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Characterization of the Sak kinase interaction with cyclin B1 and Gadd45a

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ABSTRACT

The Polo like kinase (Plk) family members are critical regulators of cell cycle progression, mitosis, cytokinesis and the DNA damage response. While much is known regarding the phenotype associated with loss or overexpression of Sak, less than a handful of interacting partners/substrates are known. Therefore, the identification and characterization of Sak interacting partners will contribute to our understanding of the essential role of Sak in the cell.

Our results show that Sak physically interacts with and co-localizes with Cyclin B1. Furthermore, we identified Gadd45a as both an interacting partner and a substrate for Sak. The site of phosphorylation was putatively identified as Tyr151 on Gadd45a. In addition, we found that endogenous Sak protein levels are decreased in response to UV and that this decrease is p53-dependent. These findings suggest that Sak in addition to Sak playing a role in centrosome duplication that it may also function in mitotic entry and DNA damage response pathways.

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LIST OF ABBREVIATIONS

Plks - Polo-like kinases

PBD - Polo box domain

SPBs - Spindle pole bodies

MPF – Maturation (M-phase) promoting factor

FEAR - Fourteen Early Anaphase Release

MEN - Mitotic Exit Network

APC/C - Anaphase-promoting complex/cyclosome

SIN - Septation initiation network

MTOC - Microtubule-organizing centers

ATM - Ataxia telangiectasia mutated

ATR - Ataxia telangiectasia and Rad-3-related

BRCA2 - Breast Cancer Type 2 susceptibility protein

Chk2 - Checkpoint kinase2

Cry-pb - Cryptic polo box

Cdk2 - Cyclin-dependent kinase 2

MEFs - Murine embryonic fibroblasts

E. coli- *Escherichia coli*

SDS-PAGE - SDS-polyacrylamide gel

TBST - Tris-buffered Saline and Tween

DAPI - 6'-diamidino-2-phenylindole

NES - Nuclear export signal

CHAPTER I

INTRODUCTION

Polo-like Family Function and Members

The polo-like kinases (Plks) are a conserved subfamily of Ser/Thr protein kinases that play pivotal roles in regulating various cellular and biochemical events at multiple stages of cell cycle progression (Glover *et al.*, 1998; Nigg, 1998; Dai *et al.*, 2003). They regulate cell cycle progression, including centrosome duplication, DNA damage response in S phase, Golgi dynamics in G2 phase as well as Mitotic entry, spindle assembly and cytokinesis in M phase.

The founding member of this family, Polo, was originally identified in the fruit fly (*Drosophila melanogaster*) and was shown to be a serine–threonine kinase that is required for mitosis (Fenton and Glover, 1993). Lower eukaryotic organisms such as the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have only one homolog of Polo, Cdc5 and Plo1, respectively (Kitada *et al.*, 1993; Ohkura *et al.*, 1995). Higher eukaryotic organisms such as the *Caenorhabditis elegans* contains three Polo structural homologues (Plk1, Plk2 and Plk3) (Ouyang *et al.*, 1999; Chase *et al.*, 2000), while vertebrates have four Plk family members (Plk1, Plk2, Plk3 and Plk4/Sak) (Glover *et al.*, 1998; Nigg, 1998). The increase in number of Plk members in higher organisms is likely a reflection for the need of tighter controls on the cell cycle regulation.

The Structure of the Polo-like Kinases

All Plks share two conserved features: first, a Ser/Thr kinase domain in the amino-terminal region of these proteins which is critical for the various cellular

functions of the Plks; second a structural motif termed the Polo box domain (PBD) in the noncatalytic carboxyl-terminal region (Li *et al.*, 1996; Elia *et al.*, 2003a). The PBD plays important roles in both Plk localization and function. Mutation of the PBD of Plk1 results in loss of Plk1 localization to the spindle pole and the bud neck. Recent studies have also revealed that the PBD functions as a docking site for various serine/threonine phosphorylated proteins (Elia *et al.*, 2003b; Lowery *et al.*, 2004). The phosphopeptide–binding function of the PBD is intricately linked to how Plk1 controls cell cycle progression through mitosis. Plks 1-3 share a common domain architecture consisting of an N-terminal kinase domain and two polo box domains at C-terminus. Sak/Plk4 differs from other polo-like kinases in that it has only a single polo box at C-terminus (Hudson *et al.* 2001; Leung *et al.* 2002; Swallow *et al.*, 2005).

This chapter mainly introduces the polo-like kinases in lower eukaryotes and Plk 1-3, Sak that is the main focus in our lab will be discussed in detail at chapter 2.

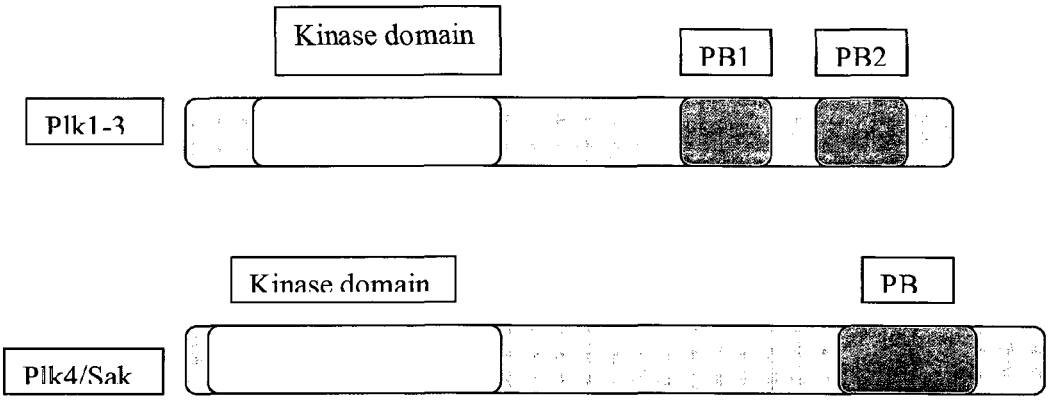


Figure 1. Polo-like kinase structure. Plk1-3 contain an amino-terminal kinase domain and two carboxy-terminal PBD. Sak/Plk4 differs from other polo-like kinases in that it has only a single polo box at the carboxy-terminus, while all other Plks have two. The kinase domain is represented by the yellow colour and the polo-box domains are showed in green colour.

Plks Expression and Kinase Activity

The Plk gene family are differentially expressed during normal cell cycle progression. In general, Plk1 expression/activity is low in G0, G1, and S, begins to increase in G2, and peaks in M phase (Roshak *et al.*, 2000; Alvarez *et al.*, 2001; Anger *et al.*, 2003). Plk2 mRNA and protein is expressed in early G1, has a fast turnover rate (half-life of B15 min) and is catalytically active (Ma *et al.*, 2003b). This finding is consistent with the function of Plk2 as a regulator of G1 progression in mammalian cells (Ma *et al.*, 2003a).The precise role of Plk3 in mammalian cells is not yet fully established, but it appears to be primarily involved in pathways involved in the cellular response to DNA damage (Barr *et al.*, 2004). Maximum levels of Plk3 mRNA expression are detected 1 h after FGF or serum stimulation of quiescent NIH 3T3 cells, and expression decreases to basal levels by 8 h (Donohue *et al.*, 1995). However, Plk3 protein levels remain relatively constant in mitogen stimulated NIH 3T3 cells (Chase *et al.*, 1998; Bahassi *et al.*, 2002)

The Plk family genes are also differentially regulated in stressed cells; for example, when DNA-damaging agents are added to cycling cells, Plk1 expression decreases, but Plk2 and Plk3 expression increases. Furthermore, Plk1 is expressed at the highest levels in tissues with actively proliferating cell populations; for example, the placenta (Wolf *et al.*, 2000), spleen (Golsteyn *et al.*, 1994), ovary (Takai *et al.*, 1999), and testis (Syed and Hecht, 1998). Plk1 transcripts are either undetectable or present at very low levels in most other adult tissues, including the liver, kidney, brain and skin (Wolf *et al.*, 2000). The tissue distribution of Plk2 and Plk3 differ greatly from Plk1. Plk2 and Plk3 transcripts have a broad tissue distribution. Finally, Plk1, 2, 3 are expressed to varying degrees in different human tissue types and it has been reported that Plk1 expression is increased and Plk3 expression is decreased in tumor

specimens. Plk2 gene expression levels in human tumor specimens have not yet been reported (Winkles JA *et al.*, 2005).

The differential regulation of Plk family member gene expression indicates one cellular strategy for controlling Plk activity in mammalian cells.

Plk Localization

The localization of individual Plks is dynamically regulated during the cell cycle. Plk proteins from yeast to mammalian undergo apparent subcellular localization differences. In unicellular organisms such as yeasts, the Plks (Cdc5 and Plo1) localize to the spindle pole bodies (SPBs) but with apparent differences in timing. Cdc5 localizes to the SPBs (Shirayama *et al.*, 1998; Song *et al.*,2000) during early G1 and remains there until late mitosis, whereas Plo1 associates with the SPBs after the activation of Cdc2 (Cdk1) and dissociates from the SPBs as Cdc2 becomes inactive (Mulvihill *et al.*, 1999). In addition, Cdc5 localizes to the septin ring filaments in G2 and remains until late mitosis (Sakchaisri *et al.*, 2004), whereas Plo1 localizes to the medial ring structures at the time of their formation (Bahler *et al.*, 1998). The founding member of this family, *Drosophila* polo is present in centrosomes early in mitosis, accumulates around the nuclear envelope until its breakdown. During prometaphase, polo localizes to the kinetochores and at metaphase associates to spindle microtubules (Moutinho-Santos *et al.*, 1999).

Studies in cultured mammalian cells revealed that Plk1 localizes to the cytoplasm and the nucleus during G2 and is specifically targeted to the centrosomes (Taniguchi E., 2002). During early mitosis, Plk1 can be found at centrosomes and kinetochores (Golsteyn RM *et al.*, 1995). In addition, a fraction of Plk1 translocates to the midzone/midbody late in mitosis. Plk2 localizes to the centrosome, indicating a

role for Plk2 in centriloe duplication (Warnke *et al.*, 2004). There are several conflicting reports about Plk3 localization in the literature. Plk3 has been reported to colocalize with the centrosome, the spindle pole and spindle microtubules (Wang Q *et al.*,), as well as with the Golgi apparatus (Ruan Q *et al.*, 2004), and actin-containing plaques (Holtrich U *et al.*, 1995, Wendy C *et al. 2007)* demonstrated that Plk3 localizes in the nucleolus.

The dynamic subcellular localization of Plk homologs suggests the functional complexity of these enzymes in various organisms. The apparent localization differences between yeast and mammalian Plks may reflect differences in the spatial and temporal regulation of the mitotic and cytokinetic machineries that these organisms utilize and the inherent need for greater controls in multicellular organisms.

The Biological Functions of Plks

Polo-like kinases play critical roles during multiple stages of cell cycle progression, they are important regulators for many cellular events critical for cell division including centrosome duplication and maturation, DNA damage checkpoint activation, mitotic onset, bipolar spindle formation, Golgi fragmentation and assembly, chromosome segregation, and cytokinesis. This review will focus on the roles of Plks in mitotic entry, mitotic exit and cytokinesis.

i) Mitotic Entry

In eukaryotic cells, initiation of mitosis requires nuclear translocation and activation of M-phase promoting factor (MPF); the complex of a Cyclin-dependent kinase Cdk1 and the Cyclin B (Nurse, 1990; Hunt, 1991). Cyclin B accumulation, phosphorylation of Cdk1 and intracellular localization of Cyclin B1 is regulated during the progression of the cell cycle (Pines, 1999; Yang and Kornbluth, 1999).

During S and G2 phases, Cyclin B accumulates and binds to Cdk1 to form heterodimers. Cyclin B facilitates the inhibitory phosphorylation of Cdk1 at Thr14 and Tyr15, which are catalyzed by Wee1, Mik1 and Myt1 kinases. At the end of G2, abrupt dephosphorylation of these sites by Cdc25 activates Cyclin B / Cdk1 complex (Morgan, 1995; Nigg, 2001) which triggers mitotic entry. Recent studies have shown that Plk1 acts as an important coordinator for MPF activation. Plk1 promotes mitotic entry by activating Cyclin B/Cdk1 at three different levels. (1) Plk1 phosphorylates one member of the Cdc25 family of phosphatases, Cdc25C. Cdc25C is phosphorylated by Plk1 in a nuclear export signal sequence during G2–M phase and this phosphorylation is necessary for the nuclear translocation and activation of Cdc25C during prophase.(Roshal AK *et al.*, 2000; Toyoshima-Morimoto *et al.*, 2002) Nuclear Cdc25C plays a role in keeping nuclear MPF active by counteracting the inhibitory activity of Wee1, which is constitutively nuclear. (2) At the same time, Plk1 phosphorylates the Cyclin B/Cdk1-inhibiting kinase Wee1 and Myt1 (Watanabe N *et al.*, 2004; Nakajima H *et al.*, 2003), leading to enhanced association with the SCF/beta-TrCP E3 ubiquitin ligase, thereby inducing its degradation. (3) In addition to, it has been shown that Plk1 phosphorylates Cyclin B1 at centrosomes, the first site at which CyclinB/Cdk1 is activated and the phosphorylation triggers Cyclin B1 nuclear translocation during prophase (Toyoshima-Morimoto *et al.*, 2001). During S and G2 phases, Cyclin B1 localized to the cytoplasm, where a subpopulation associated with microtubules. In late G2 phase, a fraction of CyclinB1 was bound to the centrosomes, and at prophase, Cyclin B1 translocates into the nucleus. The nuclear accumulation of Cyclin B1 during prophase is thought to be important for initiating and coordinating M-phase events in vertebrate cells (Pines, 1999; Yang and Kornbluth, 1999; Takizawa and Morgan, 2000).

In Xenopus, Polo-like kinase Plx1 plays an important role in initiating the G2/M transition. Data from the Xenopus system indicate that Plx1 phosphorylates Cyclin B1 on Ser 101, This is supported by data indicating that Plk1 phosphorylates Cyclin B1 on Ser 133 in human cells and Ser 147 in mammalian cells (Jackman *et al.*, 2003). This phosphorylation of Cyclin B1 initially occurs on centrosomes in prophase, and the phosphorylation does not cause Cyclin B1 to move into the nucleus (Jackman *et al.*, 2003). It has been proposed that phosphorylated Cyclin B1 facilitates the interaction between Cdk1 and Cdc25C (Peter *et al.*, 2002). In this case, centrosomes may function as sites of integration for the proteins that trigger mitosis (Jackman *et al.*, 2003). Furthermore, the studies carried out in Xenopus egg extracts and oocytes show that Plx1 is the initial phosphorylating and activating kinase for Cdc25C in vivo and the activation of Cdk1/Cyclin B also requires the activation of Plx1 as a trigger kinase to initiate Cdc25C activation (Qian *et al.*, 1998a, 2001). In addition, Plx1 and Cyclin B/Cdk1 may act in a positive feedback loop ensuring complete activation of Cyclin B/Cdk1 and mitotic entry (Alrieu A *et al.*, 1998).

Plk2 doesn't appear to play a role mitotic entry, it is identified as an early-growth-response gene. Plk2 may primarily function as a regulator of G1 and early S phase progression, a time when both Plk2 mRNA and protein levels peak (Simmons DL *et al.*, 1992; Ma S *et al.*, 2003a).

Plk3 is involved in regulation of the G1/S phase transition and is required for entry into S phase (Wedy C *et al.*, 2007). Recent studies have shown that Plk3 phosphorylates Cdc25C on serine 191, and to a lesser extent serine 198, within the nuclear export signal (NES) to promote the nuclear accumulation of Cdc25C, and that substitution of serine 191 with an alanine abrogates Cdc25C's nuclear localization (Bahassi *et al.*, 2004). Transient transfection of cells with wild-type Plk3 but not with

a kinase dead Plk3 results in a marked increase of Cdc25C in the nucleus. This nuclear accumulation of Cdc25C is inhibited when Plk3 is suppressed by siRNA, implicating the involvement of Plk3 in the nuclear accumulation of Cdc25C (Bahassi *et al.*, 2004).

Therefore, the Polo-like kinases act as important coordinators for intracellular localization of active MPF by directing nuclear entry of MPF and its activator Cdc25C, which may be essential for coordinating M-phase events.

ii) Mitotic Exit

In all eukaryotic organisms, exit from mitosis requires the inactivation of mitotic Cdk1 activity, and this step is a prerequisite for the onset of cytokinesis. Unlike other eukaryotic organisms, downregulation of Cdk1 activity in mammalian cells occurs at the metaphase/anaphase transition (Clute and Pines, 1999), while the inactivation of budding yeast Cdk1 occurs after metaphase/anaphase transition (Ghiara *et al.*, 1991; Surana *et al.*, 1993), and it is achieved through the successive actions of two cooperative pathways termed FEAR (Cdc Fourteen Early Anaphase Release) network (Stegmeier *et al.*, 2002) and MEN (Mitotic Exit Network) (Bardin and Amon, 2001; Jensen and Johnston, 2002). The FEAR and the MEN must be activated in an ordered manner, but how this order is maintained is not known. Both genetic and biochemical analyses showed that Cdc5 plays an important role in regulating both of these pathways and Cdc5 may coordinate these two pathways, perhaps by directly phosphorylating and regulating components in each pathway.

Studies show that the Xenopus Polo-like kinase Plx1 is required in mitotic exit. The metaphase to anaphase transition could be blocked by immunodepletion of Plx1, and was restored upon the addition of recombinant Plx1 (Qian *et al.*, 1999; Liu *et al.*, 2004). These results demonstrate that the metaphase/anaphase transition

requires active Plx1. Furthermore, Plx1 associates with the anaphase-promoting complex/cyclosome (APC/C). Studies so far have demonstrated three possible mechanisms that Plx1 regulate APC/C activity. First, Plx1 may activate APC/C by direct phosphorylation of several APC/C subunits (Kotani *et al.*, 1998). The second mechanism may involve the regulation of APC/C activators or inhibitors by Plx1. Thirdly, Plx1 is also required to prevent premature inactivation of the APC/C (Brassac *et al.*, 2000).

iii) Cytokinesis

Polo-like kinases have been shown to play a role in cytokinesis, and much of the evidence came from studies on the fission yeast (*Schizosacchromyces pombe* (Ohkura *et al.*, 1995; Bahler *et al.*, 1998), *Drosophila* (Carmena *et al.*, 1998), and more recently, the budding yeast (*Sacchromyces cerevisiae*) (Song and Lee, 2001). In budding yeast, besides the role in mitotic exit, Cdc5 is required for proper actin ring formation at the mother–bud-neck (Jimenez *et al.*, 1998; Frenz *et al.*, 2000; Lee *et al.*, 2001). Consistent with the role in cytokinesis, Cdc5 localizes to the neck as early as G2 and remains at this structure until late mitosis (Sakchaisri *et al.*, 2004), suggesting of additional role(s) at the neck. Furthermore, depletion of Cdc5 results in arrests at multiple points during mitosis, suggesting a failure of cytokinesis (Song and Lee, 2001).

In fission yeast, loss of Plo1 function leads to the generation of multinucleate cells with defects in actin ring and septum formation, suggesting that Plo1 activity may be required for cytokinetic events. In early mitosis, Plo1 localizes to the future division site (Bahler *et al.*, 1998; Mulvihill *et al.*, 1999), and this localization appears to be important for the placement and organization of an actin-based medial ring (Bahler *et al.*, 1998). Secondly, Plo1 activation correlates with the timing of septum

formation, which is regulated by a signaling network termed septation initiation network (SIN) (Bardin and Amon, 2001; McCollum D, 2001; Gruneberg and Nigg, 2003). The SIN in fission yeast is composed of proteins structurally related to those in the MEN and they function at each tier of the pathway. In addition, in the absence of Plo1 activity, formation of a division septum is impaired in fission yeast, whereas overexpression of Plo1 leads to formation of an ectopic septum.

In *Drosophila*, it was shown that Polo kinase and Pav-KLP coimmunoprecipitate, and they co-localize in the central part of the spindle. The Pav-KLP family of motor proteins play a conserved role in organizing the central spindle in anticipation of cytokinesis. Furthermore, cytokinesis can fail at all stages of spermatogenesis in polo mutants (Carmena *et al.*, 1998). Also, the *Drosophila* Polo was found to interact with CHO1 homolog Pavarotti, which is implicated in cytokinesis (Adams RR *et al.*, 1998). In *Xenopus,* Plx1 has been shown to play a role in cytokinesis. Similarly, Plx1 is localized to the midbody in late mitosis in *Xenopus* embryos (Qian *et al.*, 1999). Interestingly, the inactivation of Plx1 may also be required for completion of cytokinesis in *Xenopus* embryos (Qian *et al.*, 1999). It seems that the initiation of cytokinesis requires the activity of Plks, and the completion of cytokinesis requires their inactivation or degradation.

Mammalian Plk1 has also been implicated in cytokinesis, consistent with Plk localization to the midzone late in mitosis. The first evidence of the involvement of Plk1 in cytokinesis was the identification of the kinesin-like protein CHO1/MKLP-1 as an in vitro substrate of Plk1, which induces microtubule bundling and antiparallel movement in vitro (Lee KS *et al.*, 1995). On the other hand, overexpression of Plk1 does give rise to multinucleated cells (Mundt KE *et al.*, 1997), indicating the precise level of Plk1 may be important for the correct execution of cytokinesis. Evidence for

a role for Plk1 in cytokinesis was also found in overexpression studies with a dominant negative mutant of Plk1, which causes a failure in completion of cytokinesis after forced inactivation of the spindle assembly checkpoint (Seong YS *et al.*, 2002). In addition, Plk1 has been found to phosphorylate several other proteins that are involved in cytokinesis, like NudC, a component of the dynactin complex (Zhou T *et al.*, 2003). MKlp2, a kinesin localized to the midbody in cytokinesis (Neef R *et al.*, 2003). Finally, the Rho exchange factor ECT2 is another Plk1 substrate recently implicated in cytokinesis (Niiya F *et al.*, 2005).

These observations strongly suggest that Plks play a role in both the organization of the central spindle and the correct execution of cytokinesis.

The Polo Box Domain

In Plks 1-3, studies have shown that the PBD made up of polo box I (PB1) and polo box 2 (PB2) motifs, a linker of 20 residues and 45 residues upstream of PB1 is a functional domain of approximately 80 amino acids (Cheng *et al.*, 2003), It consists of a continuous six-stranded antiparallel β-sheet and an α-helix. The PBD regulates the cellular function of the Plks, subcellular localization and substrate interactions (Seong YS *et al.*, 2002; Elia AE *et al.*, 2003; Reynolds N., 2003). It is thought to localize the respective kinase to various mitotic structures during cell cycle progression, including centrosomes in early M phase, the spindle midzone in early and late anaphase and the midbody in cytokinesis, presumably to promote interaction between its catalytic domain and specific substrates and effectors (Lee *et al.*, 1998; Jang *et al.*, 2002a). For example, Polo-like kinases have been shown to interact with Cdc25C (Kumagai and Dunphy, 1996; Ouyang *et al.*, 1999), and this interaction is polo box-dependent (Elia *et al.*, 2003b). Mutation of the putative Plk1 pThr-binding

motif in Cdc25C indeed abolishes interaction between Cdc25C and the Plk1-PBD, and decreases phosphorylation of Cdc25C (Elia *et al.*, 2003).Furthermore, residues Trp-414, Val-415 and Leu-427 in PBD all contribute to proper Plk localization and function in various Plk family members, including budding yeast Cdc5, fission yeast Plo1, murine Plk and human Plk1.Trp 414 in PB1 of human Plk1 has been suggested to play a critical role in PBD substrate binding (Elia *et al.*, 2003b). Mutation of this residue to phenylalanine dramatically affects the ability of human Plk1 to complement the Cdc5-1 defect in yeast and disrupts Plk localization at spindle poles and septin ring structures (Lee *et al.*, 1998). Mutation of the residue analogous to Trp-414 in Xenopus Plx1 and Val-415 in budding yeast Cdc5 inhibits interaction with substrates, such as the APC/C subunit Cdc27 (Liu J *et al.*, 2004). In addition, Plx1 function in bipolar spindle formation (Qian *et al.*, 1998a) has also been demonstrated to be polo box dependent (Liu *et al.*, 2004). Studies identified that the optimal sequence motif recognized by the PBD is Ser-[pSer/pThr]-[Pro/X], suggesting that Cdks, MAP kinases, and other mitotic kinases might generate 'priming' phosohorylations on substrates or docking proteins to localize Plks in the vicinity of their substrates (Elia *et al.*, 2003). At present, verified *in vivo* Plk1 substrates include Cdc25C (Toyoshina Morimoto *et al.*, 2002), Brca2 (Lin *et al.*, 2003), My1(Nakahjima *et al.*, 2003), Cyclin B (Toyoshima-Morimoto *et al.*, 2001), NudC (Zhou *et al.*, 2003), NIp (Casenghi *et al.*, 2003), TCTP (Yarm, 2002), MkIp1 (Lee *et al.*, 1995; Liu *et al.*, 2004), Chk2 (Tsvetkov *et al.*, 2003), MKIp2 (Neef *et al.*, 2003), and Wee1 (Sakchaisri *et al.*, 2004; Watamabe *et al.*, 2004). All of these contain potential PBD-binding sites. Other substrates of the Plk family members have been identified are summarized and listed in Table 1.

Table 1. Summary of identified proteins interacting with the polo-like kinases

Cdc5	Plo1	Polo	Plx1	Plk1	Plk2	Plk3	Sak
Hsl1	Mid1	Asp	Cyclin B	Cyclin B1	SPAR	Cdc25C	Cdc25C
Swe1	Byr4	Cdc25	Cdc25C	Cdc25C		Chk2	p53
Cdc11				Wee1		pol	Hand1
Cdc12				Myt1		p53	Chk2
Scc1				NudC			
Bfa1				TCTP			
Bbp1				CHO1/MKLP1			
Nud1				α-tubulin			
Spc72				β-tubulin			
				γ-tubulin			
				Chk2			
				Nip			
				Emi1			
				Pin1			
				BRCA2			
				GRASP65			

Plks and Centrosomes

Centrosomes are the microtubule-organizing centers (MTOC) and are important for the formation of the bipolar spindle during mitosis. They contain a pair of microtubule-based centrioles and surrounding pericentriolar material. The centrioles duplicate once during the cell cycle to give rise to two mitotic spindle poles. So, cells normally contain one or two centrosomes depending on their phases in the cell cycle. Centrosome duplication starts at later G1 phase and proceeds through S phase (Fukida S *et al.*, 2002). Prior to mitosis, the duplicated centrosomes separate and move to the future poles of the spindle to initiate the bipolar spindle, this is required for equal segregation of chromosomes. Duplication of a single centrosome must be precisely coordinated with the duplication of the genome to ensure faithful transmission of genetic material. Aberrant centrosome numbers can result in the generation of monopolar and multipolar spindles. Centrosome abnormalities are implicated in chromosomal instability and in the development of cancer, and many cancer cells display multiple centrosomes or enhanced centrosomal activity (Li *et al.*, 2001). Extent research has indicated that polo-like kinases family plays a pivotal role in the regulation of the centrosome duplication and maturation (Glover *et al.*, 1996; Wianny *et al.*, 1998).

In budding yeast, Cdc5, a homologue of the Plks is required for spindle pole, the functional counterparts to centrosomes, and for the formation of normal mitotic spindles. The evidence indicating a role for Cdc5 in regulating the spindle pole body (SPB) came from the study of meiosis I in budding yeast (Schild and Byers, 1980). A temperature-sensitive mutant of Cdc5 fails to complete meiosis I due to arrest at a stage after SPB duplication and separation at the restrictive temperature. In these

mutant cells, SPBs lack the normal spindle microtubules that are characteristic of meiosis I in wild-type cells.

In fission yeast, Plo1 is required for the assembly and function of the mitotic spindle. Both loss of Plo1 function and over-expression of this gene results in formation of cells in which condensed chromosomes are associated with monopolar spindles (Ohkura *et al.*, 1995), indicating a failure in bipolar spindle formation.

In Drosophila, embryos from homozygous polo females display abnormal spindles which are multiple branched spindles in syncytial polo-derived embryos, spindles with broad poles and fewer circular mitotic figures in larval neuroblasts (Sunkel and Glover, 1988). While in strongly hypomorphic mutants (polo9/10), a majority of cells are arrested in a metaphase-like stage lacking asters at each spindle pole and they all possess bipolar spindles with robust arrays of microtubles (Donaldson *et al.*, 2001).

In embryonic Xenopus cells, Plx1 is localized to mitotic spindles. In early prophase, Plx1 has a bipolar localization at the spindle poles and colocalizes with γ-tubulin. Inhibition of Plx1 by microinjection of anti-Plx1 antibodies into Xenopus embryos resulted in monopolar spindles with altered patterns of α-tubulin associated with a radial distribution of chromosomes around the pole (Qian *et al.*, 1998a). The exact mechanism by which Plx1 regulates bipolar spindle formation is not clear. However, evidence from studies in mammalian cells suggests that Plx1 most likely affects bipolar spindle formation by regulating both centrosome separation and maturation. It is therefore likely that Plx1 regulates centrosome separation and maturation by recruiting specific proteins necessary for these processes to centrosomes.

Several mammalian Plks are involved in regulating centrosomal function although the molecular basis of their roles remains unclear. Plk1 was first implicated in the centrosome cycle because of its centrosomal localization during interphase and its association with mitotic spindle poles in early mitosis (Golsteyn *et al.*, 1994). Plk1 antibody injection studies show that Plk1 is also essential for centrosome separation, with defects leading to monopolar spindles and consequent mitotic arrest (Lane HA *et al.*, 1996). Plk1 depletion by siRNA significantly reduced centrosome amplification (Liu and Erikson, 2002). Plk1 interacts and phosphorylates α-, β- and γ-tubulins and the tubulin-atabilizing protein TCTP (Feng Y *et al.*, 1999; Yarm FR *et al.*, 2002), indicating that Plk1 may regulate spindle formation through centrosome maturation and separation as well as by directly regulating tubulin functions.

Plk2 also plays an important role in the reproduction of centrosomes. The study showed that the polo-box of Plk2 is required for the centrosome and centriole duplication. Mutation of Plk2 polo-box impairs centrosomal localization and centriole duplication. Plk2 is localized to centrosomes during early G1 phase and only associates to the mother centriole. Furthermore, the results suggest that Plk2 mediated centriole duplication is dependent on Sak function. In addition, the downregulation of Plk2 leads to abnormal mitotic spindles, indicating that Plk2 may have a function in the reproduction of centrioles (Cizmecioglu O *et al.*, 2008).

Plk3 primarily localizes to the microtubule organization center during interphase. In all stages of the cell cycle Plk3 is associated with centrosomes or mitotic spindle poles (Wang *et al.*, 2002). It remains unclear why both Plk1 and Plk3 are localized to centrosomal regions during interphase. Thus, Plks may fulfill different functions in regulating microtubule dynamics during the cell cycle.

Plks and DNA Damage Checkpoint Pathways

The progression of the cell cycle is tightly regulated in order to maintain genetic integrity and to ensure that genetic information is correctly passed on to daughter cells. Surveillance mechanisms, known as checkpoints monitor the integrity of cell cycle progression (Elledge, 1996). The checkpoints are activated in response to DNA damage caused by environmental stress and /or internal perturbations. Various types of DNA damage activate DNA damage checkpoint pathways that arrest cells at the G1/S border (G1-phase checkpoint), delay S-phase progression (intra-S-phase checkpoint), or prevent mitotic entry (G2-phase checkpoint), depending on the phase of the cell cycle in which the damage occurs. DNA damage induces several cellular responses including DNA repair, checkpoint activity and the triggering of apoptotic pathways depending on the severity of the damage and the cell context.

Cell cycle regulatory proteins are targets and effectors of DNA damage checkpoint pathways. Two protein kinases, ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad-3-related) are at the top of signaling cascades that transmit the signals downstream to mediators (Chk1 and Chk2) and effectors (p53, p21, Cdc25A, Cdc25C). Polo and Polo-like kinases have emerged as major regulators for various cell cycle checkpoints. Extensive research has demonstrated that the Plks are important mediators for various cell cycle checkpoints that monitor centrosome duplication, DNA replication, formation of bipolar mitotic spindle, segregation of chromosomes, and mitotic exit, thus protecting cells against genetic instability during cell division (Nigg, 1998; Dai *et al.*, 2002; Barr *et al.*, 2004). In response to DNA damage, budding yeast arrests in metaphase with active Clb–Cdc28, but eventually continues through the cell cycle even if the damage is not repaired. However, a mutation in CDC5 (cdc5-ad) completely eliminates the ability of these cells to over-

ride cell cycle arrest even in the presence of an irreparable double-strand DNA break (Toczyski *et al.*, 1997), suggesting a role for Cdc5 in overriding the DNA damage checkpoint (Sanchez *et al.*, 1999). It remains to be determined how Cdc5 downregulates the DNA damage checkpoint pathway and what it targets for adaptation.

Several studies show that the activity of Plk1 is inhibited after DNA damage (Smits *et al.*, 2000; van Vugt *et al.*, 2001; Ando *et al.*, 2004). However, expression of activation mutants of Plk1 can override the G2/M arrest induced by DNA damage (Smits *et al.*, 2000). In addition, Plk1 can bind to p53 which plays an important role in maintaining genomic fidelity by controlling cell cycle checkpoints and apoptotic process in response to genotoxic stress (Levine, 1997). Plk1 inhibits p53 transactivation activity and its pro-apoptotic function, whereas the kinase-deficient mutant form of Plk1 failed to reduce the transcriptional activity of p53. Besides, Plk1 and Chk2 have been shown to colocalize at centrosomes in early mitosis and to the mid-body in late mitosis (Tsvetkov *et al.*, 2003). Plk1 also interacts with and phosphorylates Chk2 (Tsvetkov L *et al.*, 2003). Overexpression of Plk1 enhances phosphorylation of Chk2 at T68 (Tsvetkov *et al.*, 2003), a site primarily targeted by ATM in response to ionizing radiation, leading to its activation (Matsuoka *et al.*, 2000). Another substrate of Plk1 involved in the DNA damage checkpoint is the breast cancer susceptibility protein, BRCA2, which is essential for the repair of DNA double-strand breaks (Lin HR *et al.*, 2003; Lee M *et al.*, 2004). Plk1 phosphorylates BRCA2 during mitosis (Lin *et al.*, 2003; Lee *et al.*, 2004). The phosphorylation of BRCA2 is inhibited by DNA damage, which is consistent with the finding that Plk1 activity is negatively regulated by DNA damage (Smits *et al.*, 2000). Therefore, after

DNA damage, the activity of Plk1 is inhibited, leading to activation of p53 and modulation of Chk2 and BRCA2.

Other mammalian Plks are also involved in the DNA damage checkpoint activation pathway. Expression of Plk2 mRNA is rapidly induced upon X-ray irradiation; Plk3 kinase activity is activated upon oxidative stress and DNA damage induced by ionizing-radiation mimetic drugs, and its activation is ATM-dependent (Xie *et al.*, 2001a, b). Plk3 interacts with and phosphorylates p53; targeting serine-20 of p53 in vitro (Xie *et al.*, 2001b) and the extent of this interaction is increased in response to DNA damage (Xie *et al.*, 2001b). Plk3 also physically interacts and phosphorylates with Chk2 during DNA damage checkpoint activation (Bahassi *et al.*, 2002). Together, these studies suggest that Plk3 functionally links DNA damage to the induction of cell cycle arrest or apoptosis. Furthermore, Plk1 is inhibited in response to DNA damage, Plk3 is activated suggesting that both kinases have opposing roles in checkpoint control (Xie *et al.*, 2001).

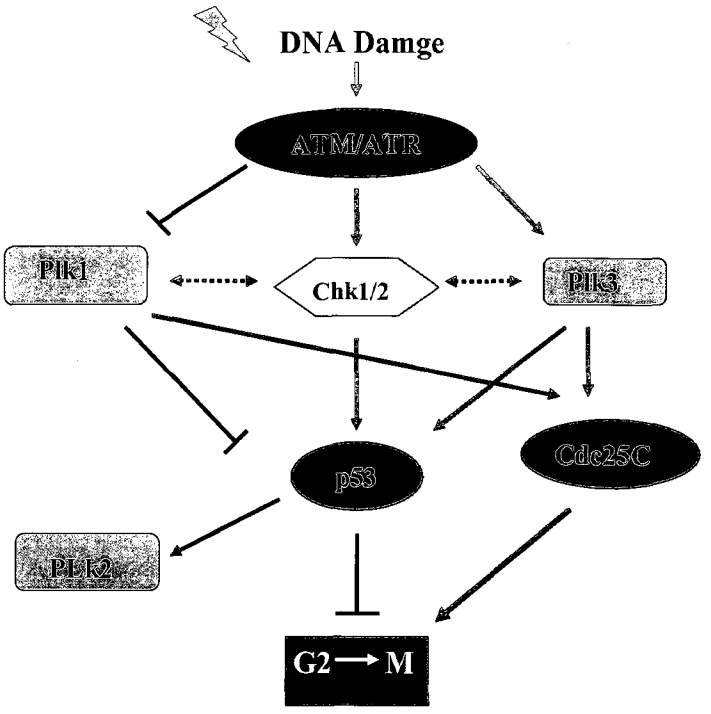


Figure 2. Major pathways where Plks may play a role in checkpoint in mammalian systems. Arrows and bars denote the positive and negative regulations, respectively. Dotted lines denote where the regulatory role in vivo between these proteins remains to be elucidated.

Plks and Oncogenesis

Polo-like kinases play an important role in the centrosome cycle. Mutations of Plks from Polo, CDC5, Plo1 to Plk1, Plk2, Plk3 resulted in aberrant centrosome numbers. Centrosome abnormalities are implicated in chromosomal instability and in the development of cancer. Extensive studies have shown that deregulated expression of Plks is detected in many types of cancer and is associated with oncogenesis.

Plk1 expression is elevated in non-small-cell lung cancer (Wolf *et al.*, 1997), head and neck cancer (Knecht *et al.*, 1999), esophageal cancer (Tokumitsu *et al.*, 1999), melanomas (Strebhardt *et al.*, 2000), breast cancer (Wolf *et al.*, 2000), ovarian cancer (Takai *et al.*, 2001a), endometrial cancer (Takai *et al.*, 2001b), colorectal cancer (Macmillan *et al.*, 2001), gliomas (Dietzmann *et al.*, 2001), and thyroid cancer (Ito *et al.*, 2004). Results from these studies indicate that overexpression of Plk1 is positively correlated with aggressiveness and prognosis in many cancers. Moreover, inhibition of Plk1 with various techniques results in growth arrest or apoptosis for the cancer cells. Thus, Plk1 could be a novel prognostic marker and a good target for chemotherapeutic intervention. The link between Plk1 and oncogenesis may be due to several interacting partners of Plk1 that are encoded by tumor suppressor genes. It has been reported that Plk1 is able to phosphorylate the p53 protein in vitro (Xie *et al.*, 2001). Over 50% of human cancers contain mutations in the gene coding for p53. Plk1 also phosphorylates the breast cancer susceptibility protein BRCA2 during mitosis. BRCA2 (Breast Cancer Type 2 susceptibility protein) is a human gene that is involved in the repair of chromosomal damage and belongs to tumor suppressor genes. The phosphorylation of BRCA2 is inhibited by DNA damage which can cause an increased risk for breast cancer. Chk2 (checkpoint kinase2) that is implicated in DNA repair processes, is also considered as a tumor suppressor protein. It has been

showed that Plk1 can phosphorylate Chk2 at Thr-68, a site that is phosphorylated by ATM. ATM and ATM- and Rad3 related (ATR) are two tumor suppressor proteins with phosphotransferase activity. ATM plays a very important role in the linkage of DNA damage and the induction of a subsequent cell cycle arrest (Zhou and Elledge, 2000). DNA damage activated ATM positively regulates the activation of other checkpoint kinases, like Chk1 and Chk2 (Matsuoka *et al.*, 2000), and phosphorylates p53, MDM-2 and BRCA1 (Khosravi *et al.*, 1999). At the G2-DNA damage checkpoint inhibition of Plk1 activity depends on ATM/ATR activity (van Vugt *et al.*, 2001). Taken together, Plk1 interacts with several tumor suppressor proteins. On the one hand, Plk1 phosphorylates p53, Chk2 and BRCA2 possibly to promote mitotic progression. On the other hand, Plk1 is inhibited by tumor suppressor proteins like ATM, ATR, BRCA1 and Chk1.

Plk2 may primarily function as a regulator of G1 progression in mammalian cells (Ma *et al.*, 2003a). Plk2 is transcriptionally regulated by p53 (Burns *et al.*, 2003) and expression of Plk2 is induced in a p53-dependent manner after DNA damage (Burns *et al.*, 2003). Silencing of Plk2 by siRNA was followed by an increase in apoptosis.

Several studies have examined Plk3 expression in human tumor specimens. Plk3 expression levels are downregulated in lung tumor specimens compared with the normal tissue (Li *et al.*, 1996). Also, Plk3 mRNA downregulation has been detected in uterus (Ando *et al.*, 2004), bladder (Ando *et al.*, 2004), and head and neck squamous cell carcinoma (Dai *et al.*, 2000) specimens. In summary, Plk3 gene expression appears to decrease during the development of some, but not all, types of tumors. Since Plk3 may primarily function in the DNA damage response pathway,

reduced Plk3 levels could contribute to the genomic instability characteristic of tumor cells.

CHAPTER II

SAK/PLK4 BACKGROUND

Sak Structure

Sak (also called Plk4) was the last member of the polo-like kinase family to be identified in vertebrates. Similar to the other Plks, Sak has a kinase domain at its N-terminus; however Sak appears to have diverged significantly from a primordial Plk early in the radiation of metazoans (Fode et al., 1994). The kinase domain of murine Sak is followed by 660 amino acids containing a single 64 amino-acid PBD at the COOH end. The Sak-pb is equally related by phylogenetic comparison to the pb1 and pb2 domains of other Plks. The polo box domain functions as a docking site for various phosphorylated proteins. The C-terminus of Sak contains 3 PEST sequences, which are commonly associated with reduced protein stability. And Sak protein displayed a short half life (2–3 h) in nonsynchronized cells (Fode et al., 1996). The removal of PEST sequences from Sak enhances the stability of the protein (Yamashita et al., 2001). The upstream region of the Sak-pb domain is designated the 'cryptic polo box' (cry-pb) (Sak596–836) which binds to the Tec tyrosine kinase and stabilizes the protein by protecting it from PEST-dependent proteolysis (Yamashita et al., 2001). Like the other Plks, Sak has been shown to be a functional kinase with known substrates including p53 (Ko *et al.,* 2005; Swallow et al 2005), Hand1 (Martindll *et al.* 2008), Cdc25C (Bonni *et al.* 2008) and Chk2 (Petrinac *et al.* in press 2009). Sak kinase activity is markedly increased by a SakT170D mutation in the T loop, while SakK41M, an inactivating mutation in the ATP-binding domain, eliminates activity.

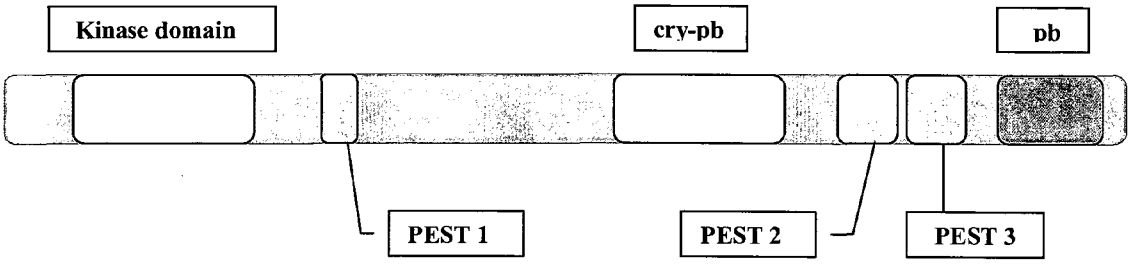


Figure 3. Structural features of Sak. Sak has a Ser/Thr kinase domain at the amino-terminus, a single PB motif at the carboxy-terminus, and three PEST destruction motifs. The conserved ATP-binding domain with the required Lys41 residue and the T-loop activation domain with the required Thr170 are indicated. The cry-pb interacts with the Sak kinase domain.

Sak Expression and Localization

Sak mRNA expression is relatively low in both quiescent, G0 phase cells and in early-to-mid-G1 phase cells and then increases beginning in the late G1 phase, then increases in S through G2 phase, and peaks in M phase. This expression pattern is consistent with the mitotic functions of Sak (Winkles JA *et al.*, 2005). Whether Sak protein levels and kinase activity are regulated in a similar manner is presently unknown. However, it was reported that Sak expression is associated with mitotic and meiotic cell division in mouse tissues (Fode C *et al.*, 1994). Sak gene expression in mammalian cells is also controlled at the post-transcriptional, protein degradation level. Sak protein is ubiquitinated and destroyed at M phase by the APC/C (Fode C *et al.*, 1994), similar in this regard to Cdc5 (Shirayama *et al.*, 1998).

Sak mRNA expression has been detected in only a few adult tissues by Northern blot hybridization analysis. In the mouse, Sak mRNA was found at the highest level in the testis, a tissue containing actively dividing somatic and germ cells, at an intermediate level in the spleen and thymus, and not at all in the brain, heart, kidney, liver, or ovary (Fode *et al.*, 1994).

The subcellular localization of epitope-tagged Sak is different from that of endogenous Sak. It is reported that GFP-Sak localized to the nucleolus in G2 and to centrosomes in early M phase (Hudson *et al.* 2001). During anaphase, GFP-Sak appeared to be dispersed throughout the cell, and at telophase the fusion protein was localized to the midbody cleavage furrow. Perinuclear localization of GFP-Sak was observed in interphase cells (Hudson JW *et al.*, 2001). This is in contrast to observations of endogenous Sak which was found to associate with centrosomes throughout the cell cycle, as indicated by its co-localization with centrin, C-Nap1 and γ-tubulin (Habedanck R *et al.*, 2005). Interestingly, a recent study showed that both

endogenous Sak and ectopic GFP–Sak localizes nucleolar in Rcho-1 cells synchronized in G2/M following nocodozole treatment and endogenous Sak localizes to a single nucleolus, whereas a GFP–Sak fusion protein is ectopically localized to multiple nucleoli (Martindill DMJ *et al.*, 2007).

Sak Null Mice

To study the role of Sak in embryonic development, a Sak null allele was generated. The murine Sak null phenotype was found to be embryonic lethal with embryos arresting at E7.5. Sak null embryos display a marked increase in mitotic and apoptotic cells. In addition, many of these cells are in late anaphase or telophase with abnormally high levels of Cyclin B1 and phosphorylated histone H3. This suggests that Sak is essential for exit from mitosis and cell viability (Hudson *et al.*, 2001).

Sak and Centrosomes

The observation that endogenous Sak localizes to centrosomes led to further functional studies on Sak function which indicate a key role for Sak in centriole duplication. Interestingly, overexpression of wildtype Sak in U2-OS and HeLa cells resulted in a multiplication of Sak-positive signals which co-localize with centriole markers (Habedanck R *et al.,* 2005). Downregulation of Sak in Drosophila cells by mutation leads to loss of centrioles (Bettencourt-Dias M *et al.*, 2005). Furthermore, depletion of Sak, but not Polo, leads to the generation of cells with a reduced number of centrioles. Centriole loss in a cycling population of cells can arise through defective centrosome duplication, abnormal separation of centrosomes at entry to mitosis, or abnormal centrosome segregation to the daughter cells in cytokinesis (Bettencourt-Dias M *et al.*, 2005). In addition, antibody-microinjection demonstrates

that suppression of Sak function interferes with centrosome overduplication. When HeLa cells were transfected with siRNA duplexes that targeted Sak, after depletion of Sak for 24 h, the vast majority of cells still exhibited bipolar spindles. However, after 48 h, many monopolar spindles were observed. The results demonstrate that cells depleted of Sak undergo a step-wise loss of centrioles through the cell cycle, indicating that Sak is indispensible for centriole duplication (Habedanck R *et al.*, 2005).

Cyclin-dependent kinase 2 (Cdk2) is localized to centrosomes and has previously been implicated in the regulation of centriole duplication in different species (Lacey KR *et al.*, 1999). Overexpression of Sak cannot cause centrosome amplification in the absence of Cdk2 activity. Conversely, Cdk2 is not able to cause centrosome overduplication in the absence of Sak, indicating that Sak acts in cooperation with Cdk2. This shows that Sak is not the sole regulator of centriole duplication and further work need to unravel the integration of the Sak pathway with cell-cycle control of centrosome duplication.

Both gain- and loss-of-function experiments demonstrate that Sak is required for the precise reproduction of centrosomes during the cell cycle. These observations strongly support a model in which Sak is a crucial regulator of centriole duplication in human and Drosophila cells.

Sak and Cell Cycle Damage Pathways

The activation of the Cdc25 and the p53 affects at least three stages at cell cycle: the G1/S transition, S phase progression and the G2/M boundary (Samuel T *et al.*, 2002; Latonen L *et al.*, 2005). Sak was identified as a p53 target gene and was shown to be repressed by p53. Furthermore, Sak interacts and phosphorylates Cdc25C

which is one of the three members of the Cdc25 family of phosphatases in mammalian cells. The Cdc25 phosphatases function as key regulators of the cell cycle during normal eukaryotic cell division and as mediators of the checkpoint response in cells after DNA damage (Busch C *et al.*, 2007). Cdc25C is active at the late G2-phase of the cell cycle. Sak has also been showed to interact with ATM, ATR, Chk1 and Chk2 (Petrinac MSc thesis; Petrinac *et al.*, in press 2009) and to phosphorylate Chk2 (Petrinac *et al.*, in press 2009) Therefore, these findings suggest that Sak may be an intermediate in the pathways regulating DNA damage responses and checkpoint arrest.

Sak and Mitosis

Sak may primarily play a role in M phase progression (Hudson *et al.*, 2001) and this possibility is supported by data demonstrating Sak mRNA expression is relatively high in the G2, and M phases. A proposed role for Sak in mitosis is supported by the observation that Sak phosphorylates Cdc25C (Bonni *et al.*, 2008). Cdc25C which regulates the activity of the mitosis promoting factor Cyclin B/Cdk1 by dephosphorylation, thus triggering G2/M transition. Additionally, both the Sak null mouse model and cells derived form the Sak mouse support a role for Sak in mitosis and mitotic exit (Hudson JW *et al.*, 2001). Null embryos display cells in late anaphase or telophase that continue to express Cyclin B1 and phosphorylated histone H3. Normally during normal mitotic progression Cyclin B1 and phosphorylated histone H3 are lost during the metaphase to anaphase transition. This implicates Sak with a role in chromosomal segregation and exit from mitosis (Hudson JW *et al.*, 2001).

Sak and Cancer

The Sak gene is found on Mouse-Chromosome 3 and Human- Chromosome 4q28. A region frequently undergoes rearrangement or loss in human cancers (Hammond *et al.*, 1999). Sak heterozygous mice are fertile. However, at age of 18-24 months, Sak $^{+/-}$ mice developed apparent tumors at the liver and the lung, suggesting that reducing levels of Sak are associated with tumor formation. The lesions of heterozgyous mice liver were primary liver carcinoma, which were typically multifocal (Ko *et al.* 2005). Additionally, studies suggest that Sak kinase regulates both Cdk1 and APC/C and, thereby, entry into and exit from mitosis (Ko MA *et al.*, 2005). Cyclin B1, phosphorylated Cdk1 and securin levels were reduced in Sak$^{+/-}$ livers after partial hepatectomy, consistent with a delay in reaching anaphase (Ko MA *et al.*, 2005). Sak RNA and protein expression were retained in both spontaneous and partial hepatectomy–induced tumors, suggesting that the increased incidence of hepatomas in Sak$^{+/-}$ mice resulted from haploinsufficiency and that Sak gene dosage is important to suppression of carcinogenesis. Murine embryonic fibroblasts (MEFs) derived from Sak $^{+/-}$ mice have an about 2 fold greater doubling time compared to Sak$^{+/+}$ MEFs which suggests that the heterozygous Sak MEFs are haplo-insufficient for cell cycle progression.

Furthermore, Sak overexpression may also play a role in tumourigenesis. It has been reported that Sak is overexpressed in colorectal tumors (Eckerdt F *et al.*, 2005). Overexpression of Sak leads to multimucleation (Habedanck R *et al.*, 2005). Increased centrosome number leads to genomic instability which in turn can contribute to oncogenic transformation.

Objectives of this Study

While there is a considerable amount of information regarding the phenotypes associated with Sak overexpression and depletion in centrosome duplication, development and tumourigenesis, little is known regarding Sak interacting partners and potential substrates. Kinases in general tend to be promiscuous, targeting and interacting with numerous other proteins, however, less than a handful of Sak targets have been established. The Plks, in general, often interact with and target the same proteins or components of the the same cellular pathways; thus placing these proteins/pathways under tighter or opposing control. Therefore, the objective of the current investigation was to identify interacting partners and substrates for Sak. In order to accomplish this, an approach was employed in which known interacting partners for other Plks or proteins known to localize and effect similar cellular functions as Sak were tested as candidates for interaction.

CHAPTER III

MATERIALS AND METHODS

Preparation *Escherichia coli* (*E. coli*) Competent Cells

Top 10 F Pilus *E. coli* cells from the frozen glycerol stock were streaked out onto LB agar plates, the plates were incubated at 37°C for overnight, then a single colony was picked and inoculated a starter culture of 10mL TYM medium which was incubated for approximately 16 hours at 37°C with vigorous shaking (approx. 250rpm). 1mL of starter culture was added to 100mL prewarmed TYM media and cells were grown in 37°C shaker, OD600 was measured every 45 minutes-1hour until OD600 equaled 0.5(log phase growth), then the cultures were placed on ice with gently swirling for 5 minutes until cold. The culture was transferred to a sterile 250mL centrifuge tube and centrifuged at 4000 rpm for 10 minutes at 4°C, then decant the supernatant and the pellet was resuspended in 30mL cold TfBI buffer gently using chilled pipettes. The cells were again centrifuged at 4°C for 10 minutes at 4000 rpm and the supernatant was carefully discarded. The pelleted cells were resuspended in 4mL ice-cold TFBII buffer and 100uL aliquots were quickly placed into sterile microcentrifuge tubes. The aliquots were frozen in liquid nitrogen and stored at -80°C.

Transformation and DNA Purification

Plasmid DNA was amplified in competent cells in order to obtain sufficient plasmid DNA needed for subsequent experimental procedures. Competent *E. coli* cells (Top 10 F Pilus) were thawed and kept on ice to ensure high transformation efficiency, 0.5-1ug of DNA was added to 100uL of competent cells and mixed gently

by pipetting up and down, then the cells were incubated on ice for at least 30 minutes, after this, the cells were heat shocked at 42°C for 40 seconds and then immediately place tubes on ice for 2 minutes. 500uL of LB medium was then added and the cells were incubated for 45 minutes at 37°C. 100uL of the transformation mix was spread onto antibiotic LB-agar plates. The cells were grown by putting the plates up side down at 37°C incubator. The next day, a single colony was picked and inoculated in 250mL of LB medium containing the appropriate selective antibiotic incubate for 16 hours at 37°C. The DNA was purified using Qiagen Plasmid Maxi Prep Kit (Qiagen Inc.) according to the manufacturer'protocol. DNA concentration was determined by UV spectrophotometry at 260nm.

Boiling Mini Preps

The transformation experiment was first performed using the desired DNA. After about 16 hours at 37°C, the colonies were observed. Then were picked and grown in 2mL of LB media containing the appropriate selective antibiotic for overnight. 2mL growth culture was transferred into an Eppendorf tube and centrifuged at 4000x for 5 minutes. The supernatant was then removed using aspirator. The cell pellets were resuspended in 100 uL of boiling mini prep solution (8% glucose, 5% Triton X-100, 50mM EDTA, 50mM TRIS pH 8.0, 10mg/mL lysozyme), and boiled for 30 seconds in a hot water bath. The lysed cells were placed on ice for 30 minutes, and centrifuged at 40°C for 15 minutes at 4000x g. The supernatant, which contained the desired plasmid DNA, was then transferred to a fresh Eppendorf tube. Finally, 5uL DNA was used in a 20 ul reaction for diagnostic restriction digests.

Cell Culture

HEK-293, NIH 3T3, U2-OS, SaOS-2 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine Serum (FBS) (Sigma), 1% penicillin-streptomycin (Gibco). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2. Sf9 cells (a kind gift from Dr. Lisa A. Porter, University of Windsor) were cultured in Grace's insect medium (Sigma) supplemented with 10% Insect Medium Supplement 10X (Sigma) and 1% penicillin- streptomycin (100ug/mL) (Gibco). Sf9 cells were grown at 27°C.

Transfection and Cell Lysis

1.0 x10^6 HEK 293 cells were seeded onto 10cm tissue culture dishes. 24 hours later, 6ug of DNA per plate was transfected using Effectene TM (Qiagen) according to the manufacturer's recommendations. Approximately 16 hours post- transfection, cells were harvested by centrifugation at 250 x g for 5 minutes. The medium was removed and the cells were washed three times with 1xPBS, then the cells were lysed with 1mL ice cold lysis buffer containing Protease Inhibitor Cocktail. The lysed cells were allowed to sit on ice for 20 minutes, then scraped off the plates and collected in eppendorf tubes. The lysates were clarified by centrifuging at 3,000 x g for 20 minutes at 4°C.

Immunoprecipitation

Immunoprecipitation of FLAG-tagged expression plasmids and endogenous Cyclin B1 was performed by incubating approximately 1mg of lysate with 1.5 ug of anti-Cyclin B1 (Sigma) at 4°C on a vertical rotator for overnight, then 70 uL of a 20 % protein G-Sepharose Slurry (GE Lifesciences) was added into immunocomplexes

and for an hour at 4°C. When the incubation time was over, the tubes were centrifuged, the supernatant was removed and the beads were washed in 500 uL of TNT buffer three times. After the last supernatant was removed, 25 uL of 2 x SDS sample buffer was added and the sample was boiled at 95°C for 5 minutes, and subseuqntly run on an SDS-polyacrylamide gel (SDS-PAGE).

Western Blot Analysis

Post electrophoresis, gels were incubated in 1x transfer buffer for 15 minutes at room temperature on a shaker. At the same time, Immobilon-P membranes were activated by methanol for 20 seconds and then equilibrated in 1x transfer buffer for 10 minutes. The protein was transferred from the gel to the membrane using a semi-dry apparatus (Biorad) at 9 volts for 35 minutes. After the transferring of the protein to the membrane was completed, the membrane was blocked with Tris-buffered Saline and Tween (TBST) containing 1% skin milk for at least 1 hour at room temperature in order to reduce non-specific binding sites on the membrane. Then the membrane was incubated with 1ug of the specified primary antibody in 10mL of TBST for one hour at room temperature. The membrane was washed three times for 10 minutes with TBST and subsequently incubated with the appropriate secondary antibody for another 1 hour at room temperature. A dilution of 1:60,000 in TBST plus 1% skim milk was used for the anti-mouse or anti-rabbit secondary antibodies which were conjugated to horseradish peroxidase (HRP) (Amersham). Then, the membrane was washed with 1xTBST for 10 minutes and was incubated with Super Signal West Femto Maximum Sensitivity Substrate (Pierce) dilution for five minutes and the proteins of interest were visualized by chemiluminescense.

Stripping and Reprobing of Western Blots

In order to detect additional proteins on the same blot, the membrane was incubated with stripping buffer at 50°C for 30 minutes with some agitation and then washed with 1x TBST for 10 minutes at 50°C. The blot was then ready for re-use in Western blot analysis.

Immunofluorescence

NIH 3T3 cells were seeded onto coverslips. 24 hours later, 3T3 cells were transfected with GFP-Cyclin B1 and various hSak constructs using Effectene TM (Qiagen) according to the manufacturer's recommendations. Approximately 16 hours post-transfection, cells were washed twice with PBS, then fixed with 3.7% paraformaldehyde in PBS pH 7.4 for 12 minutes at room temperature for 12 minutes, and washed twice with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 2 minutes at room temperature. Followed by washed again three times with PBS and blocked by 1x TBST containing 10% BSA for 30 minutes at 37°C. The blocker was removed by washing the cover slips two times in TBST and then anti-Flag antibody (Sigma) (1:1,000) was added to cells for 1 hour at room temperature with incubation in the dark in a humid atmosphere. After washing three times with 1x TBST, 1:4000 Alexa-conjugated 568 nm goat anti-mouse secondary (Molecular Probes) was added for 30 minutes, then washed three times with 1x TBST and incubated with 6'-diamidino-2-phenylindole (DAPI) (0.1mg/mL) (Molecular Probes) for 5 minutes at room temperature, After washing the cells once with PBS, they were fixed with a drop of mounting medium (Molecular Probes) and analyzed under a fluorescence microscope.

Expression and Purification of Baculovirus-expressed Protein

$5x10^6$ SF9 insect cells were seeded in 10 cm tissue culture dishes. Cells were allowed to settle and stick to the bottom of dishes 60 minutes, 20uL of recombinant baculovirus encoding GST-Cyclin B1was added (a kind gift from Dr.Helen Piwnica-Worms, Washington University School of Medicine) to 2mL Grace's media (Serum free) and mixed gently. The media was removed from settled insect cells. 2mL virus solution was added to the cells and rocked gently to disperse. The cells were incubated at room temperature for 60 minutes. After 60 minutes, 8mL Grace's complete media was added and incubated at 27°C for five days. Then the cells were harvested by gently pipetting two times to suspend as many cells as possible, transfer to sterile 15mL centrifuge tube and centrifuged at 2,500 rpm for 5 minutes. The cell pellet was resuspended in 300uL ice-cold insect cell lysis buffer containing reconstituted protease inhibitor cocktail. Cells were lysed on ice for 20 minutes. Lysate was centrifuged at 13,000 x g for 20 minutes at 4°C. The clear supernatant was added to Glutathione Sephrose Beads (GE) prepared according to manufacturer's protocol. The purification was performed as previously described.

Cloning His-Gadd45a Construct

His-Gadd45a plasmid was cloned using Creator™ DNA Cloning Kits. It uses Cre-*loxP* site to transfer Gadd45a from a Donor Vector plasmid to an Acceptor Vector containing His-tag (Figure 4).

Firstly, the Creator reaction mixture was prepared as follows:

200 ng Donor Vector

200 ng Acceptor Vector

2 µl 10X Cre Reaction Buffer

2 µl 10X BSA (1 mg/ml)

1 µl Cre Recombinase

Add deionized H2O to bring volume up to 20 µl. Then the Creator reaction mixture was incubated at room temperature (22–25°C) for 15 min and stopped by heating tube at 70°C for 5 min. 1 µl of reaction mixture was transformed into competent cells. Cells were grown at 37°C for 60 min in LB. 100 µl of transformation was plated on a LB-agar plate containing 30µg/ml chloramphenicol, and 7% sucrose (w/v) and Incubated overnight. The next day, the plate contained a mixture of larger colonies and smaller colonies. Pick larger colonies for screening by colony PCR using primers (PCP-1 and PCP-2) which only amplify across a recombination juncture. The PCR reaction was set up as follows:

18.5 µl PCR-Grade Water

2.5 µl 10X TITANIUM *Taq* PCR Buffer

1 µl Creator Reaction Mixture

1 µl PCP-1 Primer (10 µM)

1 µl PCP-2 Primer (10 µM)

0.5 µl 50X dNTP Mix (10 mM ea. of dATP, dCTP, dGTP, dTTP)

0.5 µl 50X TITANIUM *Taq* DNA Polymerase

25 µl Total volume

Primer PCP-1: 5'-GCTCACCGTCTTTCATTGCC-3'

Primer PCP-2: 5'-TCCGCTCATGAGACAATAACC-3'

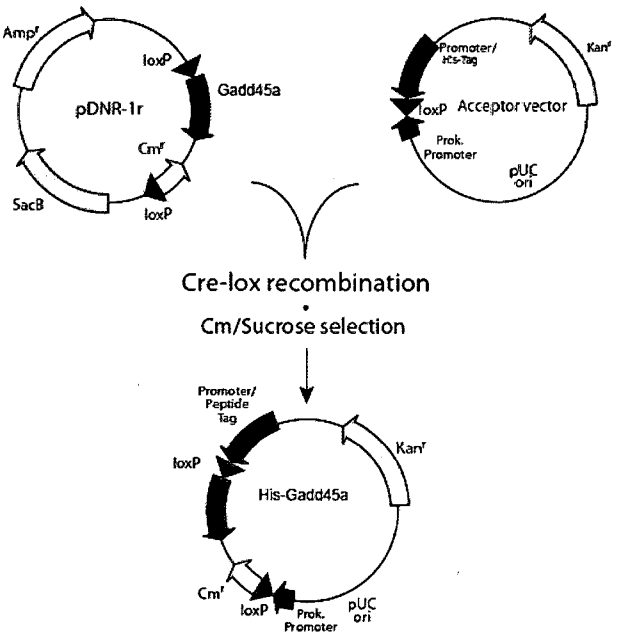


Figure 4. Flow chart of Cloning His-Gadd45a construct using the Creator™ DNA Cloning System. The Creator System uses Cre-*loxP* site-specific recombination to catalyze the transfer of a target gene Gadd45a from a Donor Vector plasmid to an Acceptor Vector, a plasmid containing His-tag.

Expression and Purification of His Fusion Proteins

E. coli BL21 cells were transformed with the His-Gadd45a plasmid. A single transformed colony was picked and inoculated in 200mL of LB medium supplemented with 100 ug/mL of ampicilin and 34ug/mL chloramphenicol for overnight at 37^0C with vigorous shaking. The following day, the 100 mL starter culture was added to 500 mL of LB medium and grown at 37^0C until the OD_{600} was upon 0.6. Then expression was induced with 250 uL of 0.5 mM IPTG (Fisher). After the cultures were grown for an additional 4 hours at 37^0C, cells were harvested by centrifugation at 10,000 x g for 30 minutes at 4^0C. Cell pellets were resuspended in 20 mL cell lysis buffer and incubated for 20 minutes on ice, cells were then sonicated on ice 10 times for 10 seconds until the cloudy cell suspension becomes translucent. The lysate was centrifuged at 10,000 x g for 30 minutes at 4^0C. The supernatant was added to the HiTrap Chelating HP (Amershan Biosciences) and processed according to the manufacturer's protocol. The flow rates were 1mL/min to allow the protein to bind to the column. Then the His elution buffer containing 5-50 mM imidazole were added to column and the eluted protein was collected in 1mL fractions. The fusion protein was then seperated on 12% SDS-PAGE gel and visualized by both coomassie blue staining and Western blot analysis.

In Vitro Kinase Assay

In order to determine whether CyclinB1 is a substrate of Sak, an in vitro kinase assay was performed. First, Flag-hSak, Flag-T170D and Flag-K41M expression plasmids were transiently transfected into HEK-293 cells. The cells were lysed 24 hours post transfection and the cell lysate was incubated with 1 ug anti- Flag antibody at 4 °C for 45 minutes. Then, 80uL of 20% protein G-sepharose slurry (GE

Lifesciences) was added to each sample and incubated at 4 °C for 45 minutes. The immunocomplexes were washed once with 500mM TNT wash buffer, once with 150mM TNT wash buffer, twice with 150mM TNT wash buffer containing 500mM of LiCl and once with kinase buffer. The immnocomplexes were resuspended in 20 uL of kinase buffer, 2.5uL of cold ATP and 4ug of the GST-Cyclin B1 protein. The kinase was started by the addition of 10uCi [γ-^{32}P] (Amersham Biosciences) and the samples were then incubated at 30°C for 35 minutes. Following that, 6uL of 6x SDS loading buffer was added to stop the reaction. Samples were boiled for 5 minutes and then analyzed by SDS PAGE gel, then the gel was transferred to a PVDF membrane as previously described. The membrane was then incubated overnight in the dark with a phosphoimager in order to visualize phosphorylation band of protein. Following this, the membrane was probed with an anti-Flag antibody in order to detect the immunoprecipitated proteins. The sane blot was subsequently probed with an anti-Cyclin B1 antibody to confirm equal loading of the protein.

Preparation of Protein for Mass Spectroscopy

In order to extract the protein for mass spectroscropy, the coomassie stained bands of interest were excised from the gel and placed in siliconized microtubes. They were then incubated in 100 uL of destain solution and incubating for 35 minutes at 37 °C, shaking at 225 RPM. This procedure was repeated twice, followed by repeated changes of 100% acetonitrile to dehydrate the excised gel until it became opaque. The pieces were then lyophilized by Speed Vac for 20 minutes to ensure that the excised gel was completely dried.

The dehydrated bands were then rehydrated in trypsin digestion buffer containing 13ng/uL promega modified trypsin 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 sealed with parafilm and incubated in a shaker at 37 °C overnight. The following morning, the samples were briefly vortexed and the remaining gel pieces spun down by centrifugation. The supernatant containing the extracted peptides was collected and transferred to a fresh siliconized microtube 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 remaining 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 microtubes. These pooled samples were then concentrated by subjecting them to the Speed Vac until only 10 uL of supernatant remained. Then Zip-Tip procedure was performed for desalting peptides. Firstly, the Zip-Tips (Eppendorf AG) were equilibrated by aspirating 10 µL wetting solution (60% Acetonitrile in 1% Formic Acid) into tips and dispensing to waste for 5 times, then peptides were bound to Zip-Tip by aspirating and dispensing 20 cycles and 10 uL of washing solution (1% Formic Acid) was aspirated and dispensed to waste for 10 times. 10 µL of elution solution (60% Acetonitrile in 1% Formic Acid) was added into Eppendorf microcentrifuge tube and aspirated and dispensed through Zip-Tip at least 20 times without introducing air. Then, the desalted peptides were purified by using the SigmaPrep. Spin Columns (Sigma). Firstly, 80 uL of the PHOS-Select affinity gel (Sigma) was added into the column and washed by 500uL of wash / equilibration buffer (250mM Acetic acid, 30% Acetonitrile) for 3 times. Followed by adding 100 uL sample (9 uL of trypsin digested peptides in 60% Acetonitrile, 1% Formic Acid and 17.5M Acetic acid) into gel and the samples were incubated for 30 minutes at

room temperature. The spin columns were placed in collection tubes which were centrifuged at approximately 3000 rpm for 30 seconds, and then the resin was washed by 500uL wash / equilibration buffer (250mM Acetic acid, 30% Acetonitrile) and 500uL of HPLC H_2O. The peptides were first eluted by 100 uL of 400mM of Ammomium Hydrotide, then eluted by 100uL of 400mM of Acetic acid and 25% Acetonitrile. Then Zip-Tip procedure was performed again for salt reduction which may improve mass signals. 1uL of each sample was spotted directly on a MALDI plate and subjected to mass spectroscopy.

UV Damage

In order to investigate the effect of DNA damage on Sak protein expression levels, U2-OS and SaOS-2 cells plated in 100-mm dishes were rinsed with PBS and irradiated with ultra-violet radiation using a Stratalink UV crosslinker (Stratagene) at a dose of 10 mJ/cm^2. After cell exposure to UV radiation, fresh medium was added in plates, and the cells were cultured in the incubator and cells were collected at the indicated times. Following lysis, Western blot analysis was performed.

CHAPTER IV

RESULTS

Sak Interacts with Cyclin B1

Based on the observation that Plk1 phosphorylates Cyclin B1 and induces its nuclear entry, we tested the possibility of an interaction between Sak and Cyclin B1 by a co-immunoprecipitation (Co-IP) based approach. Specifically, HEK-293 cells were transiently transfected with various Flag-tagged Sak constructs Flag-hSak (wild-type human Sak), Flag-hT170D (kinase active form of Sak), Flag-hK41M (kinase dead form of Sak) and Flag-YVH1 (a negative control) (Figure 6). 16 hours post transfection, the expression of the Flag constructs were detected by Western blot analysis with anti-Flag antibody (Figure 6A). Then, the co-ip was performed in which anti-Cyclin B1 antibody was used to immunoprecipitate Cyclin B1 along with any associated proteins. The presence of Flag-Sak fusion proteins in this complex was determined with an anti-Flag antibody (Figure 6B). The results indicated that Sak interacts with Cyclin B1. Furthermore, this interaction was not dependent on Sak kinase activity as both kinase dead and active forms of Sak also co-immunoprecitated with Cyclin B1. The control Flag tagged protein and Flag-YVH1 was not detected, thus indicating that the interaction was not via the Flag epitope.

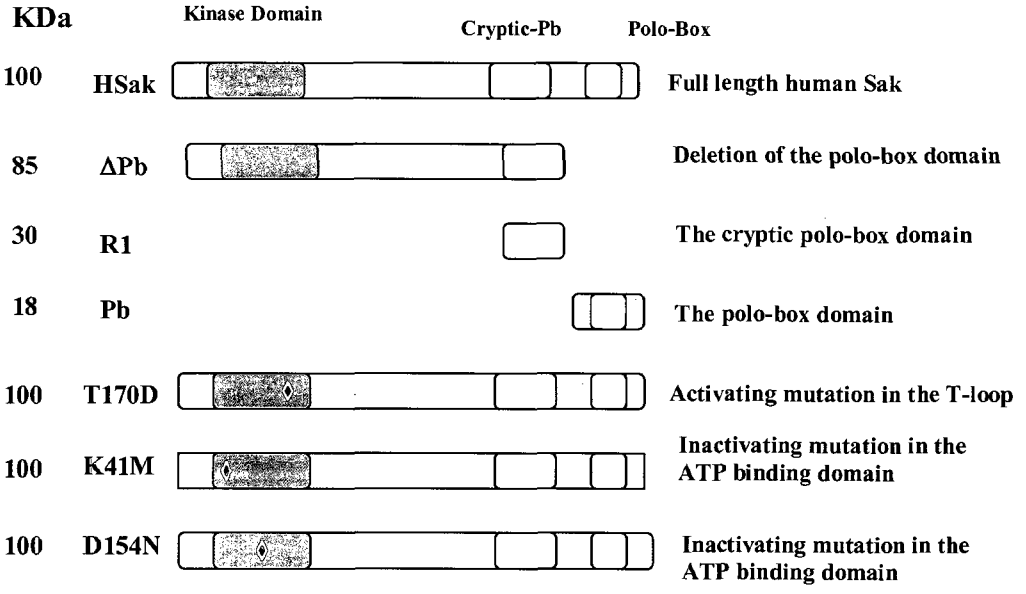


Figure 5. Sak domain specific constructs. The figure representations Sak deletion mutants and Sak kinase dead and kinase active mutants.

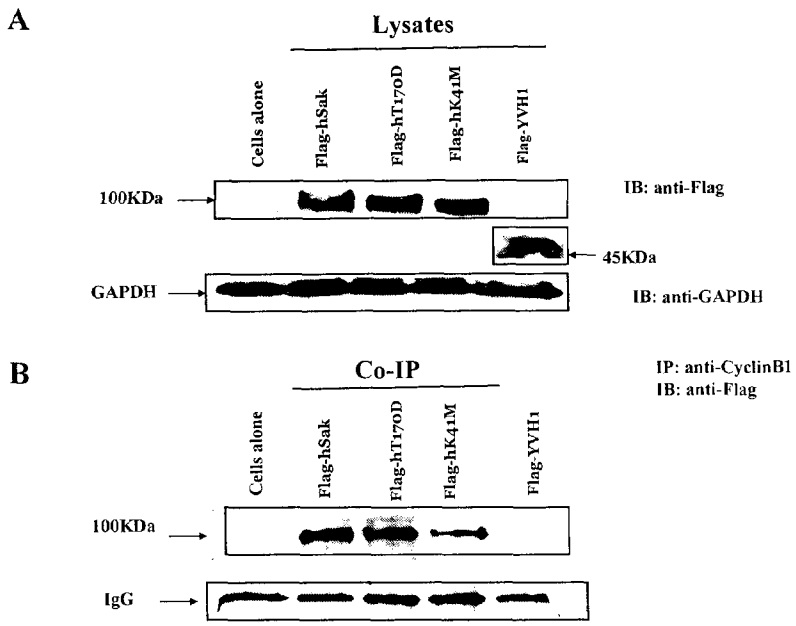
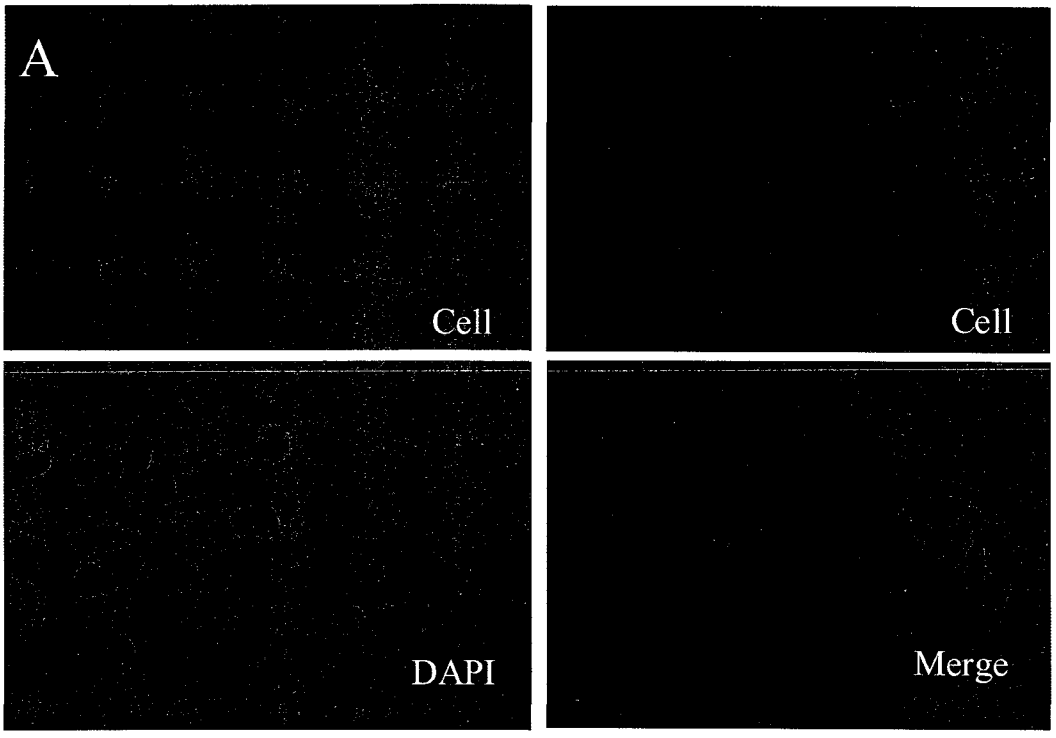
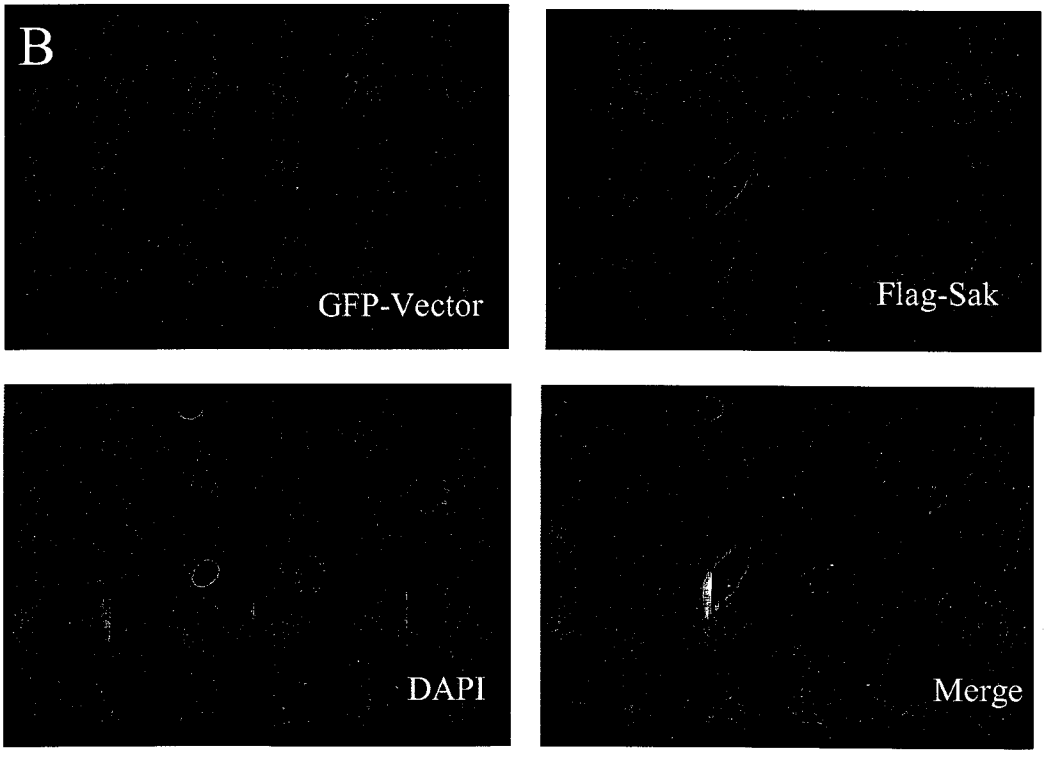


Figure 6. **Sak interacts with Cyclin B1.** HEK-293 cells were transiently transfected with various Flag-tagged Sak constructs, Flag-hSak (wild-type human Sak), Flag-hT170D (kinase active form of Sak), Flag-hK41M (kinase dead form of Sak) and Flag-YVH1 (a negative control). **(A)** 16 hours post transfection, the expression of the Flag constructs were detected by Western blot analysis with anti-Flag antibody and followed by probing with an anti-GAPDH antibody to confirm equal protein loading. **(B)** Co-ip was performed in which anti-Cyclin B1 antibody was used for immunoprecipition with Sak and anti-Flag antibody for blotting the membrane.

Co-localization of Sak and Cyclin B1 to the Centrosomes

To further confirm the interaction of Sak and Cyclin B1, co-immunofluorescence analysis was performed. Cyclin B1 localizes to the cytoplasm during S and G2 phases, From G2 to metaphase, part of Cyclin B1 is associated with duplicating centrosomes (Hagting A *et al.*, 1998), a site which Sak is also localized throughout the cell cycle. These observations prompted us to investigate whether Sak co-localizes with Cyclin B1 in centrosome. In order to test this hypothesis, NIH 3T3 cells were seeded on cover slips, then fixed with paraformaldehyde and permeabilized with Triton X-100. Followed by blocked by BSA. Then an anti-Flag antibody and Alexa-conjugated 568 nm goat anti-mouse secondary was added to cells separately, then cells were incubated with 6'-diamidino-2-phenylindole (DAPI) and analyzed under a fluorescence microscope. The results confirmed that Sak co-localizes with Cyclin B1 at the centrosomes (Figure 7).


A
Cell
Cell
DAPI
Merge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Flag-Sak
DAPI
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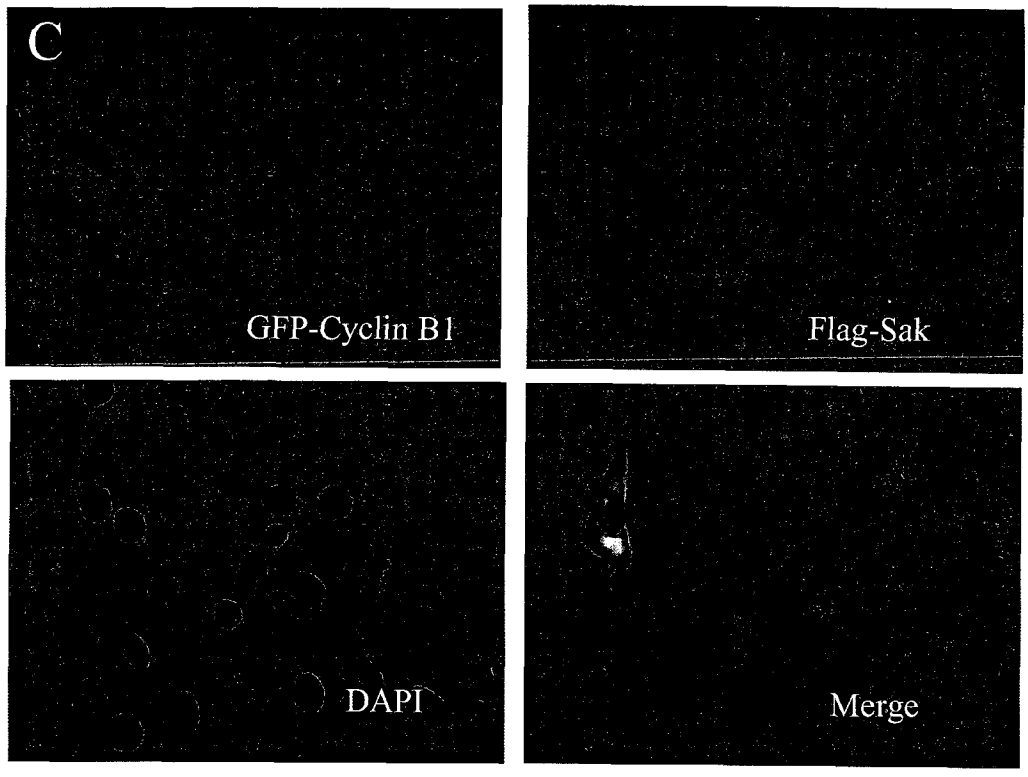
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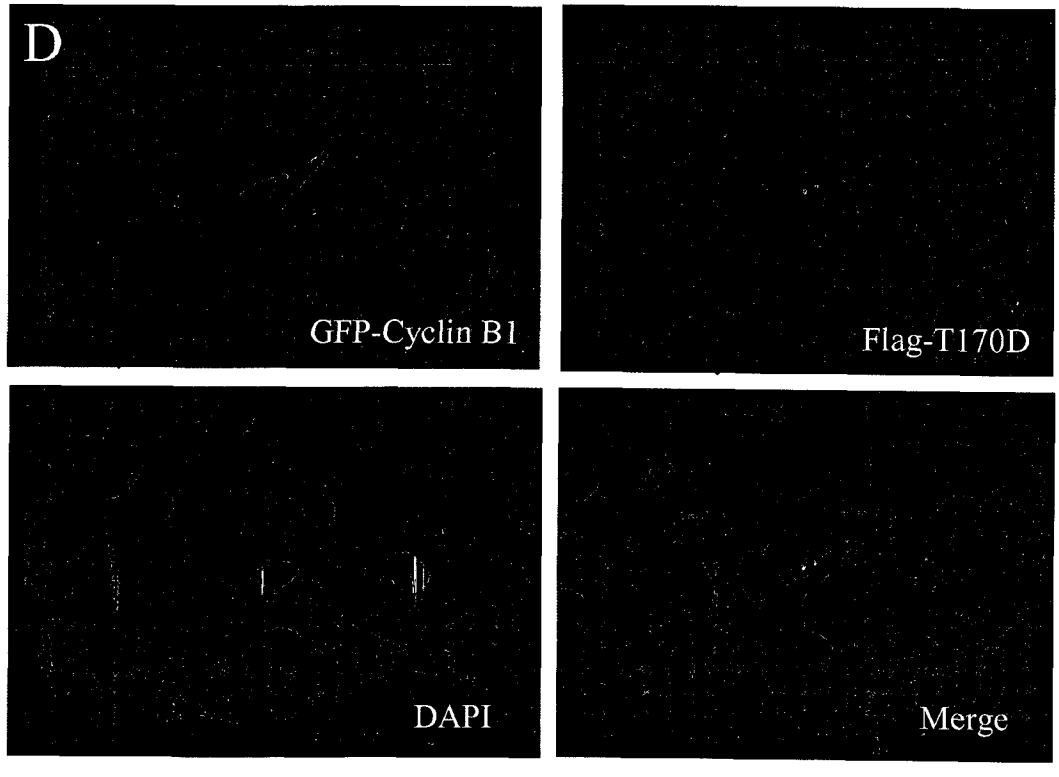
Flag-Sak
DAPI
Merge


D
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Flag-T170D
DAPI
Merge

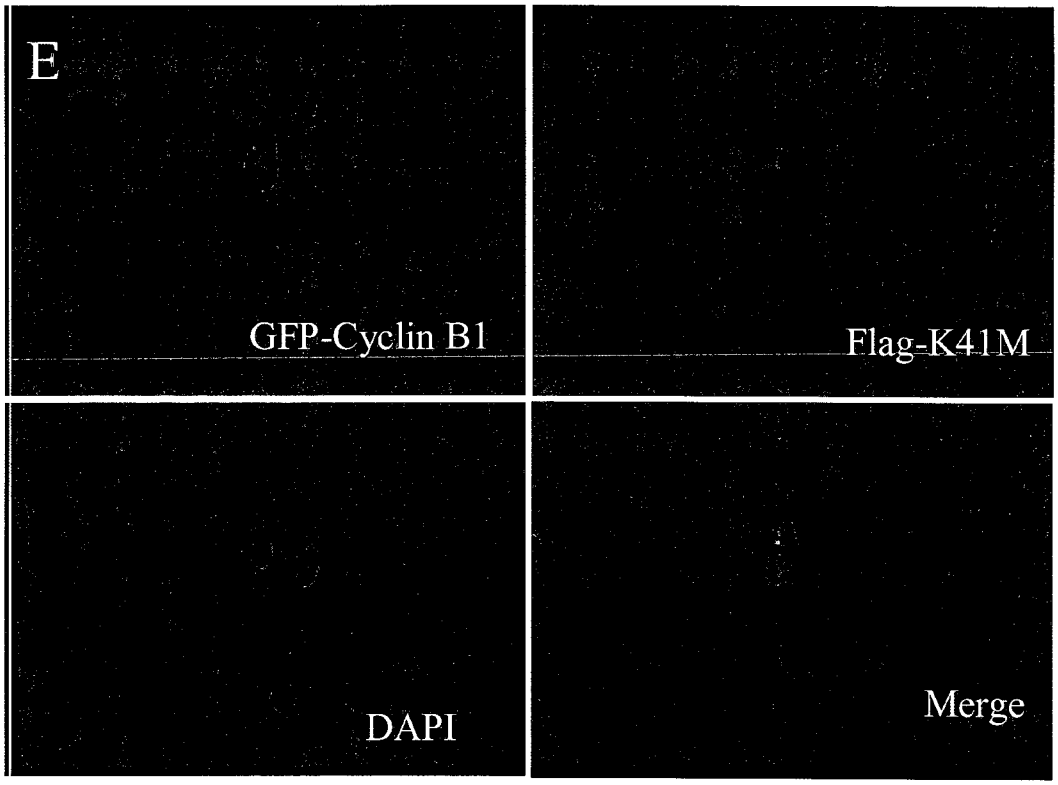
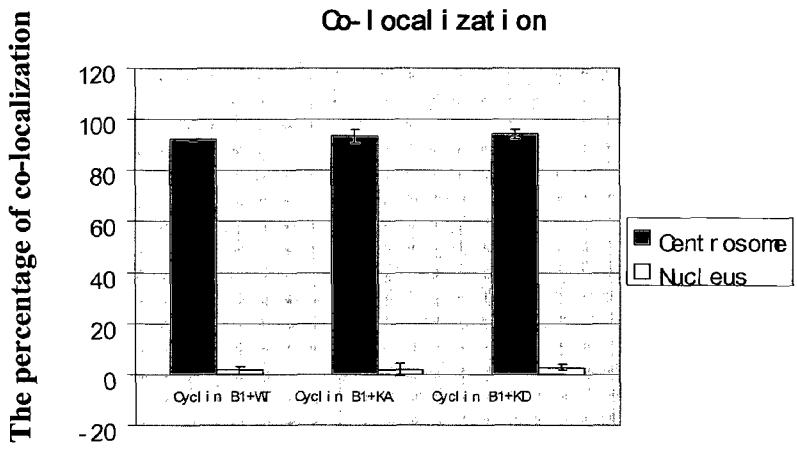


Figure 7. Sak co-localizes with Cyclin B1 at the centrosomes. NIH 3T3 cells were transfected with GFP-Cyclin B1 (Green) and various hSak constructs for 16 hours. The overexpressed kinase was detected with anti-Flag antibody (red). Cells were stained with DAPI to detect the nucleus. **(A, B)** No co-localization showed up in two negative controls. **(C, D, E)** Flag-Sak, Flag-T170D and Flag-K41M co-localized with GFP-Cyclin B1 at the centrosomes, respectively.



igure 8. Sak and Cyclin B1 mainly co-localize at centrosome. Histogram shows the percentage of co-localization between GFP-Cyclin B1 and Various hSak constructs at centrosome and nucleus. Data from three independent experiments, counting 200 cells each; error bars indicate standard deviation.

Sak does not Phosphorylate Cyclin B1 In-Vitro

The interaction between Cyclin B1 and Sak suggested the possibility that Cyclin B1 may be a substrate of Sak. To test this hypothesis, an in vitro kinase assay was performed. Firstly the GST-Cyclin B1 fusion protein was purified for use as substrate. To confirm the purity of the protein, SDS-PAGE was performed and the gel was stained by coomassie blue (Figure 9A). Immunoblotting was performed using an anti-Cyclin B1 antibody to further confirm the purity of the GST-Cyclin B1 protein. Purified GST-Cyclin B1 protein yielded a protein of 85KDa, corresponding to the size of the GST-Cyclin B1 protein (Figure 9B). Then HEK-293 cells were transiently transfected with Flag-hSak, Flag-hT170D and Flag-hK41M using Effectene TM (Qiagen). At 16 hours post transfection, the cells were lysed, the Flag fusion proteins were immunoprecipitated by incubating 500ug of protein lysate with 1ug of anti-Flag antibody. The kinase assays were performed as follows; individual immunoprecipitated Flag-Sak proteins were incubated with baculovirus expressed GST-Cyclin B1 in the presence of [γ-^{32}P] ATP (Amersham Biosciences). After 30 minutes of incubation, SDS-PAGE was performed and the membrane was exposed using a phosphoimager (Perkin Elmer). In order to ensure that both substrate and Sak protein were present, the membrane was also probed with an anti-Flag antibody and anti-Cyclin B1 antibody. The results of these assays are shown in (Figure 9C-D). The phosphorylated bands at 100KDa correspond to the autophosphorylation of Flag-Sak and Flag-T170D. Under these conditions, GST-Cyclin B1 was not phosphorylated by Sak.

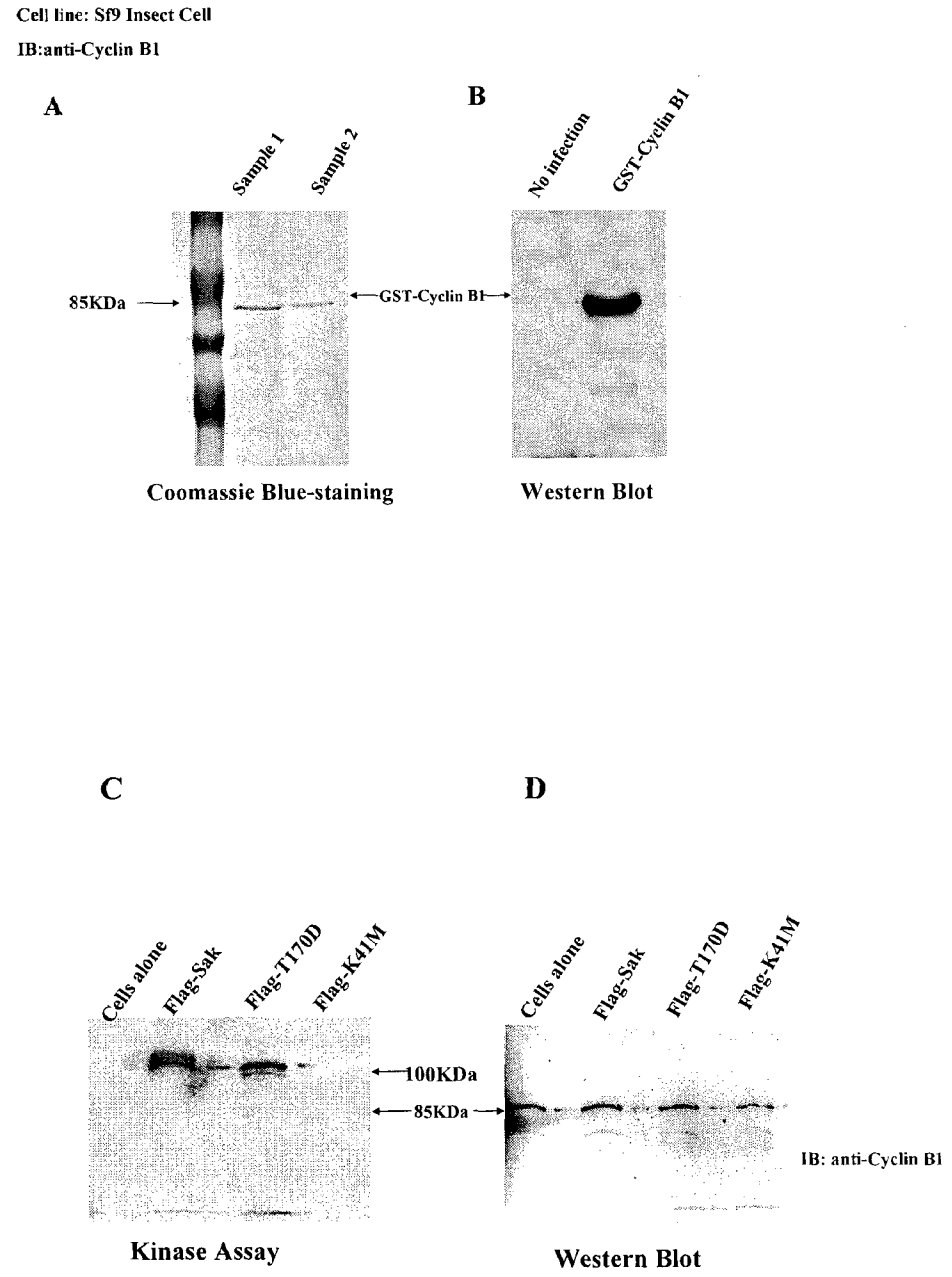


Figure 9. Sak does not Phosphorylate Cyclin B1 In-Vitro. (A) The GST-Cyclin B1 protein was produced by infecting Sf9 insect cells with baculovirus encoding GST-Cyclin B1, and then purified it using GST beads, GST- Cyclin B1 protein was

analyzed by Coomassie blue-staining. Purified GST-Cyclin B1 protein yielded a protein of 85KDa, corresponding to the size of the GST-Cyclin B1 protein. **(B)** Western blot was also performed with anti-Cyclin B1 antibody to further confirm that the band was GST-Cyclin B1. **(C)** The purified GST-Cyclin B1 protein was incubated with Flag-Sak in the presence of ATP, subjected to SDS-PAGE, transferred protein to membrane and then visualized the bands using a phosphoimager. **(D)** The membrane was then subjected to Western blot analysis with an anti-Cyclin B1 antibody to ensure the equal GST- Cyclin B1 protein loading.

Sak Interacts with Gadd45a

Gadd45a is a nuclear protein that is induced by genotoxic and other cellular stresses. It has been demonstrated that Gadd45a plays important roles in growth control, maintenance of genomic stability, DNA repair, cell cycle control and apoptosis. Furthermore, deletion of Gadd45a leads to centrosome amplification and abnormal mitosis and Gadd45a is found to interact with Aurora-A and BRCA1. Both of proteins are located in centrosome, a site which Sak is also localized throughout the cell cycle.

These observations prompted us to investigate whether Sak also interacts with Gadd45a. To test this hypothesis, HEK-293 cells were transiently transfected with domain specific expression plasmids for Flag tagged Sak (Figure 9). 16 hours post transfection, the expression of the Flag constructs were detected by Western blot analysis with anti-Flag antibody (Figure 10A). Then, co-immunoprecipitation of endogenous Gadd45a and associated proteins was performed on cellular protein extracts using an anti-Gadd45a antibody followed by Western Blotting using an anti-Flag antibody (Figure 10B). The results indicated that Flag-Sak (full length), Flag-T170D (kinase active), Flag-K41M (alternate kinase dead), Flag-154N (kinase dead), Flag-ΔPb (lacking polo-box domain), and Flag-R1 (cryptic polo-box domain) were all associated with Gadd45a. Furthermore, we did not detect an interaction between Flag-Pb (polo-box domain) and Gadd45a indicating that the polo-box region of Sak was not necessary or sufficient for this interaction to occur. These results establish that Sak interacts with Gadd45a and the cryptic polo-box domain is sufficient for the intercation.

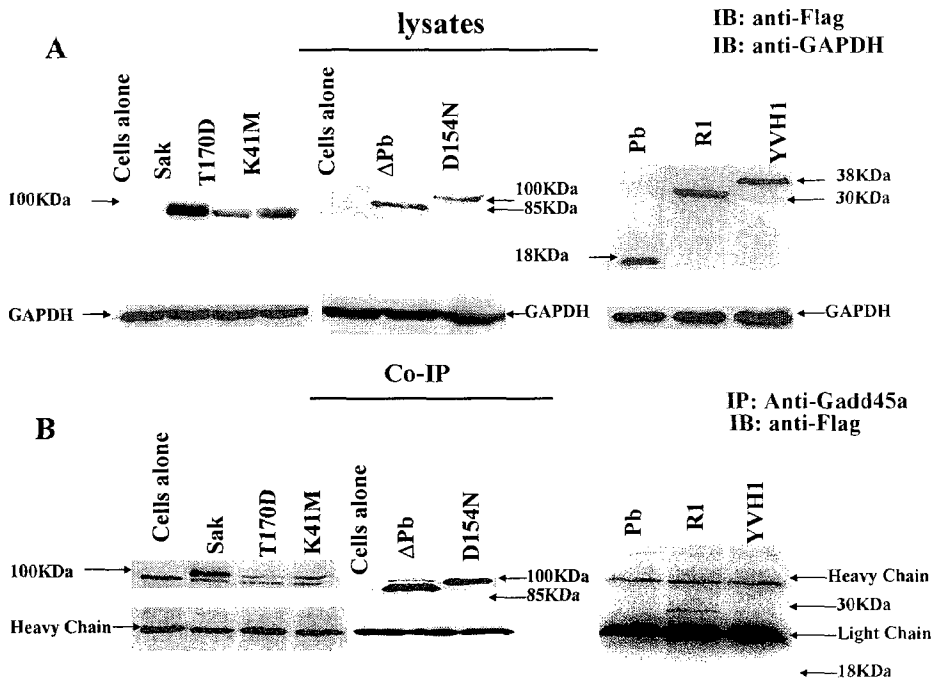


Figure 10. Sak interacts with Gadd45a. **(A)** HEK-293 cells were transiently transfected with expression plasmids for Flag-Sak domain specific constructs. 16 hours post transfection cells were lysed. Whole cell lysates were immunoblotted with an anti-Flag antibody to determine the levels of Flag-tagged proteins, followed by probing with an anti-GAPDH antibody to confirm equal protein loading. **(B)** Immunoprecipitation was performed using an anti-Gadd45a antibody on prepared lysates and western blotting was performed using an anti-Flag antibody. Full length Sak, kinase dead Sak (K41M), kinase active Sak (T170D), ΔPb-Sak (deletion of polo-box domain of Sak), an alternate kinase dead Sak (D154N) and cryptic-polo-box domain (R1) all co-immunoprecipitated with endogenous Gadd45a. The polo-box

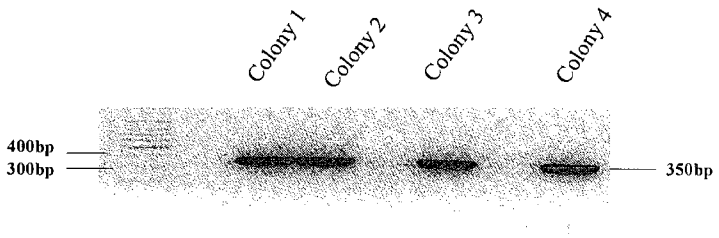
domain (Pb) was not observed. Whole cell lysates of non-transfected cells and a transfected construct encoding Flag-YVHI protein served as negative controls.

Sak Phosphorylates Gadd45a In-Vitro

The interaction of Gadd45a and Sak suggested the possibility that Gadd45a may be a substrate of Sak. Therefore, in vitro kinase assays was performed (Figure 11). First His-Gadd45a plasmid was cloned using Creator™ DNA Cloning Kits. To test whether the plasmid is successful, a PCR was set up using primers (PCP-1 and PCP-2) which only amplify across a recombination juncture. PCR band showed up at 350bp indicating that the plasmid may be successful (Figure 11A). Then the plasmid was sequenced by ACGT corp and compared to wildtype Gadd45a, The result showed that the plasmid is His-Gadd45a (Figure 11B). Then the His-Gadd45a fusion protein was produced in bacteria using IPTG and purified using His affinity column. To confirm the purity of the protein, SDS-PAGE was performed and the gel was stained with coomassie blue (Figure 11C). Immunoblotting was performed using an anti-Gadd45a antibody to further confirm the purity of the His-Gadd45a protein. Purified His-Gadd45a protein yielded a protein of 30KDa, corresponding to the size of the His-Gadd45a protein (Figure 11D). In-vitro kinase assay was performed using the purified His–fusion Gadd45a. HEK 293 cells were transfected with Flag-hSak, Flag-hT170D and Flag-hK41M and immunoprecipitation was performed using an anti-Flag antibody. The bacterially produced His-Gadd45a protein was incubated with the Flag-Sak immunoprecipitated constructs in the presence of [γ-^{32}P] ATP (Amersham Biosciences), then SDS-PAGE was performed and membrane was exposed using a phosphoimager (Perkin Elmer) (Figure 11E). Subsequently, the membrane was probed with an anti-Flag antibody and anti-Gadd45a antibody to confirm the equal protein loading (Figure 11E). The result showed that His-Gadd45a is phosphorylated by wildtype Flag-Sak and kinase active Flag-T170D, but not phosphorlated by kinase dead Flag-K41M. The phosphorylated bands at 100KDa correspond to the

autophosphorylation of wildtype Flag-Sak and kinase active Flag-T170D. The result indicates that Gadd45a is a substrate of Sak.

A



B

```
Gadd45a          AAGERQGLSCRSGACE-VQKAGARALAVAGAARTPRSLPGRPAVCNMTLEEFSAGEQKTE
His_gadd45a         MSYYHHHHHHDYDIPTTENLYFQGAMGSITSYSIHYTKLSVDTMTLEEFSAGEQKTE
                                                      * .**************

Gadd45a          RMDKVGDALEEVLSKALSQRTITVGVYEAAKLLNVDPDNVVLCLLAADEDDDRDVALQIH
His_gadd45a      RMDKVGDALEEVLSKALSQRTITVGVYEAAKLLNVDPDNVVLCLLAADEDDDRDVALQIH
                 ************************************************************

Gadd45a          FTLIQAFCCENDINILRVSNPGRLAELLLLETDAGPAASEGAEQPPDLHCVLVTNPHSSQ
His_gadd45a      FTLIQAFCCENDINILRVSNPGRLAELLLLETDAGPAASEGAEQPPDLHCVLVTNPHSSQ
                 ************************************************************

Gadd45a          WKDPALSQLICFCRESRYMDQWVPVINLPER----------------------------
His_gadd45a      WKDPALSQLICFCRESRYMDQWVPVINLPERLGSFLDHSFGARAQVSGHNHNHNHNHNHN
                 ******************************

Gadd45a          -
His_gadd45a      -
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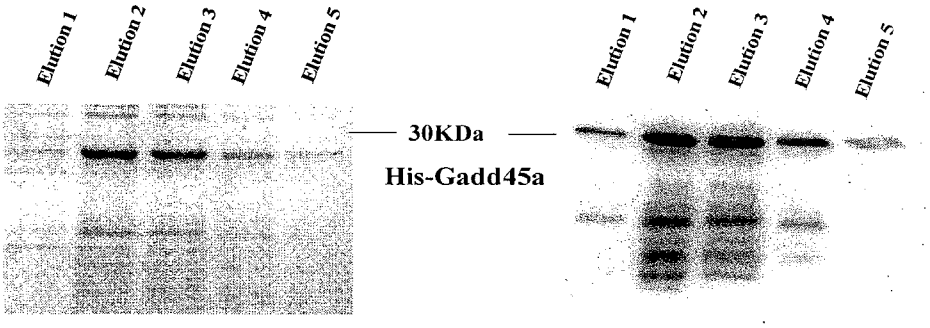
C

D

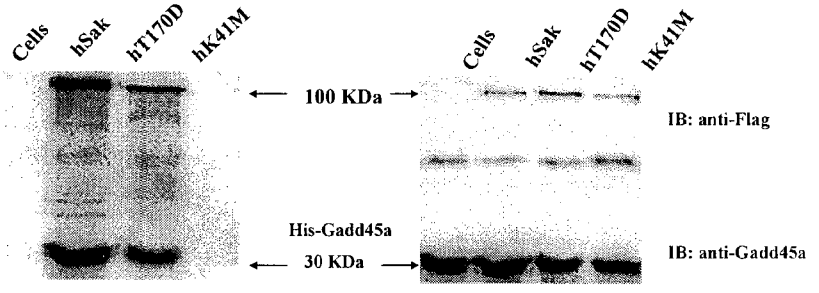


Coomassie blue-staining

Western Blot

E

F



In-vitro Kinase Assay

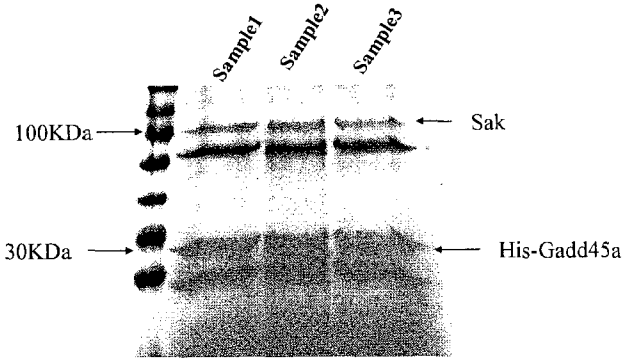
Western Blot

Figure 11. Sak Phosphorylates Gadd45a In-Vitro (A) His-Gadd45a construct was cloned and A PCR was set up using primers (PCP-1 and PCP-2) to test His-Gadd45a construct. PCR amplified product band showed up at 350bp. **(B)** The His-Gadd45a plasmid was sequenced by ACGT corp and compared to wildtype Gadd45a. **(C)** His-Gadd45a protein was expressed in bacteria using IPTG. The fusion protein was purified using His affinity column and SDS PAGE was performed. Purification resulted in a 30KDa band corresponding to His-Gadd45a protein at Coomassie blue-staining gel. **(D)** Western blot was also performed using an anti-Gadd45a antibody to ensure the observed bands were His-Gadd45a protein. **(E)** Bacterially produced His-Gadd45a was incubated with the Flag-Sak immunoprecipitated constructs in the presence of [γ-^{32}P] ATP (Amersham Biosciences), then SDS-PAGE was performed and membrane was exposed using a phosphoimager (Perkin Elmer). **(F)** The membrane was subjected to Western blot analysis with an anti-Flag antibody to ensure transfection efficiency, also using an anti-Gadd45a antibody to ensure equal protein loading.

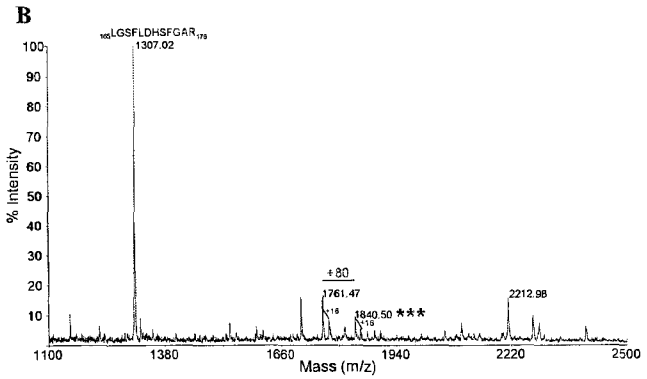
Sak Phosphorylates Tyr151 on Gadd45a

In order to identify the phosphorylation site of Sak on Gadd45a, we employed a mass spectrometry based approach in collaboration with the Vacratsis lab. An in vitro cold kinase assay was performed, protein samples were separated by SDS-PAGE gel, followed by coomassie blue staining (Figure 12A) and the particular band(s) of interest were excised from the gel, and then digested with trypsin. The extracted peptides were spotted directly on a MALDI plate and subjected to Mass Spectroscopy. The Gadd45a phosphopeptide peak was identified by MALDI-TOF mappings at 1840 m/z (Figure 12B) and the phosphorylation specific peptides of Gadd45a are YMDQWVPVINLPER (Figure 12C). Then MALDI-PSD analysis of the phosphorylation specific peptides was performed. The sequence of this peptide enabled identification of the phosphorylated residue at Tyr151 within Gadd45a (Figure 12C). So, our preliminary results suggest that Sak phosphorylates Tyr151 on Gadd45a.

A


Sample1
Sample2
Sample3
100KDa
Sak
30KDa
His-Gadd45a

B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% Intensity
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2212.98
Mass (m/z)

C

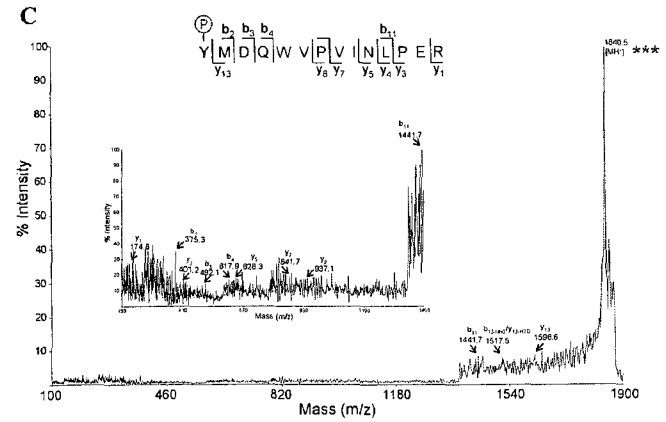

Y M D Q W V P V I N L P E R
% Intensity
1441.7
1517.5
1598.6
***
Mass (m/z)

Figure 12. Sak phosphorylates Tyr151 on Gadd45a. **(A)** An in vitro cold kinase assay was performed with the immunoprecipitated Flag-Sak protein, the purified His-Gadd45a protein and cold ATP, protein samples were separated by SDS- PAGE gel, followed by coomassie blue staining. **(B)** MALDI-TOF spectrum of His-Gadd45a tryptic digest. Bacterially expressed His-Gadd45a was subjected to kinase assay with Flag-Sak, expressed and immunoprecipitated from Hek 293T cells. The peak at 1307 m/z corresponds to Gadd45a peptide LGSFLDHSFGAR. The peak at 2212 m/z is common trypsin proteolysis peak. The peaks at 1761 m/z and 1840 m/z are separated by +80 Da, thus suggesting modification by phoshorylation. Phosphopeptide peak at 1840m/z is marked with asterisk and shifted by 80Da (HPO3 = 80 Da) relative to predicted unphosphorylated peptide peak at 1761 m/z. **(C)** MALDI-PSD spectrum of singly charged parental ion at 1840 m/z. The sequence of this peptide enabled identification of the phosphorylated residue at Tyr151 within Gadd45a.

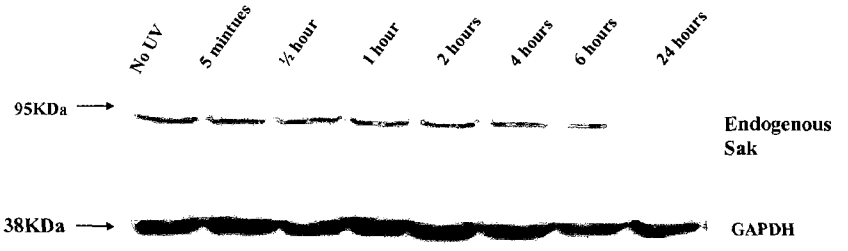
Downregulation of Sak Expression in Response to UV Irradiation is p53-dependent

Sak was identified as a p53 target gene (Swallow *et al.*, 2005) and transcriptionally repressed by p53. Furthermore, Sak mRNA levels decrease in response to DNA damage in a p53-dependent manner (Li *et al.*, 2005). So the endogenous Sak protein expression levels in response to UV irradiation were investigated in order to determine the effect of DNA damage on Sak protein expression levels. U2-OS and SaOS-2 cells were irradiated with ultra-violet radiation, then collected at the indicated time. The Sak protein levels were quantified by Western blotting analysis.

The results showed that endogenous Sak expression was significantly repressed in response to UV damage in U2-OS cells (wildtype p53) (Figure 13), whereas no significant change in Sak protein expression was observed in SaOS-2 cells (p53–null) (Figure 14). The results suggested that downregulation of Sak expression in response to UV irradiation is p53-dependent.

A

Cell line: U2-OS
IB: anti-Plk4
UV: 10mJ/cm²



B

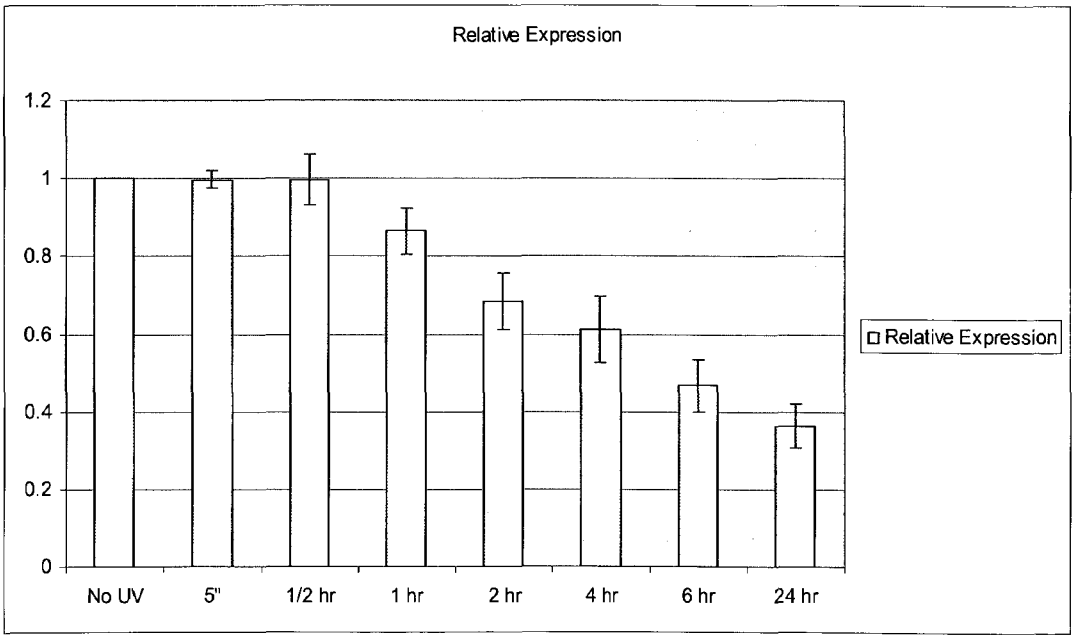
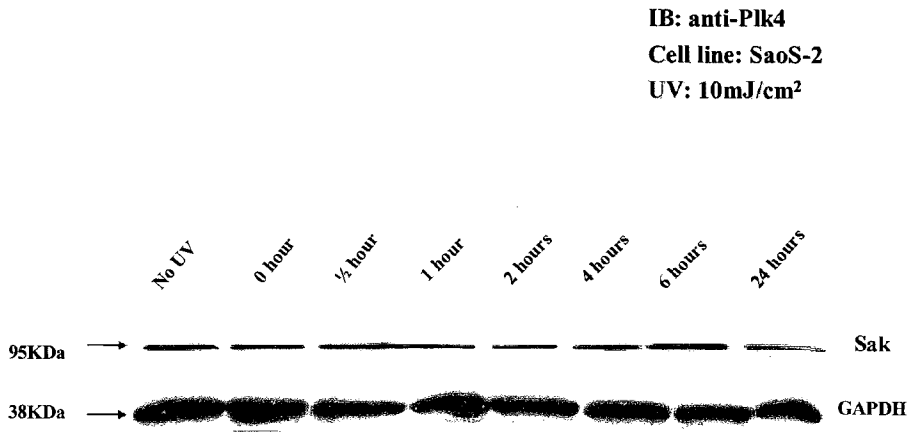


Figure 13. UV radiation decreases Sak protein expression levels in the U2-OS cells. (A) The U2-OS cells were exposed to UV irradiation and lysed at the indicated time points. The endogenous Sak protein expression levels were quantified by Western blotting analysis with anti-Sak antibody. Lysate were probed with an anti-GAPDH antibody as a loading control. **(B)** Compared to no UV damage, the endogenous Sak protein expression levels have no change at 5 and 30 minutes after UV damage, but Sak expression levels decrease after 1 hour.

A


IB: anti-Plk4
Cell line: SaoS-2
UV: 10mJ/cm²
No UV
0 hour
½ hour
1 hour
2 hours
4 hours
6 hours
24 hours
95KDa
Sak
38KDa
GAPDH

B

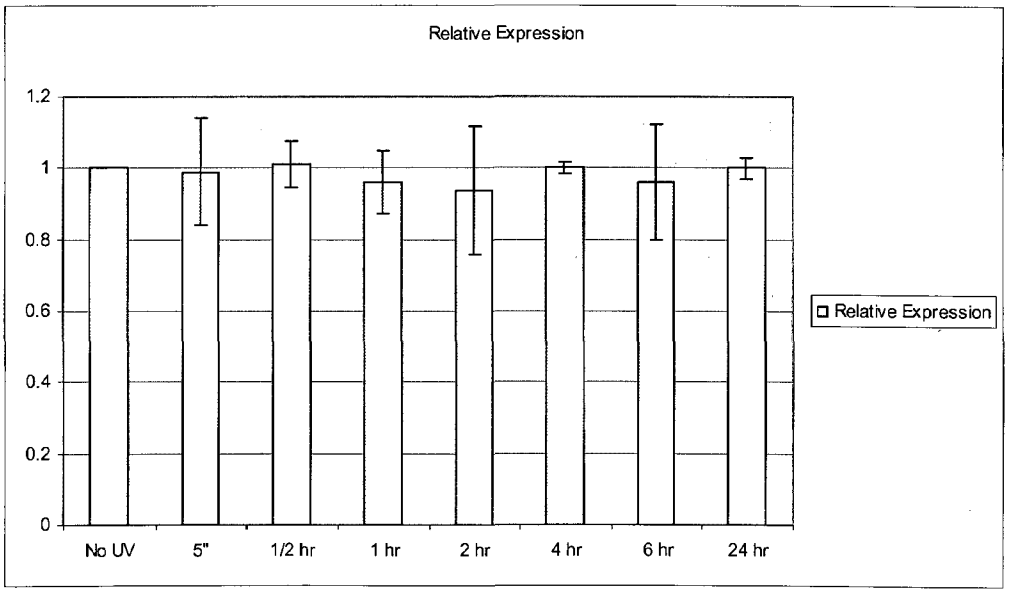

Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
No UV
5"
1/2 hr
1 hr
2 hr
4 hr
6 hr
24 hr
Relative Expression

Figure 14. The endogenous Sak protein expression levels remain constant in SaOS-2 cells. (A) The SaOS-2 cells were exposed to UV irradiation and lysed at indicated time points. The endogenous Sak protein expression levels were quantified by Western blotting analysis with anti-Sak antibody. Lysate were probed with an anti-GAPDH antibody as a loading control. **(B)** Compared to no UV damage, the endogenous Sak protein expression levels remain constant in SaOS-2 cells after UV damage at indicated time points.

CHAPTER V

DISCUSSION

Sak interacting partners

Plks are a conserved subfamily of Ser/Thr protein kinases that play pivotal roles in regulating various cellular and biochemical events at multiple stages of cell cycle progression (Glover *et al.*, 1998; Nigg, 1998; Dai *et al.*, 2003). They regulate cell cycle progression, including centrosome duplication, DNA damage response, Golgi dynamics, mitotic entry, spindle assembly and cytokinesis.

Sak was the last member of the human Plk family to be identified. Sak has been implicated in playing a role in centriole biogenesis (Habedanck R *et al.*, 2005) and has been implicated in playing a role during mitosis (Fode *et al.* 2004; Hudson *et al.* 2001). Both Sak levels and localization change throughout the cell cycle with Sak mRNA levels relatively low in both G0 and early-to-mid-G1 phase cells, an increase in S and G2 and peaking in M phase. Sak homozygous null mice are embryonic lethal at E7.5 with a marked increase in mitotic and apoptotic cells (Hudson et al 2001) and Sak haploinsufficiency causes mitotic infidelity and carcinogenesis (Ko *et al.*, 2005). Sak heterozygous mice are fertile. However at age 18-24 months Sak heterozygous mice have an increased incidence of developing liver and lung cancers when compared to wild-type mice. While there is an increasing body of evidence for phenotypes associated with Sak function, the exact functions or specifically its molecular targets remain largely unknown. The purpose of my research was to identify/characterize Sak's interaction with several key cellular proteins with the goal of helping us further understand the essential role of Sak in the cell cycle.

The interaction between Sak and Cyclin B1

Previous studies have found that Plk1 interacts with and phosphorylates Cyclin B1 thus inducing Cyclin B1's nuclear entry (Toyoshima-Morimoto F *et al.*, 2001). This is proposed to be essential for the induction and coordination of the initiation of mitosis (Toyoshima-Morimoto *et al.*, 2001). Similar to Sak, Cyclin B1 localization changes during the cell cycle. The protein is in the cytoplasm during S and G2 phases where a subpopulation associates with microtubules. Cyclin B1 accumulation in the cytoplasm up to metaphase upon which time it translocates to the nucleus. Furthermore a subpopulation of the protein associated with duplicating centrosomes (Hagting A *et al.*, 1998), a site which Sak is also localized to. These observations prompted us to investigate whether Sak also interacts with Cyclin B1. Interestingly through co-immunoprecipition and co-localization experiments, we found that Sak both physically interacts with Cyclin B1 and co-localizes with it at the centrosome. Cyclin B1 is a protein that binds to CDK1 to form a complex called MPF (mitosis promoting factor), MPF is activated by phosphatase Cdc25C in M phase, and then triggers mitosis. So Cyclin B1 is a necessary protein for mitosis entry. The finding suggests that Sak may play a novel role in mitosis entry through binding Cyclin B1.

The interaction of Sak with Cyclin B1 suggests that Cyclin B1 may be a substrate of Sak. This is supported by a recent study by Leung *et al* 2006 in which a putative phosphorylation target motif for Sak substrates was proposed to be [IFYLW]-{ILV}-[ST]-[YVFLICA]-[YVFLICA]-X-[PIFYLW] (Leung GC *et al.*, 2006). Using Expasy software, we found that the Cyclin B1 protein includes a match for the consensus phosphorylation site of Sak substrates. However, after numerous in-vitro kinase assays, we were unable to demonstrate the Sak phosphorylates Cyclin B1.

There are several possible explanations for our inability to observe Cyclin B1 phosphorylation. The simplest is that my observations are correct and Cyclin B1 is not a substrate of Sak. The possibility is that the observed association serves to place key proteins including Sak in close proximity. For example, Cdc25C is a phosphatase which can activate the Cyclin B/Cdk1 complex by dephosphorylation of Cdk1 at Thr14 and Tyr15 at end of G2 phase, thus triggering G2/M transition. This association may require the presence of Cyclin B1 in order to place Sak, Cdc25C and possibly other proteins together. Like Sak and CyclinB1, Cdc25C is also localized to centrosomes (Busch C *et al.*, 2007). In this scenario the centrosome may function as a site of integration for the proteins that trigger mitosis.

Another possible explanation is that Sak may phosphorylate Cyclin B1 under some special condition, for example genotoxic or DNA damage. Sak has been found to phosphorylate Chk2 *in vitro*, however the phosphorylation of Chk2 by Sak is abolished in response to UV (S. Petrinac, MSc thesis, 2007), which suggests DNA damage can alter the activity of Sak. Therefore, it will be interest to determine whether Cyclin B1 is phosphorylated by Sak in response to UV radiation or some other genotoxic stress. Still another alternative scenario is that, Sak may require the presence of other proteins, which together form a complex that regulates Sak activity towards Cyclin B1. Their functions may include changing the conformation of the Sak protein or possibly the substrate Cyclin B1 itself in a manner that exposes the targeted residues.

Sak interacts with and phosphorylates Gadd45a

Gadd45a, growth arrest- and DNA damage inducible gene, is involved in control of centrosome duplication and maintenance of genomic stability. Deletion of

Gadd45a leads to centrosome amplification, abnormal mitosis and aneuploidy. Furthermore, Gadd45a is found to physically interact with Aurora-A and BRCA1 (Shao SJ *et al.*, 2006) that also localize to the centrosome. These observations prompted us to investigate a possible interaction between Sak and Gadd45a. In order to test this hypothesis, a co-immunoprecipitation experiment was performed using an anti-Gadd45a antibody followed by Western blotting using an anti-Flag antibody. The results indicated that wild type, kinase active and kinase dead forms of Flag-Sak all associated with Gadd45a. We found no association between the Sak polo-box domain and Gadd45a. The polo-box regions of Plk1-3 have been implicated as phosphopeptide substrate binding motifs that play a role in both substrate recognition and localization of these proteins (Lowery DW *et al.*, 2004*)*. A truncated Sak protein that lacked the polo box was still able to associate with Gadd45a. Interestingly, the cryptic polo box region, R1 was sufficient for the interaction.

The interaction between Gadd45a and Sak suggested the possibility that Gadd45a may be a substrate of Sak. Therefore, we tested the ability of Sak to phosphorylate bacterially expressed and affinity purified His-Gadd45a fusion protein using an *in vitro* kinase assay assay. The results demonstrate that Gadd45a is a substrate of Sak as both wild type and kinase active forms of Sak were able to phosphorylate Gadd45a. Our results are also consistent with the finding that the polo-box domain of Sak may not function as a substrate docking site (Lowery DW *et al., 2005)*. The possibility exits that the cryptic polobox may fulfill this function. Future experiments would be necessary to determine this possibility.

Implications of the interaction of Sak and Gadd45a

Gadd45a is nuclear protein that is induced by genotoxic and certain other cellular stresses, including UV radiation, methyl methanesulfonate, and ionizing radiation. It is involved in growth control, maintenance of genomic stability, DNA repair, cell cycle control, and apoptosis. A previous study found that mouse embryonic fibroblasts derived from Gadd45a knock-out mice exhibit centrosome amplification and abnormal cytokinesis (Hollander M C *et al.*, 1999), suggesting that Gadd45a may play a critical role in the maintenance of centrosome stability. Gadd45a is also found to be transcriptionally activated by p53 (Kastan MB *et al.*, 1992), which has been implicated in the maintenance of genomic stability and regulation of centrosome duplication. Furthermore, Gadd45a was demonstrated to interact with Aurora-A, strongly inhibit its kinase activity and antagonize Aurora-A-induced centrosome amplification (Shao SJ *et al.*, 2006). Sak is also involved in maintenance of centrosome stability. Both gain- and loss-of-function experiments demonstrate that Sak is required for the precise reproduction of centrosomes during the cell cycle. However, the exact mechanism of how Sak play the role in centrosome duplication remains unclear. Our finding that Sak phosphorylates Gadd45a provides a novel mechanism that Sak might regulate centrosome stability probably via its effect on Gadd45a activity. Further studies are required to elucidate the relationship between Sak and Gadd45a in centrosome duplication in more detail. A broader understanding of Sak- regulated signaling pathways in maintaining centrosome stability and genomic fidelity will be invaluable.

Sak phosphorylates Tyr151 on Gadd45a

Protein phosphorylation plays a vital role in the cellular processes. Many signaling transduction pathways were formed by phosphorylation and

dephosphorylation. Phosphorylation usually occurs on serine, threonine, and tyrosine residues in eukaryotic proteins and phosphorylation of any site can change a protein' stability, activity and cellular localization. Therefore, characterizing the putative targets of Sak on Gadd45a will be useful in shedding light on Sak function.

Like many other kinases, the polo-like kinase family is also regulated by phosphorylation. The four mammalian family members share overlapping functions, with some overlap in target proteins and the residues targeted. Additionally, they also phosphorylate unique residues within common substrates as well as unique substrates. Recently, Johnson *et al* identified the predominant consensus sequence from the set of substrates identified for each Plk member. The results indicate the consensus substrate sequences for Sak include a basic residue at -3 (37%), an acidic residue at -2 (50%), Tyr or hydrophobic (Ψ) at +1 (47%) and +2 (89%), and Ser/Thr (42%) or Ala (26%) at +4. While this motif like that of leung *et al*, 2007 also predicts phosphorylation of Cyclin B1, which we didn't observe, it does not predict that Gadd45a as a potential substrate. The interaction of Sak and Gadd45a prompted us to attempt to determine the phosphorylation site of Sak on Gadd45a. Using and *in vitro* kinase assay and a mass spectrometry based approach, the preliminary results suggested that the specific phosphorylation peptides for Gadd45a is Tyr151 within the following peptide YMDQWVPVINLPER (see Figure12C). Sak kinase domain resembles a serine/threonine kinase. However, the active site loop of Sak more closely resembles that of a tyrosine kinase. Therefore the possibility exists that Sak is a dual specificity kinase be able to phosphorylate Ser/Thr and Tyr.

Sak expression levels decrease after UV irradiation

Plks regulate multiple processes in normal cell cycle progression and play key roles in DNA damage checkpoint pathways. Extensive research has demonstrated that Plks are important mediators for various cell cycle checkpoints that monitor centrosome duplication, DNA replication, formation of bipolar mitotic spindle, segregation of chromosomes, and mitotic exit, thus protecting cells against genetic instability during cell division (Nigg, 1998; Dai et al., 2002; Barr et al., 2004). Plk1 expression level is inhibited in response to DNA damage. The DNA damage-induced inhibition of Plk1 appears to be mediated by blocking its activation because expression of activation mutants of Plk1 can override the G2/M arrest induced by DNA damage (Smits *et al.*, 2000). In addition, Plk1 can phosphorylate and inactivate p53, the inhibition of Plk1 in response to DNA damage leads to the activation of p53 which plays an important role in maintaining genomic fidelity by controlling cell cycle checkpoints and apoptotic process following cell exposure to genotoxic stress (Levine, 1997). In response to DNA damage, Plk2 and Plk3 are activated, suggesting that Plks have different roles in checkpoint control (Xie *et al.*, 2001). Furthermore, Plk3 also physically phosphorylates and activates p53 during DNA damage, thus the activation of Plk3 in response to DNA damage leads to the activation of p53 (Ando *et al.*, 2004). Sak has also been implicated in DNA damage response pathways. Sak was identified as a p53 target gene (Swallow *et al.*, 2005) and transcriptionally repressed by p53. Furthermore, Sak mRNA levels decrease in response to DNA damage in a p53-dependent manner (Li *et al.*, 2005). Since Sak levels, as well as its relationship with its interacting partners, may be effected by DNA damage, we were interested in examining the effect of UV irradiation on endogenous Sak protein expression levels. Commercial antibodies that recognize endogenous Sak protein have only recently become available. Sak protein expression was significantly repressed in response to

UV irradiation in p53-wildtype U2-OS cells, consistent with the decrease of Sak mRNA levels in response to DNA damage. To further demonstrate that this was a p53 mediated event we also tested Sak protein levels after UV irradiation of a p53 null cell line, SaOS-2. SaOS-2 cells were derived from a human osteogenic sarcoma. As expected, no significant change in Sak expression level was observed in SaOS-2 cells. Taken together, the results strongly suggested that downregulation of Sak expression is p53-dependent.

Appendix A: Solutions

Competent Cell Solutions

TFBI

30mM KOAc

50mM MnCl2

100mM KCl

10mM CaCl2

7% glycerol

TFBII

10mM MOPS pH 7.0

75mM CaCl2

10mM KCl

7% glycerol

TYM broth

2% bactotryptone

0.5% yeast extract

0.1M NaCl

10mM MgSO4

Transformation

LB Media(1L)

10g tryptone

5g yeast extract

10g NaCl

Adjust pH to 7.0 and autoclave

LB-AMP Plates (1L)

10g tryptone

5g yeast extract

10g NaCl

15g Agar

Autoclave.

After the solution has cooled, ampicilin was added to a final concentration of 100 ug/ml.

Cell Culture

Cell Media

500mL Dulbecco's Modified Eagle's Medium (DMEM)

50 mL(10%) Fetal Bovine Serum

5mL (1%) Penicillin/Streptomycin

5mL (1%) Amphotericin B

500 uL Gentamycin

500mL Grace' Insect Medium

50mL(10%) Insect Medium Supplement 10X

5mL (1%) Penicillin/Streptomycin

Cell Lysis

PBS

4.3mM Na_2HPO_4

1.4mM KH_2PO_4

137mM NaCl

2.7mM KCl

pH to 7.4

Lysis Buffer

50mM Tris-HCl pH 7.4

150mM NaCl

1mM EDTA

1% Triton X-100

1 protease inhibitor tablet (Roche) was added to every 10mL of buffer

Western Blotting

Tris Buffer Saline(TBS)

100mM Tris-Cl pH 7.4

150mM NaCl

TNT

0.1% Triton X-100

50 mMTris-HCl pH 7.4

150mM NaCl

6 X SDS-PAGE sample buffer (10mL)

1M Tris pH 7.5

3.0mL 100% glycerol

1g SDS

100mg bromophenol blue

Ultrapture water was added to a final volume of 10 mL

Running Buffer

25mM Tris

250mM Glycine

0.1% SDS

Transfer Buffer

3.03g Tris base

14.4g glycine

2000mL methanol

1X TBST (Tris buffered saline and Tween)

100mM Tris-Cl pH7.5

150mM NaCl

0.1% Tween

Stripping Buffer

10mL 10%(w/v) SDS

6.25mL 0.5M Tris-HCl (pH=6.7)

342ul Beta-Mercaptoethanol

Filled to 50 mL with ddH2O

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

Immunofluorescence

3.7% Paraformaldehyde

3.7g paraformaldehyde

70ml ddH2O

10mL 10XPBS

Adjust pH 7.0

Add ddH2O to 100mL and filter though 0.45u filter

GST- Protein Purification buffers

Cell Lysis Buffer

50 mM Tris pH 7.5

100 mM NaCl

0.1 mM EDTA

0.1% Triton X-100

2mM DTT

1 protease inhibitor tablet (Roche) per 10mL buffer

Elution Buffer

100mM NaCl

50mM Tris pH 8.0

10mM Glutathione

His- Protein Purification buffers

Start Buffer

5mM Imidazole

100mM NaCl

50mM Tris pH7.5

Kinase Buffer

60mM HEPES pH 7.5

3mM MgCl2

3mM MnCl2

50mM NaF

12mM DTT

1 protease inhibitor tablet (Roche) per 10mL buffer

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