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Sak and its potential role in the DNA damage response.

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SAK AND ITS POTENTIAL ROLE IN THE DNA DAMAGE RESPONSE

by

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ABSTRACT

The Plk family of serine/threonine kinaese are key players in the cell cycle, centrosome regulation and DNA damage pathways. Sak's (Plk4) involvement in these processes is not fully established. We examined Sak's role in DNA damage pathways and found that full length wild-type Sak protein was found to be subjected to phosphorylation both in the presence and absence of UV. This post-translational modification did not occur in the Sak polo-box and cryptic polo-box domains. Furthermore, we established that Sak interacts with two key DNA damage proteins, ATR and Chk1. Additionally, another DNA damage protein Chk2, which also interacts with Sak, was found to be a substrate of Sak. Interestingly, UV exposure abolished phosphorylation of Chk2 by Sak, suggesting that Sak is in fact inhibited by UV radiation. These findings suggest that Sak may play a role in the DNA damage response pathway.

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CHAPTER I

INTRODUCTION

The Cell-Cycle

Every cell undergoes an orderly sequence of events in which its genomic and structural content is duplicated and divided into two. This process is termed the cell-cycle and is essential for every living cell. During this process, DNA is replicated into an identical copy from the mother strand, then the cell proceeds to mitosis; a phase of the cell cycle in which a series of dynamic events leads to the passage of this replicated DNA into the daughter cells. This process involving the growth, replication and division of the eukaryotic cell requires the correct function of numerous proteins and the sequential activation of cyclins and cyclin-dependent kinases (cdks) which drives this disciplined transition through the phases of the cell cycle (Obaya *et al.,* 2002).

To ensure that correct cell division occurs, cells have evolved mechanisms which make certain the completion of one cell cycle phase prior to initiation of the next stage. Termed the cell-cycle checkpoints, the cell is able to halt the progression of cellular growth, replication and division at particular phases to ensure DNA damage or cellular instability is repaired before proceeding to the following step. Without these checkpoints, faulty replicated DNA or incorrect cellular division may lead to aberrant cellular growth or tumourigenesis (Kastan *et al.,* 2004).

One of the key regulatory mechanisms that the cell utilizes to control cell cycle progression is through the phosphorylation and dephosphorylation of key proteins involved in cell cycle control by several protein kinases and phosphatases respectively. In general, protein kinases catalyze the transfer of the gamma-phosphate group of adenosine triphosphate (ATP) to the hydroxyl groups of serine and threonine residues or

the phenolic group of tyrosine residues of proteins (Schlessinger, 2002). Phosphatases are involved in the reverse reaction and catalyze the release of a phosphate group from a residue. Once proteins are phosphorylated or dephosphorylated, they may undergo a conformational change that alters their activity, affinity for substrate, stability or cellular localization.

A family of proteins known to be involved in cell cycle regulation are the Polo-like kinases and their specific roles will be examined later in this chapter (Roshak *et al.*, 2000, Qian *et al.*, 2001, Toyoshima-Morimoto *et al.*, 2002, Myer *et al.*, 2005).

Polo-like kinases (Plks)

The polo-like kinases (Plks) comprise a family of serine/threonine kinases that are highly conserved from yeast to humans (Takai *et al.*, 2005). These proteins are involved in a number of vital processes during the cell cycle such as bipolar spindle formation (Sunkel and Glover, 1988, Kitada *et al.*, 1993, Ohkura *et al.*, 1995), centrosome dynamics (Lane and Nigg, 1996, Casenghi *et al.*, 2003), activation of Cdc25C allowing entry into mitosis (Roshak *et al.*, 2000, Toyoshima-Morimoto *et al.*, 2002), regulation of the anaphase-promoting complex and cytokinesis (Lee *et al.*, 1998, Golan *et al.*, 2002, Jiang *et al.*, 2006). In addition, members of the Plk family have been implicated in roles involving DNA damage response (van Vugt *et al.*, 2001, Xie *et al.*, 2001). The founding member of this family was first identified in *Drosophila melanogaster* (fruit fly) and named Polo due to the protein's association with the spindle poles. Furthermore, mutations in Polo resulted in highly branched spindle poles leading to abnormal mitotic divisions in brain cells, as well as aberrant meiosis in males (Sunkel and Glover, 1988). Since the discovery of Polo, other Plk family members have been identified in simple eukaryotes such as Cdc5 in *Saccharomyces cerevisiae* (Kitada *et al.*, 1993), Plo1 in

Schizosaccharomyces pombe (Ohkura *et al.*, 1995), tbplk in the protist *Trypanosoma brucei* (Graham *et al.*., 1998) and PlkA in the filamentous fungus, *Aspergillus nidulans* (Bachewich *et al.*, 2005). Complex eukaryotes contain more than one Plk member and indeed, homologues of Polo have been found in various model species indicating a conserved function in ensuring species survival (Dai, 2005). In *Caenorhabditis elegans* and *Xenopus laevis*, three homologues have been identified and denoted Plc1, Plc2 and Plc3 (Chase *et al.*, 2000) and Plx1, Plx2 and Plx3 respectively (Duncan *et al.*, 2001). Furthermore, four homologues were identified in mammals and named Plk1, Plk2/Snk, Plk3/Prk/Fnk and Plk4/Sak (Eckerdt *et al.*, 2005). This chapter aims at introducing the the Polo-like kinases found in simple eukaryotes, as well as Plk1-3. However, the focus of my research deals directly with Sak (also known as Plk4). As a result, Chapter 2 will be entirely devoted to introducing this protein.

The General Structure of the Plks

In general, the Polo-like kinases are defined as having two distinct features. The first is a highly conserved N-terminal serine/threonine kinase domain that most closely resembles the kinase domains of Aurora kinases and the calcium/calmodulin-dependent kinases (Lowery *et al.*, 2005). In addition, the Plks share one or two C-terminal conserved stretches of amino acids termed the polo-boxes (Dai *et al.*, 2006). These polo-boxes function as a single unit and as a result, have been named the polo-box domain. Unlike the kinase domain, the polo-box domain is less conserved where less than half the amino acids are conserved among species (Lowery *et al.*, 2005). (Figure 1)

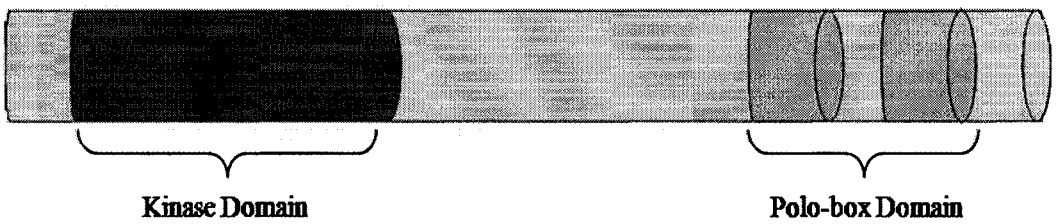

Figure 1. **The conserved structure of Polo-like kinases (Plk).** The structure of the Plks is conserved from yeast to mammals. A highly conserved protein kinase domain is located at the N-terminal and shown here in blue. The two polo boxes are shown in yellow. These two areas and the linking region between them are referred to as the polo-box domain.

The Polo-box Domain

When examining the crystalline structure of the polo-box domain for Plk1, Cheng *et al.* found that each polo-box contains an alpha-helix and a six stranded antiparallel beta-sheet. Each polo-box consists of approximately 80 amino acids and the two conserved domains are joined together by a highly variable linker region consisting of approximately 20 amino acids (Cheng *et al.,* 2003). This six-stranded antiparallel beta sheet seems to form a shallow cavity, allowing for peptides to bind. The linker region, although highly variable and disordered, joins the two polo-boxes and borders the target peptide binding site (Garcia-Alvarez *et al.,* 2006).

Studies have suggested that the polo-box domain is essential for subcellular localization. When a single point mutation was introduced changing a tryptophan to a phenylalanine at the conserved amino acid 414, Plk1 was no longer able to localize to the spindle poles and structures thought to be involved in cytokinesis (Lee *et al.,* 1998). Similarly, this same group found that a mutated polo-box and an inactivating kinase mutation were unable to rescue the cdc5-defect phenotype although kinase activity was not needed for Plk localization to the centrosomes and cytokinetic neck filaments of the budding yeast (Lee *et al.,* 1999). Hanisch *et al.,* (2006) further demonstrated that the polo-box domain localized to centrosomes, kinetochores and the spindle midzone. Expression of this construct allowed for proper bipolar spindle formation with proper chromosome segregation in HeLa S3 cells. However, this expression caused the appearance of an irregular metaphase plate and the chromosomes were unable to properly congress causing cells to arrest. That is, although the overexpression of the wildtype polo-box domain resulted in mitotic arrest similar to the depletion of Plk1, overexpression of the polo-box still allowed centrosome maturation, separation of the chromosomes and

spindle formation to occur (Hanisch *et al.,* 2006). In addition, the polo-box domain of Plk3 has also been found to localize to the centrosome during interphase, the spindle poles during mitosis and the midbody during cytokinesis. An intact polo-box domain, and not the kinase domain, was required for normal distribution of Plk3 and overexpression of the Plk3 polo-box domain induced apoptosis in U2OS cells (Jiang *et al.,* 2006).

The polo-box domain of Plk1 has further been identified as a phospho-threonine/serine binding domain allowing for Plk1 to recognize its substrates and localize to cellular structures (Elia *et al.,* 2003a). This led to the observation that all mammalian Plk homologues, as well as the Plk members from *Xenopus laevis* and *S. cerevisiae,* showed selection for the consensus sequence most generally noted as [Pro/Phe]-[φ/Ala_{Cdc5}/Gln_{Plk2}]-[Thr/Gln/His/Met]-Ser-[pThr/pSer]-[Pro/X], where φ is any hydrophobic amino acid. Upon substrate binding to the Polo-box domain of Plk1, it was observed that only His-538 and Lys-540 interact directly with the phosphate group of the substrate and mutations of these conserved residues resulted in the abolishment of phosphopeptide binding to the polo-box (Elia *et al.,* 2003b). Indeed, when these residues were mutated to alanine, the polo-box was unable to localize to the kinetochores and localization to the centrosomes was drastically decreased (Elia *et al.,* 2003b, Hanisch *et al.,* 2006), thus indicating the importance of binding of the polo-box to substrates resulting in its localization.

It has been proposed that the kinase activity of the Plks is regulated by the polo-box domain. Firstly, Jang *et al.* (2002) observed that truncated Plk protein lacking the polo-box domain had increased kinase activity. This suggested that the polo-box may inhibit the catalytic kinase domain of Plk through intramolecular interactions (Jang *et al.,*

2002a). Similarly, binding of the polo-box to the phosphopeptide motif of substates also increased the kinase acitivity of Plk1. This binding is believed to cause a conformational change, and as a result, liberates the kinase domain of Plk allowing phoshorylation of its T-loop (at threonine-210) (Elia *et al.,* 2003b). Consequent phosphorylation of Plk in this manner leads to its activation where it may phosphorylate the same protein that contains the phosphopeptide binding motif resulting in a feedback loop system. In addition, it may phosphorylate other associated proteins, acting as a key step in a phosphorylation cascade.

Characterizing the Phosphorylation Status of the Plks

Characterizing the phosphorylation site of a protein is essential in shedding light onto the activity and ability of that protein. Thus, determining the phosphorylation status of the Plk members has further given insight into how these proteins function and are regulated. By mutating threonine on amino acid 210 (a conserved site among the Plk members) to an aspartate which mimics phosphorylation, Lee and Erikson (1997) were able to greatly increase the kinase ability of Plk1 towards casein *in vitro* (Lee and Erikson, 1997). Thus, it has been suggested that this residue is contained within the "T" or activating loop in the kinase domain and phosphorylation of this residue is essential for triggering the kinase activity of Plk1 (Jang *et al.,* 2002b). In addition, the phosphorylated T210 residue increased greatly as cells proceed to mitosis, indicating that this event assists in activating Plk1 during its roles throughout this phase (Jang *et al.,* 2002b).

Nakajima *et al.* (2003) were the first group to suggest a crude consensus sequence for substrate phosphorylation by Plk1. They determined that the sequence [Asp/Glu]-[X]-[Ser/Thr]-[φ]-[X]-[Asp/Glu] (where X is any amino acid and φ is any hydrophobic amino

acid) to be the optimal phosphorylation sequence by this kinase. Further refinement determined the consensus sequence to be [X]-[φ/Glu]-[Asp/Glu]-[Asp/Glu/Ser]-[Ser/Thr]-[φ]-[Ser/ φ]-[Ser/Glu/Asp]-[Glu] and revealed that Plk2 and Plk3 have very similar substrate specificity (Johnson *et al.,* 2007).

Investigations into the phosphorylation status of Plk1-3 have determined that the kinase ability of these proteins is inhibited by Wortmannin. A potent fungal metabolite, Wortmannin was once believed to inhibit only the members of the PI 3-kinase family, such as ATM and ATR (Liu *et al.,* 2005). Although the protein level did not change, surprisingly, this metabolite was found to inhibit the activity of Plk1 in intact cells resulting in G2/M phase arrest (Liu *et al.,* 2005). Similarly, the kinase ability of Plk3 was also potently inhibited by Wortmannin, suggesting a universal ability to block all Plk member function (Liu *et al.,* 2007). Indeed, a model has been proposed explaining the binding of Wortmannin to Plk1-3. It explains that the Wortmannin molecule associates with three conserved amino acid residues (Cys 67, Lys 82 and Cys 133) and interferes with the ATP binding ability of these kinases (Johnson *et al*, 2007).

Normal Gene and Protein Expression of the Polo-like Kinases

When considering the mammalian Polo-like Kinases, each member is located at distinct chromosomal locations. Human Plk1 is located at 16p12.3 (murine Plk1 is located on chromosome 7), 5q12.1-13.2 is the chromosomal location of human Plk2 (chromosome 13 in mice), human Plk3 is at position 1p34.1 (murine Plk3 is on chromosome 4) and Sak/Plk4 is located on chromosome 4q28 (chromosome 3 in mice) (Winkles and Alberts, 2005). The diverse chromosome locations of the mammalian Plk members demonstrates the divergence of these proteins during the evolutionary process and gives further input into their distinct cellular roles.

Plk1 gene expression during the different phases of the cell cycle has been observed. Lake and Jelinek (1993) first reported the regulation of human STPK13 mRNA, which encoded a protein kinase related to polo and cdc5. They found that the mRNA expression of this gene was strictly regulated throughout the cell cycle where it was present in growing but not in non-growing cells. Furthermore, they found that the expression of STPK13 mRNA was low in G1 phase and began to accumulate in S phase. The expression of this gene reached its maximum abundance during G2/M (Lake and Jelinek, 1993). These observations were confirmed by Lee *et al.* (1995), where they showed that STPK13 mRNA corresponded to the mRNA expression of Plk1. Using NIH 3T3 cells, they were able to confirm the work performed by Lake and Jelinek, showing that STPK13 corresponded to Plk1 gene expression levels and further demonstrated that the protein expression and catalytic activity of Plk1 followed the same trend (Lee *et al.*, 1995). From there, it was observed that the expression of Plk1 was found in many other experimental systems illustrating a positive correlation between the expression of Plk1 and cell growth (Winkles and Alberts, 2005). Furthermore, Plk1 mRNA levels were detected in large amounts in mouse testis, and to a weaker extent, in the ovaries, spleen, intestine, liver, pancreas and thymus. However, no Plk1 gene expression was found in non-diseased tissues containing low or non-proliferating cells such as the heart, brain, lung, kidney and stomach (Lake and Jelinek, 1993, Winkles and Alberts, 2005).

Plk2 was first identified as an immediate-early response gene whose mRNA level was induced by growth-stimulating agents such as fetal calf serum. Indeed, it was shown that the expression of Plk2 mRNA increased dramatically within one hour after the addition of serum and decreased to almost undetected levels 6 hours post-treatment (Simmons *et al.*., 1992). In addition, Plk2 protein levels and activity seem to follow the

same trend. Cells induced with serum contained levels of Plk2 protein just one hour after treatment. These levels decreased after 2 hours and were undetectable in cells harvested 4, 8 or 25 hours post-serum induction or in cells blocked in S phase/M phase with hydroxyurea and nocodozale respectively (Ma *et al.,* 2003). Furthermore, Plk2 gene expression seems to be tissue specific (Winkles and Alberts, 2005). Levels of Plk2 mRNA were detected in tissue samples from mouse lung, heart and brain but were not detected in thymus, spleen, intestine, kidney and liver (Simmons *et al.,* 1992). Further investigation by Liby *et al.,* (2001) detected high Plk2 transcript levels in the testis, mammary gland, uterus and trachea (Winkles and Alberts, 2005).

Unlike Plk1 and Plk2, the mRNA and protein levels of Plk3 do not follow the same trend of expression during the cell cycle. First identified as a fibroblast growth factor (FGF) stimulated and early response gene in NIH 3T3 cells, Plk3 mRNA levels expressed very similar patterns to those of Plk2. That is, after the addition of serum in serum-starved cells, Plk3 mRNA levels were detected 0.5 hours post treatment and gradually decreased until they reached undetectable levels 8 hours later (Donohue *et al.,* 1995). Conversely, it has been observed that the protein level of this protein is relatively constant throughout the cell cycle. It was observed that Plk3 protein is very stable in human dermal fibroblasts (Winkles and Alberts, 2005). Further investigations have found that the nuclear import of Plk3 is required for degradation of this protein and indeed, proteosomal degradation of Plk3 occurs in the nucleus (Alberts and Winkles, 2004). As a result, sequestering of this protein in the cytoplasm would explain its relative constant levels throughout the cell cycle. Furthermore, the expression of Plk3 transcript in tissues from a newborn mouse were found in the intestine, kidney, liver, lung and skin, while

Plk3 in adult tissue was expressed at significant levels only in the brain, lungs and skin (Donohue *et al*, 1995).

Overexpression and Loss-of-Function of the Plks

The expression patterns of the Polo-like Kinases are stringently regulated throughout the cell cycle. Investigations into the roles of these members have shed insight into their functions and indeed, experiments involving the overexpression and loss-of function of Plks have been pivotal in determining their cell cycle roles. Studies involving the founding member of this group, *Drosophila melanogaster*, have shown that the original mutation in one polo gene (denoted $polo^{1}$) was recessive and larvae containing this mutation survive through embryogenesis. The few homozygous females that survived produced embryos containing abnormal spindle morphology. In addition, larvae homozygous for other polo mutations blocked in mitosis and were unable to propagate into adult structures resulting in their death (Sunkel and Glover, 1988).

Similar results were obtained from experiments involving Cdc5 and Plo1, where the roles of these members were further characterized. Investigations by Ohkura *et al.* (1995), first characterized Plo1 as a homologue of polo in *Schizosaccharomyces pombe*. From there they found that loss-of-function of Plo1 resulted in cells that arrested in mitosis. These cells contained over-condensed chromosomes with substandard spindles irradiating from a single pole. Furthermore, this phenotype was responsible for the disruption of actin ring and septum formation. As a result, Plo1 has been implemented in the proper formation of bipolar spindles and cell cleavage (Ohkura *et al.,* 1995). Overexpression of this protein resulted in similar phenotypes, primarily mitotic defects such as the overcondensing of chromosomes with the inability for cells to divide. In addition, septum formation was induced at inappropriate times during the cell cycle due

to the overexpression of this protein (Ohkura *et al.,* 1995). Cdc5 has shown very similar phenotypes where loss-of function experiments demonstrate cells that have a characteristic dumbbell shape and incomplete nuclear division (Kitada *et al.,* 1993) arresting at various phases of mitosis (Song and Lee, 2001). Interestingly, wildtype Plk1 and a kinase active form of this protein were able to completely restore the defect caused by the temperature sensitive Cdc5 mutant, similar to endogenous expression of Cdc5 (Lee and Erikson, 1997). Similarly, the expression of wildtype Plk3, but not a kinase-dead version of this protein, was also able to rescue the Cdc5 mutant defect (Ouyang *et al.,* 1997).

Overexpression and loss-of function experiments have also shed light on the roles of mammalian Plk members. When Plk1 was depleted in HeLa cells using siRNA, cells arrested at G2/M phase and contained the typical dumbbell morphology. In addition, depletion of Plk1 caused an increase in apoptosis and these results were consistent among other cancer cell lines used (Liu and Erikson, 2003). Further observations reported similar phenotypes and outlined Plk1's role in mitosis. For example, van Vugt and co-workers (2004) reported Plk1 depletion caused cells to arrest before the entry of mitosis and chromosomes in these cells failed to align, indicating improper mitotic spindle formation (van Vugt *et al,* 2004). Conversely, microinjection of Plk1 in quiescent NIH 3T3 cells under low serum incubation caused these cells to progress into mitosis and constitutive expression of this protein caused an increase in cell proliferation (Smith *et al.,* 1997).

When examining Plk2, it was determined that wildtype, heterozygous and Plk2 null mice were born at a ratio of 1:2:1, indicating that the deletion of Plk2 was not embryonically lethal (Ma *et al.,* 2003). In addition, Plk2 null mice were viable with no

significant difference in there 12 month survival rates compared to their littermates and appeared to be fertile. However, closer investigation revealed that Plk2 null mice were consistently smaller than there normal littermates. Although no significant change was associated with embryonic development of tissues, Plk2 may be involved in embryonic growth. In addition to a decrease in the size of the placenta harbouring nutrients to Plk2 null mice, examinations of Plk2 fibroblasts *in vitro* displayed a great amount of cells in S phase, indicating that Plk2 may have a role in the growth of cells during G1 (Ma *et al.,* 2003). The loss of Plk2 expression has also been characterized in many cancer cell lines supporting the notion that Plk2 is a tumour suppressor gene (Syed *et al.,* 2006).

In contrast to the overexpression and loss-of-function of Plk1, Plk3 seems to have the opposite effect. That is, examinations into the overexpression Plk3 in HeLa cells resulted in the promotion of chromatin condensation. Furthermore, the overexpression of this protein also inhibited cell proliferation and induced apoptosis. Interestingly, these phenotypes were obtained by overexpressing both the wildtype and kinase inactive forms of Plk3, indicating that kinase ability was not necessary in halting cell proliferation (Conn *et al.,* 2000).

Centrosome Dynamics and the Plk Members

The centrosome is a dynamic organelle consisting of two centrioles and a pericentriolar matrix. The centrioles are cylindrical structures consisting of nine microtubule polymers. The matrix contains a variety of proteins including the γ-tubulin ring complexes from which microtubule nucleation occurs, structural proteins, kinases and phosphatases. Similar to the replication process of DNA, centrosomes undergo a semi-conservative duplication in S phase of the cell cycle and proper centrosome function

is a vital process in cellular division. Indeed, centrosome abnormalities can be found in almost all cancer types (for review see Nigg, 2002).

When considering the Plk members, the various homologues have been seen to localize to the centrosome during early mitosis (Ji *et al.,* 2006). Further experimentation has been pivotal in shedding light on their particular function at this organelle. Injection of a Plk antibody blocking its function resulted in cells with much smaller centrosomes than normal and a decrease in the density of microtubules nucleating from these centrosomes. These results suggested a distinct role of Plk1 in centrosome maturation. Further investigation revealed that Plk1 is needed for the recruitment of γ-tubulin (a protein required for microtubule nucleation) to the centrosomes and thus blocking its function resulted in the abnormal phenotype (Lane and Nigg, 1996). Furthermore, Plk1 was observed to phosphorylate ninein-like protein (Nlp), which interacts with the γ-tubulin ring of centrosomes and promotes microtubule nucleation. On the onset of mitosis, Plk1 seems to phosphorylate this protein and promote its disassociation from the centrosomes, consequently marking another step in the initiation of mitosis (Casenghi *et al.,* 2003). Investigation into this process revealed that this phosphorylation event prevented Nlp to be shuttled to the centrosome by the dynein-dynactin motor complex. During interphase, Nlp is recruited to the centrosome by this complex and this process results in the assembly of centrosomal components at this organelle. Therefore, phosphorylation of Nlp by Plk1 prevents this from occurring and leads to mitotic entry (Casenghi *et al.,* 2005). In addition, before the onset of mitosis, Plk1 phosphorylates CyclinB leading to its translocation from the centrosome to the nucleus (Toyoshima-Morimoto *et al.,* 2001).

Plks and their Roles in Cell Cycle Regulation

The complex regulation of the cell cycle involves a number of proteins. It is understood that the entry into mitosis involves the formation of the Cdk1/CyclinB complex and this is achieved by the dephosphorylation of Thr14 and Tyr15 on Cdk1 by Cdc25C (Hoffmann *et al.,* 1993). Experiments involving Plk1 have shown that the activation of Cdc25C is a key event in this process that signals the entry of mitosis. Specifically, it was observed that Plk1 directly phosphorylates Cdc25C on serine 198 and this event leads to the nuclear translocation of this phosphatase (Roshak *et al.,* 2000, Toyoshima-Morimoto *et al.,* 2002). In addition, Plk1 phosphorylates the Cdk1 inhibiting kinase, Wee1 and this event leads to Wee1's subsequent degradation (Watanabe *et al.* 2004). Similarly in *Xenopus laevis*, Plx1 is phosphorylated and activated by Plkk1, where Plx1 then hyperphosphorylates Cdc25C thus initiating Cdk1/CyclinB complex formation leading to meiotic maturation of oocytes (Karaiskou *et al.,* 1999). Furthermore, microinjection of Plx1 into *Xenopus* oocytes results in the activation of Cdc25C and the subsequent initiation of mitosis (Qian *et al.,* 1998a), while immunodepletion of Plx1 prevents the activation of Cdc25C and the initiation of mitosis; thus emphasizing the profound role of this Plk member in cell division (Qian *et al.,* 2001)

As previously indicated, Plk1 and Plk3 often play opposite roles in regards to how they affect the activity of overlapping targets. As stated above, Plk1 phosphorylates Cdc25C on S198 leading to its nuclear translocation (Toyoshima-Morimoto *et al.,* 2002). Interestingly, however, Plk3 has also been observed to phosphorylate Cdc25C on Serine 191 and on Serine 198 leading to its nuclear accumulation (Bahassi *et al.,* 2004). Due to Plk3's constant protein level throughout the cell cycle, it has also been proposed that this Plk may interact with Cdc25A and regulate the G1/S transition (Myer *et al.,* 2005).

Plk1 is also involved in the activation of the Anaphase-Promoting Complex/Cyclosome (APC/C). Activation of the APC/C occurs at the anaphase-metaphase transition and only when all chromosomes are properly attached to the spindle (van de Weerdt and Medema, 2006). Plk1, as well as Cdk1/CyclinB, have been observed to partially phosphorylate this complex and as a result, partially restore the cyclin-ubiquitin ligase activity of dephosphorylated APC/C (Golan *et al.,* 2002). This suggests a direct role in APC/C activation. Indirectly, Plk1 has been observed to phosphorylate Early Mitotic Inhibitor 1 (Emi1). This protein is degraded in early mitosis by the Skp1-Cullin1 F-box protein (SCF) ubiquitin ligase and its degradation is necessary for the activation of the APC/C. It has been observed that Plk1 phosphorylates Emi1 targeting its degradation (Moshe *et al.,* 2004). Other substrates of the Plk family members have been observed, outlining the vital roles these proteins play during the cell cycle. A list of protein substrates has been summarized and provided in Table 1.

Table 1. **Substrates of the Mammalian Polo-like Kinase Members**

Polo-like Kinase Member	Known Substrates	Reference
Plk1	Cdc25C	Roshak *et al.*, 2000
	Wee1	Watanabe *et al.*, 2004
	Myt1	Nakajima *et al.*, 2003
	Cyclin B1	Toyoshima-Morimoto *et al.*, 2001
	NudC	Zhou *et al.*, 2003
	TCTP	Yarm *et al.*, 2002
	CHO1/mitotic kinesin-like protein 1 (MKLP-1)	Lee *et al.*, 1995
	α-tubulin	Feng *et al.*, 1999
	β-tubulin	Feng *et al.*, 1999
	γ-tubulin	Feng *et al.*, 1999
	Chk2	Tsvetkov *et al.*, 2003
	ninein-like protein (Nlp)	Casenghi *et al.*, 2003
	Early Mitotic Inhibitor 1 (Emi1)	Moshe *et al.*, 2004
	Pin1	Eckerdt *et al.*, 2005
	BRCA2	Lin *et al.*, 2003
	GRASP65	Lin *et al.*, 2000
Plk2	Spine-associated Rap guanosine triphosphatase activating protein (SPAR)	Pak *et al.*, 2003
Plk3	Cdc25C	Bahassi *et al.*, 2004
	Chk2	Bahassi *et al.*, 2006
	DNA polymerase δ (pol δ)	Xie *et al.*, 2005
	p53	Xie *et al.*, 2001
Sak	HandI	Martindill *et al.*, 2007
	Cdc25C	Bonni *et al.*, 2007, accepted
	p53	unpublished

Furthermore, Plk members are involved with the process of cytokinesis, which marks the end of mitosis and the division of a single cell into two daughter cells. Experiments with *Xenopus laevis* have shown that constitutively active Plx1 over-expressed in oocytes results in arrest at the cleave furrow stage of development This suggests that Plx1 deactivation is necessary for the exit from mitosis and emphasizes its role in cytokinesis (Qian *et al.,* 1999).

Plk1 was first suggested to play a role in cytokinesis by Lee *et al.,* (1995) when this group observed Plk1 co-localizations and co-immunprecipitation with CHO1/mitotic kinesis-like protein (MKLP-1) from anaphase to cytokinesis. CHO1/MKLP-1 is known to be involved in microtubule dynamics during cytokinesis, thus this interaction implies the involvement of Plk1 in this process (Lee *et al.,* 1995). Consistent with this, Plk1 has also been observed to interact with RhoA, a member of the Rho GTPase family. Overexpression of RhoA causes defects in the completion of cytokinesis. Both Plk1 and RhoA were found to co-localize to the midbody during telophase and the interaction between these proteins increased during mitosis (Dai *et al.,* 2007). In addition, Plk1 has been found to bind with microtubule associated protein regulating cytokinesis (PRC1) (Neef *et al.,* 2007). While these results together highly suggest that Plk1 plays a major role in cytokinesis, observations by van Vugt *et al.,* (2004) seem to present uncertainty to Plk1's direct involvement in this process. In Plk1 depleted cells, the cleavage furrow still forms and ingression still occurrs. Furthermore, proteins known to be necessary for cytokinesis still localize to the cleave furrow indicating that the defects in cell division might be an indirect consequence of defective bipolar spindle formation by Plk1 and not its role in cytokinesis (van Vugt *et al.,* 2004).

DNA Damage Checkpoints

The DNA content within cells is periodically encountered with metabolic and environmental stress. These factors may cause thymine dimers, mismatches between base pairs, or single-stranded or double stranded DNA breaks (Li and Zou, 2005). If they are not corrected, DNA damage may lead to tumourigenesis, immuno-deficiencies, neurodegenerative diseases or germ-line mutations passed on to the next generation resulting in genetic defects. DNA damage checkpoints have equipped species with a means of correcting potential harm before these defects occur. A simplified mechanism is proposed here where upon DNA damage, a cascade of events is initiated in order to protect the integrity of the genome (Figure 2).

Initially, the presence of DNA damage is recognized by sensor proteins (Niida and Nakanishi). These proteins associate near the sight of DNA damage and recruit mediator proteins. As a result, mediators act as bridges in this molecular cascade to facilitate signalling and accumulation of another group of proteins denoted transducers (Niida and Nakanishi, 2006). That is, mediators act through protein-protein interactions to recruit transducers in order to assist in DNA damage response.

The main signal transducer proteins within the DNA damage response cascade are two members of the phosphatidylinositol 3-kinase (PI-3 K) family, namely Ataxia telangiectasis mutated (ATM) and Ataxia telangiectasis and Rad3-related (ATR). ATM derived its name from the characteristic disease with the same name that results when a mutation in this protein occurs (Friend and Tapscott, 1998). Similarly, ATR was first identified in human and mouse by sequence similarity to ATM and Rad3 in *Schizosaccharomyces pombe* (Wright *et al.*, 1998). As members of the phosphatidylinositol 3-kinase (PI-3 K) family, they are characterized by their large

protein size and a conserved C-terminal catalytic domain. Yet unlike other members of the PI-3 K family, ATM and ATR do not function as lipid kinases, but rather as serine/threonine kinases phosphorylating peptides at particular SQ/TQ sites (Yang *et al.*, 2004). Ionizing radiation, telomere erosion and substances that generate double-stranded breaks activate ATM, where ultraviolet radiation, replication stress and certain other chemicals activate ATR. Upon DNA damage, activated ATM and ATR are both known to phosphorylate and co-localize with many proteins (Niida and Nakanishi, 2006).

One important set of proteins in which ATM/ATR phosphorylate are Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2). Although structurally unrelated, Chk1 and Chk2 play functionally similar roles in DNA damage response. Originally, it was thought that there existed distinct roles in response to certain types of DNA damaging agents, where ATM would phosphorylate Chk2 and ATR would activate Chk1 (Bartek and Lukas, 2003). However, recent evidence has shown that there exists intersecting communication between these kinases, particularly in the activation of Chk1 by ATM in response to ionizing radiation (Gatei *et al.*, 2003) and activation of Chk2 in an ATM-independent manner (Hirao *et al.*, 2002). Depletion of Chk1 causes chromosome misalignment, kinetochore defects and mitotic arrest (Tang *et al.*, 2006), while Chk2 depletion resulted in a failure to induce p53 mediated G1 arrest and apoptosis (Bahassi *et al.*, 2006). Furthermore, Chk1 and Chk2 are seen to phosphorylate many of the same known substrates including p53, E2F1 transcription factor (Bartek and Lukas, 2003), Cdc25A, Cdc25B and Cdc25C (Niida and Nakanishi, 2006) and Plk3 (Xie *et al.*, 2002) further emphasizing their roles in cell cycle progression, apoptosis and the DNA damage response.

In addition to Chk1/Chk2, ATM and ATR are known to phosphorylate members of the Polo-like kinase family. Studies have shown that Plk1 becomes inhibited by either ATM or ATR in response to specific types of DNA damage. This inhibition is believed to be critical in blocking DNA damaged cells from entering mitosis (Smits *et al.,* 2000, van Vugt *et al.,* 2001). In addition, studies have shown that increasing amounts of ATM blocks Plk1's ability to inhibit p53-dependent transcriptional activation. As a result, the inhibition of Plk1 by ATM restores p53 activity (Ando *et al.,* 2004).

Contrary to this, Plk3 interacts with p53 in a positive manner. That is, in response to DNA damage, the kinase activity of Plk3 is increased in an ATM-dependent manner and the physical interaction between Plk3 and p53 increases significantly (Xie *et al.,* 2001). In addition, Plk3 was found to phosphorylate DNA polymerase δ (pol δ), suggesting a potential role in responding to DNA damage and Plk2 is a transcriptional target of p53, although its role is unclear (Xie *et al.,* 2005).

Member of the Plk family have also been observed to interact with Chk1 and Chk2. Plk1 and Chk2 were found to physically interact in HEK 293 cells and colocalize to the centrosomes. Furthermore, overexpression of Plk1 induced phosphorylation of Chk2 and Plk1 was observed to phosphorylate Chk2 *in vitro* (Tsvetkov *et al.,* 2003). Plk3 has also been observed to phosphorylate Chk2 at residues S73 and S62. This phosphorylation event is thought to aid in ATM-mediated phosphorylation of Chk2 at T68. In response to phosphorylated T68, Chk2 is deemed active and able to respond to DNA damage (Bahassi *et al.,* 2006). In regards to Chk1, this protein negatively regulates Plk1 activity in response to ultraviolet radiation. Furthermore, depletion of Chk1 allows Plk1 to remain active and cells proceed to mitosis even after exposure to UV radiation (Tang *et al.* 2006).

The association of the Plk members with vital cell cycle regulators and DNA damage response proteins implies a vital role for this family of kinases in cellular growth, progression and the DNA damage response. Further characterization of these proteins can aid in increasing our understanding of cellular functions and help in our battle against diseases such as tumourigenesis.

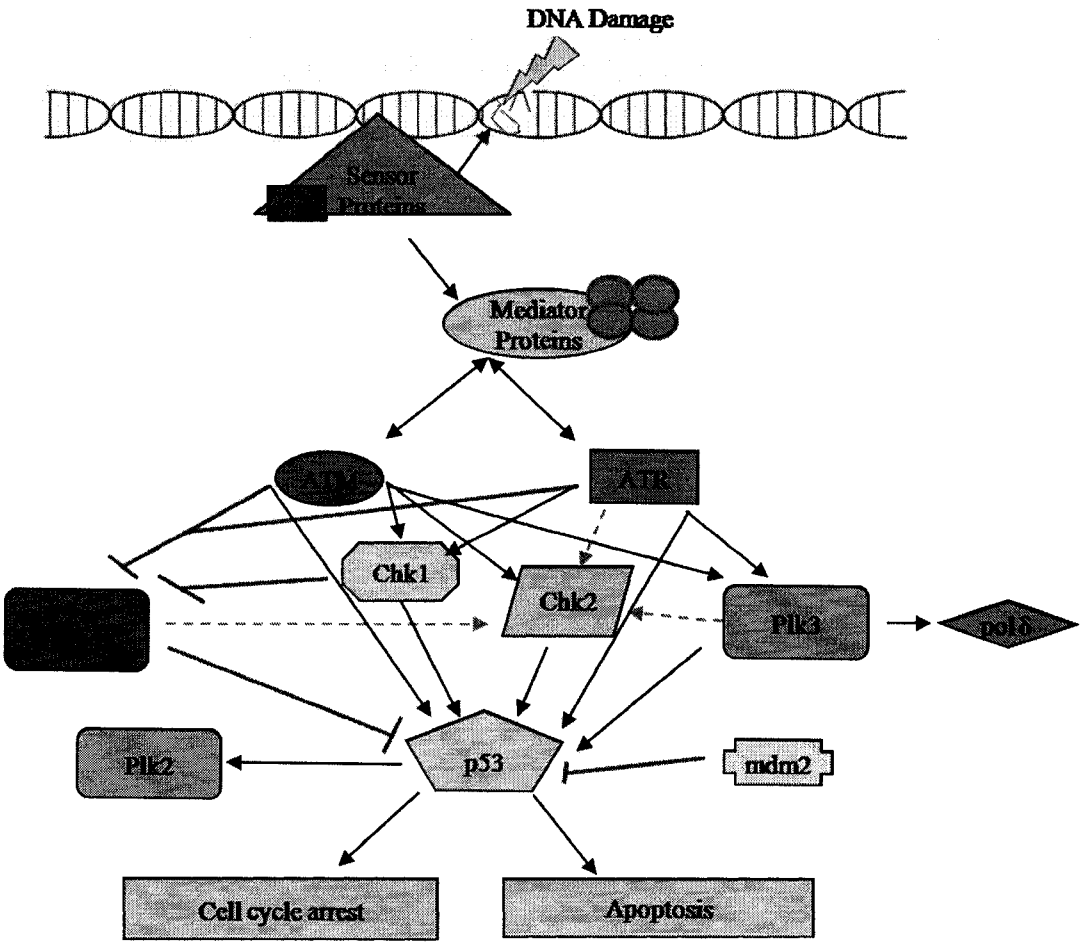

Figure 2. **The Association of Plk1-3 in DNA Damage Pathway**. The solid black lines denote a positive regulation event while the red perpendicular lines indicate a negative regulatory response. The dotted green lines represent known interactions between the proteins yet the functional significance is yet to be elucidated (Smits *et al.*, 2000, van Vugt *et al.*, 2001, Xie *et al.*, 2001, Hirao *et al.*, 2002, Bartek and Lukas, 2003, Gatei *et al.*, 2003, Tsvetkov *et al.*, 2003, Ando *et al.*, 2004, Li and Zou, 2005, Xie *et al.*, 2005, Niida and Nakanishi, 2006, Bahassi *et al.*, 2006, Tang *et al.*, 2006).

CHAPTER II

SAK/PLK4 BACKGROUND

The Structure of Sak

Sak (also known as Plk4) was the last member of the Polo-like kinase family to be identified in vertebrates and is observed to be the most structurally divergent. Indeed, unlike Plk1 and Plk3, expression of Sak was not able to rescue the temperature sensitive Cdc5 mutant from growth and mitotic defects suggesting a novel role for this kinase (Swallow *et al.,* 2005). Its N-terminal kinase domain is highly conserved when compared to the other Plk members and this observation first prompted researchers to name it Snk/Plk-akin kinase or Sak (Fode *et al.,* 1994). Within the conserved kinase domain lies a critical lysine at amino acid 41 that is required for proper ATP binding. Indeed, mutating this amino acid to a methionine leads to its inactivation rendering the protein kinase-dead. Furthermore, a mutation at the T-loop from a threonine to a phospho-mimicking aspartate leads to an increase in kinase activity (Swallow *et al.,* 2005).

The structural distinction between Sak and the other Plk family members lies within the C-terminus of these proteins. That is, unlike the other polo-like kinases that include two polo-boxes, Sak possesses only one polo box motif. Leung *et al.,* found the crystal structure of the Sak polo-box to be dimeric, containing two alpha-helices and two six-stranded beta-sheets. Harbouring a hydrophobic core, four of the six beta-strands from one polo-box interact with two beta-strands from another Sak polo-box forming an intermolecular homodimeric structure (Leung *et al.,* 2002). This organization is quite different from that of the other Plk members that form intramolecular heterodimers through the interactions of conserved hydrophilic amino acids (Cheng *et al.,* 2003, Elia *et al.,* 2003a). Furthermore, the polo-box domain of Plk1-3 is believed to play a significant

role in controlling the catalytic activity of these kinases (Jang *et al.*, 2002a, Elia *et al.*, 2003b). Recognized as a phosphopeptide binding motif, the polo-box of Plk1 has been observed to bind to particular substrates, resulting in the liberation of its kinase domain. This event causes the phosphorylation of the T-loop of Plk1 and results in its activation allowing for subsequent substrate phosphorylation. Contrary to this, no such results for Sak have been observed.

In support of the observation that the Sak polo-box domain forms a dimer, both full length Flag-tagged Sak and a Flag-tagged polo-box domain co-immunoprecipitated with the myc-tagged forms. However, a construct containing a deletion of the polo-box domain still interacted with the full length construct (Leung *et al.*, 2002). This association was dependent on a region N-terminal to the polo-box, denoted as the cryptic polo box and indeed this domain along with the polo-box are able to self associate (Leung *et al.*, 2002, Swallows *et al.*, 2005). Furthermore, the cryptic polo-box and polo-box constructs were observed to co-localize to the centrosomes and the cleavage furrow demonstrating that both were sufficient for localization. Interestingly, deletion of both drastically decreased this localization (Leung *et al.*, 2002, Habedanck *et al.*, 2005). As a result, both these domains of Sak may be needed in order for proper localization of this protein.

In addition, Sak contains three PEST sequences that are not found in the other members of the polo-like kinase family. These motifs are rich in proline, aspartate, serine and threonine and are associated with decreased protein stability (Fode *et al.*, 1994). Indeed, Sak displays a short half-life of 2-3 hours in cells and is subject to ubiquitination and consequent proteolytic degradation during mitosis (Fode *et al.*, 1996). Furthermore,

deletion of these PEST sequences result in an increase in Sak protein levels (Yamashita *et al.*, 2001) (Figure 3).

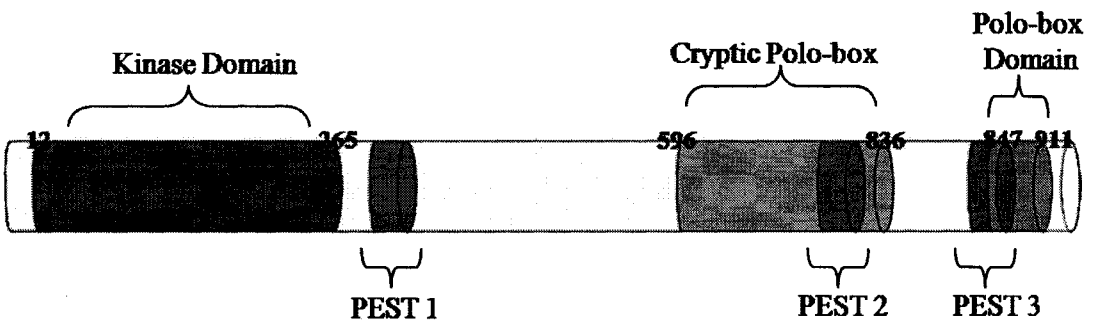

Figure 3. **Human Sak Structure.** Sak is the most structurally divergent member of the Polo-like kinases. A highly conserved kinase domain is located at its N-terminal and shown here in dark blue. The polo-box domain is located at the far C-terminus of this protein and depicted here in lighter blue. In addition, the area denoted as the cryptic polo-box is located N-terminally to the polo-box and is seen here in aqua. Sak also contains three unique PEST sequences shown here in red that are not found in the other Plk members (Swallow *et al.*, 2005).

Normal Gene and Protein Expression of Sak

The Plks likely arose as a result of gene duplication events as they are found at distinct chromosomal locations, with human Sak located on chromosome 4q28 (chromosome 3 in mice). Interestingly, this is an area that often undergoes chromosomal deletion in hepatocellular carcinomas (Ko *et al.,* 2005).

In regards to Sak, studies have revealed that its mRNA expression is similar to that of Plk1. As NIH-3T3 cells exit the cell cycle, Sak mRNA levels decrease dramatically indicating that Sak expression may be absent from cells exiting the cell cycle (Fode *et al.,* 1996). In order to confirm this result, serum starved cells that were quiescent were examined and no Sak mRNA was detected. Cells were then released from Go by introducing serum and monitored at different time points. As cells began to exit G1 and enter S phase, the level of Sak mRNA began to increase. Cells blocked in mitosis with nocodazole also displayed high levels of Sak mRNA. As these cells were released from this metaphase-block, the levels of Sak significantly decreased in G1 and disappeared by mid-G1 phase. These observations indicated that the expression of Sak mRNA is cell cycle regulated (Fode *et al.,* 1996).

In addition to being cell cycle regulated, the expression of Sak is tissue specific, with the highest levels of mRNA found in the testes. Lower levels were also detected in the spleen, thymus and ovary, while Sak mRNA levels were undetectable in the heart, liver, brain or kidneys (Fode *et al.,* 1994). It was also observed that Sak expression is developmentally regulated. The expression in testes is low until day 8 of development (a time before meiosis begins), at which time it increases significantly in correlation to meiotic activity of this tissue as the mouse ages. *In situ* hybridization of murine embryos indicated that Sak was expressed in various organs during their proliferative stages (Fode

et al., 1994). Taken together, these results indicate that Sak expression may be critical for cellular proliferation.

The murine Sak gene consists of 15 exons and this single gene encodes two alternatively spliced isoforms of this protein (Hudson *et al.,* 2000). The Sak-a transcript encodes a 925 amino acid protein of 103 kDa. The smaller isoform, denoted Sak-b, encodes a 53 kDa protein. The kinase domains of these isoforms are identical as the proteins encode the same first 5 exons or 416 amino acids. The difference between these isoforms exists in their C-terminus where Sak-a encodes the rest of the exons (exons 6-15) resulting in the full length protein. Sak-b, however, encodes the 147 base pairs contiguous with exon 5 or rather, exon 6 and the two introns flanking this exon encoding an additional 48 amino acids. The Sak-b isoform does not contain a cryptic polo-box domain or polo-box domain (Hudson *et al.,* 2000). In contrast, there is only one human Sak isoform that has been detected. Human Sak-a differs from the murine Sak-a in that it retains an intron of 102 base pairs immediately adjacent to exon 5 relative to the murine protein sequence. Interestingly, this insert encodes a 34 amino acid sequence that is quite similar to the intron sequence of murine Sak-b (Hudson *et al.,* 2000).

Catalytic Activity and Substrates of Sak

There are less than a handful of known substrates for Sak. Similar consensus substrate motifs for Sak have been proposed in an attempt to shed further light on Sak interacting partners and its potential role in the cell cycle. Leung *et al.,* performed *in vitro* kinase assays on peptide spots arrays to determine the Sak consensus phosphorylation motif. From their observation, they proposed that Sak has a potential context dependent specificity and the ability to phosphorylate several different consensus sequences. That is, depending on the net charge of the peptide sequence, Sak may prefer

either acidic or basic residues. This similar behaviour has been shown to exist in other kinases such as c-ABL protein tyrosine kinase and the Ca^{2+}-dependent protein kinases. In general, they have determined the optimal substrate consensus sequence to be [¥]-[ζ]-[Ser/Thr]-[φ]-[φ]-[X]-[¥/Pro], where ¥ represents a charged residue, ζ is any amino acid except isoleucine, leucine or valine, φ denotes a hydrophobic residue (with a preference to a large hydrophobic group) and X is any amino acid. Furthermore, it is worth noting that the charged residue is influenced by the context of the surrounding sequence. When peptides contain a net positive charge, Sak favours basic residues (such as Lysine, Histidine and Arginine), while Sak favours acidic residues (Aspartic acid, Asparagine, Glutamic acid and Glutamine) for peptide sequences exhibiting a net negative charge (Leung *et al.,* 2007).

Similarly, Johnson *et al.,* examined substrate specificity for all the mammalian Plk members. It was observed that the substrate consensus motif of Sak differed significantly from Plk1-3. They concluded that the optimal substrate consensus sequence is [X]-[Arg/Lys]-[Asp/Glu]-[X]-[Ser/Thr]-[φ/Tyr]-[φ/Tyr]-[X]-[Ser/Thr/Ala], where φ denotes a hydrophobic residue and X is any amino acid (Johnson *et al.,* 2007).

In determining the optimal substrate consensus sequence, previously determined substrates of the Plk members were used. Cdc25C is known to be phosphorylated by both Plk1 and Plk3. When Cdc25C was used as a substrate for bacterially expressed Sak, no phosphorylation was observed (Johnson *et al.,* 2007). However, recently we demonstrated that Sak and Cdc25C associate in HEK-293 cells and that Sak expressed in these cells is able to phosphorylate Cdc25C in an *in vitro* kinase assay (Bonni *et al.,* in press 2007). As a result, the substrate binding motif of Sak may prove to be inaccurate as novel substrates for this kinase emerge.

Studies involving the inhibition of the kinase ability of the Plk family members have also been examined. Through *in vitro* kinase assays using a peptide library as a substrate, Johnson *et al.* (2007) demonstrated that Wortmannin inhibits the kinase ability of Plk1-3. However, Sak was found to display a dissimilar first-order binding of Wortmannin compared to the other mammalian Plk members. Unlike Plk1-3, Sak seems not to be inhibited by this metabolite. Examination of the structure of Sak revealed that one of the cysteine residues conserved in Plk1-3 and seen to play a role in Wortmannin binding is replaced by a larger valine in Sak. As a result, this larger valine may cause steric hinderance prohibiting the binding of Wortmannin (Johnson *et al.,* 2007).

Recently, Martindill *et al.,* (2007) have observed a novel substrate for Sak. HandI is an essential protein for placenta formation and cardiac morphogenesis in the developing embryo. This protein is sequestered in nucleoli during cell proliferation and its release into the nucleus leads to the differentiation of cells. Sak was found to phosphorylate HandI in trophoblast stem cells resulting in its release from the nucleolus and committing these cells for differentiation. Overexpression of Sak prompted HandI release from the nucleoli and Sak inhibition resulted in a sequestering of HandI and continued proliferation of undifferentiated cells (Martindill *et al.,* 2007).

To date, there is only one protein that is known to phosphorylate Sak. Tec, a cytoplasmic tyrosine kinase, is found to be activated by stimulations of cell surface receptors on blood cells. Tec was found to tyrosine phosphorylate Sak and regulate Sak protein levels. It achieves this by binding to the cryptic polo-box of Sak, phosphorylating its kinase domain and thus, protecting its PEST sequences from PEST-dependent proteolysis (Yamashita *et al.,* 2001).

Sak and Embryonic Development

Sak was the first mammalian polo-like kinase member whose gene expression was depleted at the whole organism level (Hudson *et al.,* 2001). Sak null mice are embryonic lethal arresting at E7.5 with an increase of mitotic cells (Hudson *et al.,* 2001). Sak null embryos display a six-fold greater incidence of phosphorylated histone H3 in comparison to their wildtype littermates. This result indicates that Sak null embryos arrest before histone H3 is rapidly dephosphorylated following the destruction of Cyclin B by the Anaphase Promoting complex (APC), suggesting that the cells arrested before anaphase (Hudson *et al.,* 2001).

Interestingly, Sak null embryos undergo many cell divisions before arresting and many theories have been proposed to explain this observation. It has been suggested that Sak may be maternally supplied at the first stages of embryonic development. Secondly, Sak activity may not be required until the embryo begins to undergo morphogenesis (Swallow *et al.* 2005). New evidence indicating Sak phosphorylation of HandI (Martindill *et al.* 2007) suggests that this explanation may be quite possible. Lastly, it has been suggested that Sak may be required during the whole course of embryonic development. However, checkpoints in the early embryo are delayed and the outcome of no Sak expression may not be observed until this later stage of development (for review see Swallow *et al.,* 2005). Regardless of the means, the absence of Sak in the developing embryo is lethal indicating a critical role in development as well as in mitotic regulation.

Sak and Tumourgenesis

In contrast to Sak null embryos, Sak heterozygous (Sak-/+) embryos develop into viable, fertile mice displaying no obvious abnormalities in their early life (Hudson *et al.,* 2001, Ko *et al.,* 2005). However, with the onset of age, mice ages 18-24 months began

to develop tumours at a rate of 50% compared to 3% in their wildtype littermates. The development of hepatocellular carcinomas (HCC) in the liver was the most common type of tumour growth. The second most common location for tumour development in Sak -/+ mice was the lungs with the development of papillary adenocarcinoma. Lastly, large soft tissue tumours in these mice were also found in the axilla and upper chest wall (Ko *et al.*, 2005).

In order to further study Sak -/+ mice and their predisposition for increased incidence of tumour formation, a two-thirds partial hepatectomy (PH) was performed. After 44 hours the heterozygous Sak hepatocytes had a 29% incidence of aberrant spindle and mitotic complexes and cellular/nuclear hypertrophy, compared to only 4% of the wildtype cells. Examining the molecular status of the heterozygous hepatocytes revealed a loss in the levels of Cyclins D1, E and B1, prolonged phosphorylation of Aurora A and Cdk1 and suppression of the p53, p21 and BubR1 activity, as well as a delayed cellular progression through the cell cycle. In addition, normal liver mass and morphology was restored in the wildtype hepatocytes by day 7 after the partial hepatectomy while the heterozygous cells experienced poor organization and delays in cell cycle progression. By 9-12 months after the partial hepatectomy, 6 of the 7 wildtype mice had regained normal liver morphology, while all of the Sak -/+ mice displayed abnormal liver morphology. It was also observed that 4 of the 11 mice had developed multifocal anaplastic tumours containing abnormal mitotic organization (Ko *et al.*, 2005). Thus, these observations provide evidence that Sak is haploinsufficient for liver regeneration and proper gene dosage is essential for cellular progression and the suppression of tumourigenesis.

Sak and Centrosome Dynamics

First examined in NIH 3T3 cells, Sak conjugated to green-fluorescence protein (GFP) was observed to localize to centrosomes during early M phase (Hudson *et al.*, 2001). Further experimentation has confirmed this result revealing that endogenous Sak associates with the centrosomes throughout the cell cycle as observed by its co-localization with both γ-tubulin and centrin (Habedanck *et al.*, 2005).

Habedanck *et al.*, (2005) found that overexpression of Sak in U2OS and HeLa cells caused centrosome amplification while overexpression of a kinase inactive form of this protein showed only two centrioles. As a result, it was conceived that Sak catalytic activity plays a significant role in centrosome duplication (Habedanck *et al.*, 2005). In order to determine whether over-duplication occurred during the S phase of the cell cycle by Sak or through a defect in cellular division, the phenotypic effect of the overexpression of Sak was examined in the absence or presence of aphidicolin, an S phase inhibitor. Since the same phenotypic results occurred without the presence of this drug, the overduplication of centrosomes is thus caused by overexpression of this kinase (Habedanck *et al.*, 2005). Similar results were obtained in Drosophila where overexpression of Sak resulted in multiple centrosomes emphasizing Sak's role in centrosome duplication (Bettencourt-Dias *et al.*, 2005).

Scientific contributions outlining the loss-of-function of Sak have also emphasized its role in centrosome duplication. When Sak expression was blocked with siRNA in both human and Drosophila cells, a decrease in centrosome number was observed. Knocking down Sak in these cells led to a decrease in γ-tubulin at the spindle poles and proteins normally found at the centrosomes during mitosis or in the pericentriolar material were absent (Bettencourt-Dias *et al.*, 2005).

Furthermore, Habedanck *et al.,* (2005) demonstrated that the catalytic activity of Sak was needed for centrosome amplification. Constructs lacking the kinase domain could not cause centrosome overduplication. In addition, the construct lacking the cryptic polo-box and the polo-box domain could not localize to the centrosomes and overduplication did not occur. These results suggest that Sak needs to phosphorylate particular substrates at the centrosome in order to cause centrosome amplification (Habedanck *et al.,* 2005).

Interestingly, a contrary phenotype was observed in mouse embryonic fibroblasts (MEFs). Sak heterozygous MEFs grew slower than normal cells. Furthermore, five times the amount of Sak -/+ MEFs in interphase contained greater than two centrosomes compared to their normal counterparts and abnormal chromosome segregation was observed to be three times greater (Ko *et al.,* 2005). Two possible explanations have been proposed to explain this phenotype. Firstly, reduced Sak activity causes abnormal centrosome duplication resulting in cell division failure. Failure in cellular division can then lead to polyploidy and aneuploidy. This may in turn cause the increased incidence of tumour formation in Sak heterozygous mice (Habedanck *et al.,* 2005). Secondly, Sak heterozygous MEFs may cause deficient levels or activation of p53 and/or p21 resulting in uncontrolled centrosome replication by Cdk2 and cyclin E at the onset of S phase (Ko *et al.,* 2005).

Indeed, Sak and Cdk2 have been shown to cooperate in centrosome duplication. It was observed that overexpression of Sak could not cause centrosome overduplication in the absence of Cdk2 activity. In addition, in the absence of Sak, Cdk2 was also unable to cause centrosome amplification. These observations provided strong evidence indicating a connection between Cdk2 and Sak in centrosome duplication (Habedanck *et al.,* 2005).

Sak and the DNA Damage Pathways

The formation of tumours in Sak heterozygous mice and the role of Sak in centrosome dynamics emphasizes its potential role in the DNA damage response pathway. Indeed, Sak has been observed to co-immunoprecipitate with p53 (Swallow *et al.,* 2005) and has also been identified as a p53-repressed gene. p53 is a vital tumour suppressor gene that induces cellular growth arrest allowing cells to repair DNA damage. If the damage is irreparable, p53 then induces cell apoptosis (Sun, 2006). Li et al. (2005) observed the regulation of Sak expression to be p53-dependent. Although a direct interaction between p53 and the Sak promoter was not observed, the repression of Sak was found to be controlled by p53-mediated recruitment of HDAC transcriptional repressors. Furthermore, silencing Sak by siRNA contributed to p53-induced apoptosis and conversely, overexpression of Sak resulted in the attenuation of p53-induced apoptosis (Li *et al.,* 2005). These results lead to the notion that Sak regulation may be controlled by a well characterized tumour suppressor gene. Indeed this interaction may link Sak activity to the DNA damage pathway.

Objectives of This Study

In addition to Sak's interaction with p53, previous results from our lab have identified associations between Sak and well-characterized DNA damage response proteins. The role of the other Plk family members in the DNA damage pathway has been studied to a greater extent. Plk1 and Plk3 have been shown to be targets of ATM and ATR and have been observed to interact with Chk1 and Chk2 in response to DNA damage (Smits *et al.,* 2000, van Vugt *et al.,* 2001, Tsvetkov *et al.,* 2003, Bahassi *et al.,* 2006). Therefore, the purpose of this study is to elucidate the role of this Plk member in the DNA damage pathway. The phosphorylation status of Sak was thus examined in the

absence and presence of UV, as well as in the presence of Wortmannin. Furthermore, novel interactions between Sak and DNA damage response proteins including ATR, Chk1 and Chk2 were investigated with the hopes of shedding light on Sak's involvement during DNA damage.

CHAPTER III

MATERIALS AND METHODS

Preparation of Escherichia coli (E. coli) competent cells

Competent E. coli cells, Top 10 F Pilus E. Coli cells from glycerol stocks were streaked onto LB agar and incubated at 37 C overnight. A single colony was used to inoculate 10 mL of TYM media which was incubated for 16 hours at 37°C. After this initial incubation period, 1 mL of the culture was added to 100 mL of prewarmed TYM media and incubated at 37°C until an A_{600} of 0.5 was obtained. The cell cultures were then cooled by swirling the flasks on ice for 5 minutes, transferred to Oakridge tubes and centrifuged at 4000 x g for 10 minutes at 4°C. The supernatant was gently decanted and the resulting pellet was resuspended in 30 mL of TFB1. The cells were again centrifuged at 4°C for 10 minutes at 4000 x g and the supernatant was carefully discarded to avoid disturbing the pellet. While on ice, the pelleted cells were resuspended in 4 mL of ice-cold TFBII by repeated pipetting. 100 uL aliquots of the resulting cells were quickly placed into sterile mircocentrifuge tubes and flash-frozen by liquid nitrogen. The resulting compotent cells were stored at -80°C until needed.

Transformation and DNA purification

Top 10 F Pilus competent E. coli cells were transformed in order to obtain plasmid DNA required for subsequent experimental procedures. Approximately 0.1-1 ug of DNA was added to 100 uL of competent cells and allowed to incubate on ice for 30 minutes. After this time period, the cells were heat shocked at 42°C for 40 seconds and then placed immediately back on ice. 500 uL of LB media was added and the cells were incubated at 37°C for 45 minutes. 100uL was plated onto an LB-agar plate containing an appropriate concentration of antibiotic for selection of positive cells. The plate was

incubated at 37°C overnight allowing for the growth of bacterial colonies containing the desired plasmids. The following day, a single colony was picked and inoculated in 100 mL of LB media containing 100 ug/mL of ampicillin for 16 hours at 37°C. QIAGEN Plasmid Maxi Prep Kit (Qiagen Inc.) was used to purify the DNA according to the manufacturer's protocol. DNA was dissolved in ultra pure water (DIUF) and spectrometry was used to determine the DNA sample's concentration.

Cell Culture

Human Embryonic Kidney (HEK-293) cells were maintained at 37°C with 5% CO_2 to ensure optimal growth conditions. Furthermore, these cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) comprising of 10% fetal bovine serum (Sigma). Penicillin/streptomycin, Gentamycin and amphotericin B were also added to the media to prevent contamination of the cells.

Transfection of Plasmid DNA and Cell Lysis

The day prior to transfection, cells were washed with HBSS and trypsinized. They were then plated onto 10 cm tissue culture dishes at a density of 1 x 10^6 cells per plate. The following day, fresh media was added to the plates and 6 ug of DNA per plate was transfected using Effectene™ (Qiagen) according to the manufacturer's recommendations. Approximately 12 to 16 hours post-transfection, the cells were washed three times with 10 mL of cold Tris-Buffer Saline (TBS) and then supplemented with 1 mL of lysis buffer and allowed to sit on ice for 20 minutes. The lysed cells were then scraped off the plate and collected in eppendorf tubes which were spun in a microcentrifuge at 12 000 x g for 20 minutes at 4°C in order to separate the lysate from cellular debris. 5 uL of 6X loading buffer containing 5% Beta-Mercaptoethanol was added to 30 uL of lysate and the samples were boiled for 5 minutes. Following this, the

lysate samples were loaded into a gel and subjected to SDS-electrophoresis for 1 hour at 180 volts.

Exposure to UV radiation

In order to induce DNA damage, the Stratalink UV Crosslinker (Stratagene) was used to expose cells to ultra-violet radiation. Cells were subjected to various amounts of UV radiation and then were either lysed immediately or at the indicated time points.

Immunoprecipitation and Co-immunoprecipitation:

In order to enrich for the protein of interest or associated proteins, immunoprecipitation (IP) and Co-immunoprecipitation (Co-IP) reactions were conducted in which 1 ug of anti-FLAG (Sigma), anti-ATR (Santa Cruz and Calbiochem), anti-Chk1 (Sigma), anti-Chk2 (Sigma), anti-pChk2 T26 (Abcam) or anti-GAPDH (Cell Signaling) were incubated with 1 mg of total protein lysate for 2 hours at 4°C. The protein-antibody complex was then precipitated out of solution by the addition of 20 % Protein G-Sepharose slurry (Amersham) to the lysate and incubation on a vertical nutator was performed for an additional hour at 4°C. Following this period, the immunocomplexes were centrifuged at 3000 x g for 2 minutes at 4°C allowing separation of the immunocomplex from the rest of the lysate. The supernatant was then carefully decanted and the beads were gently washed three times with 400 uL of 150 mM TNT wash buffer. The immunoprecipitates were resuspended with 5 uL of 6 X SDS loading buffer and boiled for 5 minutes. 15-20 uL of each sample was loaded into the SDS-polyacrylamide gel and ran for 1 hour at 180 volts.

Western Blot Analysis

Following the completion of SDS-PAGE, the resulting gels were incubated in transfer buffer for 15 minutes. Simultaneously, polyvinylidene fluoride (PVDF)

membranes were activated by incubating the membranes in methanol for approximately 20 seconds. The membranes were then equilibriated in transfer buffer for 5 minutes. Depending on the size of the protein, transfer of the proteins from the gel to a membrane was performed using a semi-dry method at 11 Volts for 45 minutes or a wet transfer method at 45 Volts for 3 hours.

After transferring of the protein to a PVDF membrane was complete, the membranes were re-activated with methanol for 20 seconds and then blocked with Tris-buffered Saline and Tween (TBST) containing 1% skim milk for 1 hour to reduce the expression of non-specific proteins. The membranes were then incubated with primary antibody for one hour. That is, detection of the protein of interest by Western Blot Analysis was achieved in which 1 ug of each particular primary antibody was incubated in 10 mL of TBST with 1 % skim milk. The membranes were washed three times for ten minutes with TBST and then incubated with the appropriate secondary antibody for an addition 45 minutes. Anti-mouse secondary antibody conjugated to horseradish peroxidise (Amersham) was diluted to a final concentration of 1:60 000 in TBST with 1 % Skim milk. Similarly, anti-rabbit secondary antibody conjugated to horseradish peroxidise was diluted to a final concentration of 1:45 000. The different secondary antibodies used were dependent upon the specific species of the primary antibody. This was followed by an additional three washes with TBST of ten minutes each. In order to detect protein, the blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) dilution for five minutes and visualization was achieved by chemoiluminescense.

Stripping of Western Blots for Re-probing

In order to ascertain the presence/levels of additional proteins, previously probed membranes were stripped in order to remove any bound antibodies and re-probed with a different primary antibody. This was achieved by incubating the membrane with stripping buffer with gentle agitation for 30 minutes at 55°C, followed by three washes with TBST for 10 minutes at 55°C. The blots were then ready to be re-probed with the appropriate primary antibody.

Flag–Agarose Affinity Purification of Flag-Tagged Protein

HEK293 cells were plated and transfected with Flag-tagged human Sak as previously described. In order to induce DNA damage, 16 hours post transfection the cells were exposed to various amounts of UV radiation. The cells were then lysed with lysis buffer, scraped off the plates and centrifuged at 12 000 x g for 20 minutes at 4 °C in order to remove the lysate from the cellular debris. The lysate was then incubated at 4°C with 1:1 M2-Flag slurry (Sigma) and allowed to rotate on a vertical nutator for 2 hours. The samples were then centrifuged at 3000 x g for 1 minute and the supernantant was carefully decanted. Following this, the beads were washed three times with 400 uL of TNT Wash Buffer.

The desired Flag-tagged protein of interest was then eluted by the addition of 3 X Flag peptide (Sigma). Briefly, 35 uL of 3 X Flag peptide diluted in 1 X TBS to a concentration of 5 ug/uL was added to each sample and allowed to sit on ice for 1 hour with occasional agitation. This step was repeated an additional time to ensure the Flag-tagged protein was eluted from the 1:1 M2-Flag slurry beads. The samples were then spun down at 3000 x g for 1 minute and the resulting supernatant containing the eluted protein was transferred to a fresh Eppendorf tube.

In order to precipitate the eluted protein out of solution, 3 X the volume of acetone was added to the supernatant and allowed to sit at -20 °C overnight. The following morning, the samples were centrifuged at 10 000 x g for 15 minutes and three-quarters of the supernatant was carefully decanted. Samples were lyophilized (Speedvac, Biorad) in order to remove excess acetone. Following this, 15 uL of 2 X SDS loading buffer was added to each sample, the sample was boiled for 5 minutes and then subjected to SDS-PAGE.

In-Gel Detection of Phoshoproteins

Following SDS-PAGE, the gel was subjected to Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) in order to detect the presence of phosphoproteins. The gel was first immersed in 100 mL of Fix solution and incubated with gentle agitation at room temperature for 1 hour. The gel was then washed with ultrapure distilled water for 10 minutes. This step was repeated two more times for a total of three washes. Following this, the gel was subjected to 15 mL of phosphoprotein gel stain and microwaved for 10 seconds. The gel was then incubated in the absence of light at room temperature with gentle agitation for 15 minutes. This step was repeated an additional two times. The gel was then immersed in 100 mL of destain solution and allowed to incubate in the absence of light for 1 hour. Following this, the gel was then washed twice with ultra-pure water for 5 minutes. In order to obtain a phospho-image, the gel was scanned at 532 nm using the Molecular Imager FX (Biorad). After a phospho-image was produced, gels were subjected to 100 mL of Coomassie Blue stain, incubated at room temperature with gentle agitation for 1 hour and then subjected to various washes with destain solution in order to remove any background staining.

Preparation for Mass Spectroscopy

In order to extract the protein for Mass Spectroscropy, the particular band(s) of interest were excised from the gel and placed in Eppendorf tubes. They were then incubated in 100 uL of destain solution and allowed to sit for 35 minutes at 37 °C. This procedure was repeated a second time, followed by repeated changes of 100% acetonitrile to dehydrate the excised gel until it became opaque. The pieces were then lyophilized for 20 minutes to ensure that the excised gel was completely dried.

The dehydrated bands were then rehydrated in trypsin digestion buffer and incubated on ice for 30 minutes. After this period, an addition 20 uL of 50 mM ammonium bicarbonate was added to the gel pieces. Subsequently, the tubes were parafilmed and incubated in a shaker at 37 °C overnight. The following morning, the samples were briefly vortexed and the spun down. The supernatant containing the extracted peptides was collected and transferred to a fresh siliconized eppendorf tube containing 5 uL of 5% Formic Acid. In order to maximize the amount of peptide extraction, 200 uL of 60 % acetonitrile and 1% Formic Acid were added to the gel pieces and allowed to incubate at 37 °C for 45 minutes. This step was repeated an additional time. The gel pieces were then vortexed, spun down and the supernatant was collected and added to the peptides collected and placed in the siliconized eppendorf tubes. These pooled samples were then concentrated by subjecting them to the Speed Vac until only 10 uL of supernatant remained. 1uL of each sample was spotted directly on a MALDI plate and subjected to Mass Spectroscopy.

In-Vivo Phosphomapping

In order to determine which part of Sak was subjected to phosphorylation, various Flag-tagged Sak constructs were transfected in HEK293 cells as previously described.

The following morning, the transfected cells were washed three times with phosphate-free media (Gibco) and subsequently incubated with 10 mL of phosphate-free media containing 10 % dialysed Fetal Bovine Serum (Hyclone) for 2 hours. After this period, the volume of media was reduced to 5 mL and 1 mCi of ortho-phosphate inorganic P^{32} was added to each plate. The cells were thus allowed to incubate at 37 ^{o}C with 5 % CO_2 for 3 hours. Succeeding this incubation period, the cells were washed twice with 10 mL of 1 X TBS and lysed as previously described. The lysates were then subjected to immunoprecipitation and SDS-PAGE. The resulting gel was then transferred to a PVDF membrane and the membrane was exposed to a phospho storage screen overnight with visualization of phosphorylated bands on a phosphoimager (PerkinElmer). The following day, Western Blot analysis confirmed the presence of the Flag-tagged Sak constructs.

Site-Directed Mutagenesis

GST-Chk2 was a gratious gift from Dr. Bonni from the University of Calgary. In order to generate a kinase deficient version of Chk2, specific primers were designed (Primer3) to create the most optimal primers used to introduce the specific mutation at amino acid 368. The forward and reverse primers are listed respectively: 5'-gtcttataaagattactgcgtttgggcactccaag-3' and 5'-cttggagtgcccaaacgcagtaatctttataagac-3'. After the primers were designed, they were created by ACGT Corp. Subsequently, 50 ng of GST-Chk2 template DNA, 125 ng of forward and reverse primers, 1 X PFU polymerase buffer (Stratagene), 100 mM dNTP (dATP, dCTP, dGTP, dTTP), as well as 2.5 U of Pfu DNA polymerase was added together to a total volume of 50 uL. From there, the samples were subjected to Polymerase Chain Reaction (PCR) for a total of 18 cycles. Once PCR was complete in order to digest the non-mutated, methylated parent

DNA strands, 10 U of Dpn1 restriction enzyme (NEB) was added to each sample, gently pipetted and left at 37°C for one hour.

The digested PCR products were then transformed into Top 10 F' Pilus E. Coli competent cells are previously described. After an overnight incubation period at 37°C, individual colonies were picked and inoculated in 2 mL of LB media containing 100 ug/mL of ampicilin. Cultures were incubated at 37°C for 16 hours and the DNA was isolated using the QiaPrep Spin MiniPrep Kit from Qiagen according to the manufacture's protocol. The DNA product was then run on a 1% agarose gel against wildtype GST-Chk2 to ensure the insert was still intact. From there, 10 uL aliquots of the isolated DNA were sent for sequencing by ACGT.

GST-Fusion Protein Purification

BL21 E. Coli cells lack many proteases and are thus, an optimal way to express and purify GST-fusion proteins. These cells transformed with plasmid DNA allow the expression and accumulation of fusion proteins and minimize the level of degradation. As a result, BL21 RIL E.Coli cells were transformed with GST-Chk2 and GST-D368A plasmid DNA as previously described. A single colony was picked and inoculated overnight at 37°C in 100 mL of LB supplemented with 100 ug/mL of ampicilin. The following day, the 100 mL starter culture was added to 1 L of LB and grown at 37°C to an A_{600} of 0.7. After the indicated A_{600} value was obtained, IPTG (Fisher) to a final concentration of 0.5 mM was added and allowed to incubate with agitation at 25 °C for 7 hours. The drop in incubation temperature was adjusted for maximum solubility of the protein. Following this period, the cells were pelleted at 6000 x g for 15 minutes at 4 °C and the resulting supernantant was carefully decanted as to not disturb the pellet. The pellet was then resuspended in 20 mL of cell lysis buffer and sonicated on ice with 10

second pulses. The resulting lysate was then centrifuged at 10 000 x g for 30 minutes at 4 °C. Glutathione-Sepharose beads (GE) prepared according to the manufacturer's protocol were carefully added to the resulting supernatant and placed on a vertical rotator for 2 hours in order to allow binding of the fusion protein. The beads were then washed three times with column wash buffer. 1 mL of elution buffer was then added to the beads and allowed to incubate on a vertical rotator for an additional hour. The beads were spun down at 200 x g and the supernatant was removed and stored at 4 °C overnight. Simultaneously, an additional 1 mL of elution buffer was added to the beads and allowed to incubate with gentle agitation overnight. The following day, the resulting supernatant was run through spin columns in order to concentrate the eluted protein. SDS-PAGE, Coomassie Blue Stain and Western Analysis was performed as previously described in order to visualize the resulting GST-fusion purified protein.

In vitro Kinase Assay

In order to determine the kinase activity of the Flag-Sak constructs, *in vitro* kinase assay was performed. Briefly, FLAG-hSak, FLAG-T170D and FLAG-K41M expression plasmids were transiently transfected into HEK 293 cells as previously described. These cells were then lysed 16-hours post transfection and the cell lysate was incubated with 1 ug of anti-FLAG antibody for 45 minutes at 4 °C. Following this, 65 uL of a 20% protein G-sepharose slurry (GE lifesciences) was added to each sample and allowed to incubate at 4°C for 45 minutes. The immunocomplexes were precipitated out of solution as previously described and washed twice with 150 mM TNT wash buffer, twice with 150 mM TNT wash buffer containing 500 mM of LiCl and once with kinase buffer. The immunocomplexes were then resuspended in 30 uL of kinase buffer, 2.5 uL of cold ATP and 8 ug of the specified GST-fusion protein. In addition, various concentrations of

Wortmannin (1 nM, 10 nM, 25 nM, 50 nM, 100 nM and 1000 nM) were added to test its ability to inhibit Sak autophosphorylation. The kinase reaction was started by the addition of 10 uCi [γ-^{32}P] ATP (Amersham Biosciences) and the samples were then allowed to incubate at 35 ^{o}C from 35 minutes. In order to stop the kinase reaction, 6 uL of 6 X SDS loading buffer was added to each sample and boiled for 5 minutes. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and the resulting gel was transferred to a PVDF membrane as previously described. The membrane was then incubated overnight in the dark with a phospho-imager in order to visualize phosphorylation of the protein. Subsequently, the membranes were subjected to Western Blot Analysis in order to detect the immunoprecipitated proteins and the GST-fusion substrates.

CHAPTER IV

RESULTS

Characterizing the Phosphorylation Status of Sak

Sak is Phosphorylated in the Absence and Presence of Ultraviolet Radiation

Little is known about the phosphorylation status of Sak and how this affects Sak activity, stability, localization and interactions with other proteins. As an initial step, it was therefore of interest to examine and characterize the phosphorylation of Sak both under normal conditions, as well as upon exposure to DNA damaging agents. On this basis, HEK 293 cells were transiently transfected with an expression plasmid containing Flag-tagged human Sak. Untransfected, asynchronous cells were used as a control. 16 hours post transfection, the cells were exposed to 35 mJ/cm^2, 70 mJ/cm^2 or 120 mJ/cm^2 of ultraviolet (UV) radiation or left unexposed. Thirty minutes after exposure to UV, the cells were lysed and the lysates were incubated with 1:1 M2-Flag slurry. The desired Flag tagged human Sak protein was then eluted from the beads with the addition of 3 X Flag peptide and concentrated by acetone precipitation. In order to detect phosphorylated proteins, the resultant concentrated protein was then subjected to SDS-PAGE and the gel was stained using Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen). As seen in Figure 4A, human Sak is phosphorylated in asynchronous cells in the absence of UV exposure, as well as after UV exposure. Furthermore, increasing the dosage of UV exposure did not alter the phosphorylation status of Sak.

In order to ensure that the phosphorylated band seen was indeed Sak, the gel was subjected to Coomassie Blue staining (Figure 4B) and the corresponding bands were excised. Following this, the protein was extracted from the gel and trypsin digested in

order to prepare the sample for mass spectrometry. A MS peptide fingerprint was produced displaying distinct peaks varying in intensity.

Mass/charge ratios were then examined and input into MS-FIT Protein Prospect which takes the MS results and matches the peptide-mass fingerprinting data from MS to known amino acid sequences in proteins. Investigating seven distinct peaks from the peptide-mass fingerprint revealed that the corresponding sequences most likely belonged to Sak (data not shown).

Although, MS fingerprinting supported the notion that the phosphorylated bands were indeed Sak, the primary purpose of employing Mass Spectrometry was to attempt to characterize the actual site of phosphorylation of Sak. Unfortunately, the abundance of phosphorylated Sak was not great enough to detect the actual phosphorylated peptide through these means (data not shown).

Figure 4A

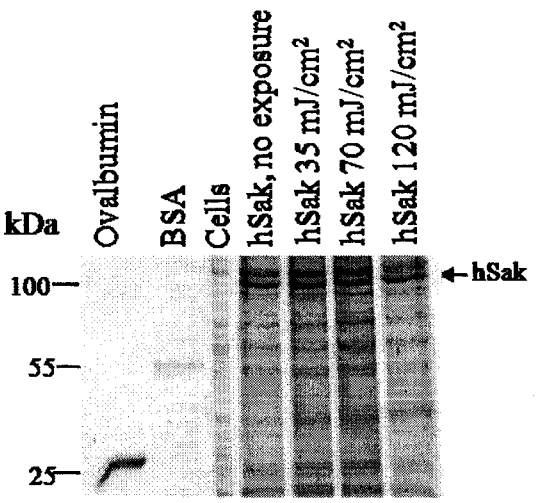

Figure 4 B

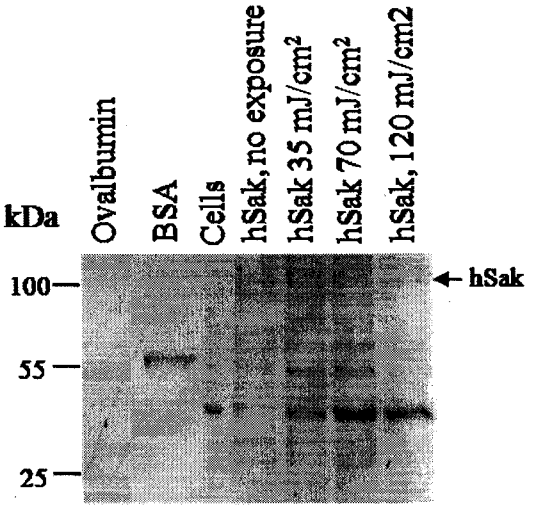

Figure 4. **Phosphorylation of Sak.** HEK 293 cells transfected with expression plasmids for Flag-tagged human Sak protein were exposed to different amounts (35 mJ/cm^2, 70 mJ/cm^2 and 120 mJ/cm^2) of ultraviolet radiation. Cells were lysed and the lysates were incubated with 1:1 M2-Flag slurry. Flag-hSak protein was eluted from the beads with 3 X Flag peptide and concentrated by acetone precipitation. The resultant concentrated protein was then subjected to SDS-PAGE. Cells alone were used as a negative control. (A) The resultant gel was subjected to Phosphoprotein Gel Staining in order to detect the presence of phosphorylated protein and exposed to a phospho-imager. Ovalbumin and BSA were used as a positive and negative control for phosphorylation respectively. A positive signal for phosphorylation was detected at 100 kDa in the lanes corresponding to immunoprecipitated human Sak both with or without exposure to UV radiation. No such band was present in cells alone. (B) The same gel was Coomassie Blue stained to ensure proper protein loading. The bands detected at 100 kDa (not seen in the cells alone lane) were then excised and subjected to Mass Spectrometry. MS-fingerprinting supported that the positive signal at 100 kDa corresponded to hSak (data not shown).

In Vivo Phosphorylation of Sak Domain-Specific Constructs

In order to characterize which domain of Sak was subjected to phosphorylation, *in vivo* phospho-mapping was employed. Flag-tagged Sak domain specific constructs were transiently transfected into HEK 293 cells (Figure 5). Specifically this included several different polypeptides. Firstly, a kinase active form of Sak (Flag-T170D) which has a single point mutation in its catalytic loop was utilized. Substitution of the threonine residue with a negatively charged aspartate mimics a phospho-residue and renders the protein kinase active. Secondly, two kinase dead forms of Sak (Flag-K41M and Flag-D154N) were utilized. Flag-K41M has a point mutation in which the critical lysine residue is mutated to a methionine; Flag-D154N also has a point mutation in which the aspartate in the activation loop was mutated to an arginine. Both of these mutations rendered the protein kinase dead .Flag-ΔPb was also utilized which consists of the Sak protein lacking the C-terminal polo-box domain. Finally, Flag-R1 and Flag-Pb were used which are proteins consisting of only the cryptic polo-box and the polo-box domain respectively.

These cells were then incubated in the presence of ortho-phosphate inorganic P^{32}, lysed and immunoprecipitation was performed with an anti-Flag antibody. Exposure to a phosphoimager (PerkinElmer) revealed distinct phosphorylation patterns. As seen in Figure 6A, all the full length Sak constructs except the mouse kinase dead protein are phosphorylated. However, a contaminating band in the cells alone and YVH1 negative control lanes at the approximate size of the full length Sak constructs was also observed. In contrast, a distinct signal for phosphorylated ΔPb construct is observed indicating that this result is conclusive. Interestingly, the cryptic polo-box domain (R1) and the polo-box domain (Pb) display no phosphorylation (Figure 6C). Taken together, these results

demonstrate that the ability for Sak to be phosphorylated is independent of Sak's own kinase ability. That is, the human kinase-dead construct was phosphorylated. This result shows that Sak is indeed being phosphorylated by an endogenous protein and not by its own ability to autophosphorylate. Furthermore, Pb and R1 are not phosphorylated and thus, indicate that under these conditions Sak is phosphorylated upstream of these domains.

Figure 5

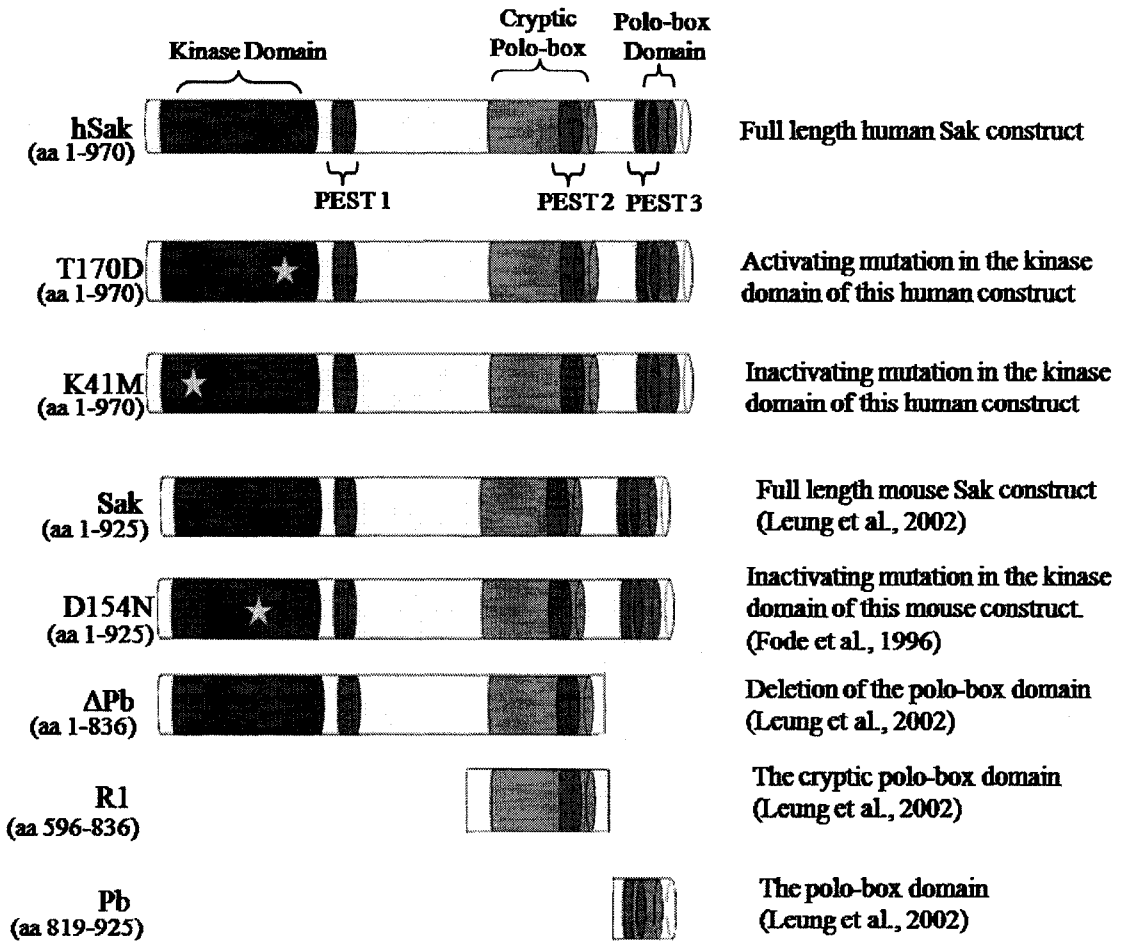

Figure 5. Schematic representation of domain specific Sak protein. cDNA encoding full length, mutated or domain specific Sak protein were cloned into the 3X FLAG expression plasmid (Leung et al., 2002; Swallow et al. 2005). Outlined above is a schematic representation of the various Sak proteins encoded by the vector used in these experiments.

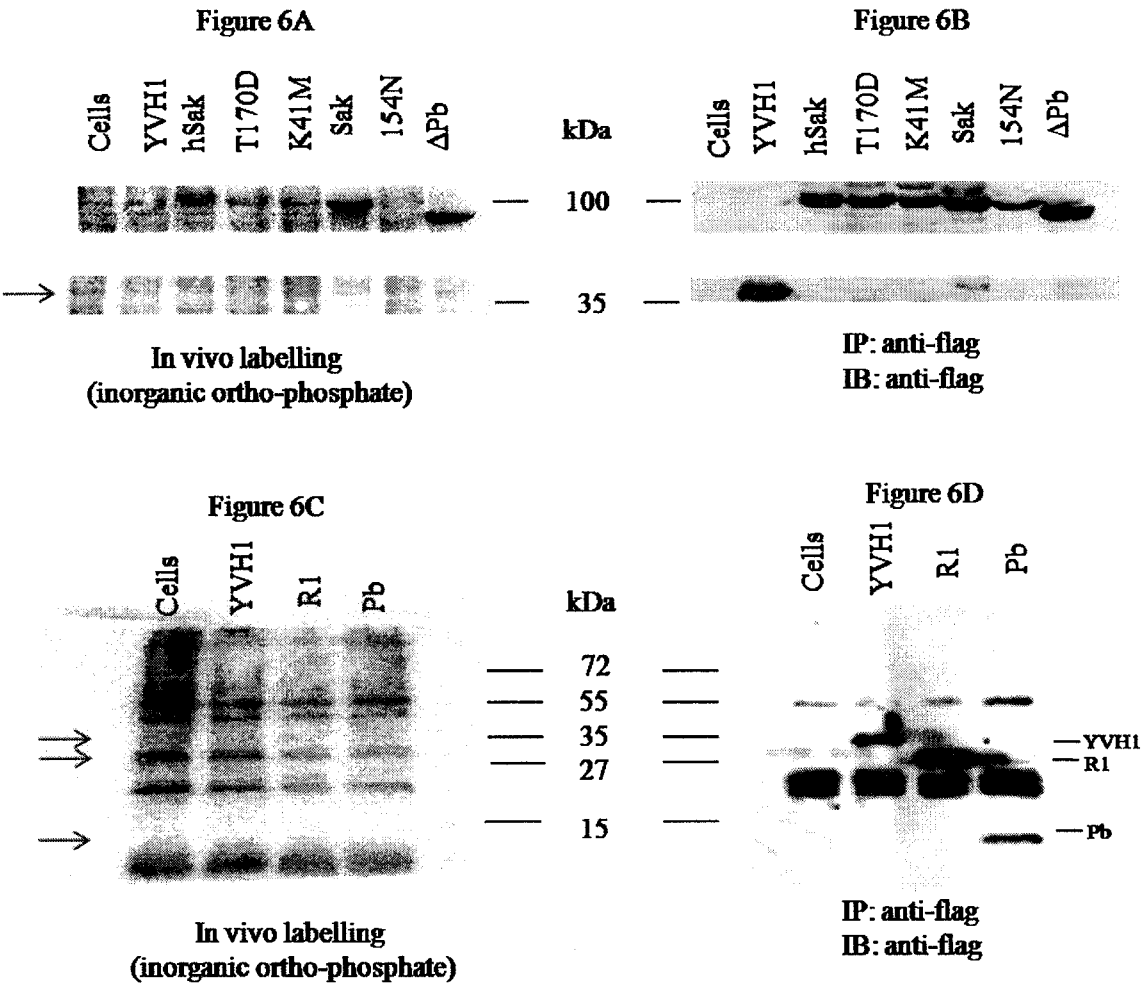

Figure 6. ***In Vivo* Phospho Mapping of Sak.** HEK 293 cells were transiently transfected with the various Sak constructs. The cells were incubated with ortho-phosphate inorganic P^{32} and subjected to *in vivo* phospho-mapping as described. Non-transfected cells and YVH1 were used as negative controls. (A) Immunoprecipitated full length Flag-Sak protein was resolved by SDS-PAGE. The corresponding membrane was exposed to a phosphoimager (PerkinElmer) in order to examine their phosphorylation status. A positive signal for phosphorylation was detected at 100 kDa for all the full length Flag-tagged Sak protein and Flag-ΔPb. (B) Western Blot Analysis using an anti-Flag antibody was performed. (C) Immunoprecipitated Flag-polo-box (Pb) and Flag-cryptic polo-box (R1) were subjected to SDS-PAGE, transferred to a PVDF membrane and exposed to a phosphoimager (PerkinElmer). No positive signal was detected for these domains. (D) Subsequent Western Blot Analysis using an anti-Flag antibody was performed in order to detect the presence of transiently expressed Flag-Pb and Flag-R1. Red arrows indicate the position of the expected positive signal in the corresponding lanes.

Autophosphorylation of Sak in the Presence of Wortmannin

Wortmannin is a potent inhibitor of the PI-3 kinase family and various studies involving its inhibitory effects on the kinase ability of ATM and ATR have been examined. Indeed, ATM/ATR kinase activity is blocked by Wortmannin with concentrations as low as 5 nM (Sarkaria *et al.,* 1999). In addition, Wortmannin has been observed to block the kinase activity of members of the Polo-like kinase family. In particular, experiments on Plk1 and Plk3 have shown a 100% inhibitory rate by Wortmannin at a concentration of 1000 nM (Liu *et al.,* 2005, Liu *et al.,* 2007). We tested the phosphorylation status of Sak in the presence of various concentrations of Wortmannin (0 nM, 1 nM, 10 nM, 25 nM, 50 nM, 100 nM and 1000 nM) utilizing casein (Sigma) as a substrate. Firstly, HEK 293 cells stably expressing Flag-hSak were induced by the addition of tetracycline. 16 hours post-induction, the cells were lysed, subjected to immunoprecipitation and incubated in the presence of [γ-^{32}P] ATP, Casein substrate and Wortmannin. SDS-PAGE was performed, the resultant gel was transferred to a membrane and exposed to a phosphoimager. Unfortunately, the image produced had various phosphorylated bands around approximately 25 kDa (corresponding to the molecular weight of Casein) in the presence and absence of Wortmannin. In addition, these bands (although not as intense) were seen in the negative control lane producing an inconclusive result as to whether phosphorylation of Casein by Sak occurred in the presence of Wortmannin (data not shown). However, Figure 7 depicts autophosphorylation of Sak in the absence and presence of this inhibitor. In addition, increasing the concentration of Wortmannin to levels known to inhibit Plk1 and Plk3, had no effect on the autophosphorylation status of Sak. Western blot analysis was performed to ensure that adequate levels of Sak expression.

At the same time this study was performed, Johnson et al. published results indicating that, unlike the other members of the mammalian Plk family, Sak was not inhibited by Wortmannin. An *in vitro* kinase was performed using a peptide library as the substrate. Wortmannin was added to the assay and time-dependent inhibition of ATP was examined. Their results indicated that Sak was not inhibited by Wortmannin (Johnson *et al.*, 2007).

Figure 7

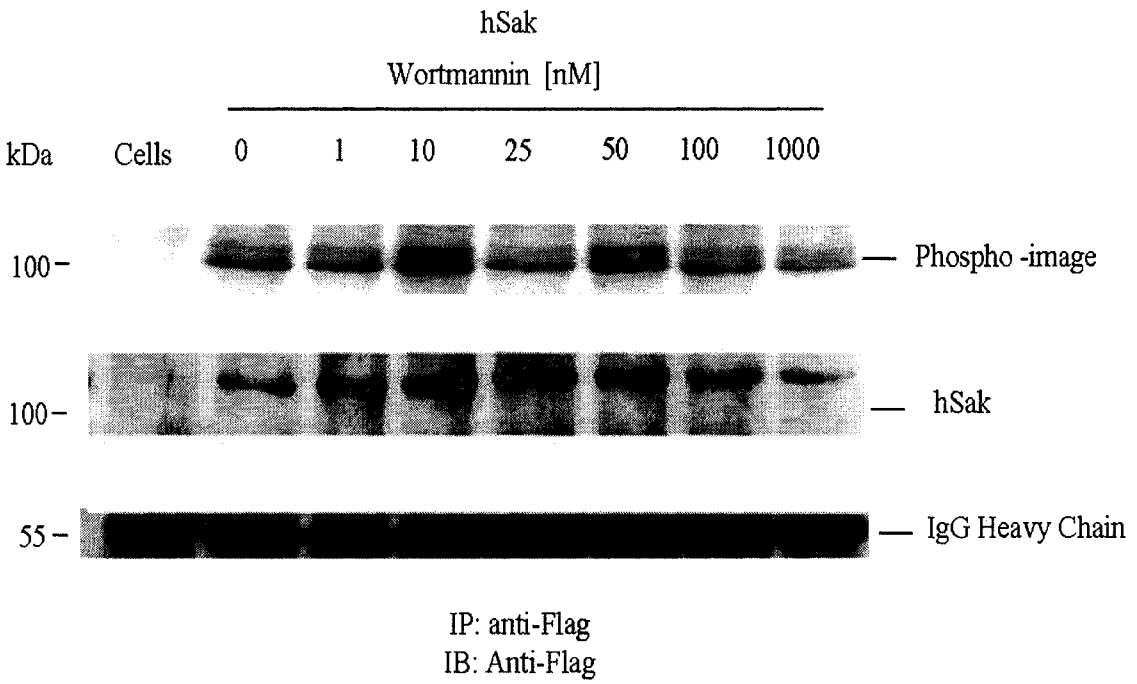

Figure 7. **Phosphorylation of Sak in the Presence of Wortmannin**. Lysates from HEK 293 cells stably expressing hSak were incubated with anti-Flag antibody. The Flag-hSak immunoprecipitates were incubated in kinase buffer containing [γ-^{32}P] ATP and the various concentrations of Wortmannin for 35 minutes at 35°C. The reactions were terminated by the addition of 6 X loading buffer, resolved by SDS-PAGE and phosphorylation was visualized by a phosphoimager (PerkinElmer). Autophosphorylation of Sak was detected in the absence and presence of Wortmannin. Subsequent Western Blot Analysis was performed using an anti-Flag antibody in order to detect Flag-hSak protein levels..

Sak and DNA Damage Response

Interaction of Sak with ATM and Rad3-Related (ATR)

Plk1 has been observed to interact and become inhibited by ATR after exposure to UV radiation and increasing amounts of adriamycin. When ATM null cell lines were exposed to UV, Plk1 activity was attenuated in the absence of ATM, suggesting that under these conditions, ATR is the primary inhibitor of this Plk member (van Vugt *et al.*, 2001). Given the known interaction between Plk1 and ATR, a previous study in our lab investigated the potential interaction between Sak and ATR, thus giving insight into Sak's role during DNA damage response. A putative interaction between wildtype, kinase active and kinase-dead hSak and ATR was identified by Melissa Ganuelas in Dr. Hudson's Laboratory (unpublished data). However, determining which domain of Sak interacts with ATR was not studied in this investigation. Therefore, in order to determine the domain specific interactions between Sak and ATR, the various domain-specific Flag-tagged Sak constructs were transiently expressed in HEK 293 cells. The cells were lysed and co-immunoprecipitation was performed using a polyclonal anti-ATR antibody (Calibiochem or Santa Cruz). Western blot anaylsis was then performed and involved probing the membrane with anti-Flag, detecting any of the Flag-Sak constructs.
As seen in Figure 8B, Flag-hSak, Flag-T170D and Flag-K41M all co-immunoprecipitated with ATR, confirming previous results. In addition, Flag-Sak, Flag-D154N, Flag-ΔPb and Flag-R1 show association with ATR. However, the polo-box construct did not co-immunoprecipitate with ATR. Flag-YVH1 was used as a negative control to ensure that these interactions were not between the Flag-tag and ATR. Furthermore, Figure 8A shows the transfection efficiency for each of the constructs. GAPDH was probed and used as a loading control. Taken together, it is observed that the kinase status of Sak does

not influence the association between these two proteins. Furthermore, the cryptic polo-box was found to be sufficient for Sak interaction as seen in Figure 8B. However, the association between ATR and Flag-ΔPb and the observation that Flag-Pb does not co-immunoprecipitate, indicates that the polo-box domain is not necessary or sufficient for this interaction to occur.

Figure 8A

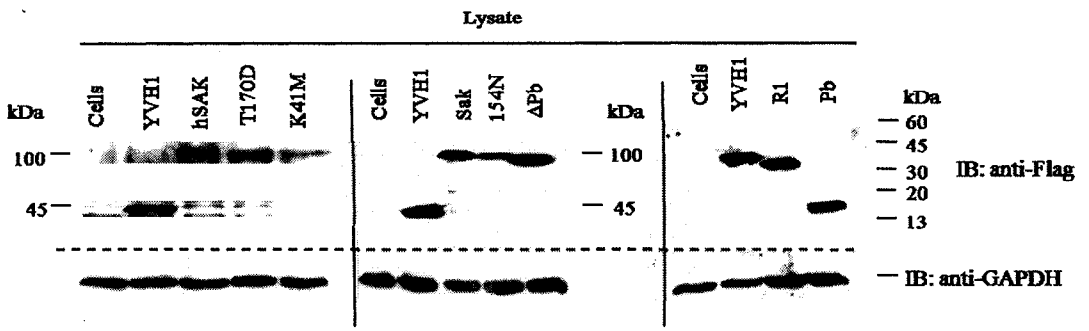

Figure 8B

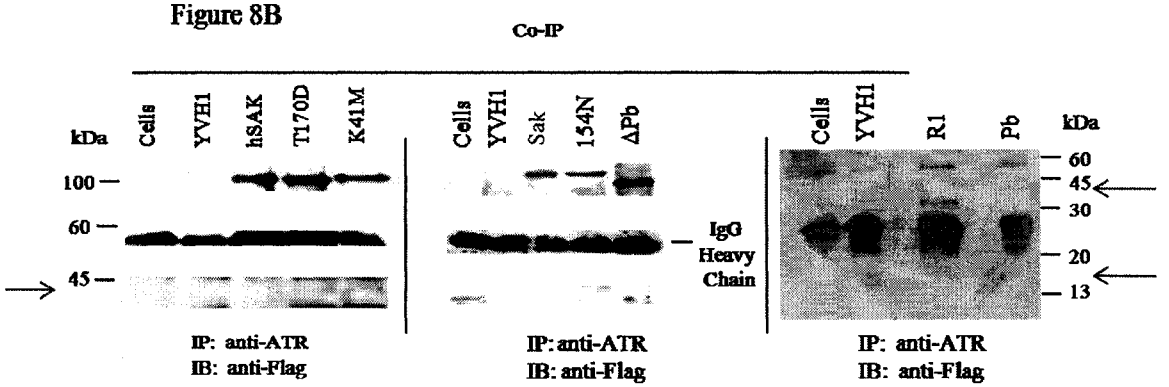

Figure 8. **Sak Interacts with ATR.** HEK 293 cells were transiently transfected with expression plasmids for various Flag-tagged Sak proteins and lysed 16 hours later. (A) Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-Flag antibody in order to determine the transfection efficiency of the Flag-tagged constructs. The blots were then stripped and re-probed with anti-GAPDH to ensure equal loading of the protein. (B) Cell lysates expressing the Flag-tagged Sak proteins were subjected to immunoprecipitation using an anti-ATR antibody. The immunoprecipitates were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted using an anti-Flag antibody. Wildtype hSak and Sak, kinase active Sak (T170D) and the two kinase dead Sak proteins (K41M and D154N) all co-immunoprecipitated with ATR. Sak lacking the polo-box domain (ΔPb) and the cyrptic polo-box (R1) also co-immunoprecipitated with ATR. The polo-box alone was not observed to interact with ATR. Cell lysate from non-transfected cells and Flag-YVH1 were negative controls. Red arrows indicate the position of the expected positive signal in the corresponding lanes..

Sak Interacts with Chk1

Chk1 has been found to block Plk1 activity in response to UV radiation preventing cells to enter mitosis (Tang *et al.,* 2006). This interaction with Plk1 led to the assumption that a potential interaction between Sak and Chk1 may exist. To test this hypothesis, the various Flag-tagged Sak constructs were transfected in HEK 293 cells and lysed 16 hours after tansfection. Cell lysates were probed with an anti-Flag antibody to ensure transfection efficiency of the various Sak constructs. This was followed by probing for GAPDH to ensure proper loading of the proteins (Figure 9A). Co-immunoprecipitation was performed on whole lysates with an anti-Chk1 monoclonal antibody (Sigma). Similar to the results obtained with ATR, Flag-hSak and Flag-Sak, Flag-T170D, Flag-K41M, Flag-D154N and Flag-ΔPb all co-immunoprecipitated with endogenous Chk1. No interaction was observed with Flag-Pb and the negative control Flag-YVH1 (Figure 9B). These results indicate that the kinase ability of Sak is independent of its association with Chk1. Flag-R1 interacts with Chk1 indicating that the cryptic polo-box is sufficient for Chk1 interaction. However, the lack of association between Chk1 and the polo-box domain suggests that this motif is not sufficient for interaction. In addition, Flag-ΔPb co-immunoprecipitating with endogenous Chk1 indicates that the polo-box domain of Sak is not necessary for this novel association.

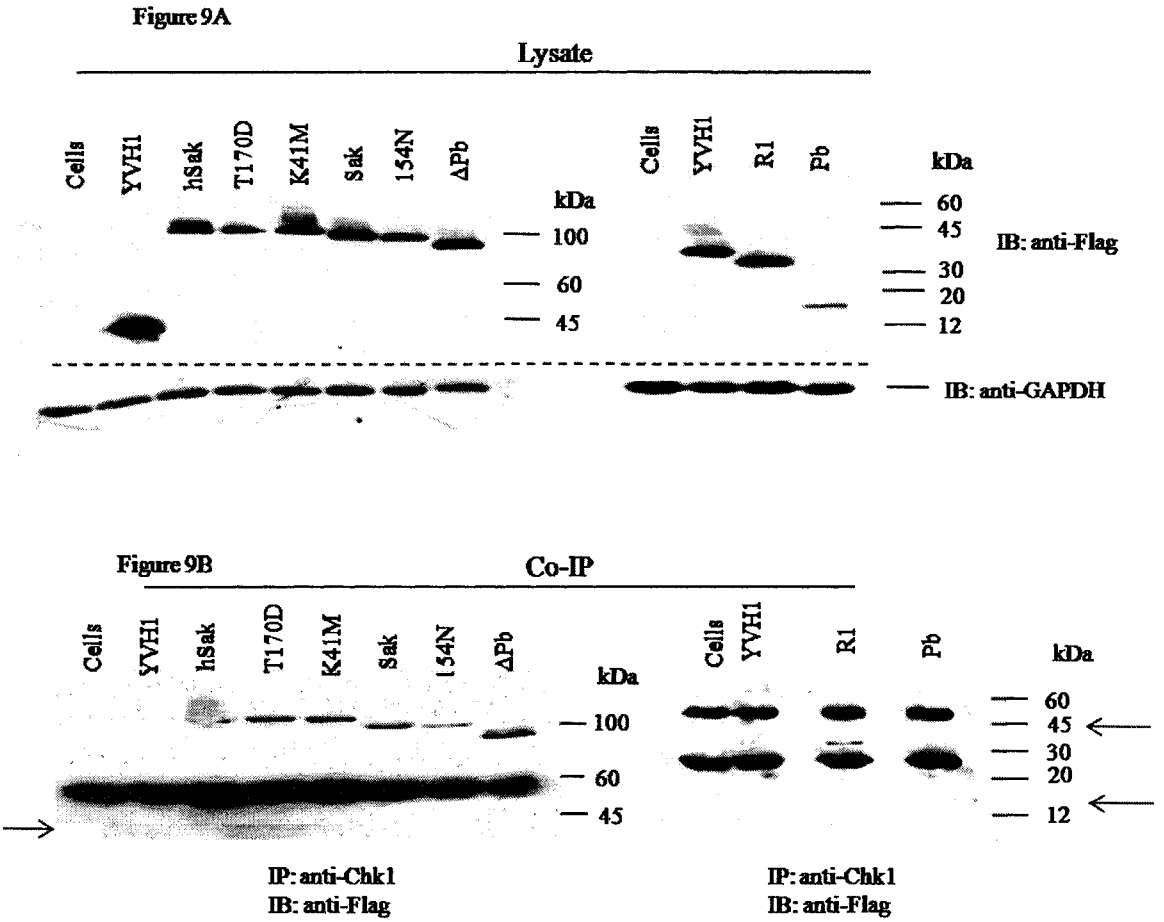

Figure 9. **Sak Co-Immunoprecipitates with Chk1.** Cells were transiently transfected with various expression plasmids for wildtype, kinase active (T170D), kinase dead (K41M and D154N) and domain specific Sak proteins and lysed 16 hours later. (A) Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-Flag antibody in order to determine the transfection efficiency of the Flag-tagged constructs. The blots were then stripped and re-probed with anti-GAPDH to ensure equal loading of the protein. (B) Cell lysates expressing the Flag-tagged Sak proteins were subjected to immunoprecipitation using an anti-Chk1 antibody. Resulting immunocomplexes were resolved by SDS-PAGE and Western Blot analysis was performed using an anti-Flag antibody. Wildtype hSak and Sak, kinase active Sak (T170D) and the two kinase dead Sak proteins (K41M and D154N), as well as the domain specific Sak protein lacking the polo-box (ΔPb) and the cyrptic polo-box (R1) co-immunoprecipitated with Chk1. The domain specific polo-box protein was not observed to interact with Chk1. Cell lysate from non-transfected cells and Flag-YVH1 were utilized as negative controls. Red arrows indicate the position of the expected positive signal in the corresponding lanes.

Protein Expression of Sak, ATR and Chk2 After Exposure to UV Radiation

In addition to ATR, novel interactions between Sak and Chk2 have been observed by Melissa Ganuelas from Dr. Hudson's Laboratory (unpublished data). The observed co-immunoprecipitation of Sak with endogenous ATR and Chk2 under normal asynchronous conditions prompted the idea that these interactions may be changed in response to UV radiation. To test this hypothesis, cells were either left alone or transfected with Flag-hSak. 16 hours post-transfection, the cells were exposed to 25 mJ/cm^2 of UV radiation and lysed at the indicated time points. Cell lysate was then subjected to SDS-PAGE and the resulting membranes were blotted using anti-Flag (Sigma), anti-Chk2 (Sigma), and anti-ATR (Santa Cruz). Lastly, the blots were probed with an anti-GAPDH antibody in order to measure protein levels. As seen in Figure 10A, protein levels of all four proteins remain relatively constant until 4 hours after UV exposure. At this time period, Sak levels remain relatively constant, while ATR and Chk2 proteins were not detectable. In addition, GAPDH levels, although present at 4 hours post UV exposure, showed a slight decrease in expression at this time, most likely the result of increased apoptosis due to UV exposure. This is in contrast to the protein levels in our control cells. Endogenous levels of ATR, Chk2 and GAPDH are relatively constant at all the times post UV exposure including 4 hours after UV. Interestingly, these results present the idea that cells over-expressing Sak and exposed to UV radiation may be more susceptible to UV radiation.

The striking difference in ATR protein levels in cells overexpressing Sak compared to cells absent of exogenous Sak expression led to the assumption that UV radiation may affect the association between these two proteins. As a result, cells were transiently expressed with Flag-Sak, exposed to UV 16 hours after transfection and lysed

at the indicated time points after exposure. Immunoprecipitation was performed using an anti-ATR polyclonal antibody (Santa Cruz) and the resulting immunoblot was probed with an anti-Flag antibody. The blot was then re-probed with an anti-ATR antibody to determine the levels of this protein. Interestingly, Flag-hSak co-immunoprecipitated with endogenous ATR at every time point. However, the level of Flag-hSak began to decrease 4 hours post UV radiation and ATR was not detected at this time (Figure 11). Taken together, the association between Sak and ATR does not change due to the presence of UV radiation. However, similar to the observations in Figure 10, the levels of ATR decrease in cells overexpressing Sak indicating that this association in the presence of UV exposure may affect overall protein levels or cell viability.

Figure 10A

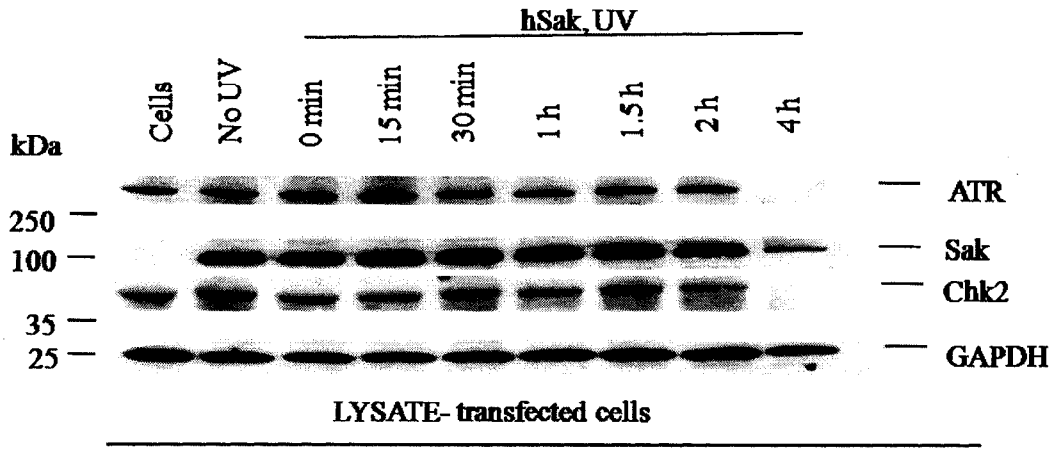

Figure 10B

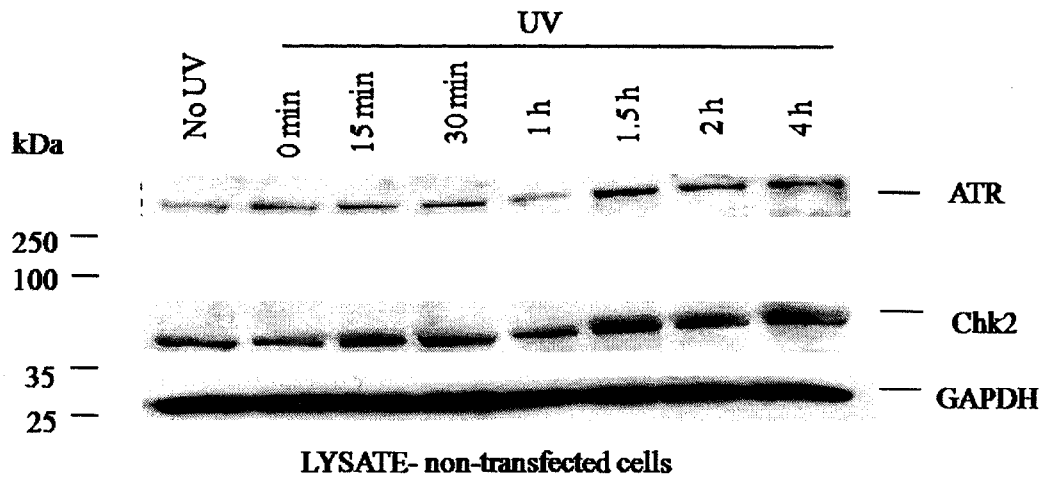

Figure 10. Time Scale Observations of the Interaction Between Sak, Chk2 and ATR. Cells were transiently transfected with the expression plasmid for hSak protein and exposed to 25 mJ/cm^2 of UV radiation 16 hours after. The cells were then lysed immediately after UV exposure or at the indicated time points (15 min, 30 min, 1 hour, 1.5 hours, 2 hours and 4 hours). (A) Whole cell lystates were immunoblotted with anti-Flag antibody in order to display Flag-hSak protein levels. The blot was then stripped and re-probed with anti-ATR, anti-Chk2 and anti-GAPDH in order to detect endogenous levels of these proteins. ATR and Chk2 protein levels were not detected 4 hours post-UV exposure. (B) Whole cell lystates from non-transfected cells were lysed at the indicated time points after UV exposure and resolved by SDS-PAGE. Subsequent immunoblotting with anti-ATR, anti-Chk2 and anti-GAPDH antibodies were done in order to detect endogenous levels of these proteins.

Figure 11

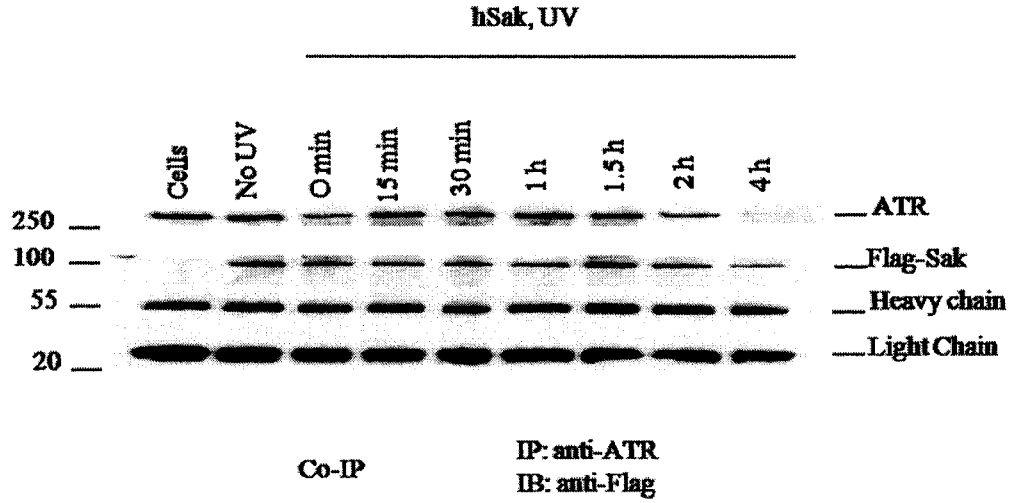

Figure 11. **Sak interacts with ATR After Exposure to UV.** Cells were transiently transfected with the expression plasmid for hSak protein and exposed to 25 mJ/cm^2 of UV radiation 16 hours after. The cells were then lysed immediately after UV exposure or at the indicated time points (15 min, 30 min, 1 hour, 1.5 hours, 2 hours and 4 hours). Whole cell lysates were subjected to immunoprecipitation with an anti-ATR antibody and the resultant immunocomplexes were resolved by SDS-PAGE. Western Blot Analysis was performed using an anti-Flag antibody. Flag-Sak co-immunoprecipitated with ATR at every time point after UV exposure. The membrane was then probed with anti-ATR in order to detect endogenous levels of ATR protein.

Creation of GST-D368A, a Kinase Inactive Form of GST-Chk2

Chk2 is a vital DNA damage response protein and is a known target of ATM/ATR (Bartek and Lukas, 2003, Hirao *et al.,* 2002). In addition, Plk1 and Plk3 were both observed to interact and phosphorylate this kinase (Tsvetkov *et al.,* 2003, Bahassi *et al.,* 2006). This prompted us to investigate the potential interaction between Sak and Chk2, and indeed an interaction has been observed. Since both these proteins are kinases, we investigated whether one or both are prospective targets for phosphorylation. In order to perform this assay, recombinant GST-Chk2 was given to us as a gracious gift by Dr. Bonni from the University of Calgary.

It has been observed, however, that bacterially expressed GST-Chk2 autophosphorylates (Xu *et al.,* 2002). As a result, in order to truly assess which protein is the substrate, a kinase-dead version of Chk2 was created. In order to do this, the Stratagene QuickChange Mutagenesis approach was utilized. Primers were designed to introduce a mutation into GST-Chk2 plasmid DNA in which the codon for an aspartate in the activation loop of the kinase domain was mutated to a codon for an alanine. Polymerase Chain Reaction (PCR) using Pfu DNA polymerase (Stratagene) allowed for the incorporation of the mutagenic primers. Following PCR, the DNA was incubated with DpnI which digested the methylated parental DNA not containing the mutation allowing for the selection of newly synthesized mutated DNA. Top 10 F' Pilus E. Coli competent cells were transformed with the resulting PCR products. Individual colonies were picked and incubated overnight in LB media containing 100 ug/mL of ampicillin. DNA was then isolated from these cultures using the QiaPrep Spin MiniPrep Kit (Qiagen) and the resulting DNA was sent for sequencing by ACGT Corp.

The inferred proteins sequence was determined by translating the sequence results from ACGT Corp and comparing it to the translated DNA sequence from the wildtype GST-Chk2 (ExPASy). As seen in Figure 12, the aspartate at amino acid 368 is mutated to an alanine indicating that site-directed mutagenesis was successful and Chk2 is theoretically now rendered kinase inactive. In order to distinguish between the two GST-Chk2 constructs, the kinase-dead construct was named GST-D368A.

This DNA was then transformed into BL21 E. Coli cells for the purpose of expressing and purifying GST-fusion protein. BL21 E. Coli cells lack many proteases, thus making them an optimal system to express protein for the purpose of purification. 0.5 mM IPTG (Fisher) was added in order to induce the cells to translate the GST-fusion DNA into protein. After an induction period of 7 hours, the cells were spun down and sonicated in order to break open the cells and remove the protein. In order to enrich for the GST fusion proteins, the resulting lysate was incubated with Glutathione-Sepharose beads (GE) which bound the GST-fusion protein. The resulting complex was washed, followed by elution of the GST-fusion protein. The resulting lysate was subjected to a spin column for the purpose of concentrating and purifying the GST-fusion protein. Through this process, GST-Chk2, the kinase defective form GST-D368A and GST-vector fusion proteins were purified and Bradford Assays were performed in order to determine their concentrations (data not shown).

Before these GST-fusion proteins were used in *in vitro* kinase assays, the protein products were run on an SDS-PAGE gel along with samples from the various stages of protein purification. This was performed in order to ensure that the proteins induced and were purified. Figure 13A depicts the resultant Coomassie Blue stained gel for GST-vector. Samples from the various stages of protein purification of GST-vector were taken

and subjected to SDS-PAGE. No GST-vector protein is detected in the uninduced lane. However, as protein purification proceeded, the band at 27 kDa increasing becomes more abundant with less contamination. Furthermore, Figure 13B depicts the final purified protein products of GST-Chk2 and GST-D368A, depicting a band at 93 kDa. Uninduced Cells were run as a negative control. In addition, the purified protein was subjected to

Western Blot Analysis where it was probed with an anti-Chk2 monoclonal antibody to ensure that the purified protein was indeed GST-Chk2 fusion protein. As seen in Figure 13C, bands in both the GST-Chk2 and GST-D368A at 93 kDa correspond to the GST-fusion proteins. These results indicate that GST-Chk2 and the kinase-dead construct, GST-D368A, have been bacterially expressed, harvested and purified.

Figure 12

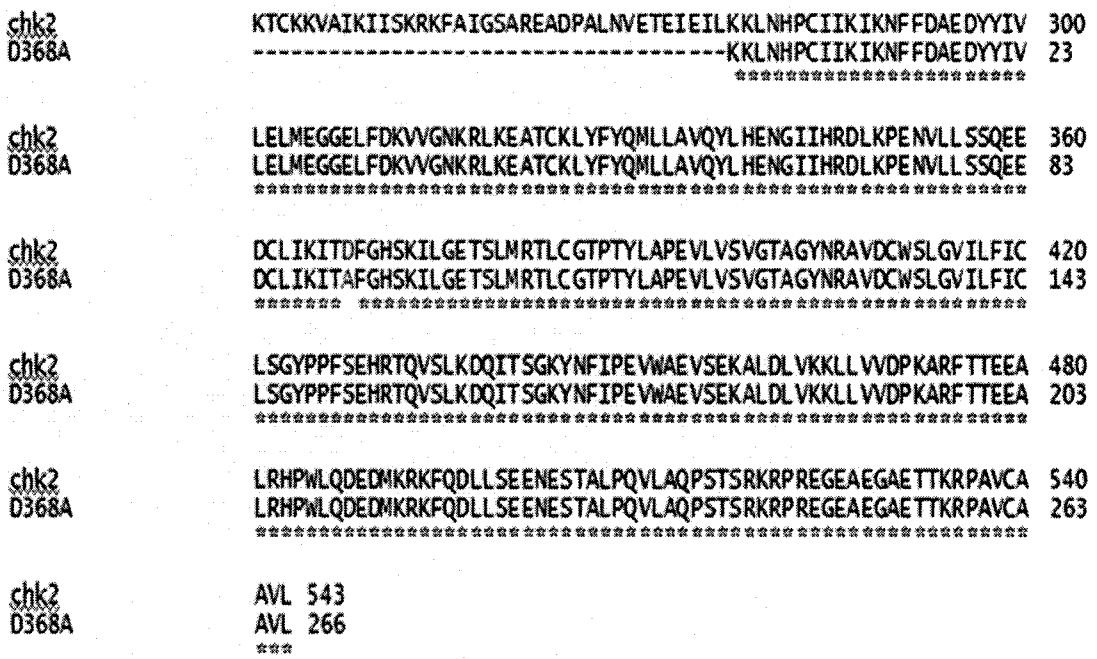

Figure 12. **The Sequencing Results From Site-Directed Mutagenesis of GST-Chk2.** The Stratagene QuickChange Mutagenesis approach was used in order to design primers introducing a mutation into GST-Chk2 plasmid DNA. Polymerase Chain Reaction (PCR) using Pfu DNA polymerase (Stratagene) allowed for the incorporation of the mutagenic primers. Sequential incubation with DpnI, digested the methylated parental DNA not containing the mutation allowing for the selection of newly synthesized mutated DNA. Top 10 F' Pilus E. Coli competent cells were transformed with the resulting PCR products. Individual colonies were picked, incubated overnight in LB media containing 100 ug/mL of ampicillin and DNA was isolated using the QiaPrep Spin MiniPrep Kit (Qiagen). The resulting DNA was sequenced by ACGT Corp., translated and compared to the translated DNA sequence from wildtype GST-Chk2 (ExPASy). The aspartate at amino acid 368 was mutated to an alanine theoretically rendering it kinase defective.

Figure 13A

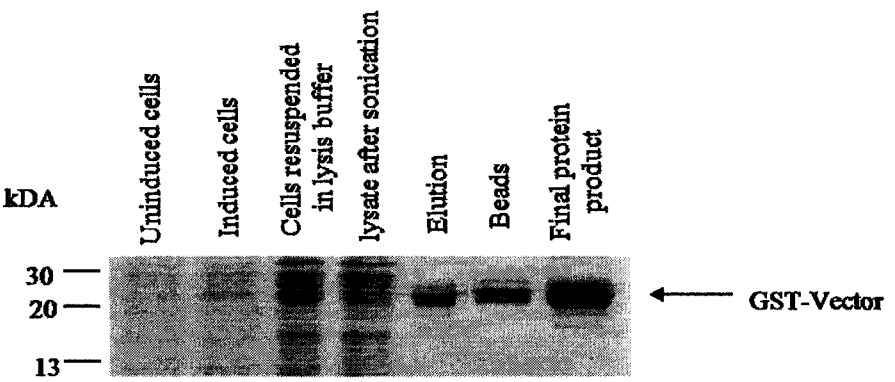

Figure 13B

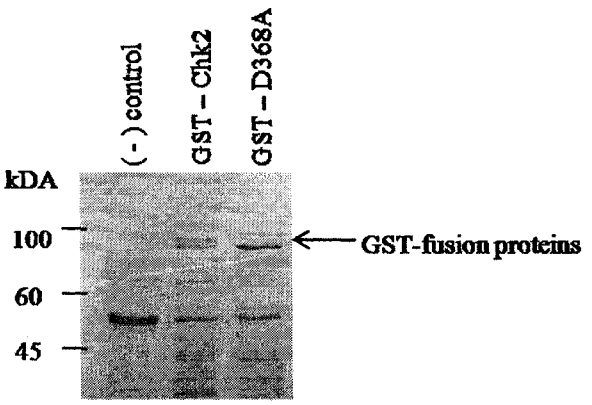

Figure 13C

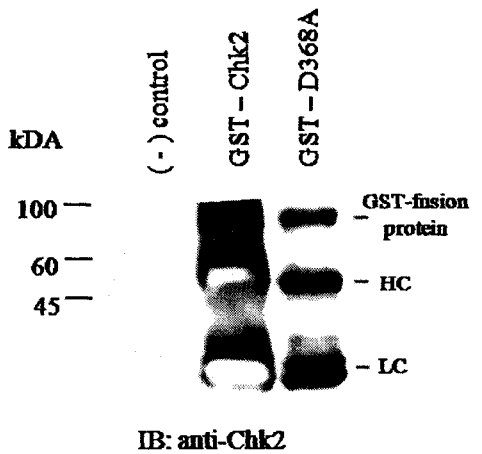

Figure 13. **Purification of the GST-Fusion Proteins.** Wildtype GST-Chk2, kinase dead GST-Chk2 (GST-D368A) and GST-vector were expressed in BL21 E. Coli cells using 0.5 mM of IPTG. The resulting fusion proteins were purified using Glutathione-Sepharose beads (GE), the beads were washed and the GST-fusion proteins were eluted. (A) Samples were taken from every step of the purification process of GST-vector, SDS-PAGE was performed and the resulting gel was then Coomassie Blue stained. Purification of GST-vector protein yeilded a band seen at 27 kDa in every lane after induction. The final protein product yields a band at the corresponding weight with very little contamination. (B) The final protein purification products of GST-Chk2 and GST-D368A were resolved by SDS-PAGE and subjected to Coomassie Blue staining. Uninduced cells were used as a negative control. Corresponding bands at 93 kD can be seen. (C) Western blot analysis was performed using an anti-Chk2 antibody to ensure that the observed bands at 93 kDa were in fact the GST-Chk2 fusion proteins. HC and LC denote heavy chain and light chain respectively.

Sak Phosphorylates Chk2 In Vitro

In order to determine whether the association between Sak and Chk2 led to the subsequent phosphorylation of one of these proteins, the purified GST-fusion proteins were used in an *in vitro* kinase assay. HEK 293 cells were transfected with Flag-hSak, Flag-T170D and Flag-K41M and lysed 16 hours after. Whole cell lysates prepared from these transfected cells were immunoprecipitated with anti-Flag antibody. From there, the bacterially expressed GST-Chk2 or GST-D368A were incubated with the immunoprecipitated Flag-tagged Sak constructs in the presence of [γ-^{32}P] ATP (Amersham Biosciences), SDS-PAGE was performed and the resulting membrane was exposed using a phosphoimager (Perkin Elmer). Subsequently, the membranes were probed sequentially with an anti-Flag antibody and anti-Chk2 antibody to confirm the presence of the Flag-tagged Sak proteins and Chk2 protein respectively. Figure 14A depicts the Flag-Sak constructs without the presence of a GST-fusion protein substrate. The phosphorylated bands at 100 kDa correspond to the autophosphorylation of wildtype Sak and kinase active Sak (T170D). Incubation of the Flag-Sak constructs with GST-Chk2 is seen in Figure 14B. Phosphorylation of GST-Chk2 is observed in every lane including the untransfected control, indicating that Chk2 is autophosphorylated. In addition, no phosphorylation of kinase-dead Sak was detected, indicating that Chk2 does not phosphorylated Sak. However, the kinase dead version of Chk2, GST-D368A, is observed to be phosphorylated by the wildtype Flag-hSak construct (Figure 14C). Interestingly, phosphorylation of D368A by kinase active T170D is not observed. Taken together, these results reveal that Chk2 is indeed a substrate of Sak.

Previous studies have also revealed that the phosphorylation of Chk2 by Plk1 occurs at T26 or S28. This phosphorylation is involved in the localization of Chk2 to

centrosomes (Tsvetkov *et al.,* 2003). Since Plks are known to target the same residues on particular targets, we examined the possibility that Plk4 may target Chk2 at the same site that Plk1 does. In order to test this hypothesis, the membranes from the previous kinase assay were stripped and re-probed with the anti-Chk2 phospho T26 specific antibody (Amersham). No bands were detected on any of these blots suggesting that a phospho-T26 residue on GST-D368A was not present. However, the lack of a positive control prohibits us stating with certainty that the site of phosphorylation of Chk2 by Sak is not T26 (data not shown).

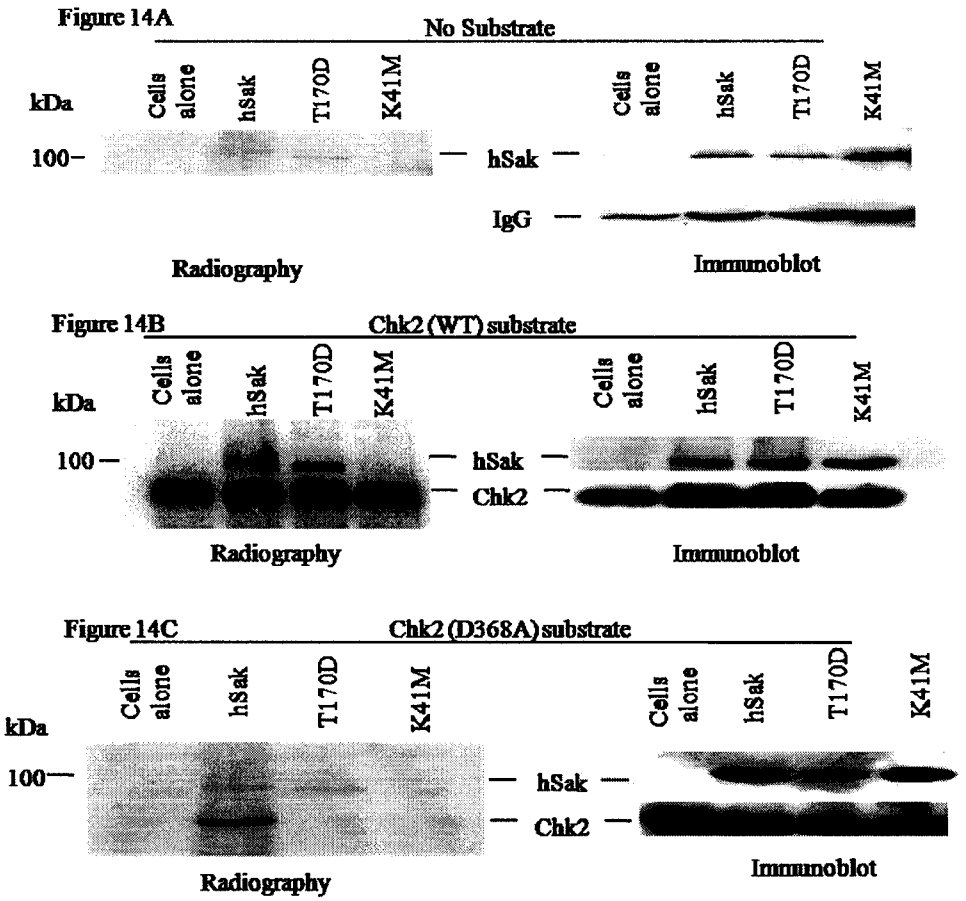

Figure 14. **Sak Phosphorylates GST-Chk2 *In Vitro*.** HEK 293 cells were transiently transfected with expression plasmids for either Flag-hSak, Flag-T170D or Flag-K41M. The cells were lysed and immunoprecipitation was performed using an anti-Flag antibody. (A) Immunoprecipitated Flag-hSak protein were incubated in the presence of ATP without any substrate. SDS-PAGE was performed and the membrane was exposed using a phosphoimager (PerkinElmer). A positive signal was detected at 100 kDa in the lanes corresponding to wildtype and kinase active hSak (T170D). The blots were then probed with anti-Flag antibody to ensure transfection efficiency. (B) Purified wildtype GST-Chk2 was incubated with or without the immunoprecipitated Flag-tagged hSak protein in the presence of ATP, subjected to SDS-PAGE, transferred to a membrane and viewed using a phosphoimager (PerkinElmer). A positive signal for phosphorylation was observed at 100 kDa in the wildtype hSak and T170D lanes and in every lane at 93 kDa. Western Blot analysis was then performed with an anti-Flag antibody to ensure transfection efficiency. The blot was then stripped and re-probed with anti-Chk2 antibody. (C) Purified kinase dead GST-Chk2 (GST-D368A) was incubated with or without the immunoprecipitated Flag-tagged hSak protein in the presence of ATP. SDS-PAGE was performed andthe subsequent membrane was analyzed using a phosphoimager (PerkinElmer). A positive signal for phosphorylation was observed at 100 kDa in the wildtype hSak and T170D lanes. A positive signal was also detected at 93 kDa in the Flag-hSak lane. Immunoblotting was performed with an anti-Flag antibody to ensure transfection efficiency, followed by re-probing with anti-Chk2 antibody. .

In Vitro Phosphorylation of Chk2 by Sak is Abolished in the Presence of UV

To further characterize the interaction between Sak and Chk2, it was of interest to determine if Chk2 is still phosphorylated by this Plk member in response to UV radiation. Classical understanding of the DNA damage pathway emphasizes that Chk2 is activated in response to ionizing radiation. However, recent finding have suggested that Chk2 is activated in an ATM-independent manner. That is, in response to UV radiation, Chk2 was found to be phosphorylated by ATR (Matsuoka *et al.*, 2000, Hirao *et al.*, 2002). As a result, cells transiently expressing the Flag-tagged constructs were exposed to 25 mJ/cm^2 UV radiation and lysed one hour later. Immunoprecipitation was performed using an anti-Flag antibody and Flag-HSak, Flag-T170D and Flag-K41M were incubated with GST-Chk2 or GST-D368A in the presence of [γ-^{32}P] ATP (Amersham Biosciences). The resulting blots were subjected to autoradiography and Western Blot Analysis to ensure protein levels. As seen in Figure 15A, autophosphorylation of Chk2 remains in the absence or presence of UV radiation. Interestingly, however, phosphorylation of the kinase inactive Chk2 construct, D368A, by Sak was abolished in the presence of UV (Figure 15B). It is also worthy to note, that Flag-Sak was also incubated with column purified GST protein, to ensure that the observed phosphorylation of GST-D368A was not with the GST tag. As seen in Figure 15C, Sak does not phosphorylate the GST protein thus confirming that the phosphorylation event was on Chk2.

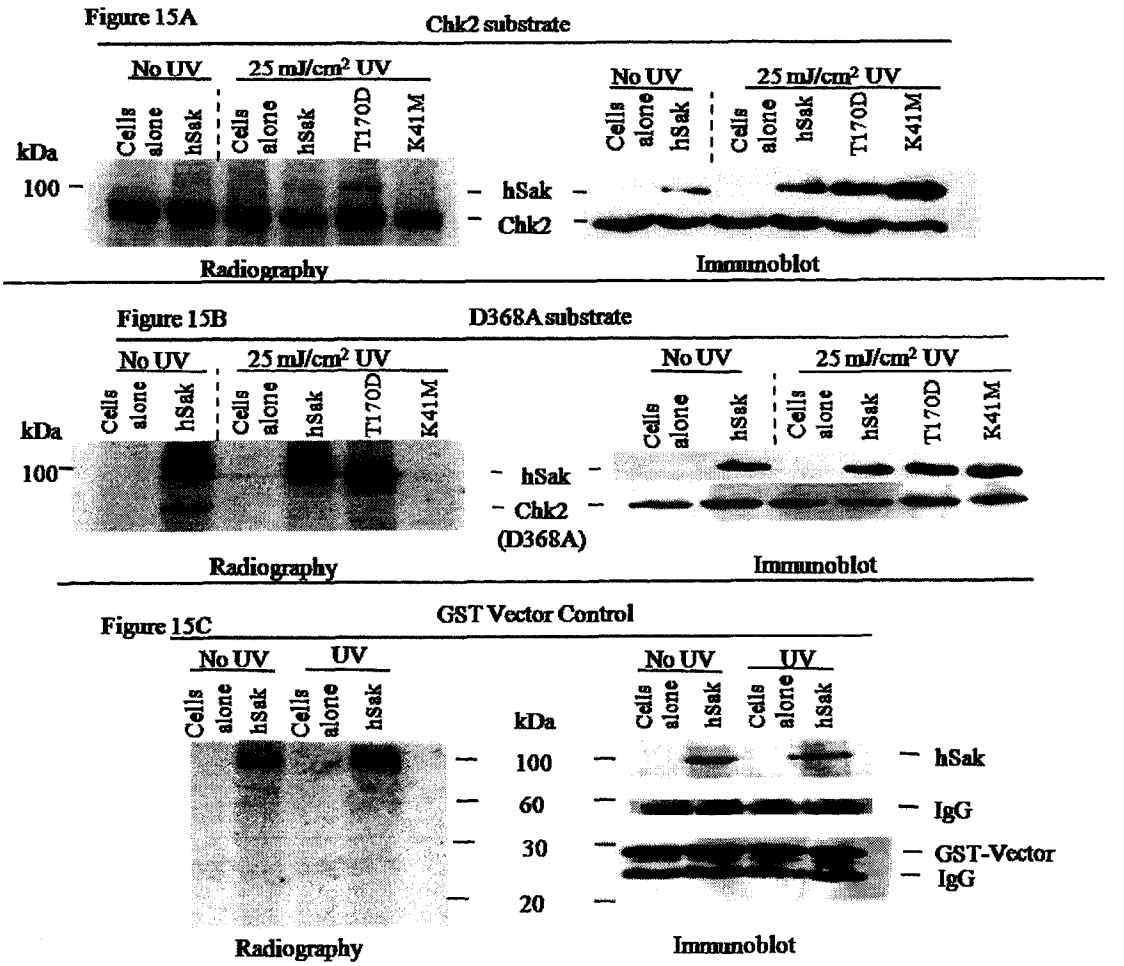

Figure 15. **Phosphorylation of Chk2 by Sak is Abolished in the Presence of UV.** HEK 293 cells were transfected with expression plasmids for either Flag-hSak, Flag-T170D or Flag-K41M. 16 hours after transfection, the cells were exposed to UV, allowed to sit for an additional hour, lysed and immunoprecipitation using an anti-Flag antibody was performed. (A) Purified wildtype GST-Chk2 was incubated with or without the Flag-tagged constructs in the presence of ATP, subjected to SDS-PAGE and viewed using a phosphoimager (PerkinElmer). The blots were then probed with anti-Flag and anti-Chk2 antibodies. (B) Immunoprecipitated Flag-tagged Sak protein was incubated with purified kinase dead GST-Chk2 (GST-D368A) in the presence of ATP, resolved by SDS-PAGE and viewed using a phosphoimager (PerkinElmer). A positive signal at 93 kDa was not detected in any of the lanes containing Flag-hSak protein that had been exposed to UV. Subsequent Western Blotting performed using an anti-Flag antibody and then re-probed with an anti-Chk2 antibody. (C) For a negative control, immunoprecipitated Flag-hSak protein incubated in the presence of purified GST-vector and ATP were subjected to SDS-PAGE and subsequent exposure using a phosphoimager (PerkinElmer). Western Blot Analysis was then performed using an anti-Flag antibody, stripped and re-probed with an anti-GST antibody.

CHAPTER V

DISCUSSION

Characterizing the Phosphorylation Status of Sak

Phosphorylation and dephosphorylation are vital mechanisms that may alter a protein's stability, activity, cellular localization and affinity for substrates. This post-translational event can alter the structure of a protein rendering it in an active or inactive state. Like many other kinases, members of the polo-like kinase family are also known to be regulated by phosphorylation. Little is known however, about the phosphorylation status of Sak and characterizing this would prove useful in shedding light on its potential interacting partners and cellular pathways that it plays a role in. Therefore, a goal of this study was to determine the phosphorylation status of this kinase and identify particular residues targeted by this event.

As an initial step we examined whether Sak is phosphorylated in an asynchronous population of HEK-293 cells. Under these conditions Sak was found to be a target of phosphorylation (Figure 4A).

In order to assess whether the observed phosphorylation of Sak was the target of other kinases or a result of autophosphorylation mutations that affected Sak activity, as well as domain-specific constructs were utilized in an *in vivo* phosphomapping experiment. Constructs encoding numerous Sak proteins were transfected into HEK 293 cells and subjected to *in vivo* phosphomapping. All the full length proteins except the mouse kinase dead version of Sak displayed a positive signal for phosphorylation. These results suggest that this phosphorylation is independent of Sak activity, therefore implying that Sak is a target of another endogenous kinase. While this is true for the human kinase dead Sak protein, a kinase dead version of the murine form of Sak did not

display a positive signal. It is possible that the mouse kinase dead Sak protein is subjected to different protein folding preventing it from being phosphorylated on a particular residue. A major problem with this study was the presence of a background signal which was close to the same size as Sak. This contaminating signal was difficult to discern from the Sak signal and it was also present in the cells alone and YVHI negative controls. We were however detecting a positive signal on the Sak protein as Flag-ΔPb was found to be phosphorylated (Figure 6A). Phosphorylation was not detected in the Flag-Pb and Flag-R1 proteins under these conditions (Figure 6B). Taken togethether these results imply that the phosphorylated residue is located N-terminally to the polo-box domain.

Further studies are needed in order to properly assess the phosphorylation status of Sak. Experiments in this study were performed under asynchronous conditions. Performing this experiment after synchronizing cells to particular phases of the cell cycle may result in different observations. For example, the phosphorylation status of Plk1 has been shown to be cell cycle dependent where it was observed not to be phosphorylated in S phase and becomes phosphorylated at the onset of mitosis (Hamanaka *et al.,* 1995, Roshak *et al.,* 2000). This phosphorylation event then activates Plk1 where it was observed to phosphorylate Cdc25C leading to the initiation of mitosis (Roshak *et al.,* 2000). As a result, performing the *in vivo* mapping approach with subsequent Mass spectrometry Analysis in synchronized cells may produce different results then those observed in this study.

Furthermore, exposure of DNA damaging agents may also profoundly change this phosphorylation pattern, as Sak may be targeted in response to DNA damage and upstream kinases may phosphorylate different residues. DNA damage has been shown to alter the phosphorylation status of numerous kinases (Smits *et al.,* 2000, van Vugt *et al.,*

2001, Bartek and Lukas, 2003, Bahassi *et al.,* 2006). For example, Plk1 and Plk3 have been shown to display changes in their phosphorylation status in response to DNA damage (Smits *et al.,* 2000, van Vugt *et al.,* 2001). Indeed, this study addressed this issue where HEK 293 cells overexpressing Flag-tagged human Sak were exposed to various amounts of UV and subjected to Phospho Gel Staining. Sak was observed to further be a target of phosphorylation (Figure 4A). These novel results show that Sak is phosphorylated both in the absence or presence of UV.

The possibility that Sak is phosphorylated on alternate residues in response to different environmental conditions and DNA damaging agents, led us to attempt to characterize the particular residues using Mass Spectrometry. Signature motifs for numerous kinases are well established and determining the residues of Sak that are phosphorylated can suggest which kinases are targeting this Plk member. As a result, the bands from the phospho-stained gel corresponding to Sak were excised, subjected to trypsin digestion and introduced to a PHOS-Select Iron Affinity Gel (Sigma). This chelated matrix is positively charged and binds to the negatively charged phosphorylated residues allowing the other peptides to be washed and removed. The phosphorylated peptides are then eluted from the matrix and subjected to Mass Spectrometry. Unfortunately, the intensity of the phosphorylated peaks was too low to give a positive result in this manner.

Low endogenous levels of Sak proved to be problematic in these experiments as well as the lack of a suitable Sak antibody. Transient expression of Sak protein proved to be a well suited substitute in determining its phosphorylation status and identifying the bands through Mass Spectrometry. However, generally speaking, the stoichiometry of phosphorylated peptides only represents a very small fraction of the total peptide pool

obtained for this procedure (Mann *et al.,* 2002). Thus, the actual amount of phosphorylated Sak protein is assumed to be much less than the total amount of protein. In addition, Sak has a very short half life of 2-3 hours (Fode *et al.,* 1996) resulting in sub-optimal experimental conditions. The phospho-stain is able to detect minimal amounts of phosphorylation, however Mass Spectrometry requires a larger amount in order to work properly. On a positive note, stable cell lines expressing Sak have recently been created in our lab and may prove to be a valuable tool in overcoming this obstacle. Presently, experimentation utilizing the stable cell lines is underway and may provide enough phosphorylated peptides to positively identify the particular residue(s).

Sak Remains Phosphorylated in the Presence of Wortmannin

Wortmannin was originally thought to be a specific and potent inhibitor of the PI3-Kinase family (Sarkaria *et al.,* 1999). Recent findings, however, have indicated that Plk1-3 are also inhibited by this fungal metabolite (Liu *et al.,* 2005, Liu *et al.,* 2007). It was therefore of interest to determine if Wortmannin had an effect on the activity of Sak. This possibility was examined by first incubating Flag-tagged human Sak with Casein as a substrate in the presence of [γ-^{32}P] ATP. Interestingly, a positive signal for phosphorylation was seen at 100 kDa in every lane except the negative control. After the membrane was subjected to Western Blot Analysis using an anti-Flag antibody, it was confirmed that this positive signal corresponded to autophosphorylation of Sak (Figure 7). Previous results from our lab and others have reported the presence of Sak autophosphorylation (Bonni et al.2007, accepted, Leung *et al.,* 2007) and this study revealed that Sak autophosphorylation was not abolished in the presence of Wortmannin (Figure 7).

In addition, many phosphorylated bands were observed at approximately 25 kDa in every lane including the lanes with cells alone or in the absence or presence of Wortmannin (data not shown). This observation led to an inconclusive result in determining how Sak phosphorylated Casein. Another possible way to overcome this was to test Sak's kinase ability with a known substrate. Unfortunately, no known substrates of Sak were known at the time this study was conducted. Yet the positive signal obtained at 100 kDa corresponding to autophosphorylation of Sak leads us to assume that this Plk member is not inhibited by Wortmannin. Indeed, at the same time this study was performed, it had been reported that the kinase activity of Sak on a peptide library substrate had not been inhibited by Wortmannin (Johnson *et al.,* 2007). In contrast to Plk1 that exhibited time-dependent inhibition by this metabolite, they observed that Sak was not be affected by Wortmannin. Based on the known binding mode of Wortmannin with members of the PI3-K family, a model for binding to the Polo-like kinases has been proposed indicating an interaction between three amino acid residues conserved in Plk1-3. Cys 67 is one of the residues and found to interact with two methyl groups of Wortmannin through van der Waals interactions. This residue is replaced with a valine in Sak and the larger size of this amino acid causes steric hinderance preventing any such binding (Johnson *et al.,* 2007).

Sak and the DNA Damage Response

The Interaction Between Sak and ATR

The DNA damage response pathway is a critical function of cells in order to retain its genomic integrity and cellular viability. A major contributor in this process is the activation of larger signal transducers which receive information about DNA damage from upstream sensor and mediator proteins and respond by targeting critical downstream

targets (Figure 2) (for review see Niida and Nakanishi, 2006., Bartek and Lukas, 2007). The key signal transducers of the DNA damage pathway include ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR). These proteins have been found to interact with various targets including members of the Polo-like kinase family. In addition, ATM/ATR, the Plk family members and other DNA damage response proteins such as Chk1 and Chk2 are all known to localize to the centrosomes. As a result, recent evidence supports the idea that the centrosome plays a major role as the centre for cellular control and DNA damage responses (Kramer *et al.*, 2004, Loffler *et al.*, 2006). As previously stated, ATM/ATR are known to inhibit Plk1 resulting in the G2/M phase arrest of cells exposed to DNA damage (Smits *et al.*, 2000, van Vugt *et al.*, 2001), while Plk3 is positively regulated by these signal transducers in response to DNA damage (Xie *et al.*, 2001).

Associations between these proteins and the observation that members of the Polo-like kinase family have overlapping protein partners, led us to believe that ATR may be an interacting partner of Sak. Indeed, through a co-immunoprecipitation based approach, we found an interaction between these two proteins (Hudson *et al.*, unpublished). Further characterization of this interaction revealed that ATR interacted with wildtype, kinase active and both kinase dead forms of Sak. This indicates that the kinase activity of Sak is not necessary for this interaction to occur.

We next examined the interaction between specific Sak domains and ATR. The ΔPb construct of Sak also associated with ATR indicating that the polo box domain was not necessary for this interaction. In support of this the Sak pb domain did not interact with ATR. Examination of the crystalline structure of the Sak polo-box suggested that this domain contains an interfacial cleft and pocket region assumed to be important for

ligand binding (Leung *et al.,* 2002). However, overexpression of Sak containing a deletion of the polo-box was still observed to localize to the centrosomes and cause centrosome overduplication, indicating that the polo-box is not necessary for this particular function (Habedanck *et al.,* 2005). The results of this particular study have implemented that the polo-box domain is not needed for this particular interaction. Thus, the polo-box domain of Sak is not necessary or sufficient for its interaction with ATR.

Although direct interactions between the polo-box domain of Plk1 and ATR have not been observed, this domain was found to play a key role in recognizing Plk1 substrates and localizing it to subcellular locations (Elia *et al.,* 2003a). Site directed mutagenesis of key amino acid residues in the polo-box of Plk1 rendered it unable to bind to particular substrates (Elia *et al.,* 2003b). Studies comparing the Polo-box domain of Plk1 to that of Sak reported striking differences in the organization, architecture and overall net charge of their polo-boxes (Elia *et al.,* 2003a). In addition, sequence alignment of the Sak polo-box with that of polo-box 1 motif and polo-box 2 motif in this domain of Plk1 revealed a sequence identity of 22% and 8% respectively (Cheng *et al.,* 2003). As a result, it is possible that the cryptic polo-box of Sak may be closer related to the polo-box domain of Plk1. In addition, it is possible that the cryptic polo-box may be responsible for protein-protein interactions. Indeed, the domain-specific cryptic polo-box protein was found in this study to be sufficient for ATR interaction (Figure 8). Co-immunoprecipitation of the cryptic polo-box with ATR further demonstrates the importance of this domain in protein interactions and function.

The previously stated co-immunoprecipitation experiments were performed in asynchronous cells under normal conditions. However, ATR is known to primarily become activated during UV-induced DNA damage (Niida and Nakanishi, 2006). In

regards to the other Plk members, ATR is known to inhibit Plk1 upon UV exposure hampering its kinase ability and preventing cells from progressing into mitosis (van Vugt *et al.,* 2001). This observation suggested that it would be interesting to examine the nature of the interaction between Sak and ATR upon UV-induced DNA damage. Co-immunoprecipitation performed after UV exposure revealed that full-length wildtype Sak continued to associate with endogenous ATR. However, the levels of ATR and Sak began to significantly decrease 4 hours post UV radiation (Figure 11). As a result, the association between these two proteins seems to diminish at this time point after UV exposure. An explanation could be that upon DNA damage, cells are prone to apoptosis and protein levels consequently decrease. Thus, the interaction between ATR and Sak protein would consequently be reduced 4 hours after UV exposure.

We then further examined the effects of overexpressing Sak in cells exposed to UV radiation. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-ATR (Santa Cruz), anti-Chk2 and anti-Flag (Sigma) antibodies in order to determine their protein levels. Similar to the results obtained from the co-immunoprecipitation, ATR protein levels were relatively constant until 4 hours after UV exposure, at which ATR protein was not detected. Chk2, a downstream effector protein that is also activated in response to UV exposure, displayed the same pattern as ATR. Levels of Sak also began to decrease 4 hours post UV exposure. Similarly, the same pattern was observed for our GAPDH loading control leading to inconclusive results regarding overexpressing Sak and ATR and Chk2 protein levels (Figure 10A). Contrary to this, in cells that only contained normal endogenous levels of Sak, the levels of ATR and Chk2 were similar at every time point (Figure 10B). It is known that overexpression of Sak is seen to have a profound effect on cell viability resulting in multinucleation (Fode *et al.,* 1996) and

centrosome overduplication (Habedanck *et al.,* 2005). Taken together, these results imply that in the presence of UV, overexpression of Sak may render cells more susceptible to UV radiation.

Further experimentation is necessary to provide greater insight into the association between Sak and ATR. This study revealed the domain specific interaction between these proteins, as well as characterized its interaction in the presence of UV-induced DNA damage. Future experimentation is needed to examine how the interaction between ATR and Sak affects their functions within the cell, as well as ATR's possible affects on the kinase ability of Sak or vice versa. Similar to other Plk family members, Sak may be a target of ATM/ATR phosphorylation in response to DNA damaging agents. These experiments could further characterize the relationship between ATR and Sak and specify a direct role for Sak in the DNA damage pathway.

Sak Interacts with Chk1

Based on our aforementioned assumptions it was also of interest to test for a potential interaction between Chk1 and Sak. For example, recent studies have indicated that Chk1 negatively regulates Plk1 in response to DNA damage (Tang *et al.,* 2006). Chk1 is a known downstream effector protein in DNA damage response and becomes activated primarily in response to UV radiation. Our results were strikingly similar to that which we found for ATR. Regardless of its kinase status, Flag-Sak interacted with endogenous Chk1. Furthermore, Flag-ΔPb associated with Chk1, while Flag-Pb did not. Therefore, as with ATR, the polo-box domain was not necessary or sufficient for Chk1 interaction. In addition, Flag-R1 was found to interact with Chk1 indicating that the cryptic polo-box is sufficient for this interaction. These results indicate that the mode of interaction between Sak and Chk1 may be the same as that between ATR and Sak.

Interestingly, similar co-immunoprecipitation experiments in our laboratory between Sak and Chk2 and Sak and ATM revealed no interation with the cyptic polo-box and these proteins (Hudson *et al.,* unpublished). Further characterization of this interaction is necessary to determine the effects resulting from this association. *In vitro* kinase assays would prove to be a useful tool to determine if Sak is regulated by this effector in the same manner as Plk1.

Chk2 is Phosphorylated by Sak

Chk2 is another important protein involved in DNA damage response. Although inactive in the absence of DNA damage, this protein is classically believed to become activated by ATM in response to ionizing radiation (for review, see Bartek and Lukas, 2003). However, recent studies have revealed that Chk2 becomes activated in an ATM-independent manner (Hirao *et al.,* 2002). Upon UV radiation, Chk2 becomes activated and this is speculated to be mediated by ATR (Shiloh, Y., 2001). Furthermore, both Plk1 and Plk3 interact with and phosphorylate this kinase (Tsvetkov *et al.,* 2003, Bahassi *et al.,* 2006). We therefore tested the ability of Sak to interact with Chk2 and indeed, novel results show that this association occurs (Hudson, unpublished). Furthermore, we have established in the present study that Chk2 is a substrate of Sak. Previous literature has established that bacterially expressed GST-Chk2 has a tendency to autophosphorylate (Xu *et al.,* 2001). As a result, a kinase dead version of Chk2 was created by mutating the critical aspartate of its activation loop to an alanine (GST-D368A). It is noted that both Sak and Chk2 are kinases, therefore the creation of this mutant allowed us to ascertain which protein was the kinase and which was the substrate in this interaction.

Figure 14 depicts the *in vitro* kinase assay where Chk2 has been identified as a substrate of Sak. As predicted, wildtype GST-Chk2 displayed autophosphorylation in

every lane. In the same experiment, a positive signal for phosphorylation was not detected for kinase dead Sak (K41M) indicating that Sak was not a substrate for Chk2 under these conditions. Furthermore, the GST-D368A mutant was unable to autophosphorylate and thus any positive signal we obtained would be a direct result of Sak activity. Interestingly, Sak wild type protein was able to target Chk2 while the kinase active form of Sak, Flag-T170D showed no phosphorylation of Chk2. In order to explain this odd occurrence, two possible explanations have been proposed. Firstly, the site-specific mutation of Flag-T170D may cause this construct to have a different confirmation than its wildtype counterpart. As a result, Flag-T170D may not be able to associate with Chk2 in a manner that allows phosphorylation to occur. Secondly, the initial phosphorylation of Chk2 may in turn create a positive feed-back loop, resulting in the phosphorylation of Sak at an unknown residue. The phospho-mimic mutation may have rendered this association from occurring.

In order to determine if this association still persists in the presence of DNA damage, cells transfected with the various Flag-Sak constructs were exposed to 25 mJ/cm^2 of UV radiation one hour prior to lysing. Findings from our lab and this study revealed that Sak expression still persists after this time and amount of UV exposure. In addition, Sak was still observed to associate with ATR after this time period post UV exposure. Thus, if Chk2 phosphorylation by Sak was dependent upon ATR, this period between initial exposure and lysing would still allow ATR and Sak to associate. Fascinatingly, phosphorylation of Chk2 by Flag-Sak was abolished when cells were exposed to UV prior to the kinase assay (Figure 15) while autophosphorylation of Sak was still present. This novel result presents many intriguing possibilities. As previously mentioned and displayed in this assay, Sak remains phosphorylated in the presence of

UV. The specific phosphorylated residue(s) of Sak is/are unknown and are quite possibily different in response to UV then in normal conditions. UV exposure may cause upstream kinases to target Sak marking it for degradation. Furthermore, in response to UV, the confirmation of Sak may change and render it unable to phosphorylate Chk2. Regardless of the means, the outcome remains the same; the abolishment of phosphorylation of Chk2 by wildtype Sak in response to UV.

Taken together, these novel results observed from the kinase assays show that under normal asynchronous condition, Chk2 is a substrate of Sak. However, further experimentation is needed to solidify these novel findings. ATR is thought to phosphorylate Chk2 (Shiloh, Y., 2001, Hirao *et al.,* 2002), and one may argue the possibility that ATR is brought down with Sak during the initial immunoprecipitation. As a result, ATR may be the active kinase. Extensive washing during the initial immunoprecipitation was done in order to prevent this from occurring. In addition, future experiments could be performed in ATR deficient cell lines or in the presence of caffeine or Wortmannin to block ATR kinase ability. However, the results presented from this study shy away from the possibility that ATR is the active kinase. That is, ATR is known to become activated during UV exposure (Niida and Nakanishi, 2006) and thus would phosphorylate Chk2 in its presence. Yet this study observed the opposite to occur. If indeed ATR was phosphorylating Chk2 instead of Sak, phosphorylation would be observed in the presence of UV.

Nevertheless, the possibility that ATR has a role in these observations is quite possible. Under normal conditions, phosphorylation of Chk2 by Sak may be inhibitory. Upon UV exposure, ATR may interact with Sak resulting in a change in its confirmation hindering it from phosphorylating Chk2. This relief from Sak phosphorylation may then

allow Chk2 to become active and proceed with its duty of repairing DNA damage (Figure 16). In addition, the phosphorylation of Chk2 by Sak under normal conditions may act as a means of localizing Chk2. This may be analogous to the situation for Plk1 where phosphorylation of Chk2 by Plk1 has also been observed and is suggested to result in localizing Chk2 to the centrosomes (Tsvetkov *et al.*, 2003). Thus, Sak phosphorylation of Chk2 may act in a similar manner.

An attempt to identify the residue targeted by Sak phosphorylation was made using a Chk2 phospho-specific antibody. Studies involving Plk1 and Chk2 have reported that this Plk member phosphorylates Chk2 on either T26 or S28 (Tsvetkov *et al.*, 2003). Thus, the membranes from the kinase assay showing phosphorylation of Chk2 by Sak were stripped and re-probed with an anti-Chk2 phospho-T26 antibody. No signal was detected when this was performed. The lack of a positive control, however, prevents us to state with certainity that Sak does not phosphorylate Chk2 at T26 (data not shown).

The results presented here open exciting new possibilities involving Sak's role in the DNA damage response. Further experimentation is currently underway in the hopes of characterizing the particular residues of Chk2 that are targeted during this phosphorylation event. Chk2 domain specific constructs may prove to be useful in narrowing down the particular residue. In addition, Mass Spectrometry may prove to be a valuable tool in discovering this new site. Yet the implementations of these results already present exciting novel assumptions. These results have placed Sak as an interacting partner with proteins vital in maintaining cellular integrity and imply that it has a novel functional role in response to DNA damage. Increasing our knowledge of Sak is essential to our understanding of DNA damage response and may help in our battle against tumourigenesis.

Figure 16A

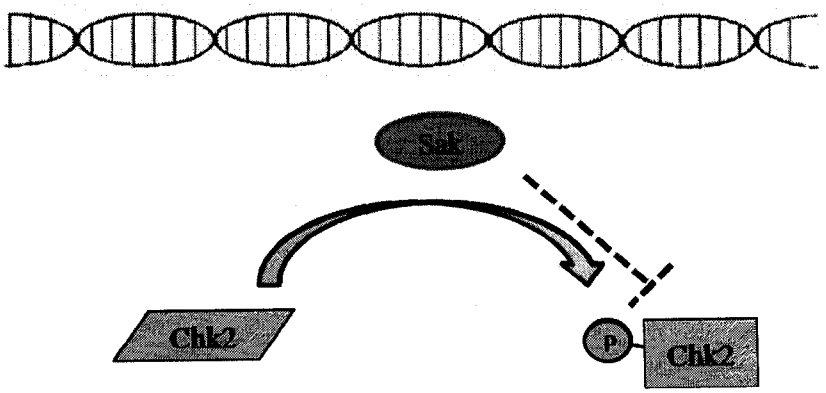

Figure 16B

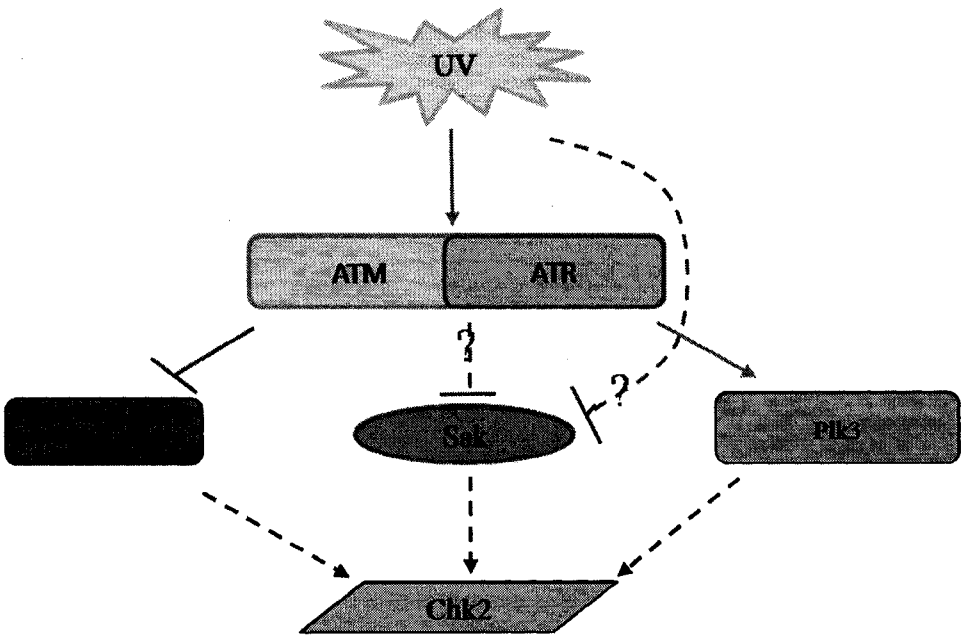

Figure 16. **Proposed Pathway through which Sak is affected by UV.** (A) In the absence of UV, Chk2 is phosphorylated by Sak. It is hypothesized that this event may inhibit Chk2 under normal conditions. (B) This proposed model places Sak in the DNA damage response pathway. Upon UV exposure, Sak phosphorylation of Chk2 is abolished. Green arrows indicate positive regulation whereas the red perpendicular lines indicates negative regulation. The dotted black arrows represent known interactions between the proteins yet the functional significance is yet to be elucidated. Dotted red lines represent proposed regulatory interactions.

Appendix A: Solutions

Competent Cell Solutions

TFB1
30 mM KOAc
50 mM MnCl2
100 mM KCl
10 mM CaCl2
7% glycerol

TFB2
10 mM MOPS pH 7.0
75 mM CaCl2
10 mM KCl
7% glycerol

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

Transformation

LB-AMP Plates (1L)
10 g tryptone
5 g yeast extract
10 g NaCl
15g Agar
Autoclave.
After the solution has cooled, ampicilin was added to a final concentration of 100 ug/mL.

Cell Culture

Cell Media
500 mL Dulbecco's Modified Eagle's Medium (DMEM)
50 mL (10%) Fetal Bovine Serum
5 mL (1%) Penicillin/Streptomycin
5 mL (1%) Amphotericin B
500 uL Gentamycin

Cell Lysis and Immunoprecipitation

Lysis Buffer
50 mM Tris-HCl pH 7.4
150 mM NaCl

1 mM EDTA
1% Triton X-100
1 protease inhibitor tablet (Roche) was added to every 10 mL of buffer

PBS
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4
pH to 7.4

Tris Buffer Saline (TBS)
100 mM Tris-Cl pH 7.5
150 mM NaCl

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

6 X SDS-PAGE sample buffer (10 mL)
1 M Tris pH 7.5
3.0 mL 100% glycerol
1 g SDS
100 mg bromophenol blue
Ultrapure water was added to a final volume of 10 mL

Western Analysis

Running Buffer
25 mM Tris
250 mM Glycine
0.1% SDS

Transfer Buffer (1 L)
3.03 g tris base
14.4 g glycine
200 mL methanol

1 X TBST (Tris buffered saline and tween)
100 mM Tris-Cl pH 7.5
150 mM NaCl
0.1% Tween

Stripping Buffer
10 mL 10% (w/v) SDS
6.25 mL 0.5 M Tris-HCl (pH = 6.7)

342 uL Beta-Mercaptoethanol
Filled to 50 mL with ddH_2O

In-Gel Detection of Phoshoproteins

Fix Solution

Coomassie Blue Stain
0.2 % (w/v) Coomassie Blue
7.5 % Glacial Acetic Acid
50 % Ethanol

Destain Solution
0.75 % Glacial Acetic Acid
10 % Ethanol

GST-Protein Purification Buffers

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

Column Wash Buffer
100 mM NaCl
50 mM Tris pH 7.5
1 mM DTT

Elution Buffer
100 mM NaCl
50 mM Tris pH 7.5
10 mM Glutathione

In Vitro Kinase Assays

Kinase Buffer
60 mM HEPES pH 7.5
3 mM $MgCl_2$
3 mM $MnCl_2$
50 mM NaF
1.2 mM DTT
1 protease inhibitor tablet (Roche) per 10 mL buffer

REFERENCES

Alberts, G. F., Winkles, J. A. Murine. FGF-Inducible Kinase is Rapidly Degraded via the Nuclear Ubiquitin-Proteosome System When Overexpressed in NIH 3T3 Cells. *Cell Cycle* 3 (5), 678-684 (2004)

Ando, K., Ozaki, T., Yamamoto, H., Furuya, K., Hosoda, M., Hayashi, S., Fukuzawa, M., Nakagawara, A. Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. *J. of Biol.Chem.* 279, 25549-25561 (2004).

Bachewich, C., Masker, K., Osmani, S. The polo-like kinase PLKA is required for initiation and progression through mitosis in the filamentous fungus Aspergillus nidulans. *Mol. Microbiol.* 55 (2), 572-587 (2005)

Bahassi, E. M., Hennigan, F. R., Myer, L. D., Stambrook, J. P. Cdc25C phosphorylation on serine 191 by Plk3 promotes its nuclear translocation. *Oncogene* 23, 2658-2663 (2004).

Bahassi, E. M., Myer, L. D., McKenney, R. J., Hennigan, F. R., Stambrook, J. P. Priming phosphorylation of Chk2 by polo-like kinase 3 (Plk3) mediates its full activation by ATM and a downstream checkpoint in response to DNA damage. *Mutation Research* 596(1-2), 166-176 (2006)

Bartek, J., Lukas, J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 3, 421-428 (2003)

Bettencourt-Dias, M., Rodrigues-Martins, A., Carpenter, L., Riparbelli, M., Lehmann, L., Gatt, M. K., Carmo, N., Balloux, F., Callaini, G., Glover, D. M. SAK/PLK4 Is Required for Centriole Duplication and Flagella Development. *Current Biol.* 15, 1-9 (2005)

Bonni, S., Ganuelas, M. L., Petrinac, S. T., Hudson, J. W. Plk4 Phosphorylates Cdc25C. *Cell Cycle*, accepted, in press.

Casenghi, M. et al. Polo-like kinase 1 regulates Nip, a centrosome protein involved in microtubule nucleation. *Dev. Cell* 5, 113-125 (2003).

Casenghi, M., Barr, F.A., Nigg, E.A. Phosphorylation of Nlp by Plk1 negatively regulates its dynein-dynactin-dependent targeting to the centrosome. *J. Cell Sci.* 118(21), 5101-5108. (2005)

Chase, D., Golden, A., Heidecker, G., Ferris, D. K. Caenorhabditis elegans contains a third polo-like kinase gene. *DNA Seg.* 11 (3-4), 327-334 (2000)

Cheng, K-Y., Lowe, D. E., Sinclain, J. Nigg, E. Johnson, N. L. The Crystal structure of the human Plk1 polo box domain and its phospho-peptide complex. *The EMBO Journal* 22, 5757-5768 (2003).

Conn, W. C., Hennigan, F. R., Dai, W., Sanchez, Y., Stambrook, J. P. Incomplete cytokinesis and Induction of Apoptosis by Overexpression of Mammalain Polo-like Kinase, Plk3. *Cancer Research* 60, 6826-6831 (2000).

Dai, W. Polo-like kinases, an introduction. *Oncogene* 24, 214-216 (2005)

Dai, W., Wang, X. Grabbing Plk1 by the PBD. *Mol. Cell* 24 (4), 489-490 (2006)

Dai, B. N., Yang, Y., Chau, Z., Jhanwar-Uniyal, M. Polo-like kinase 1 regulates RhoA during cytokinesis exit in human cells. *Cell Prolif.* 40, 550-557 (2007)

Donohue, P. J., Alberts, G. F., Guo, Y., Winkles, J. A. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. *J. Biol. Chem.* 270 (17), 10351-10357 (1995)

Duncan, P. I., Pollet, N., Niehrs, C., Nigg, E. A. Cloning and characterization of Plx2 and Plx3, two additional Polo-like kinases from Xenopus laevis. *Exp. Cell. Res.* 270 (1), 78-87 (2001)

Eckerdt, F., Yuan, J., Saxena, K., Martin, B., Kappel, S., Lindenau, C., Kramer, A., Naumann, S., Daum, S., Fischer, G., Dikic, I., Kaufmann, M., Strebhardt, K. Polo-like Kinase 1-mediated Phosphorylation Stabilizes Pin1 by Inhibiting Its Ubiquitination in Human Cells. *J. Biol. Chem.* 280 (44), 36575-36583 (2005).

Eckerdt, F., Yuan, J., Stebhardt, K., Polo-like kinases and oncogenesis. *Oncogene* 24, 267-276 (2005)

Elia, A. E., Cantley, L. C., Yaffe, M. B. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 substrates. *Science* 299, 1228-31 (2003a).

Elia, A. E., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J., Yaffe, M. B. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell.* 115, 83-95 (2003b).

Feng, Y., Hodge, D.R., Palmieri, G. Chase, D.L., Longo, D.L., Ferris, D.K. Association of polo-like kinase with a-, b- and g-tubulins in a stable complex. *Biochemical Journal.* 339, 435-442 (1999)

Fode, C., Motro, B., Yousefi, S., Heffernan, M., Dennis, J. W. Sak, a murine protein-serine/threonine kinase that is related to the *Drosophila* polo kinase and involved in cell proliferation. *PNAS* 91, 6388-6392 (1994)

Fode, C., Binkert, C., Dennis, J. W. Constitutive expression of murine Sak-a suppresses cell growth and induces multinucleation. *Mol. Cell. Biol.*, 16, 4665-4672.(1996)

Friend, S. H., Tapscott, S. J., Sibling rivalry, arrested development and chromosomal mayhem. *Nat. Genet.* 19 (01), 39-46 (1998)

García-Alvarez, B., Ibañez, S., Montoya, G. Crystallization and preliminary X-ray diffraction studies on the human Plk1 Polo-box domain in complex with an unphosphorylated and a phosphorylated target peptide from Cdc25C. *Acta. Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 62 (4), 372-375 (2006)

Graham, T. M., Tait, A., Hide, G. Characterisation of a polo-like protein kinase gene homologue from an evolutionary divergent eukaryote, Trypanosoma brucei. *Gene* 207 (1), 71-77 (1998)

Gatei, M., Sloper, K., Sorensen, C., Syljuäsen, R., Falck, J., Hobson, K., Savage, K., Lukas, J., Zhou, B.B., Bartek, J., Khanna, K. K. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *J. Biol. Chem.* 278 (17),14806-14811 (2003)

Golan, A., Yudkovsky Y., Hershko A., The Cyclin-Ubiquitin Ligase Activity of Cyclosome/APC is Jointly Activated by Protein Kinases Cdk1-Cyclin B and Plk. *J. of Biol. Chem.* 277 (18), 15552-15557 (2002)

Habedanck, R., Stierhof, Y. D., Wilkinson, J. C., Nigg, A. E. The Polo kinase Plk4 functions in centriole duplication. *Nat. Cell Biol.* 7 (11), 1140-6 (2005).

Hanisch, A, Wehner, A., Nigg, E. A., Sillje, H. H. Different Plk1 functions show distinct dependencies on Polo-Box domain-mediated targeting. *Mol. Biol. Cell* 17, 448-459 (2006).

Hirao, A., Cheung, A., Duncan, G., Girard, P.M., Elia, A.J., Wakeham, A., Okada, H., Sarkissian, T., Wong, J.A., Sakai, T., De Stanchina, E., Bristow, R.G., Suda, T., Lowe, S.W., Jeggo, P.A., Elledge, S.J., Mak, T.W. Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol. Cell Biol.* 22 (18), 6521-6532 (2002)

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., Draetta, G. Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO 12(1), 53-63 (1993)

Hudson, J. W., Chen, L., Fode, C., Binket, C., Dennis, J. W. *Sak* kinase gene structure and transcriptional regulation. *Gene* 241, 65-73 (2000).

Hudson, J. W., Kozarova, A., Cheung, P., Macmillan, C. J., Swallow, J. C., Cross, C. J., Dennis, J. W. Late mitotic failure in mice lacking Sak, a polo-like kinase. *Current Biology* 11, 441-6 (2001).

Jang Y. J., Lin, C. Y., Ma, S., Erikson, R. L., Functional studies on the role of the C-terminal domain of mammalian polo-like kinase. *PNAS* 99 (4) 1984-1989 (2002a)

Jang Y. J., Ma, S., Yasuhiko, T., Erikson, R. L., Phosphorylation of Threonine 210 and the Role of Serine 137 in the Regulation of Mammalian Polo-like Kinase. *J. Biol. Chem.* 277 (46) 44115-44120 (2002b)

Ji, J. H., Jang, Y. J. Screening of Domain-specific Target Proteins of Polo-like Kinase 1: Construction and Application of Centrosome/Kinetochore-specific Targeting Peptide. *J. of Biochem. and Mol. Biol.* 39 (6), 709-716 (2006)

Jiang, N., Wand, X., Jhanwar-Uniyal, M., Darzynkiewicz, Z., Dai, W. Polo Box Domain of Plk3 Functions as a Centrosome Localization Signal, Overexpression of Which Causes Mitotic Arrest, Cytokinesis Defects, and Apoptosis. *J. of Biol. Chem.* 281 (15), 10577-10582 (2006)

Johnson, E. F., Stewart, K. D., Woods, K. W., Giranda, V. L., Lou, Y. Pharmacological and Functional Comparison of the Polo-like Kinase Family Insight into Inhibitor and Substrate Specificity. *Biol. Chem.* 46 (33), 9551-9563 (2007)

Karaiskou, A., Jessus, C., Thierry, T., Ozon, R. Phosphatase 2A and Polo kinase, two antagonistic regulators of Cdc25 activation and MPF auto-amplification. *J. Cell Sci.* 112, 3747-3756 (1999)

Kastan, M. B., Bartek, J. Cell-cycle checkpoints and cancer. *Nature* 432, 316-323 (2004)

Kitada, K., Johnson, A. L., L. H., Sugino, A. A multicopy suppressor gene of the *Saccharomyces cerevisiae* G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. *Mol. Cell Biol.* 13, 4445-57 (1993).

Ko, A. M., Rosario, O. C., Hudson, J. W., Kulkarni, S., Pollett, A., Dennis, J. W., Swallow, J. C. Plk4 Haploin sufficiency causes mitotic infidelity and carcinogenesis. *Nature Genetics* 37, 883-8 (2005).

Kramer, A., Lukas, L., Bartek, J. Checking out the Centrosome. *Cell Cycle* 3 (11), 1390-1393 (2004)

Lake R. J., Jelinek W. R., Cell-cycle and Terminal Differentiation-Associated Regulation of the Mouse mRNA Encoding a Conserved Mitotic Protein Kinase. *Mol. and Cell. Biol.* 13 (12), 7793-7801 (1993)

Lane, H. A., Nigg, E. A. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in functional maturation of mitotic centrosomes. *J. Cell Biol.* 135, 1701-1713 (1996).

Lee, K. S., Yuan, Y. O., Kuriyama, R., Erikson, R. L. Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO/MKLP-1. *Mol. Cell. Biol.* 15, 7143-7151 (1995).

Lee, K. S., Erikson, R. L. Plk is a functional homolog of *Saccharomyces cerevisae* Cdc5, and elevated Plk activity induces multiple septation structures. *Mol. Cell. Biol* 17, 3408-17 (1997).

Lee, K. S., Grenfell, T. Z., Yarm, F. R., Erikson, R. L., Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinases Plk. *PNAS* 95, 9301-9306 (1998)

Lee, K. S., Song. S., Erikson. L. R. The polo-box-dependent induction of ectopic septal structures by a mammalian polo kinase, Plk, in Saccharomyces cerevisiae. *PNAS* 96 (25), 14360-14365 (1999)

Leung, G. C., Hudson, J. W., Kozarava, A., Davidson, A., Dennis, J. W., Sicheri, D. The Sak polo-box comprises a structural domain sufficient for mitotic subcellular localization. *Nature Struct. Biol.* 9, 719-724 (2002).

Leung, G. C., Ho, C. S. W., Blasutig, I. M., Murphy, J. M., Sicheri, F. Determination of the Plk4/Sak consensus phosphorylation motif using peptide spots arrays. *FEBS Letters* 581, 77-83 (2007)

Li, J., Mingjia, T., Ling, L., Pamarthy, D., Lawrence, S. T., Sun, Y. Sak, A new Polo-like kinase, Is transcriptionally repressed by p53 and induces apoptosis upon RNAi silencing. *Neoplasia* 7, 312-323 (2005).

Lin, C., Madsen, M.L., Yarm, F.R., Jang, Y., Liu, X., Erikson, R.L. Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. *PNAS* 97 (23), 12589-12594 (2000)

Lin, H., Ting, N.S.Y., Qin, J., Lee, W. M Phase-specific Phosphorylation of BRCA2 by Polo-like Kinase 1Correlates with the Dissociation of the BRCA2-P/CAF Complex. *J. Biol. Chem.* 278 (38), 35979-35987 (2003)

Liu, L., Zou. L., Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells. *J. Cell Biochem.* 94 (2), 298-306 (2005)

Liu, X., Erikson, R. L. Polo-like minase (Plk) 1 depletion indices apoptosis in cancer cells. *PNAS* 100 (10), 5789-5794 (2003)

Liu, Y., Shreder, K. R., Gai, W., Corrai, S., Ferris, D. K., Rosenblum, J. S. Wortmannin, a Widely Used Phosphoinositide 3-Kinase Inhibitor, also Potently Inhibits Mammalian Polo-like Kinase. *Chem. and Biol.* 12, 99-107 (2005)

Liu, Y., Jiang, N., Wu, J., Dai, W., Rosenblum, J. S. Polo-like Kinases Inhibted by Wortmannin. *J. Biol. Chem.* 282 (4), 2505-2511 (2007)

Loffler, H., Lukas, J., Bartek, J., Kramer, A., Structure meets function—Centrosomes, genome maintance and the DNA damage response. *Experimental Cell Research* 312, 2633-2640 (2006)

Lowery, M. D., Lim, D., Yaffe, M. Structure and function of Polo-like kinases. *Oncogene* 24, 248-59 (2005).

Ma, S., Charron, J., Erikson, L. R. Role of Plk2 in Mouse Development and Cell proliferation. *Mol. Cell. Biol.* 23, 6936-6943 (2003).

Mann, M., Ong, S., Gronborg, M., Steen, H., Jensen, O. N., Pandley, A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *TRENDS in Biotechnology* 20 (6) 261-268 (2002)

Martindill, D. M. J., Risebro, C. A., Smart, N., Franco-Viseras, M. D. M. F., Rosario, C. O., Swallow, C. J., Dennis, J. W., Riley, P. R. Nucleolar release of Hand1 acts as a molecular switch to determine cell fate. *Nature Cell Biol.* 9 (10), 1131-1141 (2007)

Matsuoka, S, Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., Elledge, S. J. Ataxia telangiectasia-mutated phosphorylates Chk2 *in vivo* and *in vitro.* *PNAS* 97(19), 10389-10394 (2000)

Moshe, Y., Boulaire, J., Pagano, M., Hershko, A. Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. *PNAS* 101 (21), 7937-7942

Myer, D. L., Bahassi, E. M., Stambrook, P. J., The Plk3-Cdc25 circuit. *Oncogene* 24, 299-305 (2005)

Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E., Nishida, E. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J. Biol. Chem.* 278 (28), 25277-25280 (2003)

Neef, R., Gruneberg, U., Kopajtich, R., Li, X., Nigg, E. A. Sillje, H., Barr, F. A.
Choice of Plk1 docking partners during mitosis and cytokinesis is controlled by the activation state of Cdk1.*Nature Cell Biol.* 9 (4), 463-444 ((2007)

Nigg, E. A. Centrosome Aberrations: Cause or consequence of cancer progression? *Nature Rev. Cancer* 2, 815-825 (2002).

Niida, H., Nakanishi, M. DNA damage checkpoints in mammals. *Mutagenesis* 21 (1), 3-9 (2006)

Obaya, A. J., Sedivy, J. M. Regulation of cyclin-Cdk activity in mammalian cells. *Cell. Mol. Life. Sci.* 59, 126-142 (2002)

Ohkura, H., Hagan, I. M., Glover, D. M., The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes and Development* 9, 1059-1073 (1995)

Ouyang, B., Pan, H., Lu, L., Li, J., Stambrook, P., Li, B. Dai, W. Human Prk Is a Conserved Protein Serine/Threonine Kinase Involved in Regulating M Phase Functions. *J. of Biol. Chem.* 272 (45), 28646-28651 (1997)

Pak., D.T.S., Sheng, M. Targeted Protein Degradation and Synapse Remodeling by an Inducible Protein Kinase. *Science* 302 (5649), 1368-1373 (2003).

Qian, Y. W., Erikson, E., Li, C., Maller, J. L., Activated Polo-Like Kinase Plx1 Is Required at Multiple Points during Mitosis in *Xenopus laevis. Mol. and Cell. Biol.* 18 (7), 426-4271 (1998a)

Qian, Y. W., Erikson, E., Maller, J. L., Purifcation and Cloning of a Protein Kinase That Phosphorylates and Activates the Polo-Like Kinase Plx1. *Science* 282, 1701-1704 (1998b)

Qian, Y. W., and Erikson, E., Maller, J. L. Mitotic effects of a constitutively active mutant of the *Xenopus* polo-like kinase Plx1. *Mol. Cell. Biol.* 19, 4262-4271 (1999).

Qian, Y. W., Erikson, E., Taieb, F. E., Maller, J. L., The Polo-like Kinase Plx1 Is Required for Activation of the Phosphatase Cdc25C and Cyclin B-Cdc2 in *Xenopus* Oocytes. *Mol. Biol. of the Cell.* 12, 1791-1799 (2001)

Roshak, A. K., Capper, E. A., Imburgia, C., Fornwld, J., Scott, G., Marshall, L. A. The human *polo*-like kinase, PLK, regulates cdc2/cyclin B through phosporylation and activation of the cdc25c phosphatase. *Cellular Signalling* 12, 405-411 (2000)

Sarkaria, J. N., Busby, E. c. Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M., Abraham, R. T. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Research* 59, 4375-4382 (1999)

Schlessinger, J. A solid base for assaying protein kinase activity. *Nat. Biotechnol.* 20 (3), 232-233 (2002)

Simmons, D. L., Neel, B. G., Stevens, R., Evett, G., Erikson, R. L. Identification of an Early-Growth-Response Gene Encoding a Novel Putative Protein Kinase. *Mol. and Cell. Biol.* 12 (9), 4164-4169 (1992)

Smith, M. R., Wilson, M. L., Hamanaka, R., Chase, D., Kung, H., Longo, D. L., Ferris, D. K. Malignant Transformation of Mammalian Cells Initiated by Constitutive

Expression of the *Polo*-like Kinase1. *Biochem. and Biophys. Res. Comm.* 234, 397-405 (1997)

Song, S., Lee, K. S. A Novel Function of *Saccharomyces cerevisiae CDC5* in Cytokinesis. *J. of Cell Biol.* 152 (3), 451-469 (2001)

Smits, V. A., Klompmaker, R., Arnaud, L., Rijken, G., Nigg, E. A., Medema, R. H. Plk1 is a target of the DNA damage checkpoint. *Nat. Cell Biol.* 2, 672-676 (2000).

Sun, Y. p53 and its downstream proteins as molecular targets of cancer. *Molecular Carcinogenesis* 45, 409-415 (2006)

Sunkel C. E, Glover D. M. Polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J. Cell Sci.*, 89 (Part 1), 25–38. (1988).

Swallow, J. C., Ko, A. M., Najeeb, U. S., Hudson, J. W., Dennis, J. W. Sak /Plk4 mitotic fidelity. *Oncogene* 24, 306-312 (2005).

Syed, N., Smith P., Sullivan, A., Spender, L., Dyer, M., Karran, L., O'Nions, J., Allday, M., Hoffman, I., Crawford, D., Griffin, B., Farrel, P., Cook, P. Transcriptional silencing of Polo-like kinase 2 *(SNK/PLK2)* is a frequent event in B-cell malignancies. *Blood* 107 (1), 250-256 (2006)

Takai, N., Hamanaka, R., Yoshimatsu J., Miyakawa, I., Polo-like kinases (Plks) and cancer. Oncogene 24, 287-291 (2005)

Tang, J., Erikson, R. L., Liu, X. Checkpoint kinase 1(Chk1) is required for mitotic progression through negative regulation of the polo-like kinase (Plk1). *PNAS* 103(32), 11964-11969 (2006)

Toyoshima-Morimoto, F., Taniguchi, E. Shinya, N., Iwamatsu, A., Nishida, E. Plk1 phosphorylates Cyclin B1 and targets it to the nucleus during prophase. *Nature* 410, 215-220 (2001).

Toyoshima-Morimoto, F., Taniguchi, E., Nishida, E. Plk1 promotes nuclear translocation of human Cdc25C during prophase. *EMBO Reports* 3(4), 341-48 (2002).

Tsvetkov, L., Xu, X., Li, J., Stern, D., F. Polo-like Kinase 1 and Chk2 Interact and Co-localize to Centrosomes and the Midbody. *J. Biol. Chem.* 278, 8468-9475 (2003).

van de Weerdt, B. C., Medema, R. H. Polo-like kinases: a team in control of the division. *Cell Cycle* 5 (8), 853-864 (2006)

Vugt van, M., Smits, A. J. V., Klompmaker, R., Medema, R. Inhibition of Plk1 by DNA damage occurs in an ATM- or ATR-dependent fashion. *J. Biol. Chem.* 276, 41656-41660 (2001).

Vugt van, M., Weerdt van de, B., Vader, G., Janssen, H., Calafat, J., Klompmaker, R., Wolthuis, R., Medema, R. Plk1 is Required for Bipolar Spindle Formation but is Dispensible for APC/Cdc20 Activation and Initiation of Cytokinesis. *J. Biol. Chem.* 279, 36841-36854 (2004).

Watanabe, N., Ari, H., Nishihara, Y., Taniguchi, M., Watanabe, M., Osada, H. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFβ-TrCP. *PNAS* 101 (13), 4419-4424 (2004)

Winkles, J. A., Alberts G. F.Differential regulation on polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. *Oncogene* 24, 260-266 (2005)

Wright, J. A., Keegan, K. S., Herendeen, D. R., Bentley, N. J., Carr, A. M., Hoekstra, M. F., Concannon, P. Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. *PNAS* 95 (13), 7445-7450 (1998)

Xie, S., Wu, H., Wang, Q., Cogswell, J. P., Husain, I., Conn, C, Stambrook, P., Jhanwar-Uniyal, M., Dai, W. Plk3 Functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. *J. Biol. Chem.* 276, 43305-43312 (2001).

Xie, S., Wu, H., Wang, Q., Kunicki, J., Thomas, R.O., Hollingsworth, R.E., Cogswell, J., Dai, W. Genotoxic stress-induced activation of Plk3 is partly mediated by Chk2. *Cell Cycle* 1 (6), 424-429 (2002)

Yamashita, Y., Kajigaya, S., Yoshida, K., Ueno, S., Ota, J., Ohmine, K., Ueda, M., Miyazato, A., Ohya, K., Kitamura, T., Ozawa, K., Mano, H. *J. Biol. Chem.* 276, 39012-39020 (2001).

Yarm., F.R. Plk Phosphorylation Regulates the Microtubule-Stabilizing Protein TCTP. *Molecular and Cellular Biology*. 22 (17), 6209-6221 (2002)

Zhou, T., Aumais, J., Liu, X., Yu-Lee, L., Erikson, R.L. A Role for Plk1 Phosphorylation of NudC in Cytokinesis. *Developmental Cell.* 5, 127-138 (2003)

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Bonni, S., Ganuelas, M.L., Petrinac, S., Hudson, J.W. Plk4 Phosphorylates Cdc25C. *Cell Cycle*, accepted, in press.