalian Plk and has been shown to influence centrosome dynamics and to aid in mitotic progression. Using a candidate approach, this study identified putative interactions between Plk4 and proteins known to associate with other established mammalian Plks. Furthermore, in order to facilitate the characterization of Plk4 in future experiments, a cell line that stably expresses either wildtype or mutant Plk4 was created.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. John Hudson, for endowing me the opportunity to work under his supervision. His instruction and guidance were invaluable in my career as a Master's student and have allowed me to mature into a better researcher.

I would also like to show gratitude to my committee members, Dr. Andrew Hubberstey and Dr. Otis Vacratsis, as well as my committee chair Dr. Al Warner, for taking the time to read my thesis and for providing me with helpful advice and support throughout my Master's career.

I would also like to express gratitude to my parents, family, and friends for their unwavering love and support throughout my Master's career.

Finally, I would like to express thanks to my co-workers in the Hudson Lab, both past and present, who have endured with me through the good times and bad and have now become lifelong friends. I could not have done this without them. A special acknowledgment goes to Anna Kozarova, for her generous advice and assistance.

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CHAPTER I

REVIEW OF LITERATURE

Polo-like kinases

The polo-like kinases (Plks) are a group of serine-threonine kinases that are highly conserved from yeast to humans (Glover *et al*, 1998). Plks are involved in a number of cell-cycle related events including bipolar spindle formation (Hamanaka *et al*., 1995; Glover *et al*., 1996), chromosome segregation, centrosome maturation in late G2/early prophase (Lane and Nigg, 1996), activation of Cdc2, regulation of the anaphase-promoting complex, and execution of cytokinesis, thus emphasizing the importance of this group of kinases in mitotic progression (Lane and Nigg, 1996; Glover *et al*., 1998; Nigg, 1998). In addition, Plks have been implicated in playing major roles during the G1/S transition as well as in DNA damage pathways (Barr *et al*., 2004). *Polo,* the founding member of the Plk family, was originally identified in the fruit fly (*Drosophila melanogaster)*. Mutations in the Drosophila *Polo* gene were found to produce embryos that exhibited abnormal mitotic networks of microtubules, and structural defects in centrosomal components (Glover *et al*, 1998). Plk family members have also been described in single cell organisms such as Cdc5 in *Saccharomyces cerevisiae* (Kitada *et al*, 1993), Plo1 in *Schizosaccharomyces pombe* (Okhura *et al*, 1995), CaCDC5 in *Candida albicans* (Bachewich *et al*., 2003), tbplk in *Trypanosoma brucei* (Graham *et al*., 1998), and most recently PLKA in *Aspergillus nidulans* (Bachewich *et al*, 2005), whereas in multicellular organisms such as *Xenopus laevis* and *Caenorhabditis elegans*, three or four homologues of Plks have been described denoted Plx1/Plc1, Plx2/Plc2, and Plx3/Plc3, respectively, as well as the recently identified Plx4 (Kumagai and Dunphy, 1996; Ouyang *et al*., 1999; Chase *et al*., 2000; Duncan *et al*., 2001). In mammals, four Plk homologues have been identified to date: Plk 1 (Plk), Plk2 (Snk), Plk3 (Fnk), and Plk4 (Sak), each of which are critical participants in various aspects of cell cycle progression. The increased number of Plks in higher organisms is likely directly related to

an increase in the complexity of the cell cycle during the development of multicellular organisms and metazoans (Table 1).

Plks all share a high degree of structural homology in their N-terminal kinase domain, each containing a critical threonine residue in the activation segment that must be phosphorylated for proper activity (Cheng *et al*, 2003). Another characteristic feature of the Plks is the presence of one or two highly conserved, non-catalytic motifs in their C-terminal domains termed polo boxes (PB). Structurally, Plk1, 2, and 3 display high similarity in that there are two PB sequences designated PB1 and PB2, each comprising ~80 amino acids (AA) (Lowery *et al*, 2005). These motifs are separated by a ~20 AA linker region and are composed of a continuous six-stranded antiparallel β-sheet and an α-helix. Plk4, however, is structurally divergent from the other Plks in that it contains only one PB (Cheng *et al*, 2003; Leung *et al*, 2002). In terms of the Plk family, the remainder of this chapter will focus primarily on the Plks found in single celled organisms and Plks 1-3. Since my thesis is focused directly on Plk4, the background for this protein will be dealt with separately in Chapter 2.

Polo-box domain (PBD) structure and function

Collectively, the two PBs, the linker region between them, the 45 AAs N-terminal to PB1, and the amino acids C-terminal to PB2, all comprise a functional unit designated the PB domain (PBD) (Liu and Maller, 2005). The PBD is a feature unique to the Plk members, as revealed by a BLAST search that showed the motifs within this domain do not occur in other proteins (Altschul *et al*, 1990). This is in contrast to other motifs such as the SH2, SH3, or MH2 domains that are considerably more ubiquitous (Pawson and Nash, 2003). Analysis of the crystalline structure of Plk1 revealed that its two PBs have identical secondary protein structure consisting of antiparallel β-sheets and an α-helix, as previously mentioned (Cheng *et al*, 2003).

Table 1. Selected Plks and their functions

Organism	Plk member	Function
Drosophila melanogaster	Polo	Proper spindle formation
Saccharomyces cerevisiae	Cdc5	Bipolar spindle and septal formation
Schizosaccharomyces pombe	Plo1	Assembly of mitotic spindle, prophase actin ring required for cytokinesis, and septation
Mammals	Plk1	Mitotic related events e.g. centrosome development, bipolar spindle assembly, mediating DNA damage
	Plk2	Immediate-early gene; also involved in embyonic development, centriole duplication, and cell cycle progression
	Plk3	Immediate-early gene; mediating DNA damage response, tumor suppression; regulating mitosis
	Plk4	Centriole duplication; M phase progression
Xenopus laevis	Plx1	Activation of Cdc25c for entry to mitosis; promotes bipolar spindle formation at centrosomes; activation of APC/C; cytokinesis
Caenorhabditis elegans	Plk1	Nuclear envelope breakdown and completion of meiosis

Structurally, the core of the PBD in Plk1, Plk2, and Plk3 show high resemblance, whereas the linker region found between the two PBs is quite dissimilar. As a whole, the PBD appears to be critical to the function and localization of the Plk members, as evidenced by mutation and deletion studies of this domain. In Plk1, for example, a conserved W414F mutation in the PB region of Plk1 has been shown to disrupt the enzyme's ability to migrate to spindle poles and cytokinetic filaments during mitosis (Cheng *et al*, 2003). The same mutation can also disrupt the ability of Plk1 to complement the Cdc5 temperature-sensitive mutant in budding yeast (Lee *et al*, 1998); these data suggest that W414 is one of the residues most critical to the function of the PBD. In addition, overexpression of the PBD in Plk1 resulted in impaired spindle checkpoints and defective cytokinesis (Seong *et al*, 2002). Jiang *et al* (2006) reported that the PBD of Plk3 was sufficient for proper subcellular localization, and that sequence changes to either PB motif disrupts proper migration to the centrosomes during interphase, spindle poles during mitosis, and midbody during cytokinesis. The PBD of Plk3 also proves to be crucial in regulating the G2/M checkpoint upon DNA damage such that its deletion completely eliminates the kinase's ability to induce a G2/M arrest (Jiang *et al*, 2006). Furthermore, ectopic expression of Plk3's PBD in HeLa cells caused significant changes in cell morphology such as nuclear condensation and cell fragmentation, characteristics of cells undergoing apoptosis.

The PBD has also been suggested to regulate the kinase activity of certain Plk members, primarily through an intramolecular association with the N-terminal kinase domain leading to inhibition of catalytic activity. HeLa cells expressing C-terminal deletion mutants of Plk1 exhibited increased kinase activity compared to the wildtype, suggesting that the loss of the PBD within this region led to a deregulation of the enzyme (Jang *et al*, 2001). In Plk1, C-terminal inhibition of kinase activity can be relieved through the phosphorylation of Thr210 in the N-terminal kinase domain, which impedes its interaction with the C-terminus, or through its mutation to an aspartate (Jang *et al*, 2002).

The critical influence of the PBD in localization may be indicative of its ability to recognize specific protein binding partners at particular subcellular structures. Recent studies have proposed the PBD may indeed serve as a binding site for effectors or substrate proteins (Elia *et al*, 2003). Lowery *et al* (2004) present evidence that characterizes the entire PBD as a binding module for phosphoserine/threonine molecules, and suggest that the PBDs of Plk1, Plk2, and Plk3 all recognize the same binding motif of Ser-[pSer/pThr]-[Pro/X]; this indicates that a 'priming' phosphorylation of target proteins can enhance its recognition by these Plk members. It has been found that other mitotic regulators such as the cyclin-dependent kinases (Cdks) and MAP kinases can phosphorylate proteins that in turn bind to the PB domain, prompting localization of the Plk to its own substrate thus enhancing its kinase activity. Cdc25c, for example, is a confirmed substrate of Plk1 that contains the optimal PBD binding site $ST^{130}P$, while also consisting of a Cdk phosphorylation motif; indeed, Cdk1 phosphorylates Cdc25c, which enhances its interaction with Plk1 (Toyoshima-Morimoto *et al*, 2002) (see below). A study by Garcia-Alvarez *et al* (2007) revealed that the conserved W414 within the PBD originally attributed to proper Plk1 localization is also critical to its substrate recognition. Mutation at this site eliminated Plk1's ability to identify Cdc25c as its phosphorylation target, thus preventing Plk1 from assisting in Cdc25c's nuclear translocation (Garcia-Alvarez *et al*, 2007). Plk4 differs in that it contains only one conserved PB homologous to the PBs found in other Plk members, however, a second divergent 'cryptic PB' has been suggested to exist, a matter that will be more extensively discussed below (Leung *et al*, 2002).

Analysis of Plk loss-of-function and overexpression

The Plks have been characterized as a critical family of proteins that regulate a variety of cell cycle events. Loss-of-function, mutational and overexpression studies for the various Plk members have been crucial in dissecting their function within the cell and have highlighted the

significance of these proteins in controlling mitotic progression. In *Drosophila, Polo* mutants resulting from a recessive mutation in chromosome 3 were found to be embryonic lethal; larvae homozygous for this mutation failed to develop adult structures and eventually died in later stages of development (Sunkel and Glover, 1988). In addition, mutant embryos exhibited strikingly abnormal centrosome structures such as branched mitotic spindles and broad, unfocused poles; these defects in centrosomal organization would lead to unequal separation of chromosomes and ultimately genomic instability (Sunkel and Glover, 1988). Clearly, *Polo* has a pivotal role in maintaining chromosomal integrity and thus proper development of the organism.

Cdc5 is found in the budding yeast *Saccharomyces cerevisiae* and is a critical gene involved in cell division (Lee and Erikson, 1997). Loss of function studies in CDC5 highlight the importance of its activity during mitosis whereby its depletion induced a late nuclear division arrest resulting in dumbbell-shaped cellular morphology (Kitada *et al*, 1993); this abnormal phenotype was attributed to nearly-separated nuclei from both parental cell and bud that was connected by a thin string of chromatin. Various temperature-sensitive mutants for cdc15, cdc20, and dbf2, all of which affected mitotic progression and led to a defective growth phenotype in budding yeast, were able to be rescued by cdc5 (Kitada *et al*, 1993). Removal of the catalytic domain of this Plk member, however, completely abrogated this ability, suggesting that the kinase activity of cdc5 is crucial to its proper function. A separate study conducted on a temperature-sensitive mutant of cdc5 at its restrictive temperature resulted in a cell division arrest after spindle pole body (SPB) duplication, causing a failure of the cell to complete meiosis I (Dai *et al*, 2002). Overexpression of CDC5 resulted in the production of multinucleated cells as well as the presence of ectopic cytokinetic structures within abnormally-elongated buds (Kitada *et al*, 1993; Song *et al*, 2000). The mammalian Plk members Plk1 and Plk3 are both able to rescue the cdc5 temperature-sensitive mutant, thus underlining the conserved function of these Plk members in mitosis (Ouyang *et al*, 1997; Lee and Erikson, 1997). Plk2 and Plk4, however, are not capable of

complementing the mutant phenotype in a similar manner, suggesting these two mammalian Plks likely diverged functionally at some point during the evolutionary process.

In the fission yeast *Saccharomyces pombe,* the Plk member Plo1 has several noteworthy functions, one of which is proper spindle assembly and function. Deletion of the Plo1 gene leads to a characteristic phenotype defined by monopolar spindle formation in cells that are blocked in mitosis (Ohkura *et al*, 1995). Furthermore, cells lacking Plo1 are unable to form both a prophase actin ring, which is necessary for initiating the site for cytokinesis, and a septum, the cross-wall formed between mother and daughter cell during cell division (Ohkura *et al*, 1995). Conversely, it was found that overexpression of Plo1 can induce a state of multiple septation during any particular phase of the cell cycle (Ohkura *et al*, 1995). In *Caenorhabditis elegans,* RNA-mediated interference of the Plk homologue PLK-1 significantly disrupted proper embryonic development as a result of oocytes that were unable to divide (Chase *et al*, 2000). Close examination of the oocytes revealed an incomplete breakdown of the nuclear envelope prior to ovulation, although they still remained amenable to fertilization as shown by the presence of sperm DNA within the embryo; however, the resulting embryos displayed an inability to progress through meiosis, exhibiting defects in chromosomal segregation as well as in expulsion of polar bodies (Chase *et al*, 2000). Ultimately, embryos never progressed beyond the single-cell stage and showed signs of multinucleation. In contrast to other Plk members, depletion of PLK-1 did not hinder the cell from undergoing centrosome duplication or nucleation of microtubules (Chase *et al*, 2000). Indeed, in RNAi-treated embryos, the absence of PLK-1 still permitted formation of the mitotic spindle and replication of centrosomal structures as expected. These observations suggest that the loss of PLK-1 may not be capable of disrupting all aspects of the cell cycle and that certain events such as spindle assembly and centrosomal duplication can still prevail.

Plx1 is a crucial participant in the cell cycle pathway of *Xenopus laevis*, having been shown to phosphorylate the Cdc25c phosphatase at its N-terminus resulting in its activation (Kumagai and Dunphy, 1996). Enhancement of Cdc25c's activity then triggers a

dephosphorylation event on cyclin B/cdc2 or the mitosis promoting factor (MPF), thus pushing cells past the G2/M boundary into the mitotic phase; in turn, MPF induces activity of Plx1 as part of an MPF positive feedback loop (Abrieu *et al*, 1998). Kumagai and Dunphy (1996) showed how treatment of cell extracts with anti-Plx1 antibody significantly reduced Cdc25c activation, thus demonstrating that Plx1 is critical for mitotic progression at the G2/M transition. A separate study that focused on the role of Plx1 in the MPF amplification loop showed that immunodepletion of Plx1 with antibodies against its C-terminus (thus eliminating its activation) resulted in suppression of MPF activity, arresting cells in the G2 phase and preventing entry into mitosis (Abrieu *et al*, 1998).

Ideally, to gain insight into the critical role the Plks play in mammalian embryonic development, a knockout organism would be generated that would enable observation and analysis of the resultant phenotype. Given that some of the Plks are essential proteins needed for regulating early stages of the cell cycle, however, it may not be feasible to create a null organism, since knockout of the alleles would likely be lethal to the developing embryo. Indeed, there are no established knockout organisms for Plk1 or Plk3 due to their necessity in various critical events that enable proper mitotic progression. As a result, depletion of these Plks through antibodies or RNA-interference has been the primary method in studying their respective contributions to development and cell cycle. Plk1 has been the subject of numerous studies and is therefore the most well-characterized mammalian Plk. Liu, Erikson, and colleagues (2003) transfected HeLa cells with double-stranded RNA targeting Plk1 for depletion and discovered that cell proliferation was considerably retarded, and the viability of cells was compromised. Furthermore, cells deficient of Plk1 showed signs of a block in G2/M, along with a propensity for apoptosis (Liu and Erikson, 2003). Cellular death was indicated by Caspase 3 induction (an apoptosis instigator), as well as fragmented nuclei and dumbbell-shaped chromatin due to unseparated sister chromatids. In a previous study, Lane and Nigg (1996) had originally observed a buildup of cells that were unable to enter mitosis, and that contained fragmented and/or inappropriate numbers of nuclei

when Plk1 was neutralized by micro-injected antibodies. In addition, cells lacking Plk1 displayed abnormalities in mitotic spindle formation, as well centrosomes that failed to mature and separate (Van Vugt *et al*, 2004). Conversely, overexpression of Plk1 in mammalian cells seems to instigate abnormal cellular proliferation and thus has become useful as a prognostic marker in tumor formation.

The depletion of Plk3 has also been subject to analysis to determine the function of this protein *in vivo*. One study used RNA interference to suppress Plk3 gene expression in human mammary epithelial cells and discovered that depleting Plk3 prevented cyclin E expression, a confirmed regulator of the G1/S transition, and therefore hindered cells from entering S phase (Zimmerman and Erikson, 2007). Furthermore, cells treated with anti-Plk3 lentivirus showed significant reductions in the proliferation marker Ki67, indicating these cells were no longer actively progressing through mitosis and had entered into a quiescent phase. Overexpression of Plk3 produces a contrasting phenotype to that of Plk1 in that enforced levels of Plk3 lead to inhibited cellular proliferation as a result of apoptosis (Conn *et al*, 2000). Wang *et al* (2002) confirmed the pro-apoptotic role of Plk3 upon overexpression, revealing that ectopic Plk3 expression can lead to a disruption of microtubule integrity and subsequently to changes in cell morphology such as shrunken cells and unseparated midbody structures; therefore, mitotic progression is halted and cellular death is observed (Wang *et al*, 2002).

The precise function of Plk2 in mammalian cells is relatively unknown. In adult mice, Plk2 expression has been detected in the brain. Ectopic expression of Plk2 can induce changes in cellular morphology and eventually induce apoptosis (Ma *et al*, 2003). While attempts to create knockout organisms for Plk1 and Plk3 have been futile, experimenters have been able to create a null organism for Plk2, suggesting that depletion of this protein may not completely abrogate cellular proliferation and embryonic development. Ma and colleagues (2003) generated a Plk2 –/– mouse line through targeted disruption of the Plk2 gene locus and insertion of ES cells containing the perturbed gene into a mouse blastocyst. The result after breeding was a mouse line containing

a mutation in both Plk2 alleles, which could then be assessed for defects in embryonic development, mitotic progression, and other aspects of its phenotype. Embryos derived from Plk2 –/– mice were analyzed and observed to develop at a slower rate with a subsequent delay in skeletal growth compared to wildtype embryos (Ma *et al*, 2003). Histological evalutation of Plk2 –/– embryos also indicated reduced levels of phosphorylated histone H3, a protein utilized as an indicator of cellular proliferation (Ma *et al*, 2003). Furthermore, Plk2 –/– embryonic fibroblasts exhibited a delayed G1→S progression, suggesting a possible role of Plk2 in promoting cells through the cell cycle.

Plks: regulation and cell cycle functions

Expression of the Plks is differentially regulated throughout the cell cycle (Lane and Nigg, 1996). Plk1 expression, for example, peaks during late G2 and M phase and is involved in mitotic-related events such as centrosome development (Lane and Nigg, 1996) and bipolar spindle assembly (Ohkura *et al*, 1995). In addition, Plk1 has been shown to phosphorylate cyclin B1, a component of mitotis-promoting factor (MPF) when complexed with Cdc2 kinase; the result is nuclear accumulation of MPF during prophase and subsequent initiation and coordination of M-phase events (Toyoshima-Morimoto *et al*, 2001). Cdc25c, a protein phosphatase also responsible for MPF activation, is phosphorylated by Plk1, being targeted to the nucleus upon modification (Toyoshima-Morimoto *et al*, 2002). In addition to promoting mitotic entry, Plk1 displays an active role at the metaphase-anaphase transition, primarily by activating components of the anaphase-promotic complex (APC) (Nigg, 1998). The APC is an E3 ubiquitin-protein ligase responsible for degrading anaphase inhibitors; Plk1 has been shown to phosphorylate APC components Cdc16 and Cdc27 *in vitro*, suggestive of a direct role in APC regulation by Plk1 (Glover *et al*, 1998). Plk2 [PC1]expression occurs largely outside the mitotic stage during G1 and its protein levels have a rapid turnover rate; data suggest it may aid in propelling cells

through to S phase (Simmons *et al*, 1992; Liby *et al*., 2001; Ma *et al*., 2003). Unlike Plk1, Plk2 does not contribute significantly to cell division *per se*, though it has been shown to be activated by a p53-mediated DNA-damage checkpoint in mitosis, suggesting a role in halting M-phase progression (Burns *et al*, 2003). Plk3 has been found to associate with mitotic components such as the bipolar spindles, spindle poles, and midbody (Glover *et al*, 1998). Levels of Plk3 were originally shown to remain relatively constant throughout the cell cycle, with maximal kinase activity occurring in late S and G2 phase (Ouyang *et al*, 1997). A recently published study, however, found that the level of Plk3 protein is in fact cell-cycle regulated, with maximal expression occurring during G1 phase (Zimmerman and Erikson, 2007). Data indicate that Plk3 [PC2]augments cyclin E expression, a regulator of the G1/S transition, through phosphorylation of Cdc25A and that cells depleted of Plk3 after overcoming the G1/S checkpoint can progress through the cell cycle unabated, but fail to re-initiate mitosis. Clearly, the Plks each play a unique role in controlling aspects of cell cycle progression.

Plks and centrosome regulation

The centrosome is an essential component of the microtubule organization centre (MTOC), a unique assembly of proteins that work together to ensure fidelity of cellular division. Comprised of two centrioles surrounded by pericentriolar material, the centrosomes enable formation of the bipolar spindle, facilitating segregation of chromosomal material (Blagden and Glover, 2003). Numerous families of protein kinases have already been established as key regulators of the centrosomal cycle, including the cyclin-dependent kinases (Cdks), Aurora kinases, and the NIMA family of kinases (Okuda *et al*, 2000; Hannak *et al*, 2001; Fry *et al*, 2000). Additionally, the Plks have been shown to influence centrosomal dynamics. Nearly all of the Plk members have been shown to mediate centrosome duplication, though the precise mechanism by which this process occurs remains to be clearly defined. In *Drosophila*, expression

of *Polo* allows for recruitment of essential centrosomal components and for its proper localization to the microtubule organization center (MTOC) (Dai *et al*, 2002). Without Polo, proper spindle assembly would be hindered leading to defects in chromosome segregation and cytokinesis. Further evidence for the role of Polo in centrosome regulation comes from its ability to phosphorylate Asp, a confirmed microtubule-linked protein that associates with centrosomes and assists in forming microtubule asters (Gonzales *et al*, 1998).

In yeast, spindle pole bodies (SPB) are considered to be the functional equivalent to the centrosomes found in mitotic-capable cells. The Plk Cdc5 in budding yeast has been linked to regulating SPBs, primarily through its functional interaction with verified SPB proteins such as dbf2, cdc15, and Mob1p (Luca *et al*, 2001; Menssen *et al*, 2001). It appears that Cdc5 enables translocation of certain SPB proteins to its proper structures through phosphorylation of these substrates (Dai *et al*, 2002). Plo1 in the fission yeast localizes to the SPBs during early mitosis and continues its association until anaphase, where it assists in the formation of the mitotic spindle (Ohkura *et al*, 1995). Its association with the SPB is dependent on mitosis-promoting factor (MPF), while its dissociation relies on the anaphase-promoting complex (APC) (Mulvihill *et al*, 1999). In vertebrates, Plk1 was first suggested to participate in controlling centrosome dynamics based on its localization to centrosomal structures when tagged with green fluorescent protein (GFP) (Golsteyn *et al*, 1995; Arnaud *et al*, 1998). Indeed, Plk1 was found to associate with spindle components throughout mitosis, with an eventual redistribution throughout the cell following the transition from metaphase to anaphase (Golsteyn *et al*, 1995). Evidence points out that Plk1 may be involved with promoting centrosome maturation, rather than mediating its duplication; depletion of Plk1 was shown to cause monopolar spindles derived from centrosomes that were properly replicated, but that failed to grow to size and separate (Golsteyn *et al*, 1995). Lane and Nigg (1996) confirmed the role of Plk1 in centrosomal maturation, showing that injection of anti-Plk1 antibodies into HeLa cells caused monoastral microtubule arrays stemming

from underdeveloped centrosomes that contained reduced amounts of γ-tubulin, a centrosomal protein marker.

Plk2 is another mammalian Plk that has been found to play a role in centriole duplication. Overexpression of Plk2 in CHO cells was shown to increase the number of centrosomes, while transfection of a kinase-defective Plk2 inhibited centriole duplication in a dominant-negative fashion (Warnke *et al*, 2004). Additionally, silencing of endogenous Plk2 using small hairpin RNAs (sh-RNAs) interfered with replication of the centrioles in U2OS cells, confirming the role of Plk2 in promoting centriolar duplication. Plk3 has also been linked to controlling centrosomal dynamics; it is found to localize to the MTOC during interphase, and continues its association even after centrosome duplication, eventually migrating to the spindle poles during mitosis and the midbody during cytokinesis (Dai *et al*, 2002). Wang *et al* (2002) confirmed that Plk3 maintains association with the centrosomes or spindle poles throughout all phases of the cell cycle. They further showed that centrosomal localization by Plk3 is dependent on the microtubules, since depolymerization by exposure to low temperatures and nocodazole caused Plk3 to disperse throughout the cell. Although both Plk1 and Plk3 associate with the centrosomes during interphase, the elevated abundance of Plk3 protein may indicate it has a more significant contribution in regulating centrosomes during this phase (Dai *et al*, 2002). Therefore, the Plk members may each possess a unique role in regulating centrosome dynamics throughout different stages of the cell cycle.

Plks and oncogenesis

Cancer can arise when specific genes involved in cellular proliferation become altered through mutation. For example, tumor-suppressor genes encode proteins that regulate abnormal propagation of cells by halting their progression through the cell cycle. When these genes become mutated and no longer functional, cell cycle progression may remain unchecked resulting in

tumorigenesis. DNA damage checkpoints also exist within the cell cycle to ensure fidelity of DNA replication and correct propagation of genetic material. Genomic instability can ensue when heritable changes in genes accumulate as a result of base-pair mutations, chromosomal loss or rearrangement (Draviam *et al*, 2004). One type of genomic instability, called chromosomal instability (CIN), occurs when cells suffer from a high rate of chromosomal deletion or additions, which causes inappropriate amounts of genetic material to be distributed to dividing cells (Draviam *et al*, 2004). This is a significant contributor to conditions of aneuploidy and polyploidy with a potential for tumorigenesis. Draviam *et al.* (2004) highlight two potential causes of CIN, namely defects in spindle checkpoint proteins and changes in centrosomal regulation. The spindle checkpoint acts as a sensitive detector of misaligned chromosomes, which when prompted subsequently triggers pathways leading to mitotic arrest at anaphase; this ensures that the correct number of sister chromatids have attached themselves to the bipolar spindle for eventual distribution to daughter cells following cytokinesis. Because the Plks have been implicated in bipolar spindle as well as centrosome regulation, their deregulation has the potential to contribute to CIN and the development of malignancies, and indeed this seems to be the case. As previously described, depletion of Plk1 results in monopolar spindles and underdeveloped centrosomes with a reduction in centrosomal protein recruitment, all characteristic features of the CIN phenotype (Golsteyn *et al*, 1995; Lane and Nigg, 1996). Additionally, the failure of sister chromatids to separate has also been observed in HeLa cells subject to Plk1 reduction by RNAi, which can also contribute to cases of CIN and tumorigenesis (Liu and Erikson, 2003). Excessive levels of Plk1 have been observed in malignancies derived from numerous forms of cancer including gastric cancer, melanomas, breast cancer, ovarian cancer, endometrial cancer, gliomas, and thyroid cancer (for review see Takai *et al*, 2005). Plk1 was also found to be overexpressed in human colorectal cancer, a class of malignancy for which 85% of the cases exhibit the CIN phenotype (Takahashi *et al*, 2003). Overexpression of Plk1 has been utilized as a prognostic marker for tumor development, since mRNA and protein levels of Plk1 have been correlated to accelerated

cellular proliferation (Holtrich *et al*, 1994; Wolf *et al*, 2000). Even following an arrest in G2 induced by DNA damage, overexpressed Plk1 is still able to override the block and propel cells through mitosis (Smits *et al*, 2000). Thus, maintenance of normal Plk1 levels proves crucial to preventing the abnormal proliferation of transformed cells.

Conversely, Plk3 has been negatively correlated to tumorigenesis and cellular proliferation, as evidenced by its downregulation in various cancers such as lung carcinomas (Li *et al*, 1996), rat colon tumors (Dai *et al*, 2002a), and head and neck squamous cell carcinomas (HNSCC) (Dai *et al*, 2000). Due to its activation upon DNA damage prompting immediate cell cycle arrest, Plk3 can be regarded as a tumor suppressor (Eckerdt *et al*, 2005); additional studies demonstrate how overexpression of Plk3 induces a halt in cell cycle progression leading eventually to apoptosis (Wang *et al*, 2002). In addition, Plk3 can phosphorylate the oncogene Cdc25c resulting in its downregulation, as well as phospho-activating the tumor suppressor p53, both of which result in arresting the cell cycle (Ouyang *et al*, 1999; Xie *et al*, 2001). Thus, Plk1 and Plk3 can be viewed as exerting antagonistic effects on the progression of tumorigenesis.

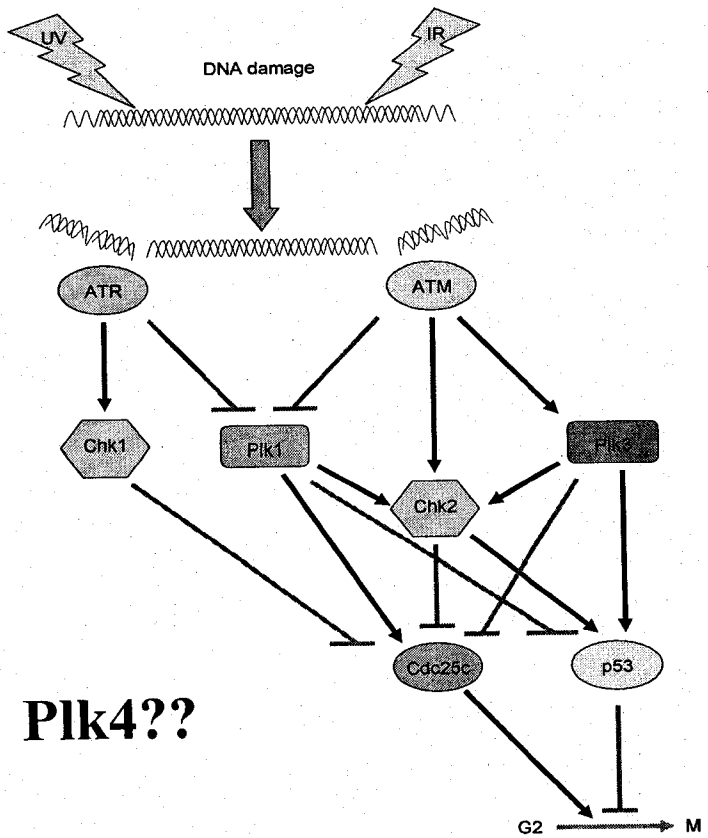
Regulating centriole duplication during the cell cycle is also a mechanism of CIN prevention. Defects in centriole reduplication have been observed in the human osteosarcoma cell line U2OS upon the introduction of small hairpin RNAs targeting Plk2, which resulted in suppression of its activity (Warnke *et al*, 2004). The overexpression of a dominant-negative mutant of Plk2 also inhibited centrosome duplication, thus confirming the role of Plk2 in regulating proper centrosome dynamics and therefore minimizing conditions of CIN (Warnke *et al*, 2004). Thus, it is evident that the Plks are essential regulators of centrosome dynamics and may provide insight into the molecular mechanisms driving tumorigenesis.

Cell cycle kinases and the DNA damage response

A cell's coping mechanism in dealing with DNA damaging agents is a critical process that ensures cell survival. Highly conserved pathways involving DNA repair mechanisms and cell-cycle checkpoints enable cells to maintain the integrity of its genetic information and ensure genomic stability. Without such devices, cancer can arise and threaten survival of the cell and, ultimately, the organism. Protein kinases are critical participants in the signal transduction pathways triggered in response to DNA damage. Through phosphorylation, these enzymes can regulate the activity of other proteins in an intricately coordinated network that enables the cell to respond appropriately to defects in genetic material. Key DNA damage proteins include phosphoinositide kinase homologs ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR), the protein kinases Chk1 and Chk2, and the tumor suppressor protein p53 (Elledge, 1996). ATM is primarily involved in repairing double-stranded DNA breaks resulting from ionizing radiation (IR), and regulates checkpoints at the G1/S and G2/M transitions; ATR is responsible for monitoring problems in DNA replication forks during S phase due to UV exposure (Guo *et al*, 2000). Activation of ATR and ATM has been shown to phosphorylate other checkpoint kinases, namely Chk1 and Chk2, which then regulate the G2/M transition through phosphorylation of a common target, Cdc25c. Chk2 is the mammalian homolog to Rad53 found in *S. cerevesiae* and upon DNA damage by IR is activated by ATM, however in cases of UV damage, activation is mediated by ATR (Matsuoka *et al*, 2000). Following damaged induced by UV, ATR can also phosphorylate Chk1 and is involved in its regulation at the G2/M checkpoint.

Plks have been shown to participate in key DNA damage response pathways (Figure 1). In mammals, Plk1 is inhibited following DNA damage prompting a subsequent arrest in the cell cycle during mitosis. Van Vugt *et al* (2001) showed that this damage-induced Plk1 inhibition can occur in an ATM- and ATR- dependent manner, which is determined by the type of damage induced upon the cell. Since Plk1 has been observed to bind and inhibit the tumor suppressor p53,

Fig. 1. DNA damage pathways and the elucidated roles of Plks. Genotoxic stress induced by ultraviolet (UV) exposure or ionization radiation (IR) triggers a signal transduction pathway that is initiated by ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) proteins. ATM phosphorylates the downstream checkpoint kinase Chk2, resulting in its activation and subsequently causing it to suppress activity of Cdc25c, but enhance activity of p53; ultimately, both result in arresting the cell cycle at the G2/M transition, thus preventing damaged DNA from being propagated. ATR phosphorylates Chk1 in a similar manner, which also results in Cdc25c being inhibited. Plk1 and Plk3 have both been implicated as intermediary participants in the DNA damage response. Plks have opposing effects on Cdc25c activity with Plk1 inhibiting Cdc25c upon phosphorylation, while Plk3 induces its activation upon phosphorylation. Plk1 and Plk3 share similar roles in phosphorylating Chk2, albeit on different residues, with both contributing to activation of this checkpoint kinase thus leading to mitotic arrest. The role of Plk4 in the DNA damage pathway has not been clearly identified; to date, no putative interacting partners for Plk4 have been described. By analyzing whether or not Plk4 has a functional association with established participants of the DNA damage response, a more accurate characterization of this relatively elusive protein may be achieved. Black arrows denote activation; red bars denote inhibition.



Plk4??

it is deemed to have a counteracting effect on DNA-damaged induced cell cycle arrest (Dai and Cogswell, 2003). Plk3 is also a key participant in the response pathways to DNA repair, becoming rapidly activated by ATM when cells are exposed to IR and oxidative stress (Xie *et al*, 2001). In contrast to Plk1, Plk3 has an activating effect when it phosphorylates p53 upon DNA damage, which contributes to the tumor-suppressing response (Xie *et al*, 2001). In addition to the phosphoinositide kinases ATM and ATR, Chk2 is another component of the DNA response pathway that has been shown to associate with certain Plk members. Studies have demonstrated that overexpression of Plk1 enhances phosphorylation of Chk2 at T68, a site that is involved in Chk2 localization to centrosomes and midbody, as well as in Chk2 activation, which ultimately induces a halt in mitotic progression; in this respect, Plk1 has a positive effect on cell cycle arrest (Tsetkov *et al*, 2003). Although Plk3 targets a different residue on Chk2 for phosphorylation, its effects on the activation of this kinase are similar to that observed by Plk1, though *in vivo* its interaction requires ATM as a mediator (Bahassi *et al*, 2002). Despite the elucidated roles of certain Plk members in the DNA damage response pathways, the role of Plk4 has yet to be clearly defined.

CHAPTER II

PLK4/SAK

Protein Structure

Plk4 is found on chromosome 3 in mice, and chromosome 4q27-28 in humans, a location that is susceptible to chromosomal deletions and reorganizations characteristic of human cancers (Swallow *et al*, 2005). Within the kinase domain is a critical Lys41 residue that, when mutated, results in complete inactivation of the enzyme (Figure 2). The T-loop at the C-terminus in turn consists of a required Thr170 residue that results in increased kinase activity as a result of mutation. Plk4 is structurally distinct from its family members in that it contains only one PB domain. Despite this structural distinction, a 'cryptic' PB domain has been suggested that encompasses amino acid residues 596-836 within the C-terminal domain; it is believed to enable self-association of the enzyme, namely through interaction with the enzyme's kinase domain (Leung *et al*, 2002). Plk4's single PB region is comprised of a cluster of hydrophobic residues that are conserved amongst several Plk family members; it also contains two conserved charged residues—Asp868 and Lys906—that interact to form a salt bridge, facilitating the dimerization of the PB domain both *in vitro* and *in vivo* (Leung *et al*, 2002). This novel dimer then localizes to various cellular structures including the nucleolus during G2, centrosomes during early G2/M phase, and to the cleavage furrow during cytokinesis (Leung *et al*, 2002; Hudson *et al*, 2001). Localization studies of the isolated PB domain of Plk4 have shown that the protein can still migrate to the centrosomes during mitosis. Expression of a truncated Plk4, which lacks the PB domain, still exhibits proper localization; however, a more extensive C-terminal deletion of the region downstream of the kinase domain up to and including the PB resulted in mislocalization of the enzyme (Leung *et al*, 2002). In this respect, Plk4 may be distinct to other Plk members in that the PB is necessary, but not sufficient, for proper localization and that regions outside the PB may assist in this process.

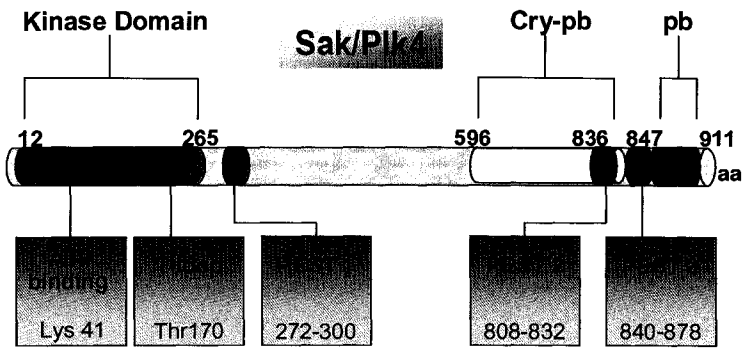


Fig. 2. Plk4 protein structure. Plk4 contains an N-terminal kinase domain, a single C-terminal polo-box domain (pb), as well as three PEST sequences associated with protein stability. A cryptic pb is also located within the C-terminus. Within the kinase domain are two conserved residues, Lys 41 and Thr 170, that when mutated render the kinase catalytically-inactive and constitutively-active, respectively. Numbers indicate the amino acids comprising the designated regions.

Regulation of Plk4 and its role in mitosis

Regulation of Plk4 expression occurs at both the pre- and post-transcriptional levels. Two isoforms of murine Plk4 exist, Plk4-a and Plk4-b, which differ in their stability at the post-transcriptional level. Structurally, these two isoforms of Plk4 are distinct in that Plk4-a comprises three PEST sequences that correlate to its susceptibility to degradation. One PEST cluster resides in the N-terminus, while the other two are in close proximity at the C-terminus. The Plk4-b isoform is significantly less abundant than its Plk4-a counterpart (Fode *et al*, 1996). Plk4-a exhibits a very short half-life of approximately 2-3 hours, being subject to multiubiquitination that targets the protein for eventual proteolysis. Removal of these sequences in human Plk4 can relieve PEST-related protein degradation and can improve the stability of the protein when overexpressed (Yamashita *et al*, 2001). In addition, Tec protein tyrosine kinase effectively thwarts PEST-dependent proteolysis upon phosphorylation of Plk4, while also increasing Plk4's kinase activity (Yamashita *et al*, 2001).

At the transcriptional, mRNA level, Plk4 expression varies throughout the cell cycle, with a gradual increase occurring from S through M phase (Fode *et al*, 1996). Following ubiquitination, Plk4 is degraded by the anaphase promoting complex (APC) and returned to resting phase levels (Swallow *et al*, 2005). Peak levels of Plk4 during M phase suggest its presence is necessary for completion of mitosis (Hudson *et al*, 2001). A murine Plk4 null mutant was generated through targeted mutation of the Plk4 gene locus to study the phenotypic effects of Plk4 loss-of-function. Cells derived from Plk4 –/– mice in fact showed signs of mitotic failure, as well as elevated levels of cyclin B1 and phosphorylated histone H3, proteins indicative of an arrest in anaphase (Hudson *et al*, 2001). Furthermore, rounded, dumbbell-shaped cells were observed in Plk4–/– blastocyst cultures, which suggests a telophase arrest (Hudson *et al*, 2001). A block in the cell cycle due to the absence of Plk4 would subsequently trigger pathways leading to apoptosis, as is in fact seen in null embryos displaying a high rate of cellular death. As a result,

observed in Plk4–/– blastocyst cultures, which suggests a telophase arrest (Hudson *et al*, 2001). A block in the cell cycle due to the absence of Plk4 would subsequently trigger pathways leading to apoptosis, as is in fact seen in null embryos displaying a high rate of cellular death. As a result, Plk4 null embryos were found to arrest 7.5 days into embryonic development (Hudson *et al*, 2001). Evidently, Plk4 is necessary for the cyclic destruction of cyclin B1 and therefore crucial for allowing cells to properly exit from mitosis.

Plk4 in tumorigenesis

The significance of Plk4 in cancer and tumorigenesis has also been observed in experiments with mice which contain only one functional Plk4 allele (Plk4 +/–). Greater than 50% of 22-24 month old heterozygous Plk4 mice develop tumours primarily in the liver and lung tumors compared to less than 3% of their wildtype littermates (Ko *et al*, 2005). In addition, cells obtained from the malignant tissue in Plk4 +/– mice displayed high mitotic irregularities such as multiple centrosomes and multipolar spindles. Examination of [PC3]Plk4 +/– mouse embryonic fibroblasts (MEFs) exhibited increased incidences of mis-segregated chromosomes and aberrant centrosome number (Ko *et al*, 2005). These cell cycle defects would likely contribute to cases of aneuploidy or polyploidy and thus enhance development of chromosomal instability and contribute to tumorigenesis. Indeed, it was found that defects in mitotic lesions excised from Plk4 +/– mice livers exhibited primary hepatocellular carcinomas, while papillary adenocarcincomas were observed peripherally in lung tissue. Multifocal tumors were prevalent in the malignant liver tissue of heterozygous Plk4 mice, displaying high disorganization of cells that were arrested in mitosis (Ko *et al*, 2005). Liver cells from Plk4 +/– mice livers that were subjected to partial hepatectomy displayed multipolar spindle structures, a disordered array of hepatocytes, as well as a high incidence of aberrant mitotic figures; this indicated a gross deficiency in cells progressing through mitosis (Ko *et al*, 2005). Additionally, cancerous lung tissue showed signs of atypical

cells when compared to normal tissue derived from wildtype mice. These observations point to a haploinsufficient condition for Plk4, in which one functional allele is not adequate to produce the normal phenotype. Undoubtedly, Plk4 is a critical factor in cell cycle progression such that its absence can lead to mitotic abnormalities and subsequently contribute to oncogenesis.

Plk4 in centriole duplication

Recent evidence highlights the role of Plk4 in regulating centriole duplication during S phase of the cell cycle. Endogenous Plk4 has been found to associate with centrioles, as evidenced by its colocalization with structure-specific proteins such as centrin and γ-tubulin (Hudson *et al* 2001; Leung *et al* 2002; Habedanck *et al*, 2005). Abrogation of Plk4 function has been shown to cause a gradual decrease in centriole numbers following successive rounds of mitosis, and/or the appearance of abnormal mitotic spindle morphology (Habedanck *et al*, 2005). This resulted in many cells left with acentriolar poles or monopolar spindles, both conditions that inhibit proper segregation of sister chromatids and contribute to aneuploidy and CIN. Bettancourt-Dias and colleagues (2005) further supplemented these findings with evidence of reduced centriolar numbers in HeLa and U2OS cells following the silencing of Plk4 using siRNA, which may be the result of defective centrosome duplication, separation, or segregation. Again, the abnormal spindle organization that would result from the loss of centrioles demonstrates the importance Plk4 may have in preserving mitotic fidelity in cells.

Candidate Approach to elucidating Plk4 interacting partners

Plks play a significant role in regulating crucial aspects of the cell cycle as well as in mediating DNA-damage responses. As part of an expansive network of proteins that participate in these regulatory events, it is no surprise that Plks have functional interactions with a variety of other kinases, which can affect the protein's activity, stability, localization, or other protein associations. Various studies have already confirmed that different Plk members can target

common substrates in pathways triggered by DNA-damage as well as common components of cell cycle regulation (Table 2). Some candidate substrates of Plks have been described in studies, including Cdc25c (Plx1, Plk1, Plk3) (Abrieu *et al*, 1998; Toyoshima-Morimoto *et al*, 2002; Ouyang *et al*, 1997), p53 (Plk1, Plk3) (Ando *et al*, 2004; Xie *et al*, 2001), and β-tubulin (Polo)

Table 2. Plks and candidate substrates

Candidate Substrate	Plk Member	Proposed Function
Cdc25c	Plx1	Activation of Cdc25c
	Plk1	Nuclear translocation of Cdc25c to allow activation of MPF
	Plk3	Nuclear translocation of Cdc25c to allow activation of MPF
p53	Plk1	Inhibits transactivation activity and pro-apoptotic function of p53
	Plk3	Promotes apoptotic function of p53 in response to DNA damage
Chk2	Plk1	Cell cycle arrest via Cdc25c inhibition
	Plk3	"
α-tubulin	Plk1	Mediating spindle assembly
β-tubulin	Polo	?
	Plk1	Mediating spindle assembly
γ-tubulin	Plk1	Mediating spindle assembly

(Tavares *et al*, 1996). In addition, Chk2, has been shown to be phosphorylated by both Plk1 and Plk3 upon DNA damage, albeit on different residues, which as a result triggers a cell cycle block through inhibition of Cdc25c (Tsetkov *et al*, 2003; Bahassi *et al*, 2002). In addition, ATM is involved in mediating inhibition of Plk1 upon IR damage, as well as in activating Plk3 to induce DNA repair and an arrest in the cell cycle (Van Vugt *et al*, 2001; Xie *et al*, 2001). Little is known about whether or not Plk4 also participates in these major pathways that control the cell cycle or the response to DNA damage. The major focus of my research was to determine whether or not Plk4 also targets some of these common substrates. This provides a basis for elucidating novel substrates of Plk4 and allows for a more defined characterization of this relatively unknown kinase.

Plk4-Expressing Stable Cell Lines

Obtaining significant expression levels of Plk4 in transient systems has been a significant and ongoing problem faced in early phases of this study. This presented set-backs when attempting to perform experiments requiring overexpression of Plk4 protein such as in co-immunoprecipitation studies that typically require ample levels of protein in order to detect transient protein:protein interactions *in vivo.* In addition studies with Plk4 have been problematic in that there are no suitable antibodies available for these co-immunoprecipation-based screens for interacting partners.

The ability to express high levels of a gene of interest is an important tool in molecular biology. To accomplish this, the foreign DNA must somehow be introduced into the cell in order to be transcribed by the host's expression machinery. Most applications of transfection require only that the gene be transiently expressed, however, inconsistent gene expression and low protein yield are common disadvantages associated with such transient methods. The establishment of a stably-transfected cell line greatly enhances the level of gene expression in

cases where high levels of purified protein are required. Therefore, another objective of this study was to establish and optimize a stable expression system for Plk4 constructs that would help to circumvent the problems experienced with minimal and inconsistent gene expression.

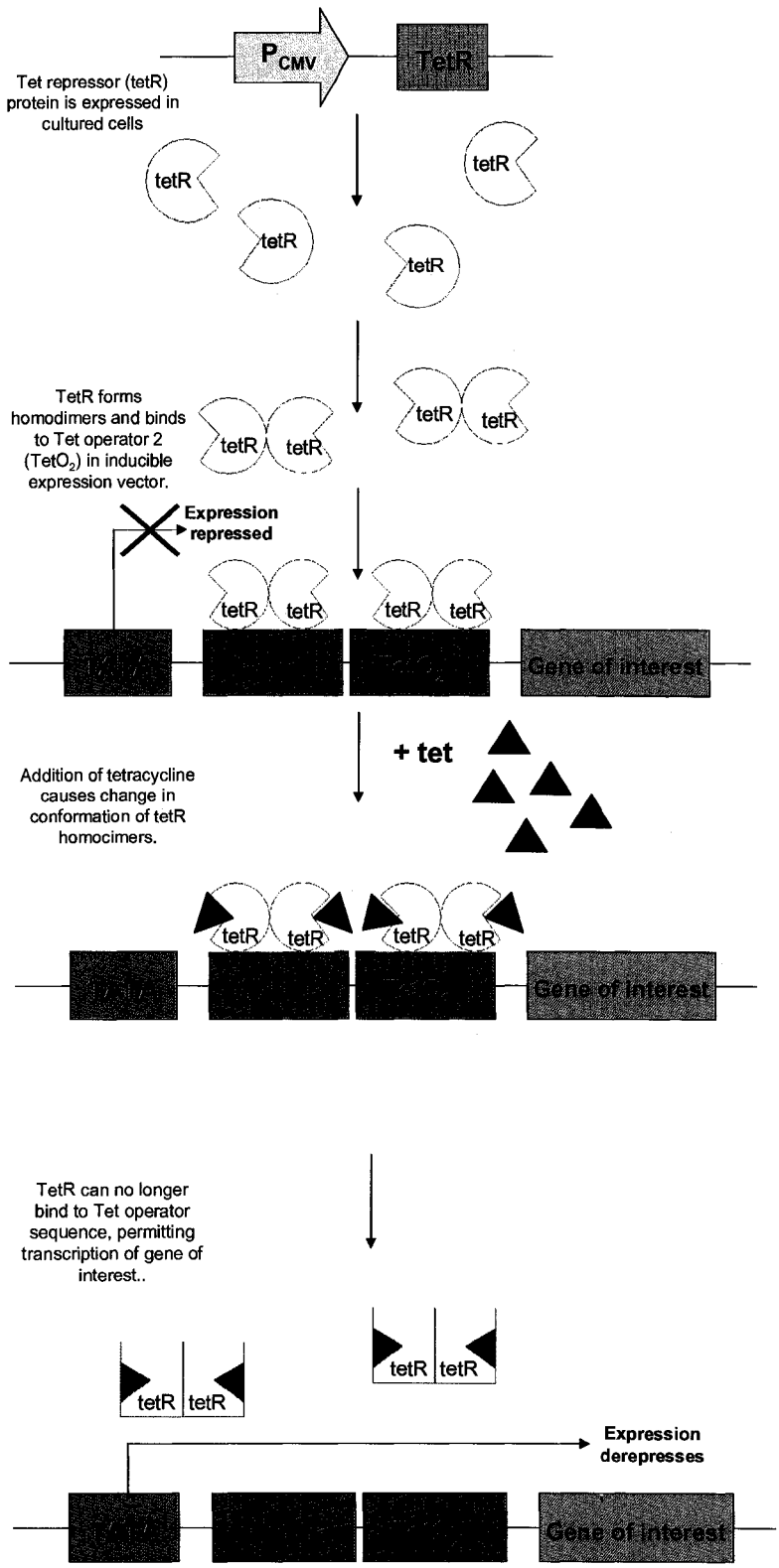
Generally, to establish a stable cell line, the gene of interest must be cloned into a vector containing a gene conferring resistance to a certain agent. The expression vector is then transfected into the desired cell line and permitted to undergo overexpression. Subsequently, those cells that successfully incorporated the foreign DNA into its genome can then be selected for upon addition of the selective agent, resulting in a population of cells stably expressing the desired gene. Additionally, the stable expression system can be made inducible through the incorporation of a repressor protein that will inhibit transcription of the foreign gene; this allows for tighter controls over the expression system, particularly when toxic proteins are generated.

One means of regulation typically employed in mammalian expression systems is based on the bacterial tetracycline operon (Figure 3). Cultured cells containing the plasmid encoding the tetracycline repressor (tetR) are grown in the absence of tetracycline. Binding of the tetR homodimers to the inducible expression plasmid prevents expression of the gene of interest. Only upon addition of tetracycline to the medium will a conformational change in the repressor protein result, permitting induction of gene expression.

A problem often encountered with the establishment of stable cell lines is that overexpression of the protein of interest creates an unfavourable condition for the cell. Studies that required imposed expression of Plks in mammalian cells presented evidence that such a condition can be detrimental to a cell's survival. For example, enforced expression of Plk3 in mammalian cells results in suppressed proliferation and inhibits colony formation, which upon further analysis is attributed to chromatin condensation and apoptosis (Conn *et al*, 2000). Thus, constitutive overexpression of Plk3 would be lethal to the cell and hinder the possibility for further experimentation. Overexpression of Plk4 has been shown to induce the formation of multiple centrosomes, a condition that would eventually give rise to aberrant chromosomal

segregation and contribute to chromosomal instability (Habedanck *et al*, 2005). It is likely that the continued production of Plk4 protein, as would be seen in a stable expression system, would have disruptive effects and greatly compromise the cell's survival. Therefore, a supplementary focus of the present study was to establish stable cell lines that are inducible rather than constitutive for Plk4 expression. This provides an invaluable tool in our ongoing studies.

Fig. 3. Mechanism of repression of the inducible expression vector utilized for stable cell line creation. Brief descriptions of the repressive mechanism are included in the diagram. P_{CMV} indicates the cytomegaloviral promoter that regulates transcription of the repressor gene. TetR represents the tetracycline repressor proteins that bind to the tandem tetracycline operator sites ($TetO_2$) situated upstresam of the gene of interest in the inducible expression vector. Red triangles represent the tetracycline (Tet) molecules that can bind tetR and induce its conformational change. Ultimately, the effect of tetR modification is to derepress expression of the gene of interest.



CHAPTER III

AIMS OF THIS STUDY

To date, no known interacting partners have been identified for Plk4; therefore, to clearly define its function in the cell cycle and in DNA damage pathways still remains an elusive task. This study aimed to investigate putative interacting partners for Plk4 in the hopes elucidating a more precise role of the protein in responding to genotoxic stress and in regulating mitosis. On the basis of the candidate approach, experiments were performed to determine if, in addition to Plk1 and Plk3, Plk4 can also interact with some of the same interacting partners. In addition, a human embryonic kidney cell line was established that stably expresses Flag-Plk4 and Flag-K41M.

CHAPTER IV

MATERIALS AND METHODS

Preparation of competent *Escherichia coli (E. coli)* cells

Glycerol stocks of Top 10 F' Pilus *E.Coli* cells were used to inoculate 10 mL of TYM media in a 50 mL Erlenmayer flask and allowed to grow in a shaking incubator for 16 hours at 37°C. After incubation, 1 mL of the culture was subsequently added to 100 mL of TYM media that was prewarmed in a 500 mL flask. Cultures were again grown at 37°C and ODs_{600} were intermittently taken until a value of 0.5 was attained. After cooling the flasks through gentle agitation on ice, the cultures were transferred to sterile Oakridge tubes and centrifuged at 4°C for 10 minutes at 4000 x g. Subsequently, the supernatant was decanted gently to prevent any disturbance to the pellet. 4 mL of ice-cold TFBII buffer (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM CaCl, 50 mM MnCl2, 15% glycerol) was added to each tube and used to resuspend the pelleted cells through repeated pipetting, all completed while tubes were on ice to ensure a cold environment for the cells. After resuspension, 100 uL of the cells were quickly but carefully aliquoted into sterile microcentrifuge tubes and subjected to flash-freezing in liquid nitrogen. The prepared cells were then stored at -80°C.

Standard Bacterial Transformation

100 uL of Top 10 F' Pilus *E.Coli* cells were transformed with 1 ug of DNA and allowed to sit on ice for 30 minutes. After incubation, the cells were then heat shocked at 37°C for 30 seconds. 500 uL of LB was added to transformed cells, which was then incubated at 37°C for 45 minutes. The sample was spun at 750 x g in a table-top microcentrifuge for 5 min, and pelleted cells were resuspended in 100 uL of LB broth. After plating the cells onto LB-Amp plates using sterile technique, the culture dishes were then incubated at 37°C overnight to permit the growth of transformed colonies.

Boiling mini preps

For a boiling mini prep protocol, a standard bacterial transformation method was first performed using the desired DNA vector. Any colonies that were observed after 18 hrs at 37°C were picked and grown in 2 mL of LB broth containing 100 ug/mL ampicillin overnight. Each 2 mL growth culture was centrifuged at 4000 x g for 5 min to pellet the cells and the supernatant was then carefully decanted. 100 uL of boiling mini prep solution (8% glucose, 5% Triton X-100, 50 mM EDTA, 50 mM TRIS pH 8.0, 10 mg/mL lysozyme) was used to resuspend the pelleted cells, which were then boiled for 30 sec in a hot water bath. Lysed cells were placed on ice for 30 minutes, and then spun in a cold microcentrifuge for 15 min at 4000 x g. The supernatant, which contained the plasmid DNA, was then transferred to a fresh Eppendorf tube.

Standard Transfection protocol and SDS-PAGE

Human embryonic kidney (HEK-293) cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) (*Sigma)* containing 10% fetal bovine serum, 2 mM L-glutamine, 1% Pen-Strep (*Sigma*), 250 ug/mL Gentamacin (*Sigma*), 250 ug/mL Fungizone (*Sigma*), and stored in a 37°C and 5% CO_2 humidified incubator. The day before transfection, cells were trypsinzed and counted on a hemocytometer, then seeded onto 10 cm tissue culture dishes at a density of 1 x 10^6 cells per plate. The next day, fresh, complete medium was replenished onto plates and 6 ug of Flag-Plk4 and its various constructs were transfected using Effectene™ (*Qiagen)* according to the manufacturer's protocol; non-transfected cells were included as a negative control. After 18 hrs, cells were lysed with 1 mL lysis buffer (50 mM Tris-Cl, 100 mM NaCl, 500 mM EDTA, 1% Triton-X) on ice for 20 min. Lysates were spun in a microcentrifuge at 4000 x g for 20 min at 4°C to remove any cellular debris. 1X loading buffer containing 5% β-mercaptohethanol was added to

20 uL of the lysates, and the samples were boiled for 5 min; 20 uL of the sample was loaded onto an 8% protein gel and subjected to SDS-PAGE for 90 min at 115 V.

Western blot analysis

After SDS-PAGE, the proteins were then transferred onto PVDF membrane using a semi-dry method at 12 V for 45 min, and the membranes were then blocked with Tris-buffered Saline and Tween (TBST) buffer for 1 hr at room temperature with gentle agitation. Blots were incubated with Anti-FLAG M2 monoclonal antibody (mouse) in TBST buffer (1:10,000) for 1 hr at room temperature, washed three times for 5 min each with TBST, and probed with anti-mouse horseradish peroxidase as a secondary antibody (1:60,000) for 45 min. Membranes were again washed 3 times and incubated with 1 mL SuperSignal West Femto Maximum Sensitivity Substrate (*Pierce)* dilution for 5 min to allow for reaction between the anti-mouse horseradish peroxidase and its substrate. Proteins were then visualized by chemiluminesence.

Stripping of Western Blots for re-probing

When a blot was required to be re-probed with a different primary antibody, a stripping protocol was used to remove any bound primary and secondary antibodies from the initial Western Blot. After washing the blot three times with TBST for five minutes each, 20 mL of stripping buffer (100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris HCl at a pH of 6.8) pre-warmed to 50 °C was added to the blot, which was then incubated with gentle agitation for 30 min at 50 °C. The stripping buffer was then discarded, and the blot was washed 3 times with warm TBST (50 °C) in 10 minute intervals. Blots were then ready to be probed with the appropriate antibody.

Creation of Stable Cell Lines

Site-directed mutagenesis

As an initial step in the creation of a tetracycline-inducible stable cell line, the Stratagene QuikChange site-directed mutagenesis protocol was used to introduce an *AflII* site into constructs encoding either a wildtype Flag-Plk4 or a kinase-dead FLAG-K41M protein. PrimerX, a web-based primer-design program (Lapid, 2002), was utilized to design the introduction of the *AflII* site (CTTAAG) upstream of the open reading frame that encoded the FLAG-tagged protein. The site was introduced in a manner that would allow the entire coding sequence, including the FLAG epitope, to be shuttled into the inducible expression vector PCDNA4/TO (*Invitrogen*). The forward and reverese primers designed are listed as follows respectively: 5'-GAGGTCTATATAAGCAGAGCTCGCTTAAGGAACCGTCAGAATTAACCATGGAC-3' (designated 'ForPFU') and 5'-GTCCATGGTTAATTCTGACGGTT CCTTAAGCGAGCTCTGCTTATATAGACCTC-3' (designated 'RevPFU'). Briefly, the mutagenesis reaction was setup as follows: each reaction contained 50 ng of Plk4 template DNA, 125 ng of forward and reverse primer, 1 X PFU polymerase buffer (*Stratagene)*, 100mM dNTP (dATP, dGTP, dTTP, dCTP), and 2.5 U of cloned *Pfu* DNA polymerase in a total volume of 50 uL. PCR cycling conditions were performed as follows: segment 1, 1 cycle at 95°C for 30 sec, and segment 2, 12 cycles of the following: 95 °C for 30 sec, 55°C for 1 min, and 68 °C for 13 min. After the PCR reaction was complete, 10 U of DpnI restriction enzyme (*NEB*) was added to the amplified samples in order to digest any methylated, nonmutated parental DNA. Samples were pipetted up and down gently, spun at 4000 x g for 1 min, and then placed at 37°C for 1 hour.

Various aliquots of the *DpnI* (*NEB*) digested PCR product (5 uL, 10 uL, and 20 uL) were individually transformed into Top 10 F' Pilus *E. Coli* competent cells, which were prepared according to standard transformation protocol. After an overnight incubation individual transformed colonies were carefully picked with a sterile pipette tip and singly dispensed into a 2 mL culture of LB-Amp containing 100 mg/mL of ampicillin in a round bottom polypropylene

tube. Cultures were placed in a shaking incubator at 37 °C for 18 hrs and plasmid DNA was isolated from grown samples using the standard boiling mini prep protocol, as described previously. To analyze the DNA for successful mutagenesis, *AflII* (*NEB*) and *EcoRI* (*NEB*) restriction enzymes were used to digest 1 ug of each DNA sample for 2 hours at 37°C. The reaction mixtures were then analyzed on a 1% agarose gel to confirm that the *AflII* restriction site had been properly introduced. Any positive clones were then propagated by transforming 5 ug of the remaining boiling miniprep sample as per standard procedure. Plasmid DNA was then isolated using the QiaPrep Spin MiniPrep Kit from Qiagen according to the manufacturer's instructions.

Cloning of Flag-Plk4 constructs into PCDNA4/TO vector

To extricate the gene of interest into the inducible expression vector, 5 ug Flag-tagged Plk4 construct (with *AflII* site introduced) and PCDNA4/TO vector (*Invitrogen*) were each digested with 1 U each of *AflII* + *SmaI*, and *AflII* + *EcoRV* (*NEB*), respectively, at 37°C for 2 hrs according to standard methods. Samples were placed on the heating block at 80°C for 20 min to heat inactivate all restriction enzymes used in the reaction. 400 ng from each digest reaction were subsequently loaded onto a 1% agarose gel and separated through electrophoresis to confirm complete digestion. The remaining digested sample was used to perform two sets of ligation reactions: 150 ng of Flag-Plk4(*AflII*) [or Flag-Plk4-K41M(*AflII*)] digest mixture was combined with 50 ng of the PCDNA4/TO digest to make a 3:1 ratio of insert:vector. Similarly, a 1:1 ratio of insert:vector was also tested using 50 ng of each DNA component. The reaction also contained 1X T4 DNA Ligase buffer and 1 U of T4 DNA ligase (*NEB)* in total volume of 20 uL, and was allowed to incubate at room temperature O/N. After approximately 18 hr of incubation, T4 DNA ligase was heat-inactivated at 65°C for 20 min. 1 U each of *BlpI* (*NEB*) and *PstI* (*NEB*) restriction enzymes were added to each ligation mixture to eliminate any remaining vector backbone, and the samples were incubated at 37°C for 2 hrs. 100 uL of Top 10 F' Pilus *E.Coli* competent cells

were transformed with the ligated samples according to standard protocol and plated onto LB-Amp plates for O/N incubation at 37°C. Plasmid DNA from any observed colonies was isolated through a boiling mini-prep procedure, then digested with *ApaI* and *EcoRI* restriction enzymes to screen for positive clones. The plasmid DNA from those positive clones were then used for transformation and subsequently isolated through the QiaPrep Spin MiniPrep Kit from Qiagen. To confirm proper ligation of Flag-Plk4 or Flag-K41M into PCDNA4/TO vector, 300 ng of plasmid DNA samples from each successful clone, as determined by restriction digest, were sent to ACGT (*Toronto, ON)* for sequencing. Reactions were performed using standard forward CMV and reverse BGH primers, as well as customized primers designed specifically to sequence critical regions of the construct. (Appendix A).

Transient transfection and induction of expression vector containing Flag-Plk4 and Flag-K41M

T-REX™-293 cells (*Invitrogen)* were maintained in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% Pen-Strep, 250 ug/mL Gentamicin, 250 ug/mL Fungizone, and 5 ug/mL of blasticidin and stored in a 37°C and 5% CO_2 humidified incubator. Cells were then transfected with either PCDNA4/TO Flag-Plk4 or PCDNA4/TO Flag-K41M according to standard transfection protocol as stated above. 24 hrs post-transfection, tetracycline at a final concentration of 100 ng/mL was added to the cells to induce expression of PCDNA4/TO-Flag Plk4; a plate of non-induced, transfected cells was also maintained as a control. After 18 hrs, cells were lysed and subjected to SDS-PAGE and Western blot analysis as previously described.

Determination of selective antibiotic sensitivity

Optimal levels of Zeocin antibiotic required to eradicate untransfected host cells were determined by subjecting cells to a range of concentrations. Seven plates of T-REx™-293 cells were each seeded to 25% confluency and maintained in complete medium containing either 0, 50,

125, 250, 500, 750, or 1000 µg/ml Zeocin™. Media was replenished every 3 days and cell survival was monitored every 24 hours. After seven days, the number of viable cells maintained in each Zeocin test plate was counted, and the appropriate concentration of antibiotic that inhibited cell proliferation was determined.

Selection of stable integrants

Once successful transfection and induction of the PCDNA4/TO-Flag hPlk4 construct was attained, the protocol was repeated to begin selection of the stable integrants and to establish a stable cell line. Again, T-REx™-293 cells were maintained in complete DMEM containing 5 ug/mL blasticidin but without Zeocin. 24 hours following transfection, the cells were washed once with Hank's Balanced Salt Solution (HBSS) and fresh medium was added. 48 hours after transfection, the cells were either split and seeded to 25% confluency in complete media now containing 250 ug/mL of Zeocin, or were just replenished with the selective media depending on cell confluency. Fresh, selective media was added every three days to maintain antibiotic potency. When distinct cellular foci were observed, these colonies were scraped, trypsinzed, and transferred to 6.4 mm cell culture plates and allowed to proliferate. These original foci were subsequently expanded onto 14 mm, 31 mm, and eventually 100 mm dishes. 100 ng/mL of Tet was added to the stably-transfected cells to induce transcription of the Flag-Plk4 and Flag-K41M genes and assayed for gene expression; a plate of non-induced stable transfectants was included in the analysis. Positive clones were then propagated to make frozen stocks.

Co-immunoprecipitation of Plk4 with putative interacting proteins

6 ug of Flag-Plk4 constructs were transfected into HEK-293 cells plated at a density of 1 x 10^6 cells per 100 mm dish. Various Flag-tagged constructs were tested, including wildtype (Flag-Plk4), kinase dead (Flag-hK41M and Flag-154N), kinase active (Flag-T170D), polo box

only (Flag-PB), Plk4 minus polo box (Flag-ΔPB), and cryptic polo box only (Flag-R1) constructs. Flag-hYVH1, a human protein phosphatase[PC4], was used as a negative control. Approximately 18 hrs post-transfection, cells were lysed and 1 mg of protein lysates was incubated with 1 ug of the antibodies listed in *Appendix B*. 60 uL of a 1:5 slurry of Protein G Sepharose 4 Fast Flow beads (*Amersham Biosciences)* diluted in TNT buffer (50mM Tris-Cl, 100 mM NaCl, 1% Triton-X) was added to each sample and incubated for 45 min with gentle rocking. Samples were spun at 4000 x g in a cold microcentrifuge for 1 min, and the beads were washed three times with TNT buffer. Samples were boiled for 5 min following the addition of loading buffer, and were separated through SDS-PAGE. Western blot analysis was performed using anti-Flag primary antibody diluted in TBST (1:10,000) for 1 hr at room temperature, and anti-mouse or anti-rabbit (1:60,000) secondary antibody for 45 min. Proteins were subjected to chemiluminesence for visualization and analysis.

CHAPTER V

RESULTS

Chk2 interacts with Plk4

On the basis that Chk2 is a physiological substrate of Plk1 and Plk3, this study attempted to investigate if Plk4 shares a physical association with this DNA-damage checkpoint kinase. Tsetkov *et al* (2003) first reported a functional interaction between Plk1 and Chk2 based on their overlapping subcellular localization patterns to the centrosomes and midbody during mitosis. Their data showed that Plk1 overexpression in the absence of genotoxic stress enhanced T68 phosphorylation of Chk2 at these critical mitotic structures, pointing to a role of Chk2 outside of the context of DNA damage. Additionally, Bahassi and co-workers (2006) revealed that Chk2 is phosphorylated at S73 and S62 residues by Plk3 as well, a modification that facilitates Chk2 phosphorylation by ATM in response to DNA damage (Bahassi *et al*, 2006). [H5] To offer a preliminary assessment of Plk4's association with Chk2, full length, truncated, and mutant forms of human Flag-Plk4 were transiently overexpressed in HEK-293 cells. Specifically, Flag-Plk4, Flag-K41M, Flag-154N, Flag-T170D, Flag-ΔPB, Flag-R1, Flag-PB, and Flag-hYVH1 constructs were utilized for transient transfection, all of which are depicted in Figure 4 (except Flag-hYVH1). A more thorough characterization of the Plk4 mutant and deletion constructs will be provided in later sections. Originally, mouse fibroblast 3T3 cells were utilized as an expression system for the Plk4 constructs, however, were observed to produce lower transfection efficiencies relative to the HEK-293 cells (data not shown). Hence, future experiments requiring overexpression of Plk4 were performed in the human embryonic kidney cell line. Following transfection and the allowance for expression of the Flag-tagged constructs mentioned above, cells were lysed and a small fraction of the collected proteins, both endogenous and overexpressed, were separated by SDS-PAGE; non-transfected cells were included as a negative control. Subsequently, Western blot analysis with an anti-Flag M2 monoclonal antibody (mouse) (*Sigma)* was performed to

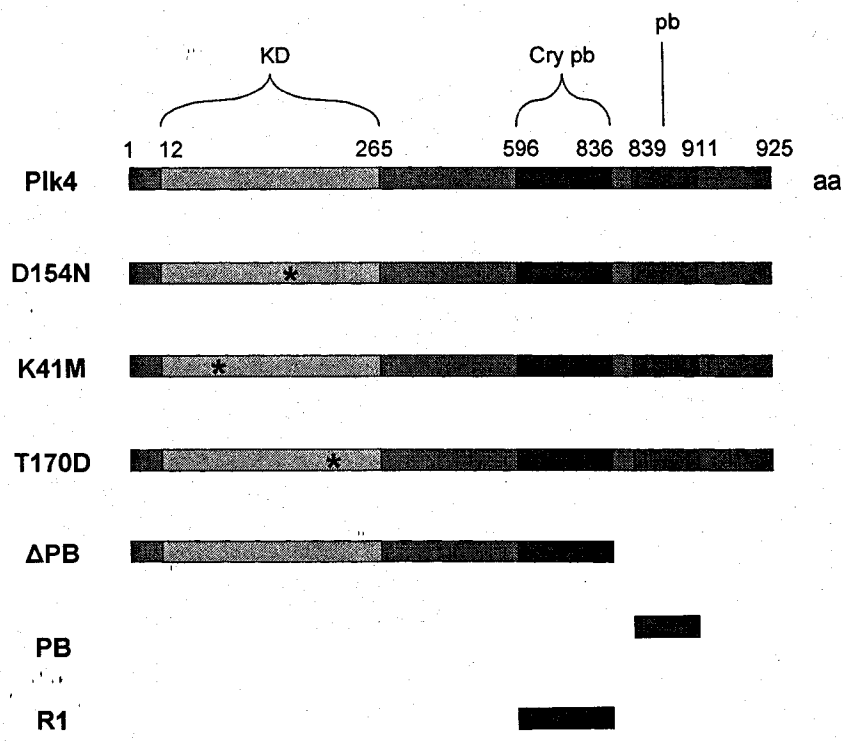
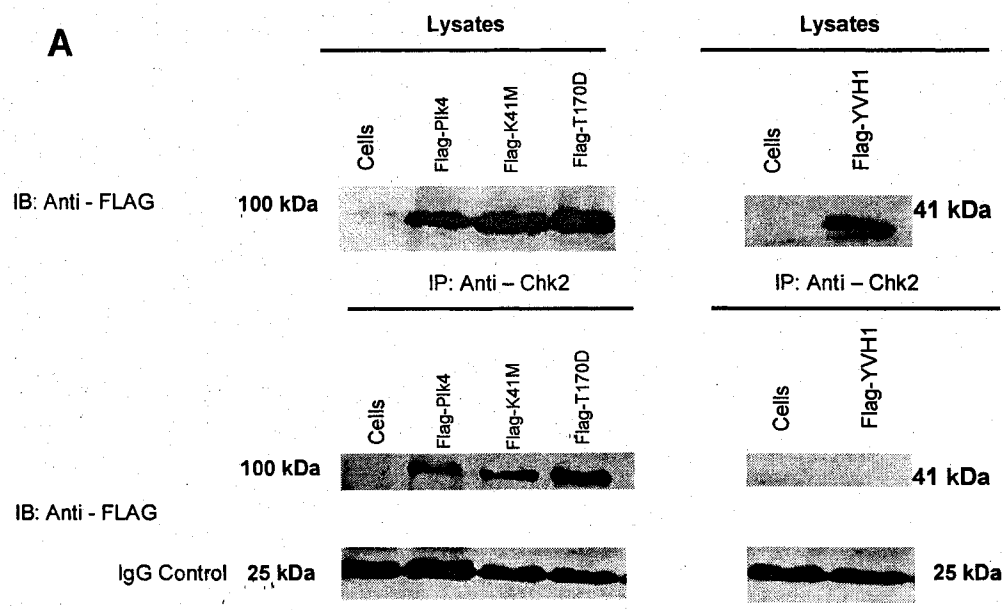
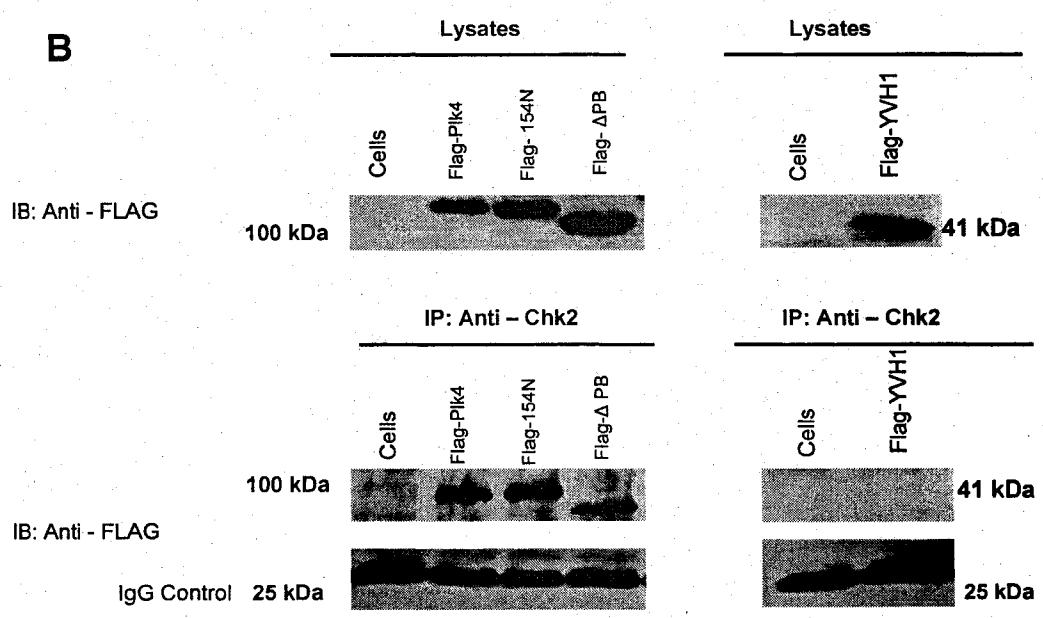


Fig. 4. Schematic representation of Plk4 constructs used in co-immunoprecipitations. Plk4 consists of a Ser/Thr kinase domain (KD) in the N-terminus, a polo-box region (pb) in the C-terminus, as well as a region defined as a 'cryptic pb' (cry-pb) upstream of pb. Cry-pb and pb are said to participate in the self-association of Plk4. The constructs were expressed individually in HEK-293 cells as fusion proteins in frame with an N-terminal with a 3X Flag-tag (*Sigma)*. Numbers indicate the amino acid position defining the specified region. Asterisks represent the relative position of the single amino acid changed to generate a kinase-defective (D154N, K41M) or kinase-activating (T170D) mutant.

confirm that transient transfection was successful. The presence of a positive band of the appropriate size in the lanes corresponding to transfected cells, verified that consistent and efficient expression of the Flag-tagged protein had occurred. (Figure 5A, upper panel). No band was detected in the lane containing non-transfected cells, confirming that the observed bands in fact depicted the overexpressed protein and not non-specific, endogenous protein (Figure 5A, upper panel, 'cells' lane). Upon verification that the various Flag-tagged proteins were indeed expressed at effective levels, co-immunoprecipitation assays with the remaining lysate sample were performed using antibodies specific for the putative interacting partners. The rationale for this approach was that that if any Plk4 protein normally interacts with a candidate partner in cells, it could theoretically remain in contact with Plk4, be co-immunoprecipitated, thus pulling Plk4 down with it, and later detected upon immunoblotting with an anti-Flag antibody. Co-immunoprecipitation experiments were performed using an anti-Chk2 monoclonal antibody (mouse) (*Sigma.)* Monoclonal antibodies were utilized when possible in co-immunoprecipitation experiments to ensure targeting of only a single epitope, thereby decreasing the chances of non-specific binding. In addition, precipitated proteins were washed at minimum three times with nonionic-detergent–containing buffers (1% Triton-X) to obtain the cleanest sample of the immunoprecipitated proteins. Western blot analysis with an anti-Flag M2 monoclonal antibody *(Sigma)* confirmed the existence of an interaction between Chk2 and various Flag-tagged Plk4 constructs. Specifically, full length Flag-Plk4, Flag-D154N and Flag-K41M (both kinase defective mutants) as well as Flag-ΔPB (which lacks the conserved PB in the C-terminus) all co-immunoprecipitate with Chk2 (Figure 5A &B, lower panels). Flag-T170D, which contains a kinase activating mutation in the T-loop, also displays a physical interaction with Chk2 (Figure



. **Fig. 5. Co-immunoprecipitation of Chk2 and Plk4.** A-C. HEK-293 cells were transiently tranfected with the expression plasmid encoding Flag-tagged domain specific Plk4 constructs. 18 h post-transfection, whole cell lysates were prepared and immunoprecipitated (IP) with monoclonal anti-Chk2 antibodies (*Sigma*). Whole cell lysates and IPs were immunoblotted (IB) with monoclonal anti-Flag antibodies to confirm expression of the Flag-tagged proteins. Flag-hYVH1 was used as a negative control. D. The blot was stripped and re-probed with an anti-Chk2 antibody to confirm equal levels of protein in each lane. Light chain immunoglobulins (IgG) are shown to verify that equal amounts of antibody were added to each sample



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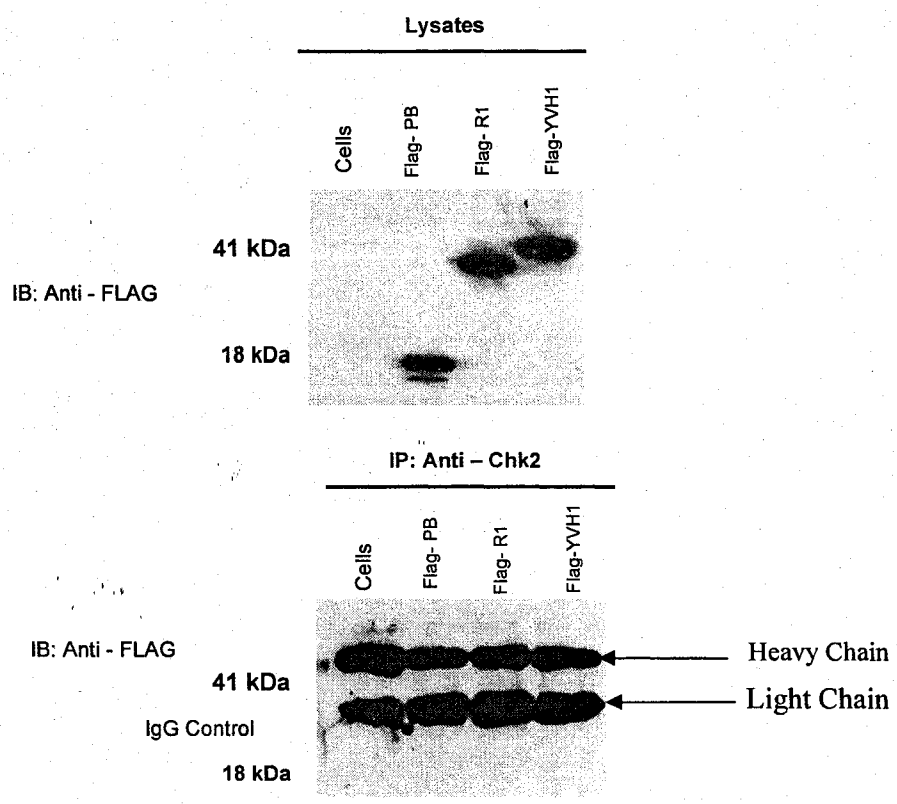

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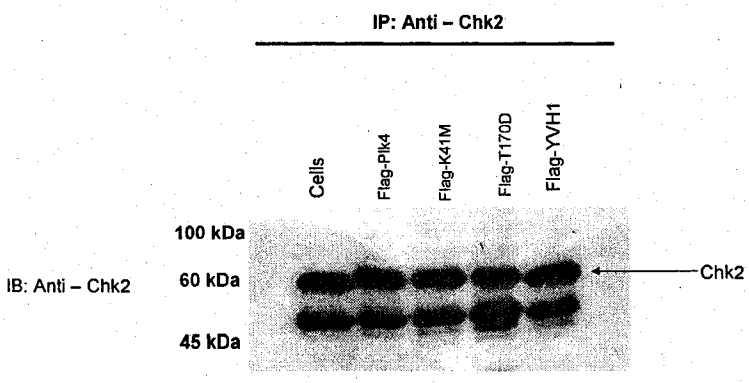


5A, lower panel). In other Plk members, the polo box has been characterized as a binding region for substrate proteins, enabling proper subcellular localization of the protein (Lowery *et al*, 2004; Elia *et al*, 2003). Therefore, in order to determine whether the polo box region is critical for interaction in Plk4, cells were transiently transfected with Flag-PB, which encodes only the polo box region of Plk4. Immunoblotting confirmed that Chk2 does not interact with the polo box of Plk4. (Fig. 5C, lower panel, 'Flag-PB' lane). Similarly, Flag-R1, which contains the region encoding the cryptic polo box of Plk4, also does not co-immunoprecipitate with anti-Chk2 (Figure 5C, lower panel). To strengthen the findings that the observed co-immunoprecipitations indeed represented *bona fide* physical interactions between Chk2 and Plk4, the experiments were performed at minimum three times, and each repeat was shown to produce identical results to what was originally observed (replicate experiments not shown). In addition, a Flag-tagged protein unrelated to Plk4 called Flag-hYVH1 was incorporated as a negative control. hYVH1 is a 41 kDa dual-specificity protein-tyrosine phosphatase that is the human counterpart to YVH1 protein found in the budding yeast *Saccharomyces cerevisiae* (Muda *et al*, 1999). The Flag-tagged hYVH1 construct was a generous gift to the Hudson Lab by Dr. Otis Vacratsis (Dept. of Biochemistry, University of Windsor, ON). Flag-hYVH1 was also overexpressed in HEK-293 cells and subjected to co-immunoprecipitation assays with anti-Chk2 antibody but did not show a physical association similar to Flag-Plk4 (Figure 5A-C, 'Flag-hYVH1' lanes). This finding enhances the significance of the interaction observed between wildtype and mutant Plk4 with Chk2, indicating that the antibody was not binding non-specifically or through the FLAG epitope. Furthermore, the blot exhibited in Figure 5A was subject to antibody stripping as described in Materials and Methods, then re-probed with anti-Chk2 antibody at a recommended dilution of 1:8000. This was done to ensure that equal levels of Chk2 protein were present in each sample, thereby eliminating the possibility that the negative results observed in the 'cells only' and 'Flag-hYVH1' lanes were simply due to reduced amounts of precipitated Chk2 protein. After stripping the blot, but prior to re-probing with anti-Chk2, chemiluminesence was performed to ensure that

the stripping protocol was indeed effective and that no remaining signal could be detected (data not shown). Subsequent immunoblotting with anti-Chk2 antibody verified that each lane was loaded with equal levels of immunoprecipitated Chk2 protein, therefore suggesting that the bands originally observed were in fact due to a *bona fide* physical association between Plk4 and Chk2 (Figure 5D).

ATM interacts with Plk4

ATM is an upstream checkpoint kinase that initiates a signaling cascade leading to cell cycle arrest in response to ionizing radiation (IR) (Rotman and Shiloh, 1999). Upon triggering of ATM activity by damage-sensing proteins, the signal is then amplified to downstream[H6] kinases such as Chk1 and Chk2, resulting in their phosphorylation and subsequent activation (Zhou and Elledge, 2000). Van Vugt *et al* (2001) provided evidence that Plk1 kinase activity is reduced in response to adriamycin-induced DNA-damage, and that this catalytic suppression is mediated by ATM. When expressed in ATM-deficient cells, Plk1 was no longer inhibited and its kinase activity remained deregulated. No defined role of Plk4 has thus far been established with respect to the DNA-damage response. This study attempted to provide insight on how Plk4 may participate in signal transduction pathways involving ATM by first examining if these two proteins can physically interact. Whole cell lysates obtained from HEK-293 cells were transiently transfected with various Plk4 constructs and 18 hrs post-transfection an anti-ATM monoclonal antibody (mouse) (*Sigma)* was used in co-immunoprecipitation assays; subsequent immunoblotting with anti-Flag (*Sigma*) confirmed an association between these two proteins. In addition to full-length Plk4, various deletion and point mutants were also overexpressed to help characterize which domains are critical for interaction. Pull down assays revealed that Flag-Plk4, Flag-K41M and Flag-T170D all interact with ATM, as evidenced by their co-immunoprecipitation (Figure 6A, lower panel). Additionally, Flag-D154N and Flag-ΔPB can also interact with endogenous ATM

protein (Figure 6B, lower left-hand panel). Although Flag-PB did not pull down with the anti-ATM antibody, the construct expressing the cryptic polo box of Plk4 (Flag-R1) did exhibit an interaction (Figure 6B, lower right-hand panel). Flag-hYVH1, expressed as a negative control vector, did not co-immunocprecipitate with ATM. (Figure 6 A & B, lower panels)

ATR interacts with Plk4

ATR is a phosphoinositide kinase structurally-related to ATM that also shares common functions in the response pathway to genotoxic stress, such as the phosphorylation and activation of tumor suppressors p53 and BRCA1 (Zhou and Elledge, 2000). ATR, however, appears to mediate repair of lesioned DNA in response to ultraviolet radiation (UV) rather than ionizing radiation (IR), the primary instigator of ATM activity (Van Vugt *et al*, 2001). ATR has also been recognized as a potential suppressor of Plk1 kinase activity following DNA-damage caused by UV irradiation (Van Vugt *et al*, 2001). Evidence showed that Plk1 activity was still attenuated in UV-treated ATM –/– cells, suggesting the presence of ATR was able to assume the inhibitory role. Following the addition of caffeine, however, which blocks activity of ATM/ATR in the DNA-damage response, Plk1 kinase activity was no longer reduced (Van Vugt *et al*, 2001). Given the evident correlation between ATR and Plk1 activity, this study analyzed the possibility of a putative relation between ATR and Plk4. Therefore, to determine if Plk4 can interact with ATR *in vivo,* various constructs encoding wildtype Plk4 as well as mutated and deleted forms, were expressed in HEK-293 cells. Co-immunoprecipitation with a polyclonal anti-ATR antibody (mouse) (*Calbiochem)* was performed on lysates obtained from these cells 18h after transfection. Western blotting with the anti-Flag antibody revealed any potential interactions between ATR and Plk4. Full length Flag-Plk4, kinase dead Flag-K41M, and kinase active Flag-T170D all co-

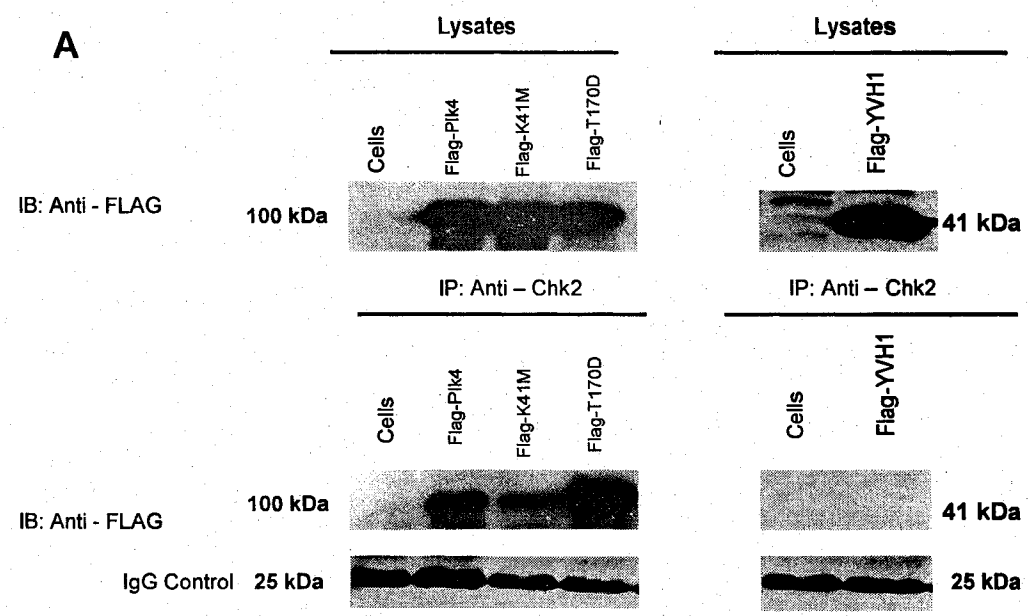
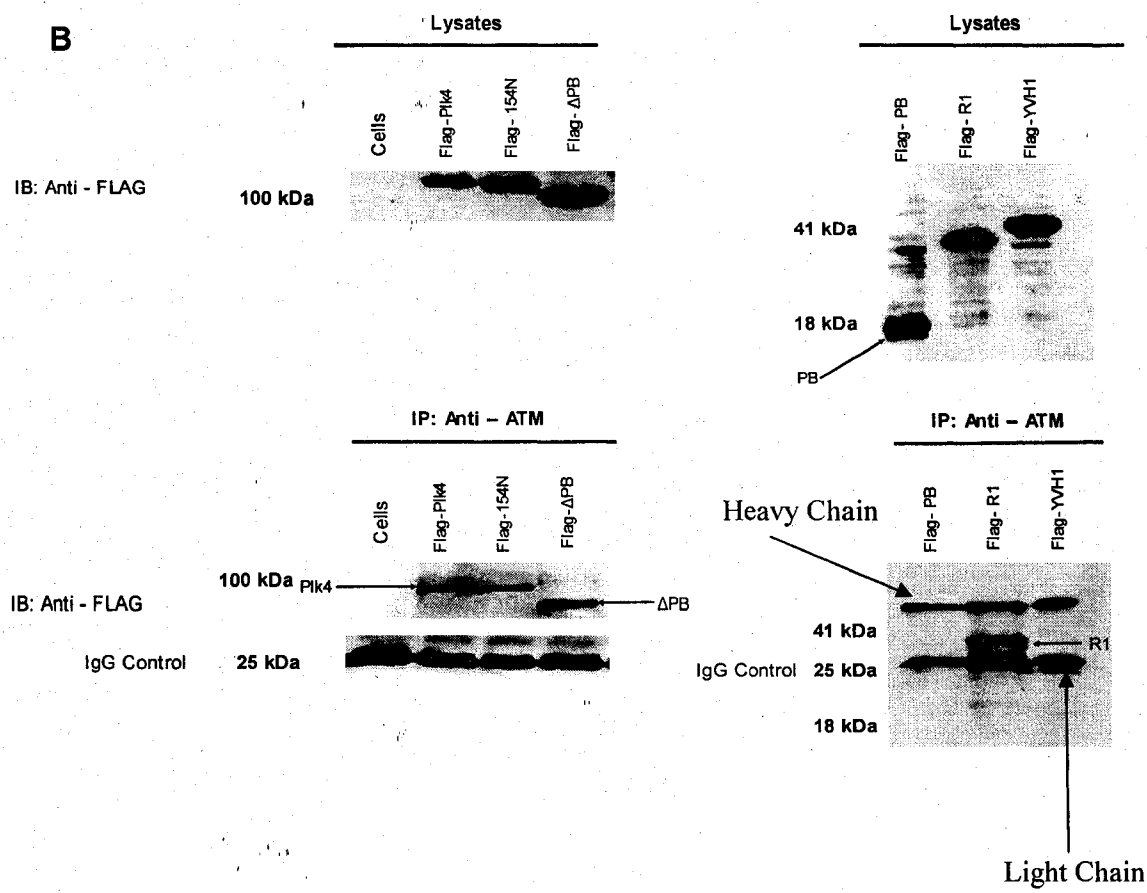


Fig. 6. Co-immunoprecipitation of ATM and Plk4. A & B. HEK-293 cells were transiently tranfected with the expression plasmid encoding Flag-tagged domain specific Plk4 constructs. 18 h post-transfection, whole cell lysates were prepared and immunoprecipitated (IP) with monoclonal an anti-ATM antibody (*Sigma*). Whole cell lysates and IPs were immunoblotted (IB) with a monoclonal anti-Flag antibody to show expression of the Flag-tagged proteins. Flag-hYVH1 was used as a negative control. Light chain immunoglobulins (IgG) are shown to verify equal amounts of antibody were added to each sample



immunoprecipitate with ATR (Figure 7). Steven Petrinac of Dr. Hudson's lab (University of Windsor, ON) followed this experiment by performing co-immunoprecipitations with anti-ATR on lysates obtained from Flag-Plk4, Flag-154N, Flag-ΔPB, Flag-R1, Flag-PB, and Flag-hYVH1 overexpression; his results indicate that Flag-154N and Flag-ΔPB show an interaction with ATR, while Flag-PB, Flag-R1 and Flag-YVH1 do no show up in co-immunoprecipitates with ATR (data not shown).

Plk4 does not interact with the tubulin proteins, BRCA2, or Rb

It has been shown that Plk family members can interact with common proteins resulting in either similar or opposing effects on that substrate's regulation. Many of these targeted proteins have been linked to regulating various aspects of mitotic progression and in mediating DNA-damage responses. Plk1, for example, has been shown to physically associate with α- and β-tubulin, the monomeric subunits that comprise the microtubules of the mitotic spindle, as well as γ-tubulin, which enables the microtubules' nucleation and polar orientation (Feng *et al*, 2001). It was found that Plk1 exists in a stable complex with the tubulins and that during mitosis, can phosphorylate the proteins, suggesting a role in regulating microtubule dynamics and spindle assembly. In addition, the breast cancer susceptibility protein BRCA2, an essential protein in repairing DNA double-strand breaks and preserving chromosomal stability, has also been found to be a substrate of Plk1, and that its phosphorylation is enhanced by mitotic progression but inhibited by DNA damage (Lee *et al*, 2004). Retinoblastoma protein (Rb) is a crucial cell cycle regulator at the G1 restriction point of the cell cycle whose inactivation can lead to uncontrolled proliferation (Wiman, 1993). Gunawardena *et al* (2004) demonstrated that Plk1 is regulated by the Rb tumor suppressor pathway and that activation of Rb pocket proteins p107/p130 suppresses Plk1 activity. Since the tubulins, BRCA2, and Rb have been demonstrated to associate with Plks, each

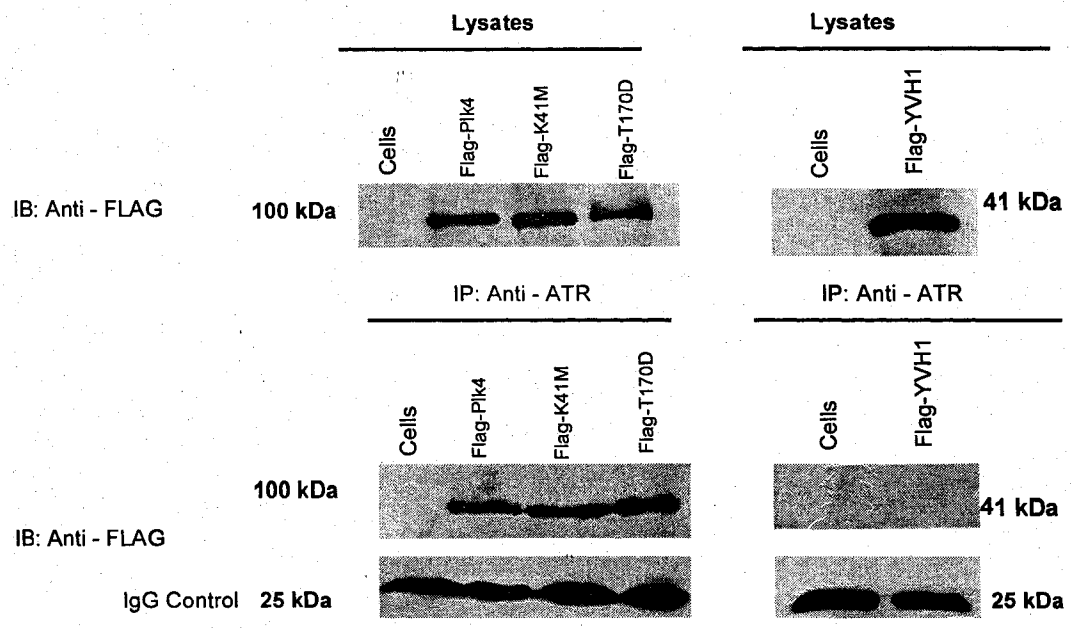


Fig. 7. Co-immunoprecipitation of ATR and Plk4. HEK-293 cells were transiently tranfected with an expression plasmid encoding Flag-tagged domain specific Plk4 constructs. 18 h post-transfection, whole cell lysates were prepared and immunoprecipitated (IP) with polyclonal anti-ATR antibodies (*Calbiochem*). Whole cell lysates and IPs were immunoblotted (IB) with monoclonal anti-Flag antibodies to show expression of the Flag-tagged proteins. Flag-hYVH1 was used as a negative control. Light chain immunoglobulins (IgG) are shown to verify equal amounts of antibody were added to each sample.

with unique consequences, these proteins provide a basis for which to ascertain putative interacting partners for Plk4.

In an attempt to identify potential interacting partners with Plk4, various Flag-tagged constructs encoding the protein as represented in Figure 4 were transiently transfected into HEK-293 cells. Lysates obtained from transient overexpression of full-length, truncated, and mutant forms of Flag-Plk4 were then used in co-immunoprecipitation experiments with the following antibodies: monoclonal anti-α-tubulin from mouse (*Sigma)*, monoclonal anti-*β*-tubulin from mouse (*Sigma),* monoclonal anti-*γ*-tubulin from mouse (*Sigma)*, polyclonal anti-BRCA2 from rabbit (*Sigma)*, and monoclonal anti-Rb from rabbit (*Sigma).* Western blot analysis with anti-Flag M2 monoclonal antibody (*Sigma)* indicated that no physical interaction was detected between any of the Plk4 constructs and these candidate interacting partners (data not shown).

Establishment of a stable cell line for inducible Plk4 expression.

Creation of PCDNA4/TO Flag-Plk4 and PCDNA4/TO Flag-K41M vector

To establish cell lines that stably express wildtype and kinase-dead Plk4, Flag-tagged constructs encoding these proteins first had to be released from their existing vector for eventual sub-cloning into the PCDNA4/TO (*Invitrogen)* expression vector. Plk4 and its kinase-defective counterpart K41M were originally cloned into separate p3XFlag-CMV™-7.1 Expression Vectors (*Sigma)* through a *BamHI* site located within the multiple cloning region (Figure 8A & B). These constructs were designed and created by Dr. Hudson and co-workers (Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, ON). To facilitate the introduction of the Flag-tagged gene encoding wildtype and kinase dead Plk4 into the inducible expression vector, mutagenic primers were first designed against Flag-Plk4 and Flag-K41M. These synthetic, oligonucleotide primers were so designed that they would mutate three nucleotides within the six-base-pair region 'TTTAGT' into '***C***TTA***AG***', which corresponds to an

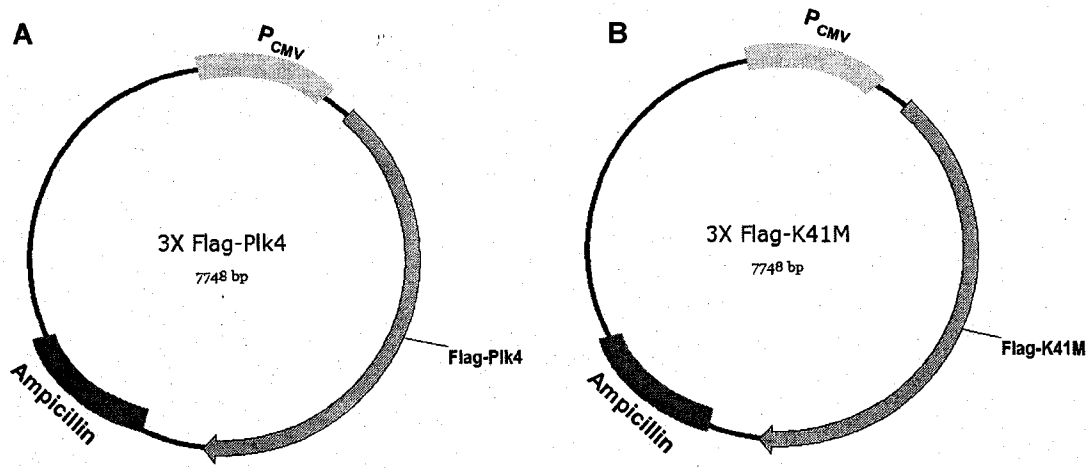


Fig. 8. A & B. Schematic diagrams of 3X Flag-Plk4 and 3X Flag-K41M constructs. Selected key components of the vectors are depicted. Base pairs 877-3999 (red arrow) represent the region encoding Flag-Plk4 (A) (wildtype) or Flag-K41M (B) (kinase-dead) protein. P_{CMV} is a cytomegalovirus promoter that regulates transcription of the Flag-Plk4 and Flag-K41M genes. The ampicillin gene confers antibiotic resistance to the vector allowing for its selection.

AflII restriction site (Figure 9A). *PfuTurbo® DNA* polymerase and the QuikChange Site-Directed Mutagenesis method (*Stratagene*) were utilized in a polymerase chain reaction (PCR) that would extend the mutagenic primers and replicate the parental plasmid strand. After being subject to PCR, the product was digested with *DpnI* enzyme, which specifically recognizes methylated, parental DNA and thus enables the selection of newly-synthesized, mutated DNA (Nelson and McClelland, 1992). It is worthwhile to mention that the *AflII* site was introduced 25 base pairs upstream of the 'Met' start site of the 3X Flag epitope in Flag-Plk4 and Flag-K41M vector (Figure 10). This was done to ensure that Plk4 and K41M retained the Flag tag when released from their original vectors, enabling them to be utilized in future experiments that may require immunoprecipitation of the protein with a commercially-available antibody after sub-cloning; PCDNA4/TO inducible expression vector itself does not encode a fusion tag that may be exploited in such a manner. Once the replicated DNA containing the *AflII* site was selected for, it was transformed into *E. coli* cells in order to propagate the vector, which was then purified through a standard boiling mini prep procedure. Restriction analysis of the DNA with *AflII* and *ApaI* enzyme (*NEB)* produced a 3221 bp and 4925 bp digest product, verifying the *AflII* site was properly introduced (Figure 9B). Following confirmation that the new site was indeed present, the Flag-Plk4(*AflII*) and Flag-K41M(*AflII*) vectors were again propagated and purified through a plasmid maxi prep kit (*Qiagen)*. The resultant plasmid DNA was subsequently cut with *AflII* and *SmaI* in order to extricate the Flag-tagged genes. The fragments were then ligated into the inducible expression vector PCDNA4/TO (*Invitrogen)* that would eventually be used for establishing the stable cell lines; the multiple cloning site contained recognition sequences for *AflII* and *EcoRV*, which were both exploited to facilitate the introduction of the newly-released Flag-Plk4(*AflII*) and Flag-K41M(*AflII*) segments (Figure 11). Ligation reactions were performed in ratios of 3:1 and 1:1 of insert:vector to maximize the potential of successful cloning. After transformation of the ligation mixtures, plasmid DNA isolated from numerous colonies was subjected to restriction digest with *ApaI* and *EcoRI* enzymes; successful clones were found to

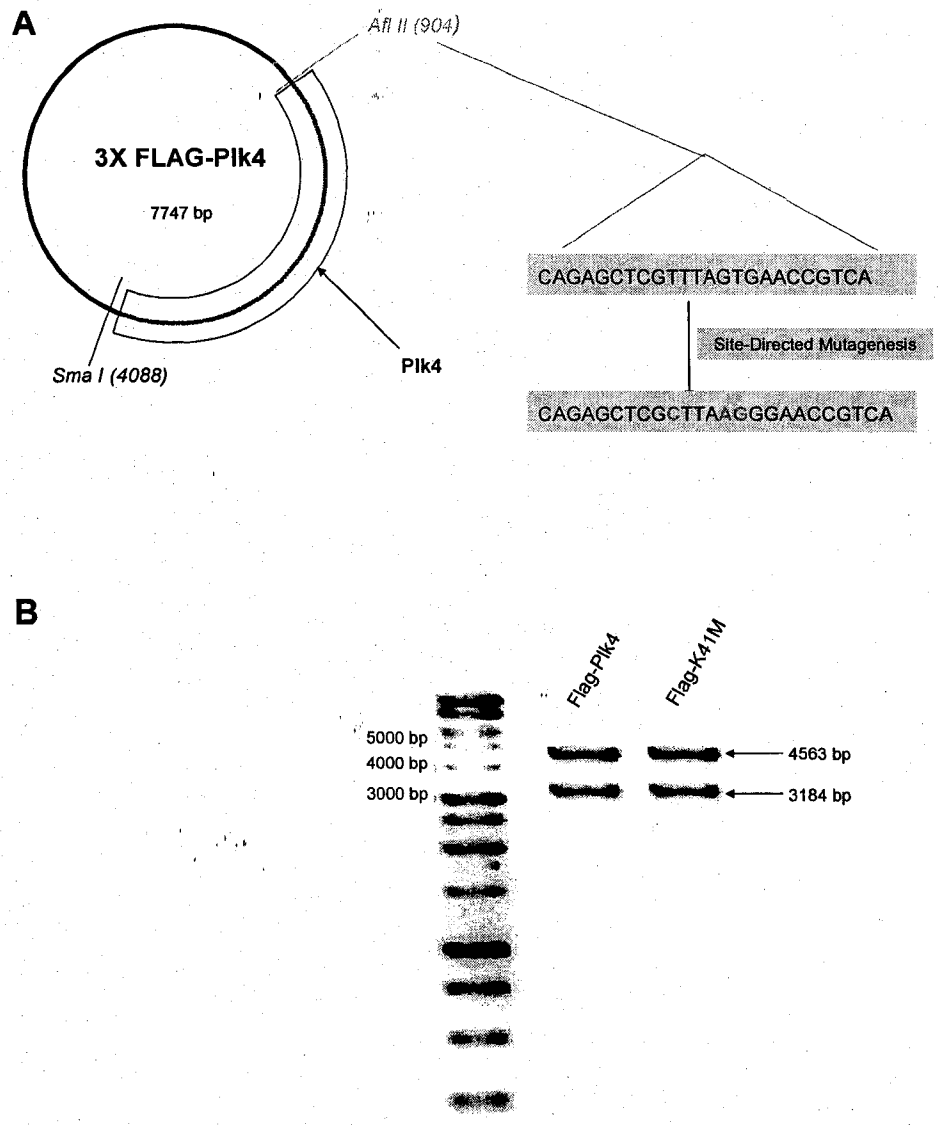


Fig. 9A &B. A, Schematic diagram of 3X Flag-Plk4 vector with *AflII* recognition site. The 3X Flag vectors (*Sigma*) encoding either Plk4 or K41M (kinase dead) were subjected to site-directed mutagenesis using Pfu polymerase (*Stratagene*). An *AflII* restriction site (CTTAAG) was introduced at position 904 of the Flag vector to facilitate the subcloning of Flag-Plk4 or Flag-K41M into the inducible expression vector PCDNA4/TO (*Invitrogen*). The position of the *SmaI* restriction site is also indicated and was used, in addition to the *AflII* site, for diagnostic digests of the PCR product. The correct restriction digest yielded a 4563 bp and a 3184 bp product.

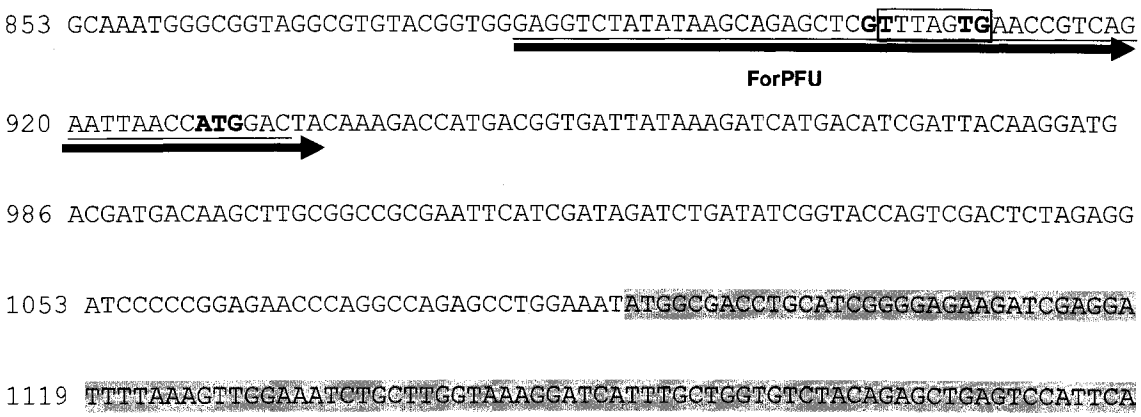


Fig. 10. Partial nucleotide sequence of 3X Flag-Plk4. The region corresponding to positions 853-1185bp of the 3X Flag-Plk4 construct are represented. The thick, bolded arrow indicates the binding region of only the forward mutagenic primer 'ForPFU' utilized in the QuikChange Site-directed mutagenesis reaction (*Stratagene*) (Reverse primer not shown). The nucleotides bolded in red correspond to the region targeted for mutation, transforming the six-base pair region (boxed area) into an *AflII* restriction site. Highlighted yellow is the ATG start site of the 3X Flag epitope, indicating its close proximity to the intended *AflII* restriction site to be utilized for extricating the Flag-Plk4 and Flag-K41M gene for sub-cloning. The region shaded grey indicates the initial region encoding the Plk4 protein. Sequence is read 5' →3'.

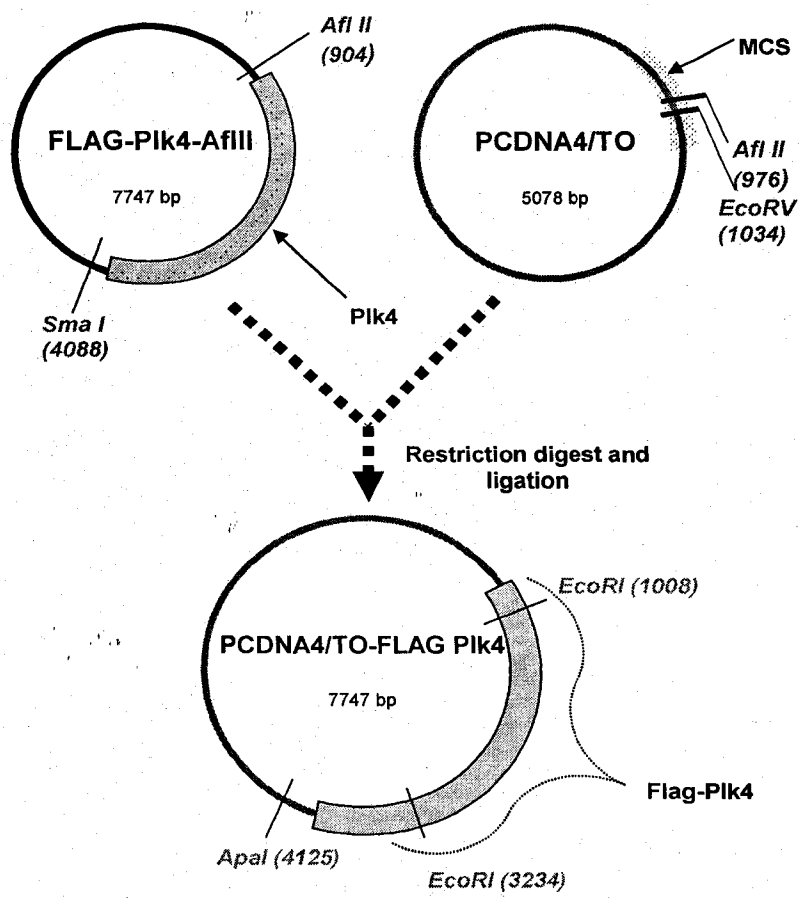


Fig. 11. Schematic representation of the subcloning of Flag-Plk4-*AflII* into the PCDNA4/TO vector. Restriction sites in red indicate those used for subcloning reactions; sites in green represent those used for diagnostic purposes. Numbers in brackets indicate the relative positions of the restriction sites on the vector.

generate a 5089 bp, 2226 bp, and 891 bp band (Figure 12), indicating that Flag-Plk4 and Flag-K41M were properly ligated in frame upon cloning. Samples from both clones were then sent to ACGT Corporation (Toronto, ON) for sequencing of the entire vector; results showed that both Flag-Plk4 and Flag-K41M were cloned in proper frame with the PCDNA4/TO vector (Figure 13).

1) Transfection of PCDNA4/TO-Flag-Plk4 and PCDNA4/TO-Flag-K41M

The newly-synthesized PCDNA4/TO-Flag-Plk4 and PCDNA4/TO-Flag-K41M vectors were then transiently transfected into the T-Rex™ 293 cells (*Invitrogen)*, which stably express a PCDNA6/TR vector (Figure 14). The plasmid contains a gene that encodes a tetracycline repressor (tetR), which is under the control of a CMV promoter, as well as a gene for blasticidin antibiotic resistance . When cultured in the presence of blasticidin-containing media, the T-Rex™ 293 cells maintain the PCDNA6/TR vector and continually express tetR. Because the PCDNA4/TO vector contains two tetracycline operator 2 (TetO2) sites within the human cytomegalovirus immediate-early (CMV) promoter, expression of the gene of interest (ie. Flag-Plk4 and Flag-K41M) can be repressed by binding of tetR protein expressed from PCDNA6/TR (Yao *et al.*, 1998). Transfected cells were grown in the presence of Zeocin™ antibiotic (*Invitrogen)* in order to select for successful integrants of the inducible expression vector. Zeocin™ is a broad-spectrum antibiotic derived from a family of related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces,* and exhibits strong toxic effects against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989). In this study, Zeocin™ is used as a selection agent for non-resistant cells, thus allowing propagation of those cells that effectively retained the PCDNA4/TO-Flag Plk4 and PCDNA4/TO-Flag-K41M vectors. A Zeocin sensitivity assay was performed to determine the minimum concentration needed to eradicate untransfected cells, and was found to be 250 μg/mL. Sensitive cells exhibited abnormal morphology upon exposure to Zeocin, as well as an increase in size, and the formation of large vesicles and the appearance of

holes within the cytoplasm. Prolonged Zeocin exposure (~ 7-10 days) caused any sensitive cells to eventually disintegrate due to a complete breakdown of cellular structures such as the endoplasmic reticulum, Golgi apparatus, and plasma membrane. Isolated colonies of those stable transfectants were then picked and subcultured onto 6.4 mm plates, and eventually propagated onto 14 mm, 31 mm, and 100 mm dishes. Cells required passaging every 3-5 days. 17 clones of PCDNA4/TO-Flag-Plk4 and 23 clones of PCDNA4/TO-Flag-K41M were competent to reach the 100 mm dish stage. Before expression of PCDNA4/TO-Flag-Plk4 and PCDNA4/TO-Flag-K41M could be quantified, the CMV promoter region controlling transcription of both genes' needed to be derepressed. Addition of tetracycline (Tet) to the medium would cause the TetR homodimers that were bound to the operator sites of the expression vector to change conformation. Upon modification of the repressor protein, it would no longer be able to block expression of the Flag-Plk4 and Flag-K41M gene, thus allowing for their transcription to occur. Initially, 100ng/mL of Tet was added to the media of each of the clones to induce gene expression, and incubated for 18 hrs prior to lysing cells. Proteins obtained from lysed samples were separated through SDS-PAGE and subjected to immunoblotting. Of the 40 clones that were tested in total for both wildtype and kinase-dead Plk4, two clones for each construct were chosen (designated Plk4-E and Plk4-F; K41M-B and K41M-S) that effectively produced a protein of ~100 kDa, corresponding to the protein size of Flag-Plk4 and Flag-K41M (Figure 15, upper panel). Non-transfected (+Tet and –Tet) as well as transfected (-Tet) T-Rex™ 293 cells were used as negative controls and in fact did not express a 100 kDa protein that would indicate the presence of Flag-Plk4 or Flag-K41M. These results provide further validity that the observed proteins in the transfected, induced lanes were Flag-Plk4 and Flag-K41M. Furthermore, immunoprecipitation of the lysates and subsequent immunoblotting with monoclonal anti-Flag M2 antibody (mouse) *(Sigma)* confirmed the observed bands were in fact the desired Flag-tagged protein and not a non-specific band coincidentally running at the same size (Figure 15, lower panel). After the

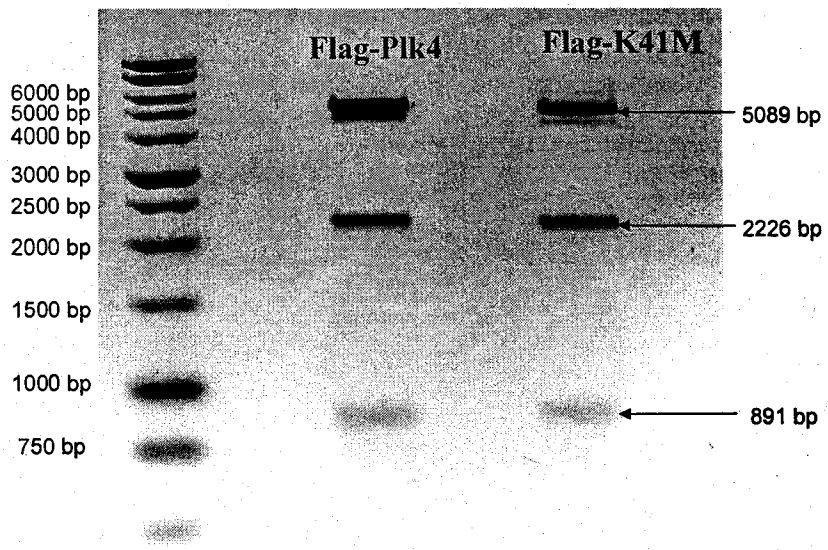


Fig. 12. Diagnostic restriction digests of ligated PCDNA4/TO-Flag-Plk4 and PCDNA4/TO-Flag-K1M plasmid DNA. After the introduction of the *AflII* restriction site, Flag-Plk4 and Flag-K41M were each digested with 1 U of *AflII* and *SmaI (NEB),* while PCDNA4/TO was subjected to *AflII* and *EcorV (NEB)* digestion. Ligation reactions in both 3:1 and 1:1 ratios of insert:vector were tested and subsequently transformed into *E. coli* cells. Plasmid DNA was isolated and digested with *ApaI* and *EcoRI (NEB)* to confirm proper ligation of Flag-Plk4 and Flag-K41M into the inducible expression vector. Digested products were run on a 1% agarose gel, which indicated the presence of a 5089 bp, 2226 bp, and 891 bp fragment for each vector, as would be expected.

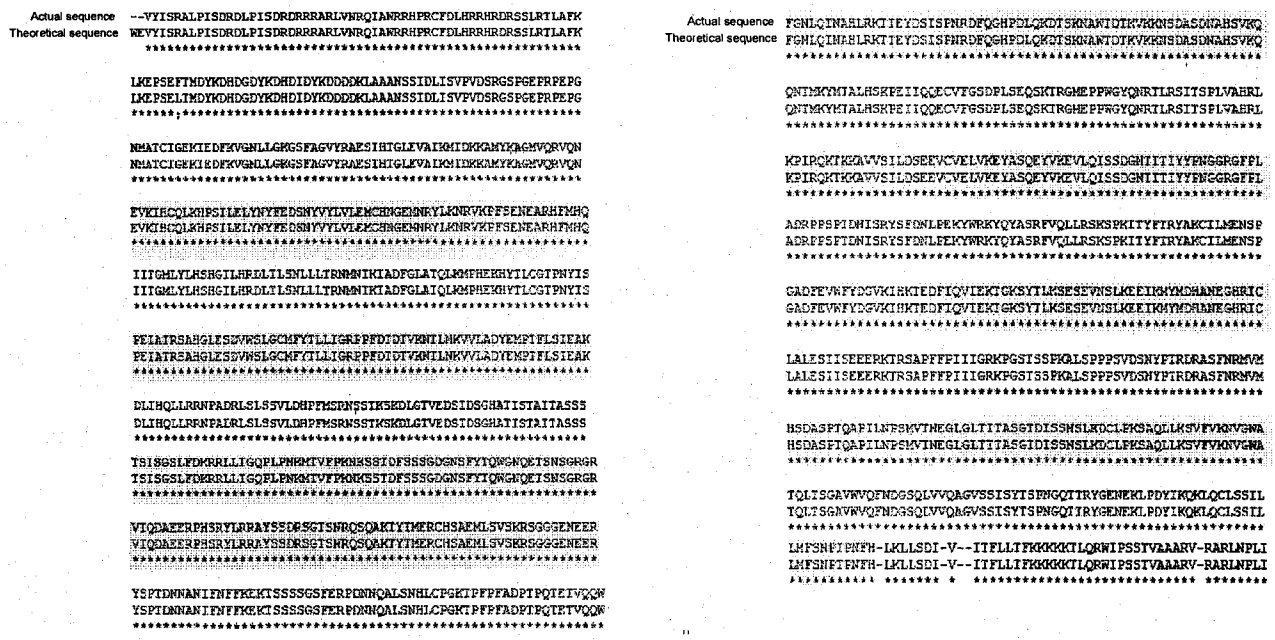


Fig. 13. Alignment of amino acid sequence of PCDNA4/TO-Flag-Plk4. After subcloning of Flag-Plk4 into the inducible expression vector PCDNA4/TO, the plasmid DNA was purified and sent to ACGT (*Toronto, ON)* for sequencing. Standard CMV and BGH primers, as well as customized primers, were utilized to fully sequence the entire region encoding Plk4. The resultant DNA sequence was inputted into a protein translation program, *Expasy*, in order to obtain the corresponding amino acid (AA) sequence. A bioinformatics program called *Vector NTI (Invitrogen)* was utilized to generate the theoretical DNA sequence that should be obtained following proper ligation of Flag-Plk4 into the PCDNA4/TO vector. This sequence was also translated and then aligned with the AA sequence obtained from ACGT. AAs are represented by their single letter codes. Asterisks indicate a matching residue. The region highlighted yellow represents the actual Plk4 protein sequence. Similar alignments were performed with PCDNA4/TO-Flag-K41M (data not shown).

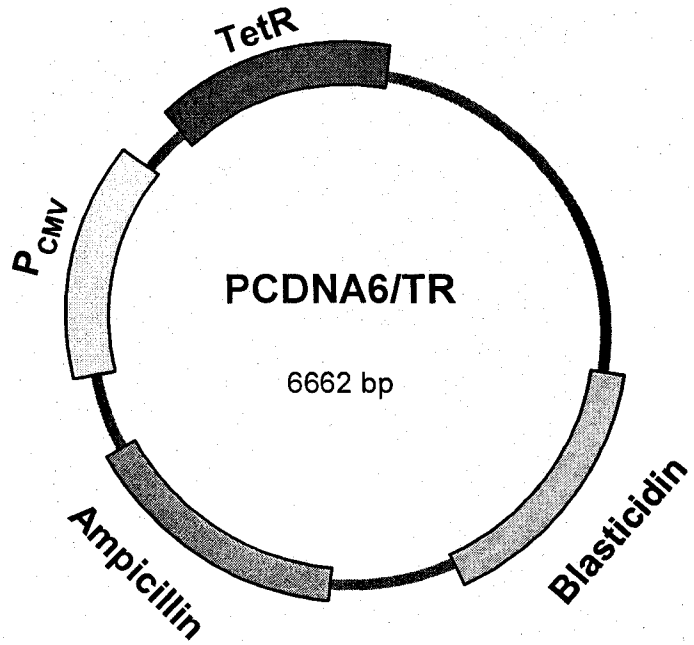


Fig. 14. Schematic representation of the PCDNA6/TR vector. Select key components of the vector are depicted. The TetR gene (bases 1684-2340) encodes the tetracycline repressor responsible for regulating transcription of the gene of interest when co-transfected with the PCDNA4/TO inducible expression vector. PCMV (bases 232-819) is the cytomegalovirus promoter which governs TetR transcription. Ampicillin (bases 5666-6526 on complementary strand) and blasticidin (bases 3782-4180) are genes that confer resistance to the plasmid and allow for its selection in the presence of these antibiotics.

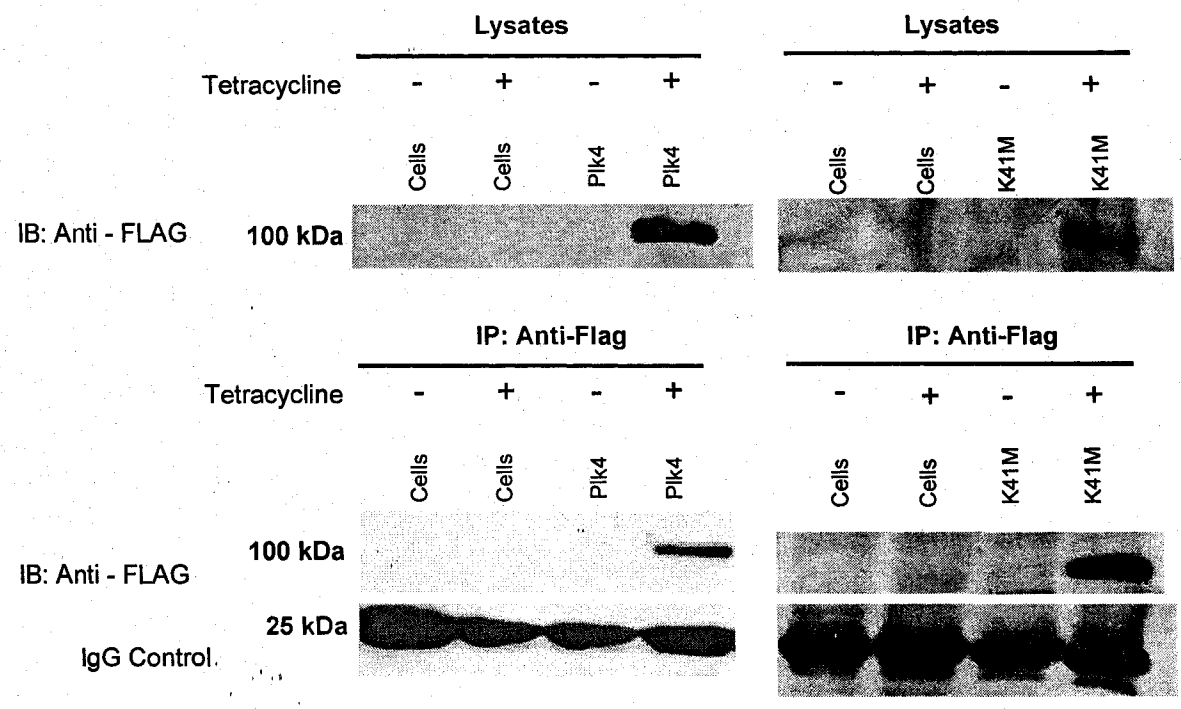


Fig. 15. Stable expression of Flag-Plk4 and Flag-K41M in T-Rex™-293 cells. T-Rex™-293 cells (*Invitrogen*) stably expressing a tetracycline repressor (Tet) were transfected with either PCDNA4/TO-Flag-Plk4 or PCDNA4/TO-Flag-K41M, which both contain Tet binding sites. 24 h post transfection, tetracycline was added at a final concentration of 100 ng/mL to induce expression of the gene. After 18 h, whole cell lysates were prepared, subjected to SDS-PAGE and then immunoblotted with an anti-Flag M2 monoclonal antibody (*Sigma)* to confirm stable expression of Flag-Plk4 and Flag-K41M (upper panel). Subsequently, an immunoprecipitation with an anti-Flag antibody (*Sigma)* was performed to confirm the observed bands were indeed overexpressed Flag-tagged proteins (lower panel).

establishment of the successful clones, cells were propagated to generate frozen stocks for future use. As a supplementary experiment, various concentrations of tetracycline (0, 25, 50, 100, and 200 ng/mL) were administered to one clone (Plk4-F) to determine the optimal concentration to induce expression. After 18 h, cells were lysed and the proteins were separated through SDS-PAGE and subject to immunoblotting. Western blot analysis revealed that 100 ng/mL produced the highest level of induction for PCDNA4/TO-Flag Plk4 expression (Figure 16).

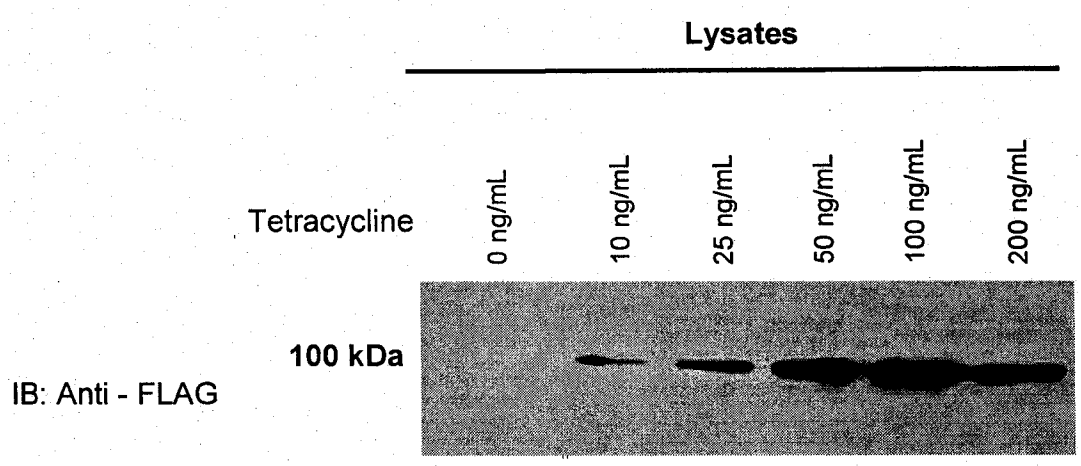


Fig. 16. Optimization of PCDNA4/TO-Flag-Plk4-F gene expression. Once positive clones for Plk4 and K41M gene expression were obtained, one clone was chosen (Plk4-F) to assay for optimal tetracycline-induced expression. Varying concentrations of tetracycline were added to each plate (0, 10, 25, 50, 100, and 200 ng/mL) followed by induction of protein for an 18 hr period. Cells were lysed, the proteins separated through SDS-PAGE, and then detected by Western blotting. A positive signal at 100 kDa corresponds to the expressed Flag-Plk4 protein.

CHAPTER VI

DISCUSSION

Putative interacting partners of Plk4

The major goal of this study was to identify and characterize putative interacting partners for Plk4. Plk4 is the least well-characterized member of the mammalian Plks. The protein partners with which it physically and/or functionally interacts with *in vivo,* as well as the mechanisms by which it is regulated, still remain to be elucidated. Because Plk family members have been found to target the same or functionally-related proteins, thereby exerting either similar or opposing effects on the interacting partner, it was worthwhile to ascertain whether Plk4 also associated with these candidate proteins and, if they exist, to define the mode of these interactions.

A key area of interest was the putative interactions of Plk4 with proteins involved in the the cellular response to DNA-damage. The Plks have been shown to be involved with the coordination of a multitude of protein pathways that function to induce cell cycle arrest, promote apoptosis or DNA repair. Checkpoints are implemented at the G1, S, or G2/M phases in order to halt further progression when activated and prevent lesioned DNA from being propagated, thus ensuring proper repair of damaged strands and promoting genomic stability. The earliest step in the pathway to DNA-damage repair is the detection of impaired DNA by sensor proteins, crucial initiators of the damage response whose identities currently remain elusive (Zhou and Elledge, 2000). More extensively characterized are the transducer proteins, which function by relaying the initial signal detected by the sensor proteins, and the effector proteins, ultimately responsible for executing the response by arresting the cell cycle. Key transducers of the DNA-damage response include the phosphoinositide kinase homologs ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR), as well as the more downstream protein kinases Chk1 and Chk2.

i.) Chk2 physically associates with Plk4

The results presented in this study provide novel evidence for Chk2 being an interacting partner for Plk4, implicating a role of Plk4 in key DNA damage pathways. Chk2 is activated in cases of double-stranded breaks caused by ionizing radiation (IR), but is also induced upon ultraviolet exposure (UV) and treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU) (Matsuoka *et al*, 2000). The Plks have been implicated as intermediary regulators in DNA-damage response pathways common to Chk2. Previously, Plk1 and Plk3 were shown to phosphorylate Chk2 at distinct residues (Tsetkov *et al*, 2003; Bahassi *et al*, 2006). In the case of Plk1, Chk2 phosphorylation occurred at residues normally phosphorylated by ATM and ATR in the DNA-damage response (Thr-68 and Thr-26 or Ser-28), but in the case of Plk1 modification of these residues occurred in the absence of DNA-damage at the centrosomes and midbody (Tsetkov *et al*, 2003). These findings suggest a role of Plk1 and Chk2 in regulating chromosomal dynamics outside of the DNA-damage response. Similarly, Plk3 phosphorylates Chk2 at residues Ser-62 and Ser-73, which results in the enhancement of DNA-damaged induced phosphorylation by ATM at Thr-68 (Bahassi *et al*, 2006). Upon mutation of Ser-73 to alanine in Chk2, Plk3 phosphorylation at this site was prohibited, leading to a decrease in Chk2 activity, as well as an inability of the checkpoint kinase to be activated by ATM's phosphorylation at Thr-68.

To further map out the region required for Plk4's interaction with Chk2, full-length Plk4 as well as deletion and point Plk4 mutants were expressed in HEK-293 cells, and the resultant lysates were subject to co-immunoprecipitation assays with anti-Chk2 antibody. Wildtype Plk4 was found to associate with Chk2 as revealed by immunoblotting analysis (Figures 5A & B, 'Flag-Plk4' lanes). The two kinase dead constructs utilized in the assays—D154N and K41M—were rendered catalytically-inactive through alteration of a charged residue (either Asp or Lys) to an uncharged one (Asn or Met) within the ATP-binding domain of Plk4; the resulting effect of this mutation is to incapacitate the enzyme from properly phosphorylating its substrate. Various point mutations in the kinase domain did not seem to hinder any interaction between the

candidate protein and Plk4 (Figure 5A, lower panel, 'Flag-K41M' lane; Figure 5B, lower panel, 'Flag-154N' lane) as evidenced by their co-immunoprecipitation. A previous study conducted by Tsetkov *et al* (2005) also showed that a kinase defective Plk1 mutant was still able to pull down with Chk2. In addition, a study of the Plk homolog Plo1 in fission yeast showed that a kinase-dead mutant, as well as Plo1 lacking the entire kinase domain, were still able to associate with known interacting partners Cut23 and Dmf1/Mid1 (Reynolds and Ohkura, 2002). Additionally, co-immunoprecipitation studies with Plk1 and the tubulin proteins showed that a kinase-dead Plk1 mutant was greatly enriched in the tubulin immunoprecipitates (Feng *et al*, 1999). The results in this study imply that the kinase activity of Plk4 is not imperative for its binding to Chk2. A defective kinase domain does not preclude the possibility that these potential interacting partners may still be a substrate of Plk4; immunoprecipitation assays are limited in that they can demonstrate a physical interaction between two proteins, but fail to characterize the nature or consequence of that interaction. Therefore, further analysis on the interaction between Chk2 and Plk4 would be required to conclusively identify the manner in which the two proteins associate.

In addition to a catalytically-inactive mutant, a kinase-activating Plk4 mutant was also shown to co-immunoprecipitate with Chk2 (Figure 5B, lower panel, 'Flag-T170D' lane). Flag-T170D contains a point mutation in the T-loop activation domain of Plk4 that modifies a Thr at position 170 to an Asp; replacement of Thr by a charged residue mimics the effect of T-loop phosphorylation. Previous studies revealed that in Plk1, intramolecular association of the PBD with the T-loop in the kinase domain suppresses Plk1 catalytic activity (Tsetkov *et al*, 2001). Phosphorylation of Thr170 in the T-loop relieves the autoinhibitory effect of the PBD, resulting in a kinase that is hyperactive (Jang *et al*, 2001). Indeed, Swallow *et al* (2005) confirmed this mutation in Plk4 renders the kinase constituitively active. One explanation for the physical interaction that was observed with Chk2 is that a constitutively-active Plk4 mutant may indicate an inability of the kinase to dissociate from its targeted protein. This would cause a virtually

permanent association between Plk4 kinase and its substrate that would be detected by a co-immunoprecipitation experiment, as was observed in this study.

Previous studies have defined the PB of Plk4 as a critical region for the formation of intermolecular homodimers of the kinase (Leung *et al*, 2002); it is also an essential component to the domain that enables proper subcellular localization of the protein. Pull down assays with only the PB region of Plk4 did not show an interaction with Chk2, suggesting it may not be required for their physical interaction *in vivo* (Figure 5C, lower panel, 'Flag-PB' lane). Indeed, deletion of the PB of Plk4 did not affect the protein's ability to bind to any of the proposed interacting partners (Figure 5B, lower panel, 'Flag-ΔPB' lane). A study conducted on Plk1 and its interaction with α-, β-, and γ-tubulin, however, showed that deletion of the PB from Plk1 did not significantly affect its association with the tubulin proteins (Feng *et al*, 1999). These results concur with the observations of this study, which confirmed that FLAG-ΔPB maintained a physical interaction with Chk2 despite the absence of the PBD. In a separate study, a yeast-two hybrid assay was performed that revealed an interaction between full-length Plk2 and calcium- and integrin-binding protein (CIB); the evidence showed that sole expression of the C-terminal domain, which retained an intact polo-box and other conserved sequences, was no longer able to physically associate with CIB (Ma *et al*, 2003). These results agree with the findings obtained in this study that showed Chk2 did not pull down with the Flag-PB construct of Plk4. Some contradictory evidence does exist, however, obtained from previous studies on other Plks, such as the findings published by Reynolds and Ohkura (2003), which showed that mutations of conserved residues within the PB region of Plo1 disrupted the association between this Plk member and its potential interacting partners. Similarly, inactivating point mutations in the PBD of Plk1 also hindered its immunoprecipitation with Chk2, as well as with known substrates Cdc25C and Mklp2, as demonstrated in previous experiments (Tsetkov *et al*, 2005; Elia *et al*, 2003; Neef at al, 2003). The fact that Plk4 was not able to associate with Chk2 when expressed solely as the PB suggests that regions outside this conserved motif are necessary for their physical

interaction. One possibility is that these kinases phosphorylate Plk4 within its catalytic domain; thus, the loss of this fundamental region from the Flag-PB construct would eliminate any potential for physical association. Perhaps Chk2 exists merely as an 'adaptor' protein rather than a direct substrate/kinase to Plk4. Therefore, regions outside the PB may act as attachment sites for these proteins; without these regions present, they cannot bind and recruit *bona fide* substrates/kinases of Plk4. Thus, the mechanism of interaction between Plk4 and these potentially associated proteins merits further investigation.

Plk4 is unique from the other Plk members in that it contains a single conserved PB, and a divergent PB region designated as the cryptic PB (cry-pb). Leung *et al* (2002) propose that the cry-pb and PB together are essential for Plk4's self-association *in vivo*. Results indicate that the cry-pb alone does not pull down with Chk2 (Figure 5C, lower panel, 'Flag-R1' lane), suggesting this region is not sufficient for interaction. Previous studies support the idea that the PB motifs individually expressed are not sufficient for proper function of the Plks, but rather the C-terminus expressed as a whole unit (ie. regions upstream of the PB, the linker region, as well as both PBs) that contributes to its phosphopeptide binding ability (Tsetkov *et al*, 2005). This may explain why Chk2 was not able to co-immunoprecipitate with only the cry-pb of Plk4, indicating a necessity of the other PBD components.

Reciprocal co-immunoprecipitations were also performed to confirm the validity of the interactions that were observed between Plk4 and Chk2, but unfortunately were unsuccessful. Originally, pull down assays were conducted by immunoprecipitating Chk2, followed by immunoblotting with an anti-Flag antibody to detect the presence of Flag-Plk4. Subsequent to immunoblot analysis, it was shown that wildtype, kinase-dead, kinase-active, and PB-deleted Plk4 proteins effectively co-immunoprecipitated with Chk2, as depicted in Figure 5A & B. Numerous attempts to detect an interaction by immunoprecipitating Flag-Plk4 constructs and probing with anti-Chk2, however, proved futile. Possible explanations for the observed phenomenon may be that endogenous Chk2 levels were too minimal to be co-immunoprecipitated

with Plk4, making it difficult to experimentally capture a physical interaction between the two proteins. Originally, monoclonal anti-Chk2 antibody was employed to immunoprecipitate any endogenously-expressed Chk2 protein, while the *overexpressed* Plk4 was examined for a potential interaction. Conversely, the reciprocal experiment involved pulling down an already overexpressed Plk4 protein with a monoclonal anti-Flag antibody, then attempting to detect a potential association with *endogenous* Chk2. In the former case, both proteins are likely more abundant due to antibody-targeting of Chk2 coupled with the overexpression of Plk4, relative to the latter case. Chk2 remains relatively inactive in the absence of DNA damage; only upon exposure to genotoxic stresses such as UV or IR does the kinase become activated upon phosphorylation by more the upstream checkpoint kinases ATM and ATR (Tsetkov *et al*, 2003). It is possible that inducing DNA-damage prior to co-immunoprecipitation assays would greatly enhance endogenous Chk2 levels, thereby increasing the likelihood that a physical association can be detected upon pulling down Plk4. In addition, a positive interaction result from overexpressing a tagged Chk2 construct and performing co-immunoprecipitations with an antibody against endogenous Plk4 would also enhance the validity that Chk2 and Plk4 physically interact *in vivo*. Currently, however, no effective endogenous antibody for Plk4 exists, precluding a means of detecting Plk4 other than through an overexpression system.

ii) ATM physically associates with Plk4

As mentioned earlier, ATM is crucial for the initiation of signaling pathways in mammalian cells following exposure to ionizing radiation (IR) and to other agents that introduce double-strand breaks into cellular DNA (Kastan and Lim, 2000). Upon activation, ATM triggers a signal transduction pathway that ultimately leads to repair of DNA lesions, one pathway being the phosphorylation of the tumor suppressor p53; this results in increased stability of the protein and more pronounced transcriptional activity, eventually giving rise to a G1 cell-cycle arrest. (Zhou and Elledge, 2000). Additionally, activation of the checkpoint kinases Chk1 and Chk2 by ATM is

an intermediary step in the pathway leading to mending unstable DNA. Previous studies have provided evidence that ATM can mediate inhibition of Plk1 in response to DNA damage (Van Vugt *et al*, 2001). It was shown that when ATM –/– cells are exposed to the DNA-damaging agent adriamycin, Plk1 is no longer effectively inhibited, implicating ATM as a key regulator of Plk1 repression. In this study, similar co-immunoprecipitation experiments to those conducted with Plk4 and Chk2 were performed with ATM to determine if it also is a candidate interacting partner. Evidence showed that full-length Flag-Plk4, kinase-dead Flag-K41M and Flag-154N, and kinase-active Flag-T170D can all pull down with immunoprecipitated ATM (Fig. 6A & B, lower panels). One notable observation was that the kinase-active mutant of Plk4 showed higher levels of immunoprecipitated ATM protein relative to the other constructs (Figure 6B, lower panel, 'Flag-T170D' lane). As mentioned earlier, Plk1 kinase activity is suppressed by the intramolecular association of the PBD with the T-loop, and that phosphorylation of Thr170 in the T-loop relieves the autoinhibitory effect (Tsetkov *et al*, 2001; Jang *et al*, 2001). It is possible that Plk4 experiences a similar effect and that release from autoinhibition upon a T170D mutation (as exhibited in the Flag-T170D construct) favours a more open structure of Plk4. This in turn may facilitate its association with other proteins, and possibly explain why co-immunoprecipitation assays with ATM revealed a stronger interaction with the kinase-active Flag-T170D mutant of Plk4. Deletion of the PB did not hinder an interaction with ATM and Plk4, as was also observed with Chk2 (Figure 6B, lower left-hand panel, 'Flag ΔPB' lane.). However, ATM was able to bind with Flag-R1, the construct expressing the cry-pb of Plk4 (Figure 6B, lower right-hand panel, 'Flag-R1' lane). Conversely, Chk2 did not associate with the cry-pb. This indicates that the cry-pb region of Plk4 may be critical to its physical association with ATM, and suggests that Plk4 may interact in a different manner with ATM than with Chk2. It would be interesting to observe whether expression of a deletion construct that lacks the cry-pb would preclude an interaction between Plk4 and ATM. A construct expressing Flag-Δcry-pb was not available at the time experiments for this study were being performed, though a HEK-293 cell line stably expressing

this protein is currently being developed by Doreen Ezeife (University of Windsor, ON). Expression of this construct and co-immunoprecipitations with anti-ATM would confirm whether or not this region is indispensable for associating with ATM.

iii.) ATR physically associates with Plk4

ATR is structurally similar to ATM, with a similar role in inducing a G2/M cell cycle arrest in cases of genotoxic stress, however is primarily triggered upon UV exposure rather than IR, which is the case with ATM (Van vugt *et al*, 2001). As with ATM, ATR was also found to be a direct regulator of p53 through its phosphorylation of the tumor suppressor, implying it is an important link between the DNA damage response and cell cycle arrest (Tibbetts *et al*, 1999). Van vugt and co-workers (2001) showed in their study that ATR mediates inhibition of Plk1 in response to UV damage. In ATM –/– cells that were subjected to UV treatment, Plk1 activity was still suppressed, suggesting the presence of ATR was able to maintain its inhibition; however upon administration of caffeine, which blocks activity of ATM/ATR in the DNA-damage response, Plk1 was no longer suppressed. This study found that full-length, kinase-dead, and kinase-active Plk4 all display a physical interaction with ATR Experiments were performed in the absence of UV damage, which is the key inducer of ATR activity; despite this fact, an association with Plk4 was still detected, indicating that that the interaction between ATR and Plk4 may not be entirely DNA-damage-dependent. These two proteins may form an association that maintains itself throughout the cell cycle and upon UV-induced damage, may alter their interaction with each other. The precise effects of genotoxic stress to the interaction between Plk4 and ATR remains to be elucidated.

As with Chk2, reciprocal co-immunoprecipitations were also attempted with ATM and ATR, namely by pulling down Flag-Plk4 and analyzing if ATM/ATR was able to co-immunoprecipitate with the targeted protein. Again, the co-immunoprecipitations performed in this manner were ineffective and no ATM/ATR protein was able to be detected by Western

blotting (data not shown). One rationalization for this may be that because ATM and ATR both have relatively high molecular masses of ~370 kDa (Chen and Lee, 1996), these proteins may not have been effectively transferred from the polyacrylamide gel used in SDS-PAGE onto the PVDF membrane during the semi-dry transfer. Indeed, the large size of ATM and ATR introduces some difficulties when performing immunoprecipitation assays, therefore scrupulous measures need to be undertaken to optimize experiments with these proteins in the future.

iv.) α-, β-, γ-tubulin, BRCA2, and Rb do not physically associate with Plk4

Numerous other proteins were tested as putative interacting partners with Plk4 on the basis of existing data showing direct or indirect association with other Plks. Feng and colleagues (1999) presented evidence that Plk1 can physically associate with the tubulin proteins in a stable complex that persists during interphase and mitosis. It was found that Plk1 was able to phosphorylate the tubulins, which functions to adjust the stability and organization of microtubule structures that form the mitotic spindle (Feng *et al*, 1999). This is a particular significant event during mitosis since it ensures proper spindle assembly and thus accurate segregation of chromosomes to dividing cells. Spindle assembly initiates from the centrosomes, which are designated the 'microtubule organizing centre' (MTOC). γ-tubulin is particularly ubiquitous at these structures, since it exists as part of a meshwork of proteins within the pericentriolar material of the centrosome; the spindle itself is composed of α- and β-tubulin protein as its monomeric subunits (Varmark, 2004). Plk4 has been confirmed to localize to centrosomes where it mediates its duplication during mitosis; overexpression of Plk4 can produce an excess of centrioles, while depletion of the protein through siRNA can cause progressive centriolar reduction (Bettancourt-Dias *et al*, 2005; Habedanck *et al*, 2005). The fact that Plk4 can localize to centrosomes makes the tubulin proteins reasonable candidates for interaction with Plk4. Co-immunoprecipitation assays, however, showed there was in fact no physical association between Plk4 and any of the tubulin proteins (data not shown). These results do not completely exclude the possibility of a

putative association between Plk4 and α-, β-, or γ-tubulin. The centrosomes and mitotic spindle are structures that mature concomitantly with cell cycle progression. As the bipolar spindle progressively lengthens, the need for α- and β-tubulins to polymerize the microtubules is greater, which should prompt an increase in their protein levels during M phase. Studies have confirmed a twofold increase in tubulin protein synthesis during mitosis in HeLa cells (Bravo and Celis, 1980). If an interaction does exist between Plk4 and any of the tubulin proteins, and that association is highly transient, it may be necessary to enrich for cells in the mitotic phase through the addition of nocodazole, for example; this would capture cells in a phase when maximal tubulin protein synthesis is occurring, and could increase the likelihood of capturing short-lived interactions between Plk4 and the tubulins.

BRCA2 is a tumor suppressor protein that functions by repairing double-stranded breaks in DNA, thus helping to maintain genomic stability and cell cycle progression (Lin *et al*, 2003). Evidence shows that Plk1 is able to phosphorylate BRCA2 and that this association results in modulation of the tumor suppressor's activity (Lin *et al*, 2003). A physical interaction between Plk4 and BRCA2 was not observed to occur based on the presented co-immunoprecipitation results (data not shown). In the study conducted by Lin and co-workers, however, cells were initially synchronized in G2/M phase prior to assessing for any putative interactions between Plk1 and BRCA2; this represents the stage of maximal Plk1 expression. Similarly, Plk4 exhibits the highest proteins levels at M phase (Hudson *et al*, 2001). It is possible that no physical association was observed between Plk4 and BRCA2 because the majority of cells were asynchronous, suggesting that expression of either gene may not be at its maximum potential. Cell synchronization through the addition of nocodazole, a microtubule-depolymerizing agent that induces G2/M block (Cooper, 2003), followed by release arrested cells, may enhance the potential for BRCA2 to associate with Plk4 if it is indeed a *bona fide* interacting partner .

Retinoblastoma (Rb) is a classical tumor suppressor protein that suppresses the transcription of critical targets in a cell cycle-dependent manner and thus prevents abnormal

cellular proliferation (Wiman *et al*, 1993). In early G1, Rb exists in an unphosphorylated state; however, the protein progressively attains a hyperphosphorylated condition in late G1 that persists until the end of M phase (Weinberg, 1995). One study showed how Plk1 is a target of the Rb tumor suppressor pathway such that Rb activation leads to suppression of Plk1 activity (Gunawardena *et al*, 2004). Conversely, loss of Rb deregulates Plk1 repression. To determine if Plk4 shows an association with Rb, various constructs encoding Plk4 protein were transfected into mammalian cells and lysates were subjected to co-immunoprecipitation. No physical association was detected between Rb and Plk4. Although Plk4 was not found to interact directly with Rb, it does not rule out the possibility that Plk4 still participates in the Rb transduction pathway and that intermediary proteins may be involved linking the two proteins in an indirect manner. Plk1 repression by Rb was discovered to require a chromatin remodeling complex called SWI/SNF, which was in fact responsible for the histone deacetylation of the Plk1 promoter region that prevented Plk1 transcription (Gunawardena *et al*, 2004). Without SWI/SNF present in cells, Rb was no longer able to mediate inhibition of Plk1 activity. It is possible that Plk4 is indirectly related to Rb activity rather than through a direct physical association and that third-party proteins are required for a functional link between Rb and Plk4 to be witnessed (Table 3).

Table 3: Potential Candidate Interacting Partners of Plk4

Candidate Partner Tested	Interaction with Plk4 observed? (+/-)	Region of Plk4 not required for interaction
Chk2	+	Polo box Cryptic polo box
ATM	+	Polo box
ATR	+	Polo box
α-tubulin	-	N/A
β-tubulin	-	N/A
γ-tubulin	-	N/A
BRCA2	-	N/A
Rb	-	N/A

Establishment of stable cell line expressing Plk4 and kinase dead K41M mutant

Previous experiments involving transient transfection of Flag-Plk4 vectors into various cell lines such as HEK-293, U2OS, and mouse fibroblast 3T3 cells displayed very poor and inconsistent transfection efficiencies, as revealed by immunoblotting analyses. Decreased cell viability due to contamination or overconfluency may have contributed to such low gene expression, which often posed difficulties in performing further experiments that required ample protein levels; establishing a HEK-293 cell line that consistently retained the Plk4 gene would help circumvent those problems. Creating a cell line that stably expresses a gene of interest provides numerous advantages over the more transient methods typically used in molecular biology studies. Habedanck *et al* (2005) were able to establish inducible osteosarcoma epithelial U2OS cell lines that stably express both Flag-Plk4 and a kinase dead Flag-D154A mutant Plk4, which were then utilized to study the effects of Plk4 overexpression on centrosome duplication. Two separate human embryonic kidney 293 (HEK-293) cell lines containing either a tetracycline-inducible Flag-Plk4 or a kinase defective Flag-K41M vector were generated in this study. Full-length Flag-Plk4, as well as the kinase defective Flag-K41M mutant, were both cloned into an expression vector that was under control of a tetracycline repressor. By making the expression of Plk4 as part of an inducible system, potential toxicity to the cells due to prolonged Plk4 expression could be avoided. For example, previous experiments have shown that exogenous Plk4 expression can induce the formation of multiple centrioles, which can cause aberrant mitotic spindle assembly and subsequently contribute to genomic instability (Habedanck *et al*, 2005). Without a means of temporally modulating the expression of Plk4 in the stable cell lines, it is possible that mitotic defects will amplify with each round of cell division, thus making the cells severely compromised for further experimentation. Therefore, by making the expression of Plk4 under control of a tetracycline (Tet) repressor, one can manipulate when the protein is expressed and limit the exposure of the cell to potential adverse effects associated with overexpression.

CHAPTER VII

FUTURE DIRECTIONS

The results described in this study provide preliminary evidence for Chk2, ATM, and ATR being interacting partners with Plk4. To extend the relevance of these findings, one important question that should be addressed is: does Plk4 phosphorylate these candidate partners or vice versa? Plk4, Chk2, ATM, and ATR are all protein kinases, which function by adding a phosphate to their respective substrates, generally leading to a change in the substrate's regulation, activity, or stability. As previously mentioned, Plk1 and Plk3 both physically interact with Chk2, leading to its phosphorylation (Tsetkov *et al*, 2003; Bahassi *et al*, 2006). *In vitro* kinase assays examining Chk2 as a substrate of Plk4 would supplement the observed physical association with a functional relevance. Similar investigations on potential kinase:substrate relations between Plk4 and ATM/ATR can also be conducted. Conversely, the possibility remains that Chk2, ATM, and ATR may in fact phosphorylate Plk4, leading to potential changes in its regulation within the cell. Tec, a protein tyrosine kinase (PTK) critically involved in mediating cytokine and antigen receptor signaling, currently remains the only enzyme known to phosphorylate Plk4 (Yamashita *et al*, 2001). Yeast two-hybrid analysis and immunoprecipitation detected Plk4 as an interacting partner of Tec kinase, while *in vitro* kinase assay confirmed Tec can phosphorylate and induce activity of Plk4 (Yamashita *et al*, 2001). Therefore, it seems that *in vitro* kinase assays are a logical next step to the presented findings. The experiments conducted in this study reveal that Plk4 can interact with each of these proteins independently of one another. Chk2, ATM, and ATR, however, are well-characterized participants in the DNA-damage response pathway with clearly defined functional associations. ATM and ATR are both responsible for phosphorylating Chk2 at Thr-68, a modification that transforms Chk2 into its active form (Matsuoka *et al*, 2000). Whereas Chk2 is phosphorylated by ATM upon IR exposure, ATR interacts and phosphorylates Chk2 when prompted by UV damage (Tsetkov *et al*, 2003).

Despite the findings of this study, the possibility remains that the association of Plk4 with these checkpoint kinases cannot simply be defined as a direct, linear interaction, but rather as a component of a larger complex of proteins consisting of bridging associations. To investigate this possibility, interactions between Plk4 and a particular candidate partner should be analyzed in the absence of the other proteins to determine if the physical association is retained. For example, ATM-deficient cell lines can be employed as an overexpression system for Plk4 followed by pull-down assays of the whole cell lysates with Chk2 antibody; if Plk4 and Chk2 are still able to co-immunoprecipitate in cells lacking ATM, this would corroborate the findings that these two kinases have a physical interaction. Analyzing the effects of ATM- and ATR-inhibiting agents such as caffeine (ATM and ATR) and Wortmannin (ATM) on the associative status between Plk4 and Chk2 would offer similar evidence. Furthermore, co-expression of differentially-tagged constructs for Plk4 and Chk2 (or ATR or ATM) in HEK-293 cells and subsequent *in vitro* co-immunoprecipitation and immunoblotting analyses would reveal if the observed physical interactions persist independently. In addition, the effects of radiomimetic agents such as adriamycin on Plk4 kinase activity should be explored. This would lend itself to investigating how genotoxic stress would affect Plk4's physical and potentially functional association with ATM, ATR, and Chk2, should these proteins be revealed to be putative substrates. Reciprocal co-immunoprecipitations would also significantly bolster the findings of a physical interaction with Plk4 and the candidate partners. Efforts to reciprocally immunoprecipitate Chk2 with Plk4 were futile. In addition, attempts to pull down Plk4 and detect a co-immunoprecipitation with ATM or ATR were also unsuccessful, likely due to the large size (370 kDa) of these kinases. It would be worthwhile to rigorously optimize this experiment so as to provide further validity to the physical interaction that was originally observed. Although no physical interaction was detected with Plk4 and BRCA2, Rb, α-, β-, or γ-tubulin in this study, sub-optimal conditions may have precluded the detection of possibly low-affinity or transient interactions. As described previously, the enrichment of cells arrested at the G2/M phase through nocodazole addition may greatly enhance

the likelihood of revealing a physical association, since Plk4 is expressed at maximal levels during this phase.

Finally, the creation of the Plk4 stable cell lines as achieved in this study will be an invaluable tool in pursuing the aforementioned experiments. The enhanced and consistent gene expression afforded by these cell lines facilitate further studies that require the presence of a tagged Plk4 protein such as in protein interaction assays, as well as localization experiments, kinase assays, cell cycle analyses, and DNA damage experiments.

APPENDICES

APPENDIX A

Customized primers used for sequencing of PCDN4/TO Flag Plk4

Primer name	Sequence	T_m
ET281	5' GAT AAG AAA GCC ATG TAC AAA GCA GGA ATG G 3'	60 °C
ET282	5' CAG GTC TTT CAA AAG ATC CAG AAC TAC TGG 3'	60 °C
ET283	5' TGT CGT AAT AAC CCC GCC CCG 3'	58°C
ET284	5' CGC ATC TTC AAT CAC TCT CCC CCG 3'	61 °C

* Primers were designed using Primer3, a web-based program used to facilitate the creation of oligonucleotide sequences utilized specifically for the sequencing of desired regions within DNA (Rozen and Skaletsky, 2000).

APPENDIX B

Antibodies against putative interacting partners used in co-immunoprecipitation assays

Antibody	Polyclonal or Monoclonal	Origin	Company
Anti-ATM	Monoclonal	Mouse	*Sigma*
Anti-ATR	Polyclonal	Mouse	*Calbiochem*
Anti-Chk2	Monoclonal	Mouse	*Sigma*
Anti-α-tubulin	Monoclonal	Mouse	*Sigma*
Anti-β-tubulin	Monoclonal	Mouse	*Sigma*
Anti-γ-tubulin	Monoclonal	Mouse	*Sigma*
Anti-BRCA2	Polyclonal	Rabbit	*Sigma*
Anti-Rb	Monoclonal	Rabbit	*Sigma*

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VITA AUCTORIS

NAME: Melissa Lauren Ganuelas

PLACE OF BIRTH: Grosse Pointe, MI

YEAR OF BIRTH: 1981

EDUCATION: St. Joseph's Catholic High School, Windsor, Ontario

1995-2000

University of Windsor, Windsor, Ontario

2000-2004 B.Sc.

University of Windsor, Windsor, Ontario

2004-2007 M.Sc.