# The Modulation of Tumour Suppressor MST2 and Proto-oncogene Raf-1 Kinases by the Scaffold Protein CNK1

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Sept 2011

In partial fulfilment of the requirements for the degree of
Doctor of Philosophy to the
University of Glasgow


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# Abstract

An emerging concept in the regulation of signal transduction specificity is the mediation of scaffold proteins embedded in the circuitry of signalling pathways. The multidomain-based architecture of scaffold proteins facilitates the assembly and modulation of protein complexes to regulate cellular signals to bring about an exacting biological output. The work presented in this thesis aimed to investigate the mechanisms of the protein scaffold CNK1 (connector enhancer of Ras 1) in the pro-apoptotic MST2 pathway and the pro-oncogenic Raf-1 signalling pathways. Here, by using several molecular, biochemical and cell biology techniques, I demonstrated that CNK1 regulates the interaction of the proto-oncogene Raf-1 and the tumour suppressor MST2 kinase. Perturbations of CNK1 levels exhibit a biphasic signalling response typical of a scaffold protein. Transient expression of CNK1 upon growth factor withdrawal results in a concentration-dependent increase of the Raf-1/MST2 complex, thus preventing apoptosis, but this complex dissociates at higher expression levels, hence promoting an apoptotic response. Moreover, CNK1 is involved in the regulation of Fas-induced apoptosis via the MST2/RASSF1A pathway by influencing the time-scale kinetics of MST2 docking and release from the Raf-1/CNK1 complex and its eventual activation. SiRNA-silencing of CNK1 destabilizes the Raf-1 and MST2 interaction, and enhanced MST2/LATS1 interaction that promotes apoptosis. Thus, CNK1 is required for Raf-1 inhibitory function, but is also necessary for MST2-mediated apoptosis. Remarkably, CNK1 selects and switches complex formation of opposing anchored proteins depending on the stimulus. In response to Fas ligand stimulation, MST2 is released, whereas Raf-1 is retained in complex with CNK1. Conversely, CNK1 retains MST2 and whilst releasing Raf-1 from the complex following growth factor treatment. Mapping the multidomain binding sites of CNK1 using peptide array demonstrates specific interaction sites of client protein complexes. Specific CNK1 point mutants were generated, and found to alter wild-type regulation of client protein complexes. Thus, the work described in this thesis may reveal a regulatory crosstalk between the MST2 apoptotic pathway and the Raf-1 proliferative pathway through CNK1 by coordinating assembly of appropriate pathway components to possibly drive discrete stimulus-specific responses.

# Acknowledgements

I would like to thank all the previous members of the R7 group at Beatson and at the Pitt Lab, especially my Supervisors Walter Kolch (previous co-supervisor) and Andy Pitt. Thank you for providing insights and inspiration that has fostered my scientific curiosity.

However, married with two children and keeping up with PhD life was quite a journey and there are many people that I want to thank for their support and help to get me through this point. During my time at Beatson and Pitt Lab, I have the opportunity to meet and work with some of the best people especially Dr. Sarah Cumming who has been very supportive of my thesis and PhD life. I thank them all for their kind support and friendship.

Also, thank you Annemarie for all your support and encouragement.

My Mom for her prayers although no longer aware that I have finally decided to do her wish for me i.e. having a PhD, to my wife Tina for putting up with me and her patience and of course for Robyn and Kristoff for the inspiration and as well as making my PhD quite a journey.

*To Kristoff, Robyn, Tina*

*and*

*Mom*

# Declaration

I hereby declare that I am the author of this thesis, that it is a record of work done during my PhD study, and that it has not been presented in any previous application for a higher degree.

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# Table of Contents

# List of Abbreviations

| | |
|---|---|
| APS | Ammonium Persulphate |
| ATP | Adenosine Triphosphate |
| Bcl-2 | B-cell lymphoma 2 |
| Bcr-Abl | Bcr-Abl gene (breakpoint cluster region –Abelson) |
| bp | base pair |
| BSA | Bovine Serum Albumin |
| cDNA | complementary DNA |
| c-Myc | cellular-myelocytomatosis |
| CNK1 | Connector enhancer of KSR-1 |
| CRD | Cysteine Rich Domain |
| DLK | Dual leucine zipper kinase |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl Sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| DTT | Dithiothreitol |
| ECL | Enhanced Chemiluminescence |
| E. coli | Escherichia coli |
| EDTA | Ethylenediaminetetra-acetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-related kinase |
| FACS | Fluorescence Assisted Cell Sorting |
| FBS | Foetal Bovine Serum |
| FOXO3a | Forkhead box-3a |
| GDP | Guanosine diphosphate |
| GEF | GTP exchange factor |
| GRB2 | Growth factor receptor-bound protein 2 |
| GTP | Guanosine triphosphate |
| HER-2 | Human Epidermal growth factor Receptor 2 |
| Hsp90/70 | Heat-shock protein 90/70 |

| | |
|---|---|
| Hpo | Hippo |
| H-Ras | Harvey Ras |
| HRP | Horseradish peroxidise |
| H2B | Histone 2B |
| IP | Immunoprecipitate |
| IQGAP1 | Ras GTPase-activating-like protein 1 |
| JIP1 | C-Jun-amino-terminal kinase-interacting protein 1 |
| JNK | Jun N terminal kinase |
| K-Ras | Kirsten-Ras |
| KSR | Kinase suppressor of Ras |
| LATS1 | Large tumour suppressor 1 |
| LB | Luria-Bertani medium |
| MAPK | Mitogen activated protein kinase/ERK |
| MAPKK | MAPK kinase/MEK |
| MAPKKK | MAPK kinase kinase/Raf |
| MBP | Myelin basic protein |
| MEF | Mouse embryonic fibroblast |
| MEK | MAPK kinase/ERK kinase |
| MgCl2 | Magnesium Chloride |
| MKK7 | MAP kinase kinase 7 |
| MLK2/3 | Mixed Lineage Kinase 2/3 |
| MORG1 | Mitogen-activated protein kinase organizer 1 |
| MP1 | MEK partner-1 |
| MST2 | Mammalian sterile 20-like kinase 2 |
| NP40 | Nonidet-40 |
| N-term | Amino-Terminus/ NH2 -Terminal end |
| PAGE | Polyacrylamide gel electrophoresis |
| PARP | Poly (ADP-ribose) polymerase |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with Tween-20 |
| PCR | Polymerase chain reaction |
| PI3-K | Phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |

| | |
|---|---|
| PKB | Protein kinase B |
| PI | Propidium Iodide |
| PMSF | Phenylmethylsulfonyl fluoride |
| PP2A | Protein phosphatase 2A |
| PUMA | p53 upregulated modulator of apoptosis |
| PVDF | Polyvinylidine difluoride |
| Raf | Rapidly growing fibrosarcoma/ Rat fibrosarcoma |
| Ras | Rat sarcoma |
| RASSF1A | Ras associated family 1A |
| RBD | Ras binding domain |
| Rho | Ras homologous |
| RKIP | Raf-1 kinase inhibitor protein |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| RNase | Ribonuclease |
| ROKα | Rho-associated Kinase-alpha |
| RT | Room temperature |
| RTK | Receptor tyrosine kinase |
| SDS | Sodium dodecyl sulphate |
| SiRNA | Small interfering-RNA |
| SOS | Son of sevenless |
| Src | Src gene (Sarcoma) |
| TAE | Tris Acetate EDTA buffer |
| TBS | Tris-buffered saline |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TGFß | Transforming Growth Factor-beta |
| TSGs | Tumour suppressor genes |
| TL | Total lysate |
| Tris | Tris (hydroxymethyl) aminomethane |
| Tween-20 | Polyoxyethylene sorbitan monolaurate |
| UV | Ultra violet |
| Wts | Warts |
| WT | Wild type |

| | |
|---|---|
| YAP | Yes-associated protein |
| Yki | Yorkie |

Units:

| | |
|---|---|
| ºC | degrees centigrade |
| g | grams |
| hr | hour |
| kDa | kilo Dalton |
| µg | microgram |
| µl | microlitre |
| µM | micromolar |
| ml | millilitre |
| min(s) | minutes |
| sec(s) | seconds |
| hr | hour |
| rpm | revolutions per minute |
| V | volts |

Amino Acid code:

| | |
|---|---|
| Ala (A) | Alanine |
| Arg (R) | Arginine |
| Asn (N) | Asparagine |
| Asp (D) | Aspartic Acid |
| Cys (C) | Cysteine |
| Glu (E) | Glutamic Acid |
| Gln (Q) | Glutamine |
| Gly (G) | Glycine |
| His (H) | Histidine |
| Ile (I) | Isoleucine |

| | |
|---|---|
| Leu (L) | Leucine |
| Lys (K) | Lysine |
| Met (M) | Methionine |
| Phe (F) | Phenylalanine |
| Pro (P) | Proline |
| Ser (S) | Serine |
| Thr (T) | Threonine |
| Trp (W) | Tryptophan |
| Tyr (Y) | Tyrosine |
| Val (V) | Valine |

# List of Figures

# CHAPTER 1

# Introduction

## 1.1 Motivation

The study presented here involved fundamental biological mechanisms that are characteristics of cancer. However, current knowledge is overwhelmingly enormous and complex, and more importantly new insights and discoveries are ever rapidly emerging. I therefore resolved to start with a general background on cancer and highlighting underlying biological processes relevant to this thesis. Next, signalling pathways involved with tumour suppressor MST2 and proto-oncogene Raf-1 were discussed leading to the overview of the scaffold protein CNK1 including current perspectives on scaffold proteins.

### 1.1.1 Cancer

Cancer is the second most common cause of death in the western world and accounts for about 13% of all human deaths worldwide. It is believed that increasing global cancer rates have been primarily due to an aging population and lifestyle changes in the developing world (Jemal et al., 2010). Cancer is an old and a very complex disease, or better yet a collection of diseases both at the molecular and system-wide level. The practices of classical biology have led to a realisation that the reductionist approach may not be adequate to explain the dynamics of complex biochemical systems within a cell or population of cells therefore introduces a bottleneck in the pursuit to understand cancer (Nurse, 2008). On the other hand, it is still extremely crucial in elucidating fundamental processes and an important tool for validation of new novel discoveries. Hence, from this realisation, the emergence of new fields of studies such as genomics, proteomics, systems biology and synthetic biology have sprung. Essentially, these are collaborative studies from different disciplines using high-throughput state-of-the-art tools to acquire new knowledge. The aim is to integrate collective complex data to advance the understanding of biological phenomena and to attempt in progressing treatment and prevention of diseases like cancer. While previously scientists have unravelled valuable knowledge of specific normal molecular functions, we are now beginning to understand better the intricacies and complexity of the mechanisms of normal cell regulation through cancer research (Gilbert and Sarkar, 2000; Rosenfeld and Kapetanovic, 2008).

Indeed, collaborative approaches with computer scientists and engineers that aim to understand the breakdown or failure of cell mechanics has been very fruitful in advancing biological research (Ideker et al., 2001; Zhong and Sternberg, 2007). It is perhaps also true in cell biology that by learning from failures of cellular regulation, such as cancer, we may gain a better understanding of regulation in normal cells and tissues.

### 1.1.2 Hallmarks of Cancer

It is now apparent that complex biological activity does not arise from specific individual molecules, but rather interactions among several components and involves different processes. Development of cancer is a multistep process resulting from the accumulation of failures or errors in the normal regulatory mechanisms initiated due to mutations of one or several genes in a cell. This altered cell then divides and multiplies along with normal cells. For survival, they eventually acquire additional aberrations including the ability to proliferate independently of normal environmental signals, gaining a growth advantage over its neighbouring normal cells, and allowing it to develop and form a malignant tumour. As the seminal review by Hanahan and Weinberg proposed the six hallmarks of cancer which has been a guide in the scientific community of cancer research since it was published 10 years ago, the following discussion was therefore adapted from this report. These six essential alterations of normal cell physiology that collectively results in cancer are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of apoptosis or programmed cell death, limitless replicative potential, sustained angiogenesis (blood vessel formation for nourishment), and tissue invasion and metastasis (Hanahan and Weinberg, 2000). A modified diagram is illustrated in Figure 1.A and will be outlined briefly in the following chapters. They represent the crucial processes in the transformation of normal cells to malignant cells via a process called clonal evolution which suggests that each time a genetic change occurs it confers a growth advantage to the cell to expand and out-compete other cells (Nowell, 1976; Merlo et al., 2006). This genetic instability facilitates acquisition of other mutations as a result of defects in the normal DNA repair mechanism (Cahill et al., 1999). The incidence of this instability

also rises dramatically with age, most likely due to a build up of risk factors from the individual's genetic make-up, exposure to carcinogens and external agents such as chronic infections for specific cancers (e.g. HPV infection for cervical cancer), radiation, diet, tobacco and alcohol use (Jemal et al., 2010; Benz and Yau, 2008).

**Self-sufficiency in growth signals**

Normally, most soluble mitogenic growth factors are synthesized by specific cell types in order to signal other cells to divide and proliferate. Cancer cells however are able to produce many of their own growth signals, often termed autocrine stimulation, thereby eliminating the dependence on their normal tissue micro-environment, thus the hallmark "self-sufficiency in growth signals" (Hanahan and Weinberg, 2000). Moreover, this independence from the exogenous growth signals also changes the homeostatic micro-environment, thereby instigating perturbations among surrounding normal tissues and cells. This acquired autonomy is usually caused by alteration of extracellular receptors (e.g. receptor tyrosine kinases), trans-cellular transducers of signals and intra-cellular circuits that translate growth signals into action (Robinson et al., 2000; Hunter, 2000). One of the most studied mutations that confer an oncogenic activity is the epidermal growth factor receptor (EGFR). EGFR is a member of the subclass I of the transmembrane receptor tyrosine kinase superfamily that plays a major role in the regulation of cell proliferation and other important processes for proper regulation of many developmental, metabolic, and physiological mechanisms of cells (Voldborg et al., 1997; Waterman and Yarden, 2001; Holbro and Hynes, 2004). Binding of EGF or other growth factor ligands to the receptor causes homo- and hetero-dimers, subsequent autophosphorylation and activation of the kinase (Jiang and Hunter, 1999; Schlessinger, 2000). This leads to stimulation of several complex intracellular signalling cascades, particularly RAS/RAF/ERK, a major signalling pathway of cell proliferation which will be discussed later in more detail. Mutations, amplifications or overexpression involving EGFR could lead to its constitutive activation, elicit ligand-independent activation or hyperresponsiveness to ambient growth factors, which can result in uncontrolled cell division (Honegger et al., 1990; Yarden and Sliwkowski, 2001). Consequently, deregulation of EGFR has been identified in several types of cancer, and it is the target

of an expanding class of anticancer therapies (Fan et al., 1993; El Sheikh et al., 2004; Green, 2004; Chinnaiyan et al., 2005)

**Insensitivity to growth inhibitory (anti-growth) signals**

In normal conditions, cells are maintained in a temporary non-dividing "quiescent" state by anti-growth signals, and then they resume proliferation upon appropriate signals. The most widely studied of the anti-growth factors is TGFβ (transforming growth factor-beta) which upregulates the expression of cell cycle inhibitors such as the cyclin-dependent kinase inhibitors p15, p21 and p27 (Hannon and Beach, 1994; Reynisdottir et al., 1995) and therefore has a crucial role in tissue homeostasis. However due to genetic instability, cancer cells have evolved to evade this signal, enabling the characteristic "insensitivity to growth inhibitory (anti-growth) signals". Tumour cells evade normal TGFβ functions by selectively inhibiting its anti-proliferative response by downregulating TGFβ receptors and acquiring mutations in the TGFβ signalling pathway components (Parsons et al., 1995; Chen et al., 1998). Several studies have also demonstrated other mechanisms that are implicated in the disruption of TGFβ in promoting cell cycle arrest; such as mutations and deficiencies in retinoblastoma protein (pRb) and protein 53 (p53) which normally prevent replication of cells with damaged DNA, mutations in CDK (cyclin-dependent kinases), and perturbations in (mothers against decapentaplegic) SMADs (Tucker et al., 1984; Derynck et al., 2001; Heldin et al., 2009). Moreover in tumours, TGFβ not only loses its anti-growth function but can also induce proliferation, angiogenesis, invasion, metastasis and immune suppression (de Caestecker et al., 2000a; Shi and Massague, 2003; Seoane, 2006).

**Resistance to Apoptosis**

Cells are continually exposed not only to a variety of growth and anti-growth signals but experience stress and damage from exogenous and endogenous sources. Under this variety of circumstances, balancing physiological processes such as cell proliferation, development, differentiation and apoptosis are tightly controlled, which is essential to ensure normal growth and functions of organs, and tissue homeostasis of multicellular organisms (Hanahan and Weinberg, 2000). A major mechanism in this homeostatic

regulation is the process of apoptosis (Kerr et al., 1972). Programmed cell death or apoptosis is a normal regulatory process that cells promote in response to stresses such as nutrient or growth factor deprivation, heat, radiation, viral infection and other intra and extracellular signals that can trigger DNA damage or abnormalities that endanger the cells (Ameisen, 2002; Conradt, 2009). Apoptosis is characterized morphologically by contraction of cell mass, blebbing, loss of membrane asymmetry and attachment, nuclear fragmentation and formation of condensed cell bodies (Saraste and Pulkki, 2000). Phagocytic cells consume these apoptotic bodies and its constituents are recycled (Kurosaka et al., 2003).

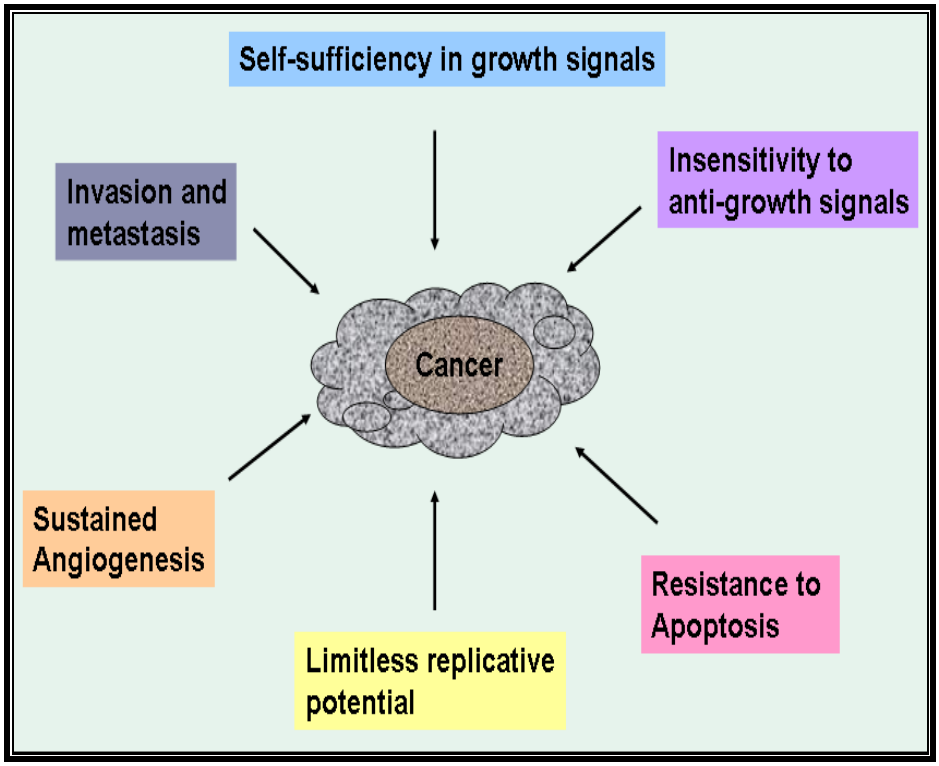



**Figure 1.A. Outline of cellular processes affected by genetic alterations acquired in cancer cells.** Illustration has been adapted from Hanahan and Weinberg, 2000. Other emerging hallmarks of cancers or characteristics of cancer have been proposed, such as evasion of inflammatory surveillance, genomic instability and altered cell metabolism (reviewed by Hsu and Sabatini, 2008; Mantovani, 2009; Negrini et al, 2010).

The underlying mechanisms of apoptotic cell death are highly regulated and complex. Briefly, apoptosis is an energy-dependent process that proceeds via two main pathways which are the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. Induction of either pathway eventually leads to activation of caspases (cysteine aspartate proteases) that function as common death effector molecules by cleaving a range of cytoplasmic and nuclear substrates (Ellis et al., 1991; Elmore, 2007). The extrinsic pathway is activated by binding of a death ligand such as CD95L/FasL or TNF (tumour necrosis factor) –related apoptosis-inducing ligand (TRAIL) to their

corresponding death receptor which induces the formation of a death-signalling complex (DISC) (Locksley et al., 2001; Fulda and Debatin, 2006). This complex is capable of recruiting several procaspase-8 molecules through an adaptor molecule Fas-associated death domain (FADD), which results in cross activation of caspase-8 (initiator) and the release of active caspase-8 into the cytosol (Reed et al., 2004). Activated caspase-8 initiates a caspase cascade by processing the (effector) caspases-3, 6, and 7 which in turn cleave a number of protein substrates (Cohen, 1997; Mandal et al., 2005). Alternatively, at low levels of caspase-8 the proapoptotic Bcl-2 family member BID (BH3-interacting domain) is cleaved, translocates to the mitochondria and causes release of apoptogenic factors such as cytochrome c to trigger the mitochondrial pathway (Cory and Adams, 2002). The intrinsic or mitochondrial pathway can be triggered by toxic chemicals, DNA damaging agents, growth factor withdrawal, hypoxia, and ionizing irradiation (Herr and Debatin, 2001). Following cellular stress, the pro- and anti-apoptotic Bcl-2 family proteins interact in a complex fashion to mediate permeability of the mitochondrial outer membrane allowing the release of cytochrome c to the cytosol, triggering the commitment step of the mitochondrial apoptotic cascade (Igney and Krammer, 2002). Once released into the cytoplasm cytochrome-c binds to Apoptotic protease-activating factor 1 (Apaf-1), recruits procaspase-9 to create an "apoptosome", a multi-protein complex that activates caspase-9 in the presence of ATP, which in turn activate caspase-3 and caspase-7, leading to widespread activation of other caspases and the execution phase of apoptosis (Chinnaiyan, 1999; Hill et al., 2004). Cleavage of caspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis. It is therefore expected that one of the characteristic features of a cancer cell is the inability to undergo apoptosis in response to stimuli that would otherwise trigger apoptosis in sensitive cells, and to allow them to become more proliferative than normal. Cancer cells that harbour genetic alterations from important components of a normally functioning apoptosis machinery disrupts the ability of the cell to respond appropriately. Upon replication this faulty apoptotic mechanism is inherited by its progeny, increasing the capability to "evade programmed cell death" leading to the accumulation of aberrant cells (Hanahan and Weinberg, 2000). For instance, anti-apoptotic Bcl-2 proteins are over-expressed in a variety of tumours, and their expression is associated with resistance to chemotherapy (Sartorius and Krammer, 2002; Wesarg et al., 2007). The up-regulation of death receptors often occurs following chemotherapy and sensitizes tumour cells to

Fas ligand or other apoptotic-inducing ligands, rendering them less resistant to apoptosis (Dai and Grant, 2007). Thus, overexpression or underexpression of anti- and pro-apoptotic gene products or mutations affecting key regulating genes of the apoptotic cascade may lead to evasion of, or resistance to, programmed cell death (Fulda and Debatin, 2006). However, it is important to note that other forms of programmed cell death such as autophagy and necrosis have been described, and are equally important and can also be exploited by cancer cells (Mizushima et al., 2008; Kroemer et al., 2009).

**Limitless replicative potential**

Most mammalian cells can only undergo a limited number of cell divisions due to progressive shortening of telomeres every cell cycle (Shay and Wright, 2002). Telomeres are a region of DNA which code for no proteins but are simply a repeated code on the end region of DNA strands. During the process of DNA replication, small segments of DNA at each telomere are unable to be copied and are lost after each time DNA is duplicated (Blackburn et al., 2006). It has been suggested that when the telomeres become too short, they unfold from their closed structure, and cells could detect this uncapping as DNA damage and therefore proceed to cellular senescence. This is also known as replicative senescence or the Hayflick phenomenon (in honour of Leonard Hayflick who first published this information in 1965), and may result in growth arrest, or even apoptosis, depending on the cell's genetic background, such as p53 or pRb integrity (Shay and Wright, 2000; Chen et al., 2006). This is a regulatory mechanism that allows a cell to prevent replicating errors that cause mutations in DNA. Telomeres are indeed essential in protecting the ends of chromosomes from degradation and preventing chromosomal end fusions and recombination (Masutomi et al., 2003). Aberrant cells that bypass this arrest become immortalized by telomere maintenance and extension due mostly to the activation of telomerase, the reverse transcriptase enzyme responsible for synthesis of telomeres (Blackburn et al., 2006). Many cancer cells are considered immortal because of high telomerase activity and are able to restore telomere length, conferring “limitless replicative potential” (Kelland, 2007). The high expression of telomerase in a wide range of human cancers is of major interest in cancer therapy, as it could be used as a diagnostic and prognostic biomarker (Gertler et al., 2008; Bisoffi et al., 2006).

**Sustained Angiogenesis**, **Invasion and Metastasis**

In order for cells to function and survive they must be supplied with oxygen and nutrients. Cells must also be located within 100-200 μm of blood vessels, which is the diffusion limit of oxygen, for proper nourishment, survival and overall tissue homeostasis (Hanahan and Weinberg, 2000). It is therefore critical for the surrounding tissues to regulate and maintain a vascular network of blood supply for the delivery of adequate and important factors for growth and sustenance. Angiogenesis is a highly ordered process used by tissues in response to demands due to physiological or pathological conditions such as cancer or heart disease; it is the outgrowth of new capillaries or blood vessels from pre-existing vessels (Baluk et al., 2005). The development and formation of the normal vascular network is regulated by exchange equilibrium of anti- and pro-angiogenic factors. Deregulation of this process leads to underdeveloped or leaky vessels, and over growth or uncontrolled sprouting angiogenesis (Bergers and Benjamin, 2003).

Cancer cells can therefore exploit this process to support their survival and growth by the process of “sustained angiogenesis", allowing tumours to progress to a larger size. This ability during tumour development is acquired via an initial “angiogenic switch”, an imbalance in the production of angiogenesis inducers and inhibitors (Hanahan and Folkman, 1996). Tumour angiogenesis is a multistep process which includes release of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factors (bFGF) which diffuse to surrounding tissues and bind to corresponding receptors (Baeriswyl and Christofori, 2009). Upon this initiation phase activated endothelial cells change shape; pericytes (mural cells of microvessels) detach from the blood vessel walls; degradation of extracellular matrix occurs (ECM) by the production of proteolytic enzymes; endothelial cells migrate and proliferate; and remodelling, stabilisation and maturation of new vessels occur (Bergfeld and DeClerck, 2010) As tumours progress to increased malignancy, primary tumour cells eventually acquire the ability to undergo "invasion and metastasis" whereby cells invade into surrounding normal tissues and travel through tissue boundaries and distant sites in the body to form new colonies (metastases) at sites distinct from the primary tumour (Talmadge and Fidler, 2010).

### 1.1.3 Emerging Hallmarks of Cancer

The steps that cells take toward becoming malignant cancers are variable, so that the order in which the hallmarks are acquired can vary from tumour to tumour and not all hallmarks have to be fulfilled for malignant cellular transformation to occur (Mantovani, 2009). In fact, other emerging hallmarks of cancers or rather characteristics of cancer have been proposed such as evasion of inflammatory surveillance, genomic instability and altered cell metabolism and cancer stem cells (Hsu and Sabatini, 2008; Negrini et al, 2010; Singh and Settleman, 2010). Even more recent is the observation of global reduction of microRNA (miRNA) levels in cancer (Esquela-Kerscher and Slack, 2006) particularly changes or alteration of gene expression is an emerging hallmark of cancer. MiRNAs are a class of short ribonucleic acid (RNA) molecules, on average only 22 nucleotides long, noncoding RNAs that has been shown to regulate gene expression of cancer-related genes in various tissues, therefore they can be regarded to function as oncogenes or tumour suppressors, thus the link to tumourigenesis (Davalos and Esteller, 2010).

### 1.1.4 Genes involved in the onset of Cancer

Induction and eventual deregulation of the normal cell processes that results in the above hallmarks of cancer are generated by two broad classes of genes; they are grouped into proto-oncogenes or tumour suppressors that play a crucial role in carcinogenesis as described below (Lee and Muller, 2010). These genes encode many kinds of proteins that regulate many key physiological processes such as cell cycle, growth, proliferation and apoptosis. Mutations that either increase the potential activation of proto-oncogenes or override the functional activity of tumour suppressors predispose cells to cancer development (Hanahan and Weinberg, 2000). Some tumour suppressor genes are classified as the stability or caretaker genes that maintain the integrity of the genome by ensuring fidelity of information transfer from one generation of cells to the next, and recognising or repairing DNA mismatch and damage (Levitt and Hickson, 2002). Their deregulation causes genetic instability that leads to a higher mutation rate of all genes including proto-oncogenes and tumour suppressor genes. However, no single mutation

gives rise to tumourigenesis, since mammalian cells have evolved a number of safeguard mechanisms to protect them from fatal effects of mutations that transform them into highly malignant cells but rather accumulation of mutations in these several types of genes will manifest the onset of cancer (Hanahan and Weinberg, 2011).

**Proto-oncogenes/ Oncogenes**

Proto-oncogenes are normal genes that encode proteins involved in different processes crucial for normal human cell development, growth and overall maintenance of tissues and organs. They become oncogenes due to mutations, often dominant because only one mutated allele is sufficient, that result in a constitutively activated protein product and leading to excessive and continuous cell proliferation (Lee and Muller, 2010). Conversion or activation of a proto-oncogene to an oncogene can be a consequence of gene duplication and chromosomal translocation that causes inappropriate 'gain-of function' expression of the encoded gene, which affects normal growth-regulatory processes such as increasing cell cycle, loss of differentiation, inhibition of cell death and eventual induction of cancer (Cantley et al., 1991; Weinstein & Joe, 2006). Examples of proto-oncogenes include HER-2, Ras, Myc, Src, Bcr-Abl, Raf and ERK. A number of oncogenes that are important in the thesis will be described later.

**Tumour Suppressor genes**

Tumour suppressor genes (TSGs) regulate diverse cellular activities including cell cycle checkpoint responses, detection and repair of DNA damage, gene transcription, mitogenic cell signalling, differentiation and apoptosis (Simpson and Parsons, 2001; Sherr, 2004). Fundamentally, TSGs are growth regulating genes that inhibit or suppress inappropriate cell division and excessive proliferation. Importantly, these genes also promote apoptosis or cell death to maintain homeostasis of body tissues. Like proto-oncogenes, tumour suppressor genes undergo a variety of mutations. 'Loss-of-function' or inactivation due to mutations and deletions of prototypic TSGs are usually recessive, that is it involves "two-hit" damage of both alleles of a gene to progress tumour formation (Knudson, 1971, 1973; Comings, 1973). For instance, retinoblastoma is a childhood form of retinal cancer that is caused by two mutations, one in each copy of the tumour suppressor gene now called *RB1* (Friend et al., 1986). However, more and more

reports shows a number of TSGs do not conform to this classic model, such as haploinsufficient (single copy is not enough to confer normal function) genes requiring inactivation of only one allele (Santarosa and Ashworth, 2004), and silencing genes not by mutation but rather epigenetic hypermethylation (Ducasse, 2006). TSGs also appear to exhibit dominant negative (antagonise the wild-type allele) and gain-of-function (new and abnormal function) mutations which indicate a more complex view than originally thought (Payne and Kemp, 2005). This complexity is well exhibited by the p53 tumour suppressor gene (Menendez and Resnick, 2009). As it plays a crucial role in cell cycle regulation (growth arrest, DNA recognition, transcription of repair genes) (Marine et al., 2006) and apoptosis (suppress replication of damaged DNA, induce cell death of damaged cells) (Zhang et al, 2007), loss of p53 function or inactivation abolishes apoptosis and allows abnormal cell division (genomic instability) (Rivlin et al., 2011), increased proliferation and increased susceptibility to carcinogenesis (Vousden and Prives, 2009). Hence, it is dubbed as the "guardian of the genome" (Lane, 1992; Efeyan and Serrano, 2007).

### 1.1.5 Cell Signalling

Oncogenes and mutated or inactivated tumour suppressor genes code for proteins that constantly interact in a dynamic fashion that includes a series of steps referred as the signal transduction cascade or cell signalling pathway. In normal cells, such cascades carry a signal from the cell membrane by receptor tyrosine kinases, usually activated by dimerisation (Heldin, 1995) and autophosphorylation of tyrosine sites (Hunter, 2000). In response, cytoplasmic effectors are activated and regulate internalisation of receptors or translocation of signals into the nucleus, to stimulate a regulated process such as cell growth (Pawson and Nash, 2000). Each step includes interaction of proteins such as recruitment of protein kinases by adaptors with specific activating domains that recognized by phosphorylated RTKs (Lowenstein et al., 1992), import or export of nuclear proteins and transcription factors to generate and process the signal (Chuderland et al., 2008). On the other hand, altered proteins encoded by mutated TSGs and oncogenes either disrupt this process or activate the signalling constitutively, for

example by over-production of growth factors which results in uncontrolled proliferation (Spivak-Kroizman et al., 1992). Cell signalling at the cellular level is complex, and involves many proteins and intermediary molecules including enzymatic activities (Metallo and Vander Heiden, 2010), movement of ions such as exit or entry of $Ca^{+2}$ through ion channels (Clapham, 2007), changes in protein conformation to induce interaction (Zhang et al., 2006), and gene expression such as induction or repression of transcription factors (Karin and Hunter, 1995). All cellular events such as cell survival and growth are a consequence of the dynamic interplay of these signalling cascades (Pawson and Nash, 2000). Multi-component cell signalling pathways also allow for feedback loops, signal amplification, and interactions between multiple signals and signalling pathways (Kolch, 2005). Some signalling transduction pathways process and respond differently, depending on the type and amount of signalling received by the cell (Hlavacek and Faeder, 2009). They respond to a multitude of stimuli from fluctuating environmental conditions which are required for homoeostasis to balance between life and death. In recent years, one of the most exciting new areas of study in the mitogen-activated protein kinase (MAPK) signalling cascades has been the elucidation of putative scaffold proteins (Nishimoto and Nishida, 2006). Scaffold proteins regulate signal flux by coordinating protein complexes and distribute diverse signals to modulate and specify an appropriate response (Burack and Shaw, 2000). Their ability to facilitate such complex mechanisms is due to their intrinsic multidomain structure. Contributions to this research have further revealed a plethora of insights into our understanding, the complexity of intracellular signalling circuitry pathways and networks in mammalian cells (Dhanasekaran et al., 2007).

## 1.2 Towards the study of CNK1

### 1.2.1 The MAPK Pathway

An evolutionary conserved signalling module, the mitogen-activated protein kinase (MAPK) pathway, is a key signalling pathway that when deregulated leads to development of cancer and other human diseases (Davies et al., 2002; Marais et at.,

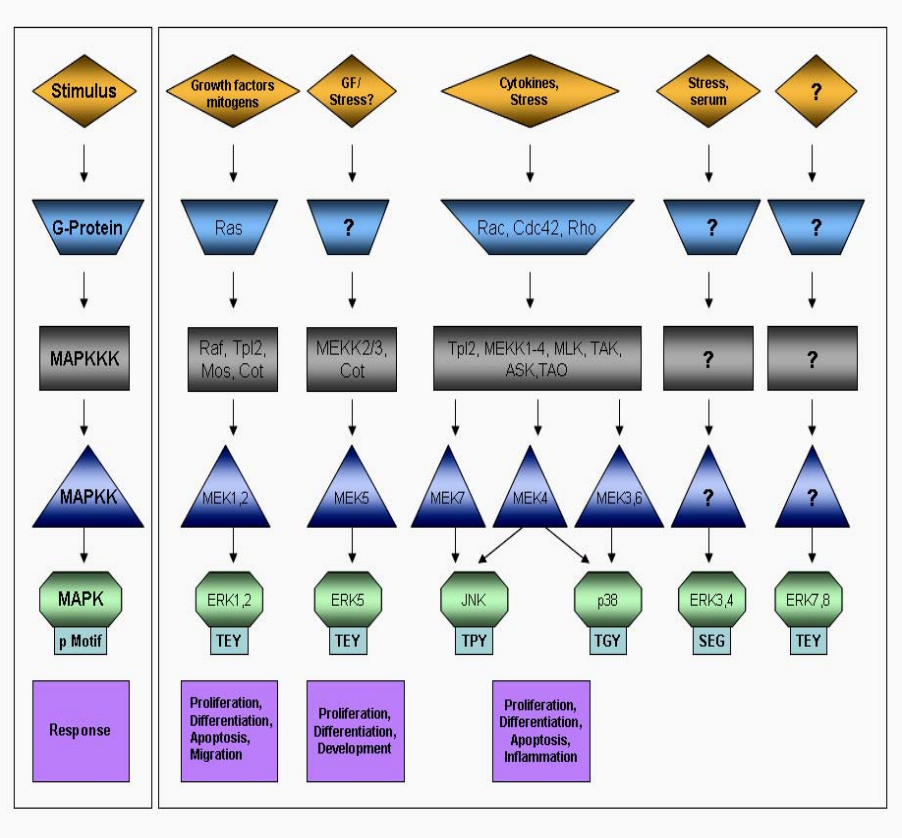
1998). Intensive research has been carried-out on the mechanisms of these pathways for a number of years in the hope of identifying and developing a drug for the treatment of cancer (Morrison et al., 2003; Reddy et al., 2003; Dhillon et al., 2007). A fundamental module downstream of a G-protein comprises a MAPK which is phosphorylated and activated by a dual specific MAPKK which is also phosphorylated and activated by a MAPKKK. Mitogens were the first group of stimuli shown to activate these pathways, hence the name given to this cascade of kinases (Ray and Sturgill 1987). A plethora of signals are now known to trigger specific response or crosstalk between pathways to elicit cellular events. A schematic overview in Figure 1.B depicts the complexity and organisation of MAP kinase cascades.

When MAP kinases are activated, a variety of membrane and cytoplasmic intracellular targets and nuclear substrates, including transcription factors, are phosphorylated or mechanistically interact with adaptor proteins and other protein kinases as well. There are currently nine distinct MAPK modules identified. They are p38MAPKα/β/δ, Jun N-terminal kinase (JNK) 1/2/3, extracellular signal-regulated (ERK) 1/2, ERK3/MAPK3, ERK4/MAPK4, ERK5/MAPK7, ERK6/p38MAPKγ, ERK7 and ERK8. In mammalian cells, six are well characterised to date. These are ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3, p38 isoforms α/β/δ/γ41 (Dhanasekaran et al., 2007). The ERK1/2 or classical MAP kinases are serine/ threonine kinases that are ubiquitously expressed intracellular signalling molecules and are generally activated in response to growth factor-induced cell surface receptors (Boulton et al, 1991; Chang and Karin, 2001). The Jun N-terminal kinase (JNK) 1/2/3, also known as stress-activated protein kinases (SAPK), phosphorylate c-Jun on Ser63/Ser73 and p38 (Reddy et al., 2003). They are involved in apoptosis, inflammation and cell differentiation which are activated by stress stimuli such as cytokines, oxidation, UV radiation, heat and osmotic shock (Yoshioka, 2004). ERK5 has recently been shown to be activated both by stress and growth factors and translocates to the nucleus where it regulates gene expression by phosphorylating different transcription factors (Turjanski et al., 2007; Raman et al., 2007). As the MAPK pathways are involved in multiple cellular processes, deregulation in the control of the MAPK signalling pathways can lead to carcinogenesis. (Chang and Karin, 2001; Dhillon et al., 2007)

**Figure 1.B. Schematic overview of the MAPK pathways.**

These pathways rely on signals that are processed through common mode of activation by phosphorylation of distinct motifs as shown below. However, the biological consequence varies widely**.**

| **Legends:** | |
|---|---|
| Phospho Motif | **TEY,** Thr-Glu-Tyr activation motif (ERK1, 2, 5, 7, 8)<br>**TPY,** Thr-Pro-Tyr activation motif (JNK)<br>**SEG,** Ser-Glu-Gly activation motif (ERK3, 4)<br>**TGY,** Ser-Glu-Gly activation motif (ERK3, 4, p38) |




This schematic diagram is an oversimplification, and the actual roles of each MAPK cascade are highly complex, cell type and context dependent. Importantly, these pathways are activated by an enormous array of stimuli, and regulated by phosphatases, kinases and other enzymes including specialized proteins such as adaptors and scaffolds; they influence gene expression, cell cycle, metabolism, immune response and other critical biological processes as shown above.

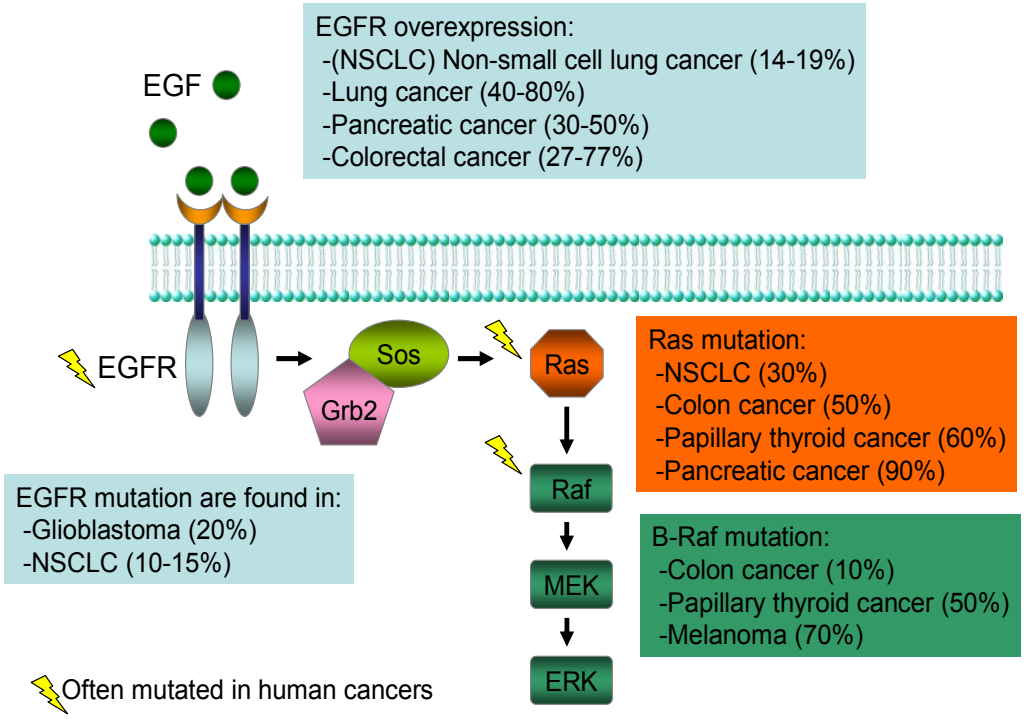
### 1.2.2 The Ras/ Raf/ MEK/ ERK Pathway

Among all the MAPK modules, the Ras-Raf-MEK-ERK (ERK/MAPK) pathway is one of the first described and best understood signal transduction pathways. It exemplifies how an extracellular stimulus is processed and transduced into the nucleus, which ultimately decides cell fate such as proliferation, differentiation and cell death. It is involved in about one third of all human cancers (Davies et al., 2002). This ERK/MAPK module consists of ERK (ERK1 and ERK2), which is activated upon phosphorylation by MEK (MEK1 and MEK2), which is activated upon phosphorylation by Raf (A-Raf, B-Raf, Raf-1). The ERK/MAPK cascade signalling is initiated by binding of growth factors to (RTKs) Receptor Tyrosine Kinases (Hunter, 2000). This triggers guanosine-triphosphate (GTP) loading of the Ras GTPase, which then binds and recruits Raf to the cell membrane for activation (Campbell et al., 1998). While at this time Raf and MEK appear to have only one substrate, ERK has been shown to have as many as 80 substrates (Kolch, 2000). Early on, studies have shown that most cancer-associated aberrant activation of this pathway occurs at the level and upstream of Ras, including overexpression and mutations of RTKs and frequent mutation of Ras (Schlessinger, 2000; Malumbres and Barbacid, 2003). Newer studies have shown that activation of this pathway in cancer can occur at different levels. More importantly, this increased expression of RTKs is associated with a poor prognosis (Repasky et al., 2004; Gollob et al., 2006). These cancer-causing aberrations of proteins are summarized in Figure 1.C. For instance, the recent discovery of B-Raf mutations in prevailing human cancers such as melanomas and colon cancer has proven that this module is far more complex than initially perceived (Wellbrock et al., 2004). Also, in the absence of clear genetic mutations, the ERK pathway has been reported to be hyperactivated in over 50% of acute myelogenous leukaemias and acute lymphocytic leukaemias (Garnet and Marais, 2004; Chong et al., 2003; McCubrey et al., 2007)

One of the most essential components of this pathway is the serine/threonine kinase, Raf (MAPKKK). The mechanism that regulates the small GTPase Ras as well as the downstream MEK and ERK is well understood, compared to the regulation of Raf itself

(Kolch, 2000). The Raf family consists of three isoforms; A-Raf, B-Raf and C-Raf (also known as Raf-1), all of which serve as immediate effectors of Ras and share MEK as a common substrate. They also share a common structure; each isoform contains three conserved regions; CR1, CR2 and CR3. The regulatory domain consists of the CR1 and CR2 which are located in the amino terminus. CR1 covers the RBD (Ras binding domain) and the CRD (Cysteine-rich domain). The kinase domain is at the C-terminus and comprises the CR3 region; however each demonstrates unique mechanisms of regulation and exhibits different physiological functions (Winkler et al., 1998; Okada et al., 1999; Kolch, 2000). For instance, activation by Ras of each isoform reveals common and different key individual phosphorylation sites which suggest that they can be regulated differently (Marais et al., 1997).

**Figure 1.C. Schematic overview of the Ras-Raf-MEK-ERK pathway and its cancer- associated lesions (modified from Wellbrock et al., 2004).**



All three isoforms induce MEK/ERK activation, but differ in strength. MEK activity is highest when activated by B-Raf, followed by Raf-1 then A-Raf. Moreover, preferential activation of MEK species also exists among these isoforms. A-Raf has been shown to have preference to MEK-1 activation, while Raf-1 and B-Raf show no preference for either MEK-1 or MEK-2 *in vitro* (Wu et al., 1996). Knock-out mouse experiments show that A-Raf gene deletion resulted in neurological and gastrointestinal postnatal defects (Pritchard et al., 1996). Meanwhile, Raf-1 knock-out mice display a variety of abnormalities depending on the genetic background and mostly die during embryogenesis due to increased apoptosis (Huser et al., 2001; Mikula et al., 2001). Although MEFs from this mouse show no change of MEK/ERK activity, it was proposed that Raf-1 functions are compensated by B-Raf (Huser et al., 2001). Raf isoforms also vary in expression dependent on cell or tissue type. A-Raf emerged as the main isoform in urogenital cells while B-Raf is highly expressed in neuronal tissues, but at lower levels also in most other tissues. Raf-1 is ubiquitously expressed (O'Neill and Kolch, 2004). Collectively, these data suggest overlapping and distinct functions among individual isoforms. Since the main focus of this project is in association and at the level of Raf-1, it shall be discussed in more detail below.

Although most studies have focused on Raf-1, the mechanism and function upon activation is still incompletely understood. Raf-1 activation is a complex multi-step process that involves dynamic changes in its phophorylation (serine, threonine and tyrosine residues), intermolecular dimerisation, protein-protein interactions and localisation as well as integration of other signalling pathways (Kolch, 2000; McCubrey et al., 2007). Accumulating evidence has demonstrated the significance of phosphorylation on different domains that control the regulation of Raf-1. For instance, Raf-1 in quiescent cells adopts an inactive conformation in the cytosol through the binding of 14-3-3 proteins to phosphorylated residues S259, and release from repression by 14-3-3 occurs upon Ras binding and displacement of 14-3-3 from pS259, which is subsequently dephosphorylated by PP1 or PP2A (Yip-Schneider et al., 2000; Ory et al., 2003). This initiates further activation steps resulting in full activation of Raf-1. There are at least thirteen phosphorylation sites that are involved in the regulation of Raf-1 (Dhillon and Kolch, 2002). They include S43 (targeted by PKA-Protein kinase A and ERK), S259 (targeted by AKT/Protein kinase B, PKA, PP2A-dephophorylation), S233

(phosphorylated by PKA), and S621 (PKA, AMPK) (Dhillon et al., 2002; Light et al., 2002; Dumaz et al., 2003). Residues S43, S259 and S621 are constitutively phosphorylated (Dhillion et al, 2002). Raf-1 activation comprises the dephosphorylation of pS259 followed by phosphorylation of S338, Y340 and Y341 (Diaz et al., 1997; Chong et al., 2001). Phosphorylation at S338 can be mediated by p21 activated protein kinase (PAK1) (King et al., 1999), and reversed by protein phosphatase 5 (PP5) (von Kriegsheim et al., 2006). Src family kinases phosphorylate Y340 and Y341 (Fabian et al., 1993). S497 and S499 residues were identified as Protein kinase C (PKC) targets, but their role in activation is not entirely clear (Chong et al., 2003; Dhillon et al., 2007). Despite controversies over the details of Raf-1 activation through phosphorylation, it is clear that the regulation of Raf-1 reveals even more complexity to its mechanism.

Protein: protein interactions also play a major role in the regulation of the Ras/Raf/MEK/ERK pathway. Interestingly, recent studies have shown that B-Raf interacts with and activates Raf-1. B-Raf/Raf-1 heterodimerization is part of the physiological activation mechanism that can be stimulated by mitogens, enhanced by 14-3-3, and either isoform can activate the other (Rushworth et al., 2006). Raf-1 associated proteins described in the literature through biochemical and genetic approaches catalogue more than 35 proteins of different types and characteristics. These include chaperones (Hsp90/70), adapters/scaffolds (BAG-1, KSR, MP1, CNK1), phosphatases (PP2A, PP5), cytoskeleton and other kinds of proteins that are required for Raf-1 activation (Kolch, 2000) or act as inhibitors (e.g. RKIP-Raf kinase inhibitor protein) (Yeung et al., 1999); and orchestrate localisation and assembly of multi-complexes for regulation of signals (Morrison and Davis, 2003; Shin et al., 2009). Notably, specialised proteins such as scaffolding proteins are now emerging as critical regulators of signal transduction pathways. Furthermore, as it will be discussed later, scaffold proteins play a major role in the processing, integration, duration and strength of signals and proper localisation of protein complexes (Kolch, 2005).

Another intriguing feature of Raf-1 is that it has functions independent of MEK/ERK signalling. For instance, it has been shown that mitochondrially-targeted Raf-1 interacts with BCR-ABL to alter the phosphorylation of Bad in haematopoietic cells (Dhanasekaran et al., 2007). Raf-1 also interacts with the proapoptotic stress-activated

protein kinase ASK1 *in vitro* and *in vivo* thereby inhibiting apoptosis independent of its kinase function (Chen et al., 2001). ASK1 is a mediator of apoptosis induced by cytotoxic stresses including tumour necrosis factor (TNF), Fas and reactive oxygen species (ROS) (Hoeflich et al., 1999). Other non-catalytic functions include suppression of apoptosis in the membrane expression or clustering of Fas as well the regulation of Rho effector ROKα to stimulate cell migration (Yamaguchi et al., 2004). In addition, Raf-1 inhibits a novel proapoptotic pathway known as the MST2 signalling pathway, also independent of MEK activation (O'Neill et al., 2004).

### 1.2.3 Raf-1 regulation of apoptosis

The regulation of apoptosis is tightly controlled in parallel to cell growth and proliferation. Deregulation of this process can cause excessive and uncontrolled cell growth and proliferation, which is a common trait of cancer (Reddy et al., 2003). The classical Ras-Raf-MEK-ERK pathway has primarily been associated with cell proliferation. But now it is firmly established that this pathway is linked to several responses and it appears to have diverse roles such as the control of cell proliferation, differentiation and apoptosis (Wellbrock et al., 2004). As previously mentioned above, Raf-1 has been described to be involved in the inhibition of other pro-apoptotic proteins via direct binding and independent of its kinase activity. Moreover, deletion of Raf-1 in mouse models enhanced apoptosis while ablation of the proapoptotic kinase ASK1 could prevent this response (Yamaguchi et al., 2004; Ehrenreiter et al., 2005). In other knock-out mice experiments, ablation of the Raf-1 gene also causes widespread apoptosis and embryonic lethality independent of MEK. But when a Raf-mutant (kinase-dead) that cannot be activated was knocked-back in, it resulted in viable mice, which suggests that Raf-1 kinase activity is not required for its suppression of apoptosis (Mikula et al., 2001). In pursuit of this novel Raf-1 regulation of apoptosis, mammalian sterile 20-like kinase (MST2) was identified as an interacting partner of Raf-1 by a proteomic approach under serum starvation. It was demonstrated that Raf-1 binds to proapoptotic MST2 kinase via the N-terminus of MST2. Conversely, B-Raf does not associate with MST1 or

MST2 and therefore has no direct involvement in the regulation on this pathway as Raf-1 does (O'Neill and Kolch, 2004). The binding regions were mapped along the CR2 region to amino acid sequence 151 to 301 that are unique to the Raf-1 isoform, which is consistent with the result that MST2 does not bind B-Raf. Sequential immunodepletion experiments also showed that Raf-1 under serum starvation recruits the entire pool of MST2. However, only a fraction of the total Raf-1 pool is bound to MST2 (O'Neill et al., 2004). Collectively, upon serum starvation Raf-1 binds to MST2, sequesters MST2 monomers and prevents its dimerisation that is required for activation. In addition, Raf-1 recruits a phosphatase (possibly PP2A) that dephosphorylates the activation loop phosphorylation site of MST2, thereby maintaining in its inactive state (O'Neill et al., 2004). It was also shown that MST2 is constitutively active in Raf-/- cells and that its activity can further be enhanced by apoptotic stimulants such as Fas or Staurosporine. This suggested that the absence of Raf-1 leads to MST2 hyperactivation and eventual apoptosis. Moreover, kinase-dead and wild-type Raf-1 efficiently interacted with MST2, abrogating its phosphorylation and kinase activity, verifying that Raf-1 exerts a kinase independent prosurvival function (O'Neill et al., 2004). Studies on the regulation of pathways upstream of MST2 are emerging. Regulation of MST2 by Raf1 is not the only mechanism for control of this proapoptotic kinase. MST1 and MST2 have also been shown to interact with members of a tumour suppressor family known as Ras association family (RASSF) in the regulation of apoptosis (Matallanas et al., 2007).

### 1.2.4 MST2 in a tumour suppressor pathway

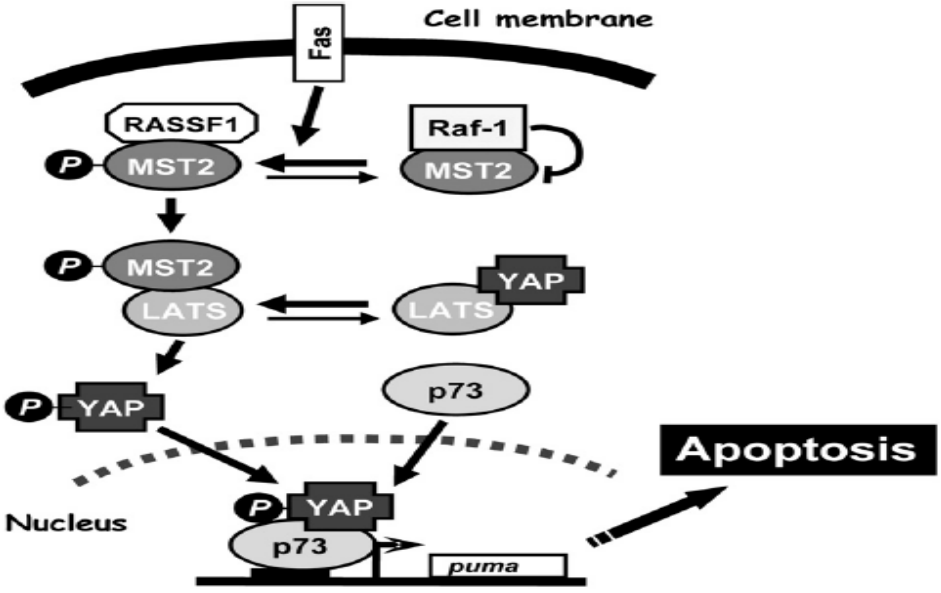
Mammalian sterile-20 (MST) kinases were first isolated as homologues of yeasts Ste20 kinases. They consist of a catalytic domain in the N-terminal region, followed by a non-catalytic region then an autoinhibitory segment and a coiled-coil domain that mediates dimerisation. MST1 (also known as Stk4 and Krs2) and MST2 (also known as Stk3 and Krs1) isoforms are very close homologues that are members of the class II germinal centre kinase (GCK) Ser/Thr kinases that promote apoptosis when overexpressed in mammalian cells (Creasy and Chernoff, 1995). Both homologues have been

demonstrated to be activated by stress and Fas signalling. They can activate the JNK stress response pathway either in a caspase-dependent or caspase-independent manner, and they may also translocate to the nucleus to phosphorylate histone H2B, resulting in DNA fragmentation (Jang et al., 2007; Radu and Chernoff, 2009). MST has also been described to inhibit the prosurvival AKT kinase. Conversely, MST1 is a substrate of AKT; it phosphorylates MST1 at S387 located at the C-terminal, blocking proapoptotic function and propensity for caspase activation (Guo et al., 2007). Recently, it was reported that in response to oxidative stress, MST1 can also induce apoptosis by phosphorylation and eventual activation of the transcription factor FOXO3a in neuronal cells (Lehtinen et al., 2006). The most widely known substrate of MST is the large tumour suppressor (LATS) kinase that belongs to the NDR/LATS subfamily of AGC (protein kinase A/PKG/PKC) which have been recently shown to regulate yes – associated protein (YAP1/2), a proapoptotic transcriptional regulator (Hergovich and Hemmings, 2009). LATS kinase is a tumour suppressor gene originally isolated from *Drosophila* and has also been identified in mice and humans (Zhang et al., 2008). In *Drosophila* LATS/*Warts* has been described as a central player in an emerging tumour suppressor pathway called the *Hippo* (MST 1/2 orthologue) pathway (Reddy and Irvine, 2008). All known components of the *Hippo* signalling pathway are conserved in vertebrates.

Mammalian homologues of Drosophila proteins in the Hippo pathway are as follows: FATJ for *fat*, NF2/Merlin for *merlin*, FRMD6 for *expanded*, RASSF1A for *RASSF*, MST1/2 for *hippo*, LATS1/2 for *LATS/Warts*, hWW45 for *Sav/Salvador*, MOB1A/B for *MATS*, and YAP for *yorkie* (Zhang et al., 2009). However, based on recent findings, not all mechanisms and connections seem to be conserved between *Drosophila* and vertebrates (Bao et al., 2011). It has been proposed that genes could have evolved and adapted to serve more complex requirements for signal transduction in mammals. However, discoveries made in *Drosophila* resulted in the elucidation of the core signalling mechanism of this MST/*Hippo* tumour suppressor pathway. Consistent with this core module, a mammalian counterpart has been reported (Mattalanas et al., 2007). Figure 1.D depicts a mammalian model of this well conserved MST2/*hippo*-LATS1/*Warts*-YAP1/*Yorkie* tumour suppressor pathway. It is noteworthy that unlike in *Drosophila* where upstream of *hippo* is *Merlin* and *Expanded*, upstream of MST2 is

RASSF1A and downstream *Yorkie* appears to be strictly anti-apoptotic while YAP1 functions are more diversified in mammalian cells (Mattalanas et al., 2007; Dong et al., 2007).

**Figure 1.D. A Schematic overview of the MST2/*Hippo* signalling pathway through RASSF1A-induced apoptosis.**




**RASSF1A-induced apoptosis through activation of Fas death receptor disrupts inhibitory Raf-1/MST2 complex by releasing MST2 and binding with RASSF1A. RASSF1A activates MST2 and promotes interaction with its substrate LATS1 leading to phosphorylation of YAP1 by LATS1. Upon phosphorylation of YAP1 it dissociates from LATS1 and directs its translocation to the nucleus forming a complex with p73 which induces the transcription of the proapoptotic *puma* gene (Matallanas et al., 2007).**

MST kinases also associate with other members of the RASSF tumour suppressor family through a leucine zipper-like motif known as SARAH (Sav-RASSF-Hpo) protein-protein interaction domain including the aforementioned RASSF1A (Hwang et al., 2007). There are 8 members of the RASSF family of proteins, which are RASSF1-8. RASSF1-6 contains a Ras domain (RA) of the Ral-GDS/AF6 type apart from the

SARAH domain at the C-terminus. Small GTPases such as Ras and Rap have been shown to interact with RA domains of the RASSF family of proteins (van der Weyden and Adams, 2007). Studies suggest that a number of Ras signalling molecules in association with additional apoptotic pathway protein complexes may play a role in proapoptotic MST signalling through RASSF tumour suppressors (Agathanggelou et al., 2005). RASSF1A and RASSF5 (also known as Nore1a, novel Ras effector 1) have been described to activate MST kinases by mutant Ras and Fas, respectively (Vos et al., 2000). RASSF1A is made of 340 amino acids and contains a cysteine-rich domain (CRD) similar to that of the CRD domain of Raf-1 which is absent in RASSF1C isoform. Both isoforms have been subjected to extensive studies, in contrast to other RASSF family members. RASSF1A is one of the most frequently silenced genes in a wide variety of human cancers (Sherwood et al., 2010). A number of studies have demonstrated that overexpression of RASSF1A induces apoptosis or cell cycle arrest (Song et al., 2005). In addition, it decreases tumourigenicity of several cancer cell lines (van der Weyden and Adams, 2007). Knockdown experiments by RNAi results in genetic instability and loss of cell cycle control including resistance to K-Ras and TNFα (tumour necrosis factor α) induced-apoptosis (Guo et al., 2007). It has also been observed that RASSF1A can regulate BAX (Bcl2-associated X protein) activity through binding to MOAP1 (modulator of apoptosis 1). BAX, a MOAP1 binding protein is a member of the Bcl2 (B cell CLL/ lymphoma 2) family that plays a major role in protecting against apoptosis (Baksh et al., 2005). Thus, RASSF1A has the ability to bind and coordinate multiple pathways (Richter et al., 2009). Intriguingly, RASSF1A lacks enzymatic activity, but has multiple binding partners, suggesting that it might function as a scaffold protein.

### 1.2.5 Connector enhancer of KSR (CNK)

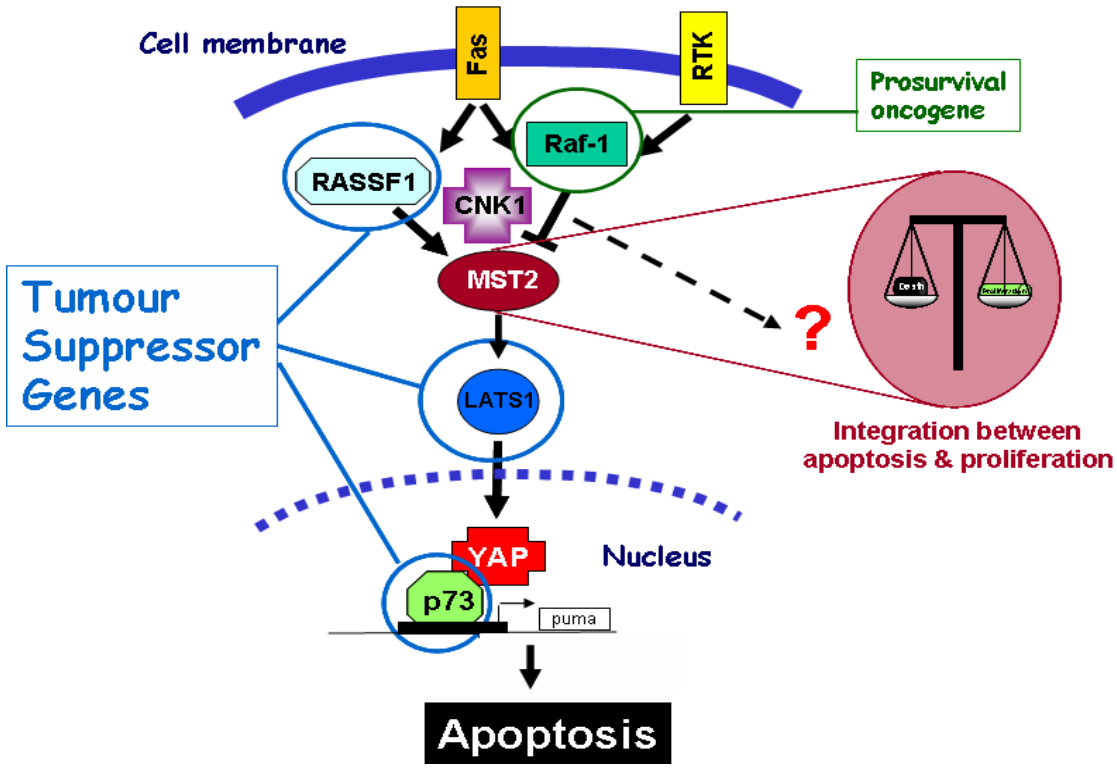
Interestingly, a scaffold protein known as CNK1 which has been described to activate Raf-1 through Src appears to be involved in the activation of MST kinases by RASSF1A and Ras (Ziogas et al., 2004). The interaction of RASSF and MST SARAH domains are

under the negative regulation of Raf-1 (Matallanas et al., 2007). But the exact mechanism of this interaction is still unclear. As depicted in Figure 1.E, given the role of CNK as a scaffold protein, it may possibly facilitate high local concentrations of RASSF and MST to enhance SARAH domain interactions. Another possible role of CNK is to exclude the negative signal of Raf-1, or it may facilitate Ras regulation of the Raf-MST complex. Fas stimulation also activates MST by removal of an inhibitory Raf-1 interaction (O'Neill et al., 2004). It is therefore of great interest to examine the mediation of CNK1 in reference to opposing physiological stimuli. How receptor signalling transmits signals to activate the MST signalling pathway either via localisation or protein interaction is still less understood. A proposed mechanism could involve compartmentalisation of effectors and adaptors to regulate such protein complexes, as it appears to be a common theme among established signalling pathways such as the ERK/MAPK pathway. For instance, the relationship of cell proliferation and apoptosis is immensely complex. Accumulating evidence details that nature has developed a mechanism to finely tune this regulation and this mechanism has been attributed to a specialised type of proteins that contain a multidomain structure and which are classified as scaffold proteins (Levchenko et al., 2000; Bhattacharyya et al., 2006).

The scaffold protein Connector enhancer of Kinase suppressor of Ras (CNK) was discovered in a genetic screen in *Drosophila* as an enhancer of the dominant negative rough-eye phenotype, which occurs due to the inhibition of photoreceptor development caused by a mutation in KSR (Therrien et al., 1998). CNK has no apparent catalytic motifs, however it has several protein: protein interaction domains. Four domains that indicate possible interaction sites with other proteins were identified. They comprise a sterile alpha motif domain (SAM) located at the N-terminus; a CRIC domain, a PSD-95/DLG-1/ZO-1 (PDZ) domain; and the pleckstrin homology (PH) domain in the middle of the protein. Although only a few SAM domain containing proteins have been characterised, they are found in a wide variety of proteins. SAM domains have distinct functions; they can homo or hetero-oligomerize, bind to RNAs, or associate with membrane lipids (Qiao and Bowie, 2005). The SAM domain of *Drosophila* CNK has been functionally studied. It was found to be essential for viability as well as Ras-mediated ERK activation through a novel protein called HYP (Hyphen)/AVE (Aveugle),

a step upstream of D-Raf and downstream of Ras. It was shown that the SAM domain on CNK and the SAM domain of HYP heterodimerise, recruit KSR (kinase suppressor of Ras)/MEK complex and activate D-Raf (Douziech et al, 2006). PDZ domains are also a prevalent class of domains that are often involved in scaffolding signal transduction complexes and localising proteins to the cytoskeleton, mostly through their ability to recognise and bind to short polypeptide motifs (X-S/T-X,-hydrophobic X, X-hydrophobic X-X-hydrophobic X, X-D/E-X-hydrophobic X) at the C-terminal of the target proteins (Sheng and Sala, 2001; Appleton et al., 2006).

**Figure 1.E. A proposed model where CNK1 may well be situated in the RASSF1A/MST2/Raf-1 signalling pathway.**




Growth factor stimulation restricts the binding of Raf-1 to MST2, while Fas receptor stimulation activates MST2 by removal of inhibitory Raf-1 in the presence of RASSF1A (Mattalanas et al., 2007). Exactly how this task is achieved is not clear. To add to this complexity, the mechanism parallel to Raf-1 or upstream of this signalling cascade is not well understood. Previously identified as an interacting protein of each of these proteins, the scaffold protein CNK1 may play a crucial role in the coordination of MST2 binding to the inhibitory Raf-1 or activating RASSF1A protein complexes.

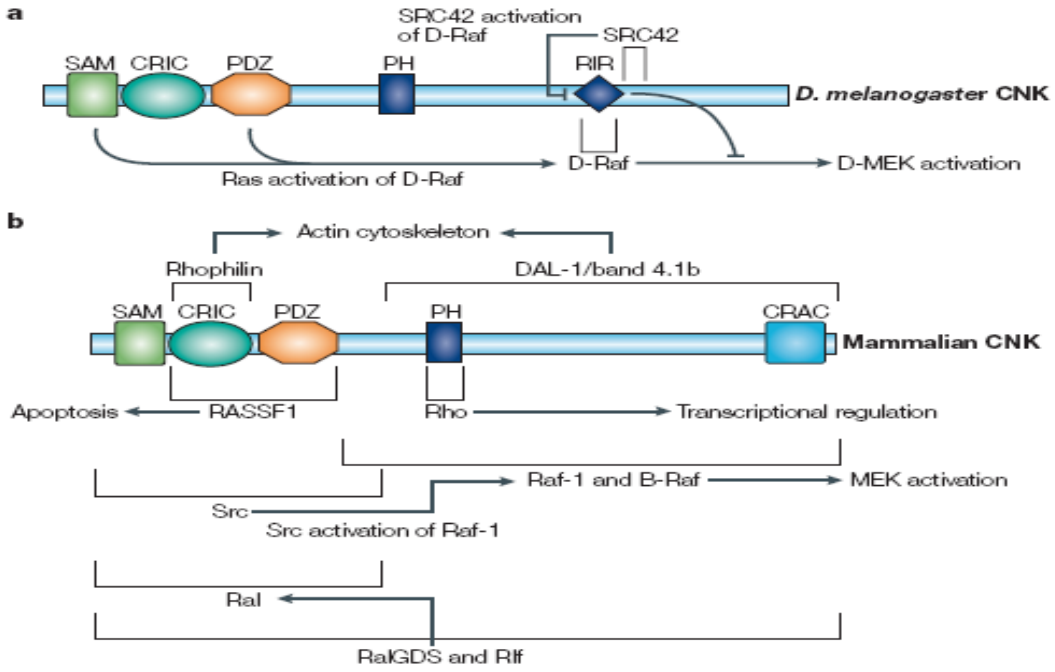
PH domains, like PDZ, domains generally function as regulated binding modules (approximately 100-130 amino acids) that are involved with many regulatory pathways (Lemmon, 2004). A diverse array of proteins contains PH domains. These include serine/ threonine kinases (such as AKT), phopholipase Cs, GTPases, adapter/scaffold proteins, cytoskeletal proteins and kinase substrates (Blomberg et al., 1999).

The PH domain of hCNK1 (human CNK1) has been found to interact specifically with the GTP-bound form of Rho (Jaffe et al., 2004). Human CNK1 overexpression of a mutant containing a single amino acid substitution (CNK W493A) in the PH domain (aa 404 to 504) abolishes Rho binding and inhibits Rho-dependent transcriptional activation. In addition to these three domains, a conserved region in CNK (CRIC) is described as another binding interface. The hCNK1 CRIC domain has been shown to associate with AT2 (Angiotensin 2) receptor, MLK 2/3 (mixed-lineage kinase) and Rhophilin (Fritz and Radziwill, 2005; Jaffe et al., 2004). The conserved region among chordate (CRAC) is found only in vertebrates and it has also been described as a binding site that is required for the interaction of IPCEF1 (Interaction protein for cytohesin exchange factors 1) with the coiled-coil motif of Cytohesin-2 (Venkateswarlu, 2003). The Raf inhibitory region (RIR) only exists in *Drosophila* (Therrien et al., 1998). The *Drosophila* CNK protein has a unique mechanism of Raf-1 regulation not found in mammalian CNK proteins. In *Drosophila*, CNK (1557 amino acids) mediates Ras activation of *D-Raf* through their SAM and CRIC domains, but inhibits *D-Raf* through the RIR motif in the C-terminus. In addition, binding of *SRC42* (Drosophila homologue of mammalian Src) to CNK relieves *D-Raf* from repression by RIR, enhancing *D-Raf* activity (Douziech et al., 2003).

This bimodal mechanism again is consistent with a typical scaffold property, which is integration of two opposing functions. CNK proteins also contain short motifs such as proline-rich and tyrosine residues that are within binding sites for consensus SH3 (Src homology 3) and SH2 (Src homology 2) domains respectively (Kolch, 2005). A schematic diagram of the multidomain structure of *Drosophila* CNK compared to mammalian CNK is shown in Figure 1.Fa and 1.Fb respectively. This structural organization indeed suggests CNK as a putative scaffold protein, and predicts an ability

to interact with several proteins and assemble various proteins into unique complexes to promote specific signalling responses. In contrast to *Drosophila and Caenorhabditis elegans*, mammalian CNK has three isoforms (Therrien, 1998). Namely; CNK1 which is widely expressed, CNK2 found mainly in neurons is also known as MAGUIN1 (membrane-associated guanylate kinase-interacting protein 1) that has shown to associate with Raf signalling complexes but independent of the ERK/MAPK pathway (Yao et al., 1999; Yao et al., 2000), and CNK3, which has yet to be functionally characterised.

**Figure 1.F. A schematic diagram of the multidomain scaffold protein CNK in (a) Drosophila and (b) mammals (modified from Kolch, 2005).**




Two variants have been identified for CNK3, namely CNK3A and CNK3B. CNK3B, also known as PIP-3E (Phosphoinositide- binding protein 3E), was identified to associate with IPCEF1 in a yeast two-hybrid screen (Krugmann et al., 2002). CNK2 appears to be the closest homologue of CNK found in fly and worm that encodes two isoforms with distinct C-termini. They are referred to as CNK2A and CNK2B. Unlike CNK2B, CNK2A ends with a PDZ binding motif (ETHV) that is necessary for interacting with Densin-180, PSD-95 and S-SCAM which suggests a role for the

assembly of a postsynaptic protein complex (Ohtakara et al., 2002). CNK2 also associates with Raf-1 and B-Raf isoforms. However, the mechanism of CNK2/ B-Raf interaction is not well understood. Also, CNK2 binds to Raf-1 but is unable to precipitate Ras, MEK, ERK or KSR which suggests that its function may not be restricted to the ERK/MAPK pathway, or that it could assemble components that do not interact with ERK/MAPK complexes (Lanigan et al., 2003). In addition, studies done on PC12 cells showed that CNK2 is required for ERK/MAPK activation by NGF (nerve growth factor) as well as neurite outgrowth, in contrast to EGF (epidermal growth factor (Bumeister et al., 2004).) However, the authors also showed that CNK2 might be also involved with actin remodelling by binding to a tumour suppressor protein, DAL-1/band 4.1b (differentially expressed in adenocarcinoma of the lung 1) but was independent of ERK/MAPK. DAL-1 is a member of the band 4.1 family of proteins implicated in regulation of cytoskeleton and plasma membrane organization (Tran et al., 1999). Therefore, CNK2 may be involved with Raf-1 in ERK/MAPK-dependent and ERK/MAPK-independent manner. CNK2 was also found to associate with RAL (Ras-like protein) GTPase and RLF (RalGDS-like factor) which is a member of the RALGDS (Ral guanine nucleotide dissociation stimulator) family of GEF (guanosine exchange factor) that activate RAL (Lanigan et al., 2003). However, CNK2 overexpression did not significantly activate RAL or cause RLF membrane localisation.

Although few studies have now addressed the role of CNK in mammalian cells, the most studied of all mammalian CNK isoforms is CNK1. Mammalian CNK1 binds Raf-1 and Src concurrently, which results in a trimeric complex that enhances Raf-1 activity and promotes phosphorylation at Y341, a Raf-1 activation site (Ziogas et al., 2005). It was demonstrated that CNK1 modulates the Src-dependent activation of Raf-1 in a concentration dependent manner typical of a scaffold protein. In addition, siRNA depletion of endogenous CNK1 interfered with Src-dependent VEGF (vascular endothelial growth factor) activation of ERK Clearly, CNK1 and CNK2 isoforms are implicated in RTK (receptor tyrosine kinase) induced Ras/ERK signalling indirectly or directly in association with Raf-1. In *C. elegans,* it was shown that CNK1 is not an essential component of Ras signalling, but could promote Raf activation as an accessory protein (Rocheleau et al., 2005). The group showed through genetic studies that CNK1 is downstream of Ras at a step leading to LIN-45 (abnormal cell LINeage-45; *C. elegans*

Raf-1 homologue) activation, consistent with data from *Drosophila*. Also, by using various activated forms of LIN-45, it was shown that CNK1 promotes activation of LIN-45/Raf-1 after dephosphorylation of the inhibitory sites and before phosphorylation in the kinase domain. Therefore, these data suggest a model in which CNK1 may promote Raf-1 membrane localisation, oligomerization or interaction with an activating kinase. Moreover, CNKs may have additional functions and phenotypes that have not yet been observed in model organisms dependent or independent of Ras/ERK signalling. For instance, human CNK1 has been identified as an effector of the Rho GTPase (Jaffe et al., 2004). Rho family GTPases act as molecular switches that regulate cellular responses such as cell transformation, cytoskeleton rearrangement and gene expression (Ellenbroek and Collard, 2007). Rho proteins cycle between an inactive GDP-bound and an active GTP-bound form. GTP loading is mediated by guanosine nucleotide exchange factors (GEFs). CNK1 was shown to interact with two Rho-specific GEFs (Net1, p115RhoGEF) and assemble components of JNK (c-Jun N-terminal kinases) signalling pathway that includes MKK7, MLK2 and MLK3 to activate JNK in a Rho-dependent manner (Jaffe et al., 2005). It was also found to associate with Rhophilin a Rho effector protein and RalGDS (Ral guanine nucleotide dissociation stimulator) Ral associated proteins are involved in membrane trafficking, transcriptional regulation and cell transformation (Jaffe et al., 2004). Although these interactions have been observed, the functional correlation remains to be elucidated.

Another CNK1 interacting protein, AT2 (Angiotensin II Type 2) receptor, has been found, but again no function has been demonstrated. AT2 receptor is a G protein-coupled receptor that antagonizes receptor tyrosine kinases to inhibit cell proliferation or promote neuronal differentiation and apoptosis (Fritz and Radziwill, 2005). Intriguingly, one of the most recent additions to this various array of CNK binding partners is RASSF1A. As mentioned above, RASSF1A is a tumour suppressor that is implicated in cell cycle arrest and apoptosis, which has been found to interact with CNK1 through MST1/2 (Rabizadeh et al., 2004). It was observed that CNK1 contributes to Ras-mediated apoptosis by its ability to bind RASSF1A and the proapoptotic kinases MST1/2. Thus, apart from Src-mediated activation of Raf-1, mammalian CNK is also involved in other signalling pathways by interacting with a variety of proteins which includes; RalGDS (Ral guanine nucleotide dissociation stimulator), RLF (RalGDS-like

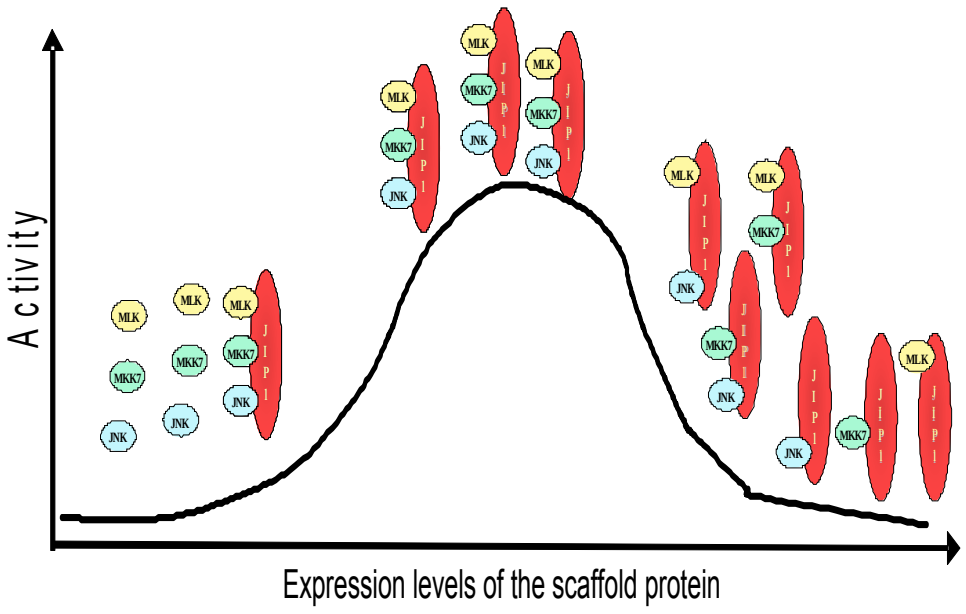
factor), Rhophilin and DAL-1/band 4.1b, a tumour suppressor which regulates the actin cytoskeleton, Rho-dependent JNK pathway, RASSF1A (Ras-association domain family 1) and MST2, which regulates apoptosis. Mammalian CNK therefore regulates at least four signalling pathways. Recently, it was demonstrated that CNK1 is also involved in PI3K/AKT (Lim et al., 2010), NFκB (Fritz and Radziwill, 2010) and AKT/FOXO signalling (Fritz et al., 2010). All together these findings suggest that CNK1 could mediate crosstalk between signalling pathways, indeed establishes CNK1 as a true scaffold protein. However, much has yet to be learned about its mode of action, including its functional interdependency. Hence, to determine whether the pathways are regulated independently and, if linked, whether they cross-talk in a combinatorial manner, will be an enormous endeavour. A proposed approach to tackle this challenge that will dramatically improve our ability to understand this molecular complexity is the application of mathematical computer modelling that enables a system-wide analysis of intertwined signalling networks (Heinrich et al., 2002; Orton et al., 2005; Kolch et al., 2005). More intriguingly, recent studies have also proven that exploiting experimentally the domain-based modular structures of scaffold proteins to generate synthetic signalling pathways, artificial control mechanisms and re-wiring gene expression is essential in elucidating the regulation of signalling pathways (Pryciak, 2009). Alternatively, it may translate to possible new directions for the future development of compounds or complexes to targeted-cancer therapy (Bhattacharyya et al., 2006; Pawson and Warner, 2007).

### 1.2.6 Perspective on scaffold proteins

Over the past several years, studies in signal transduction have revealed that intra- and extracellular signalling involves assembly of multiple protein complexes, specific localisation of proteins and dynamic regulation of kinases and phosphatases to carry-out intricate tasks within the cell (Morrison and Davis, 2003; Yoshioka, 2004). The mechanism of achieving this multifaceted function has been attributed to scaffold proteins. The localisation and compartmentalisation roles of scaffold proteins are now

widely accepted in cellular signalling, which has been demonstrated in the literature (Dhanasekaran et al., 2007). The concept and potential function of scaffolding proteins was postulated more than a decade ago through computational modelling. These theoretical simulations have been very helpful in understanding at least some of the many complex functions of scaffold proteins (Kolch, 2005). These proposed principles that govern scaffolding properties also provided conceptual guidelines in experimental work (Burack and Shaw, 2000). A typical signature of a scaffold protein is the manifestation of a non-monotonic behaviour (bell-shaped) of a signal output as a function of scaffold concentration in a titration curve. This phenomenon is often referred to as 'prozone-effect' or combinatorial inhibition (Ferrell, 2000). A schematic diagram of this behaviour is shown in figure 1.G. As depicted in the diagram, the relative stoichiometry of a scaffold to its client proteins can have an opposing effect on the signalling module. At low levels of scaffold as compared to its client proteins the binding of kinases to their substrate is inefficient, so signalling is below the optimum activation. When kinases and substrates are bound to scaffolds at an optimum stoichiometric level, they are tethered in nearest neighbour positions.

**Figure 1.G. Combinatorial inhibition of kinase activity by scaffold proteins.**



Moreover, scaffold protein can also orient and allosterically activate kinases; therefore signal enhancement is achieved (Pawson and Nash, 2003). However when the scaffold level is in excess in comparison to the kinases and substrates, non-functional kinase-scaffold/substrate-scaffold complexes are formed. Thus, signalling is impaired. At this high level of scaffold, it becomes an inhibitor of the signalling complex. A good example of this phenomenon has been demonstrated with the scaffold protein JIP-1 (JNK-interacting protein 1). In the JNK (c-Jun N-terminal kinase) cascade; MKK7, DLK, MLK3 and JNK associate with JIP-1. JIP-1 can enhance JNK activation but when overexpressed, it acts as an inhibitor (Whitmarsh et al., 2001). In the ERK/MAPK pathway, KSR-1 (kinase suppressor of Ras 1) scaffold protein also exhibits this behaviour. At an optimum level, KSR interacts with Raf-1, MEK and ERK. However, at the endogenous level, Raf-1 is difficult to immunoprecipate with KSR. At a higher level of KSR, ERK activity is inhibited. Moreover, in KSR-1 null fibroblasts ERK activation is severely compromised (Kortum and Lewis, 2004). Analogous observations have been made with other scaffold proteins such as IQGAP1 (Roy et al., 2005) and MORG1 (Vomastek et al., 2004). Mathematical models also predict that scaffold-mediated protein complexes can effectively modulate signal efficiency and specificity as well as the amplitude (Ferrel, 2000; Locasale et al., 2007). Indeed, recent evidence has validated some of these theoretical predictions of scaffold-mediated signal transduction. Although a number of studies have shown that scaffold proteins play an essential role in facilitating signal transduction, the specific mechanistic induction of dynamic scaffold-formation complex and disassembly is poorly understood. Moreover, there seem to be more questions arising from this ostensible mechanism that scaffold proteins exhibit, either through theoretical simulations or experimental approaches.

### 1.2.7 Aims of PhD

The overall objective of this thesis was to investigate a possible role of CNK1 in the regulation of MST2 and Raf-1. To determine whether CNK1 manifests a concentration-dependent property that is typical of a scaffold protein and to examine its role in mediating the agonist functions of proto-oncogene Raf-1 and tumour-suppressor MST2.

# CHAPTER 2

# Materials and Methods

# Materials and Methods

## 2.1 Materials

### 2.1.1 Antibodies

All anti-serum was used at 1:1000 unless stated otherwise here.

#### BD Transduction Laboratories

Anti-hCNK1 (1:500) (Mouse, Catalog number: 611734)
Anti-c-Raf-1 (Mouse, Catalog number: 610152)
Anti-PARP (Mouse, Catalog number: 556362)

#### Cell Signalling

Anti-P-Threonine (Rabbit, Catalog number: 9381)
Anti-phospho-MEK1/2 (Rabbit, Catalog number: 9121)
Anti-MEK1/2 (Rabbit, Catalog number: 9122)
Anti-Phopho-Raf S259 (Rabbit, Catalog number: 9421)
Anti-Rabbit IgG (HRP) (Goat, Catalog number: 7074)

#### Epitomics

Anti-MST2 (N-term) (Rabbit, Catalog number: 1943-1)
Anti-pMST2 (pT183/pT180) (Rabbit, Catalog number: 2111-1)

#### MBL

Anti-Fas/CD95 (Mouse, Catalog number: SY-001)

### Santa Cruz Biotechnologies

Anti-MST2 (C-19) (Goat, Catalog number: SC-6211)
Anti-Tubulin (1:2000) (Mouse, Catalog number: SC-8035)
Anti-LATS1 (G-16) (Goat, Catalog number: SC-12494)
Anti-Mouse IgG (HRP) (Goat, Catalog number: SC-2060)
Anti-Goat IgG (HRP) (Donkey, Catalog number: SC-2020)

### Sigma

Anti-phospho-ERK1/2 (1:20000) (Rabbit, Catalog number: M5670)

### Upstate

Anti-Myc (Mouse, Catalog number: 05-724)

## 2.1.2 Primers

### Beatson Institute Sequencing Service

CMV-F (5' CGC AAA TGG GCG GTA GGC GTG 3')
T7- (5' TAA TAC GAC TCA CTA TAG GG 3')

### Site-directed mutagenesis

**CNK1 S377A**
Forward (5'-3') TTG GTC GGA AGA AAG CAA AAG GCC TGG CGA CC
Reverse (5'-3') GGT CGC CAG GCC TTT TGC TTT CTT CCG ACC AA

**CNK1 S377D**

Forward (5'-3') AAG AGT CCT GTT GGT CGG AAG AAA GAT AAA GGC CTG GCG ACC
Reverse (5'-3') GGT CGC CAG GCC TTT ATC TTT CTT CCG ACC AAC AGG ACT CTT

### 2.1.3 RNAi

**Dharmacon**

5X siRNA buffer

Non-targeting control (NTC; D-001810-01)

Human CNK1 (SMARTpool M-012217-01-0010)

Human MST2 (J-J 004874-07)

Human Raf-1 (M-003601-00)

Human RASSF1A (J-017219-05-0010)

### 2.1.4 General Reagents and consumables

All reagents were purchased from Sigma-Aldrich unless stated otherwise here.

**Amersham Biosciences**
[$^{35}$S] methionine
PVDF membrane

**Becton Dickinson**
Insulin-like Growth Factor
15 ml conical tubes
50 ml conical tubes
Falcon 2054 tubes

Transfer pipettes

Tissue Culture plates

Tissue Culture flasks

**Biorad**

Western blot modules

Semi-dry transfer module

Precision Plus Protein Standards (Dual Color)

β-mercaptoethanol

**Calbiochem**

Nonidet P-40

**Costar**

6-well tissue culture plates

**Fisher Scientific**

Ethanol

Methanol

Glycine

Glycerol

KCl, $MgCl_2$, $CaCl_2$

$MgSO_4$, NaCl

SDS

**Fuji**

RX medical X-Ray Film

**Gibco**

Dulbecco's modified eagle medium

EDTA/ Trypsin

L-Glutamine

**Hyclone**

Foetal Bovine Serum

**Invitrogen**

Lipofectamine 2000 reagent

TOP10 Oneshot competent cells

Novex XCell SureLock Mini-Cell Module system

NuPAGE® 4-12% Bis-Tris Gels

NuPAGE® Running Buffer

NuPAGE® Transfer Buffer

**Kodak**

Ammonium Persulfate

Kodak medical X-Ray film

**Melford Laboratories**

Tris Base

**Molecular Probes**

Propidium Iodide

**New England Biolabs**

Calf Intestine alkaline phosphatase

**Promega**

Caspase 3/7-Glo Assay Kit

TNT Quick Coupled Transcription/Translation Systems Kit

**Promocell**

Cryo-Serum Free Media

**Promokine**

Epidermal Growth Factor

**Qiagen**

RNAseA

Restriction enzymes, BamH1 and Ecor1

QIAprep Miniprep Kit

QIAGEN Plasmid Maxi Kit

QIAEX II Agarose Gel Extraction Kit

**R&D Systems**

Human TGF-ß1

**Roche**

DNA Ligation Kit

Western Blocking Buffer

Complete Protease Inhibitor cocktail tablet

PhosSTOP Phosphatase Inhibitor cocktail tablet

BM Chemiluminescence substrate POD

**Severn Biotech Ltd.**

30% Acrylamide

**Stratagene**

QuickChange II Site-Directed Mutagenesis Kit

XL Blue competent cells

**Thermoscientific**

Microplate 96 well optical flat bottom plate

Cryo Freezing microtubes

Tissue culture flasks

**Whatman**

3MM Chromatography paper

### 2.1.5 General Solutions/Buffers

**PBS**
137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4$
1.47 mM $KH_2PO_4$
(Adjust to pH 7.4 if necessary with HCl or NaOH)

**TBST**
137 mM NaCl
20 mM Tris base
0.1 % (v/v) Tween-20
(Adjust to pH 7.6 with concentrated HCl)

**LB-medium**
1.0 % Bacto-Tryptone
0.5 % Bacto-yeast extract
1.0 % NaCl
(Adjust pH to 7.5 with 5M NaOH if necessary, Autoclaved)

**TE buffer**
1mM EDTA
10 mM Tris-HCl pH 7.4

**PBT**
0.5%BSA
0.1% Tween-20
PBS buffer pH 7.4

**NuPAGE MOPS running buffer**
50 mM MOPS
50mM Tris base
0.1% SDS
1 mM EDTA
(pH 7.7)

**NuPAGE transfer buffer**
25 mM Bicine
25 mM Bis-tris (free base)
1 mM EDTA
(pH 7.2)

# Methods

## 2.2 Cell Culture

### 2.2.1 Growth conditions

HeLa cells and MCF-7 cells were maintained in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% (v/v) Foetal Bovine Serum (Hyclone) and 2mM L-Glutamine (Invitrogen/ Life Technologies). Cells were grown in a humidified atmosphere containing 5% $CO_2$ at 37ºC. Cells were passaged when sub confluent (~80%) using buffered trypsin (0.05% trypsin, 0.02% EDTA, obtained from Gibco) and diluted with fresh media at a ratio of 1:3. Cell culture was performed using aseptic technique and sterile equipment and reagents in a class II hood.

### 2.2.2 Cell storage

Cells were stored by cryo-freezing. Trypsinised cells were resuspended in cryo preserving medium (Cryo-SFM, Promocell) at a concentration of 1-4 X $10^6$/ml. Cell suspension was divided into aliquots and frozen at -80ºC. Cells were transferred to liquid nitrogen for extended storage. Thawing of cells for use was performed by placing the cryo-vials in a 37ºC water bath until the frozen suspension is close to melting and swirled slowly by hand until cell suspension is completely thawed. Vials were sprayed briefly with 70% ethanol to avoid contamination then cells were transferred in a culture flask with fresh pre-warmed media, placed in the incubator overnight and the medium replaced to remove floating dead cells.

## 2.3 Cell Counting

### 2.3.1 CASY 1 Cell Counter

Cell numbers were determined using a CASY 1 TT cell counter and analyzer (Schärfe system, GmbH). Prior to counting, a cleaning cycle was performed with Phosphate

buffered saline to clear carry-over background. Using the cell counter appropriate cup (CASYcup), 400 µl of sample was used and diluted to a volume of 10 ml with PBS. Samples were run 3X and reported as number of cells per ml.

### 2.3.2 Haemocytometer

Cells were diluted as appropriate with 0.4% Trypan blue solution (Sigma) in total of 200 µl. Cell suspension was pipetted (approximately 10 µl) at the edge of the cover-slip and allowed to run onto the haemocytometer. Excluding dead cells (stained with trypan blue), the average number of cells in one large square x dilution factor x $10^4$ = number of cells per ml.

## 2.4 Transfection of Cells

### 2.4.1 Plasmid DNA Transfection

HeLa cells and MCF-7 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Cells were seeded out ($2x10^6$ per 100 mm plate) and left to adhere overnight. DNA (0-1.5 µg) and Lipofectamine 2000 were mixed separately in serum-free media before being combined to a total of 1 ml per 10 cm plate. Combined DNA and the transfection reagent were incubated at room temperature for 20 minutes to allow complexes to form. Meanwhile, cells were washed once in PBS and 5 ml of serum-free media was added. Complexes were then added drop wise to the cells and left to incubate for 4-5 hours at $37^0$C. Following incubation cells were then washed and fresh complete media was added. After 24-48 post-transfection cells were washed with PBS and either lysed or incubated in serum-free media for 16-24 hours prior to stimulation.

### 2.4.2 RNA interference (RNAi)

HeLa cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Cells were seeded out ($1x10^6$ per 60 mm plate) and grown overnight. Short RNAi oligomers were diluted with appropriate buffer (siRNA

Dharmacon buffer) to a stock of 20 μM stock. SiRNA and Lipofectamine were mixed separately with serum-free media for 5 min at room temperature. Combined siRNA-Lipofectamine master mix to a total of 500 μl was incubated for 20 minutes. Cells were washed and 2.5 ml of serum-free medium was added. Following incubation, siRNA master mix was added drop wise to each plate (total siRNA 100-140 pmol/ plate) and incubated for 5 hours at $37^0$C. After incubation, the medium was changed to fresh complete media. After 24h-post transfection cells were washed with PBS and either lysed or incubated in serum-free media for 24 hours prior to stimulation.

## 2.5 Propagation and Preparation of plasmid DNA

### 2.5.1 Transformation of competent cells

50-100 μl of competent *E. coli* were thawed on ice and 0.5-1.0 μg plasmid-DNA was added. Cells were incubated for further 30 minutes, heat-shocked at 42°C for 45 seconds and immediately put back on ice for 2 minutes, and 1 ml of LB-medium was added and cells were shaken at 37°C for 1 hour. Thereafter, 100 μl of the suspension were plated on an antibiotic-containing LB-plate and incubated overnight at 37°C. Colonies were picked from plates and used in overnight cultures for DNA purification using the Qiagen plasmid miniprep or maxiprep kits.

### 2.5.2 DNA purification and verification

Plasmid Kits (Maxi or Mini) to isolate DNA are used prior to sequencing for verification (primers are listed on Chapter 2.1). Briefly, an alkaline lysis of cells is followed by the binding of the plasmid-DNA to an anion-exchange resin while the supernatant passes through by gravity flow. RNA, proteins and other impurities are removed in salt washes and the purified DNA is eluted with high salt conditions. To precipitate the DNA, ice-cold 100% isopropanol was added and centrifuged. The supernatant was discarded and the DNA pellet washed with 70% ethanol and air-dried. DNA is resuspended in sterile distilled $H_2O$ or TE buffer (10 mM Tris-HCl pH 7.4, 1mM EDTA) and stored at 4ºC.

Sequencing to verify expression plasmid was performed by the Beatson Institute for Cancer Research, Glasgow, UK. DNA concentration was determined by measuring absorbance at 260 nm.

### 2.5.3 Subcloning of Plasmids

The plasmids used were gifts from Dr. David Mattalanas (pCDNA3.1 HA-RASSF1A, pcDNA3.1 Flag-MST2, pRK5 Myc-CNK1/ pRK5 empty vector), Dr. David Romano (pCDNA3.1 mRFP-Raf-1) and pCDNA3.1 vector (Ms. Joan Grindlay) from Beatson Institute, Glasgow, UK/ University College Dublin/ Conway Institute, Ireland). Plasmid pRK5Myc-CNK1 was subcloned to pCDNA3.1 vector for *in vitro* Transcription/ Translation. To create an expression vector for the production of pCDNA3.1-CNK1, CNK1 was removed from pRK5 using flanking restriction sites BamH1-EcoR1. The restriction digest from vectors was run on an 1% agarose gel and the resulting cDNA fragment was isolated using an Agarose Gel Extraction Kit (QIAEX II, Qiagen) as directed by the manufacturer. Ligation of DNA was performed by mixing vector DNA (pCDNA3.1), insert DNA (CNK1), ligation buffer and T4 ligase both from Promega. Ligation reaction mixture was incubated for 2 hours at room temperature. The construct was transformed into *E-coli* OneShot TOP10 (Invitrogen) competent cells. Overnight cultures were prepared from clones and plasmid mini or maxi preps were carried out. Constructs were verified by sequencing using pCDNA3.1 specific primers (T7 sequence in Chapter 2.1).

## 2.6 In vitro Transcription-Translation

Proteins were synthesised *in vitro* using a TNT Quick Coupled Transcription/Translation Kit (Promega) following the manufacturer's protocol. TNT Quick Master Mix was rapidly thawed by hand and placed on ice. Components of TNT Reactions were all assembled on ice. In a reaction tube, a mixture of 40 µl of TNT T7 Quick Master Mix, 2 µl of 1 µg plasmid DNA and 2 µl of [$^{35}$S] labelled-methionine were added to a total

volume of 50 µl with nuclease-free water. TNT Reaction mixture was gently mixed and incubated at 30ºC for 90 minutes. 5-10 µl of each sample was analysed by SDS-PAGE. Subsequently, the gel was dried and visualised by autoradiography.

## 2.7 Peptide Array

Cellulose membrane spotted with overlapping synthesised 25-mer peptides from human CNK1, off-set by five amino acids were obtained from Prof. Miles Houslay (College of Medical, Veterinary and Life Sciences, University of Glasgow). The array was blocked in 5% BSA for 1 hour at room temperature. Radioactive labelled protein (by *In vitro* Transcription/ Translation) was added on the peptide array and incubated in a rotator for 2 hours at room temperature. After incubation, the blot was washed 4-5X with TBST for 5 minute each wash. Finally, the peptide array was exposed on film. To be re-probed, the blot was stripped using a buffer containing 20mM DTT, 70mM SDS and 70mM Tris (pH 6.8) for 1 hour.

## 2.8 Site-directed Mutagenesis

Mutant forms of CNK1 were synthesised using a QuickChange II site-directed mutagenesis PCR kit from Stratagene according to manufacturer's instructions and with primers listed in Chapter 2.1. Briefly, a reaction mixture of DNA template, oligonucleotide primers, dNTP mix, DNA polymerase, reaction buffer in sterile distilled water was prepared to a volume of 50 µl. PCR programs used are as follows: 95ºC 30 sec, 55ºC 1 min, 68ºC 15 min and cycle was repeated 15X. After PCR, the reaction samples were digested with Dpn I restriction enzyme for 1 hour at 37ºC. DNA from each sample was transformed (see Transformation of competent cells), purified and was confirmed for the presence of mutation by DNA sequencing.

## 2.9 Western Blotting

### 2.9.1 Preparation of Cell Lysates

Incubated cells (transfected and/or treated/ untreated) were washed once with ice-cold PBS and lysed in buffer containing 20mM HEPES, 0.5mM EGTA, 0.5mM EDTA, 1% NP40 (v/v) and 150mM NaCl supplemented with protease and phosphatase inhibitors (1 mM PMSF, 2mM NaF, 1mM sodium orthovanadate, 2.2 µg/ml aprotinin, 2mM sodium pyrophosphate, 5 µg/ml leupeptin, 10mM β–galactosidase) or Complete protease inhibitor cocktail tablet/ PhosSTOP phosphatase inhibitor cocktail tablet from Roche. Lysed cells were scraped using a cell scraper and transferred to 1.5 ml microcentrifuge tubes on ice and vortexed. The microcentrifuge tubes were centrifuged at 14000 rpm at 4 °C for 8 minutes. The supernatants were transferred to a fresh microcentrifuge tube and either resolved by SDS-gel electrophoresis or immunoprecipitated.

### 2.9.2 SDS-PAGE

Proteins from lysates and immunoprecipitates were resolved in denaturing sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels according to molecular weight. Either pre-cast Invitrogen NuPAGE Novex 4-12 % Bis-Tris gels in a Novex XCell SureLock Mini-Cell Module system along with reagents (4X NuPAGE® LDS Sample buffer, MOPS Running Buffer) was used or manually made gels using Amersham Pharmacia mini-gel caster [10% acrylamide (v/v), 2mM EDTA, 0.33mM Tris pH 8.9, 0.07% ammonium persulphate (w/v), 0.1% TEMED (v/v)]. Distilled water was pipetted to cover the gel. Once the gels were set, excess water was removed using Whatman 3MM paper. 5% acrylamide stacking gels [5% acrylamide (v/v), 125 mM Tris pH 6.8, 4mM EDTA, 0.1% SDS (w/v), 10 % ammonium persulphate (w/v)] were poured on top of the polymerized gel, a comb inserted and allowed to set. Prior to loading of samples, 5X Laemmli sample buffer [62 mM Tris pH 6.8, 2% SDS (w/v), 10% Glycerol (v/v), 0.1% bromophenol blue (w/v), 0.5M DTT] was added to the sample and boiled (100 °C) for 5 minutes to denature protein. The precision plus dual colour protein standard from BioRad was used to estimate molecular weights of the proteins of interest. Electrophoresis was performed in 1X SDS-PAGE running buffer (25mM Tris, 192 mM

glycine, 0.1% SDS w/v) at an initial voltage of 100V. Once bromophenol blue dye had passed through the stacking gel, the voltage was set to 140V for 1 to 1.5 hours.

### 2.9.3 Immunoblotting

Following resolution of proteins by electrophoresis, proteins were transferred to a PVDF membrane (Hybond-P, Amersham Pharmacia) using an Invitrogen Novex XCell II Blot Module system along with reagents (NuPAGE® Transfer Buffer) or Biorad Transblot electrophoretic transfer cell system. The membrane was soaked in methanol and placed on the gel sandwiched with Whatmann 3MM paper and sponge and set in a transfer blotting cassette. Transfer was carried out in 1X Transfer buffer [200 mM Glycine, Tris 25 mM, 20% methanol (v/v), 0.01% SDS (w/v)] at 30V for 2 hours. After transfer, membranes were blocked with 5% Roche blocking buffer in 1X TBST for 1 hour at room temperature with gentle rotation. The membrane was then incubated with relevant primary antibodies for 2 hours at room temperature or overnight at 4 °C diluted with blocking buffer. The primary antibodies utilised for western blotting are listed in Chapter 2.1. The membranes were then washed 3X with TBST for 10 minutes each to remove excess primary antibody and incubated with appropriate horseradish peroxidise-conjugated secondary antibody (1:10,000 dilution with TBST) for 1 hour at room temperature. After 3X 10 minute washes with TBST, bands were detected using enhanced chemiluminescence (ECL) method and exposed to Fuji X-Ray Film. To re-probe, membranes were stripped with stripping buffer [0.2M glycine pH 2.5, 1% SDS (w/v)] or Roche stripping buffer for 30 minutes and re-blocked before incubating with another primary antibody.

## 2.10 Immunoprecipitation

Lysates were obtained as described above. A small sample (5%) of lysate was set aside to resolve on a gel. The remainder of the lysates were transferred to a separate eppendorf tube and kept on ice. Meanwhile, protein G sepharose beads (Sigma) were washed 2X with lysis buffer and resuspended to 50% slurry with lysis buffer. Lysates were precleared with 50 µl of the 50% slurry and rotated for 30-60 minutes at 4°C. After

preclearing, beads were pelleted by centrifugation at 14000 rpm for 45 seconds at 4°C. Precleared lysates were transferred into new sample tubes and 1-2 µl of antibody were added to each tube and rotated at 4°C for 3 hours. After the incubation, 30 µl of the 50% slurry beads were added and rotated for another 1 hour at 4°C. After the incubation, the beads were pelleted by centrifugation at 12000 rpm for 45 seconds at 4°C. The beads were then subjected to three washes with lysis buffer (beads pelleted at 12000 rpm for 45 seconds at 4°C). After aspiration of last wash, bead bound immunoprecipitates were released by the addition of equal volume of 5X sample buffer [62 mM Tris pH 6.8, 2% SDS (w/v), 10% Glycerol (v/v), 0.1% bromophenol blue (w/v), 0.5M DTT] or 4X NuPAGE® LDS Sample buffer. Samples were heated to 100°C for 5 minutes and analysed by SDS-PAGE and Immunoblotting.

## 2.11 In-Gel kinase Assay

MST2 immunoprecipitates were separated by SDS PAGE co-polymerised with 0.5mg/ml myelin basic protein (MBP). To control for autophosphorylation, samples were resolved in parallel on gels not containing MBP. Resolving gel was placed in 50 ml of SDS removal solution [20% 2-propanol (v/v), 50 mM Tris pH 8.9], agitated gently for 20 minutes, 3X at room temperature. Succeeding steps were all performed at room temperature unless specified. Gels were washed 3X in a solution of 50 mM Tris pH 8.0 with 5 mM β–mercaptoethanol for 20 minutes. The proteins were denatured by incubating the gel in 50 ml of denaturation buffer (6M guanidine HCl, 50 mM Tris pH 8.0, 5 mM β–mercaptoethanol) for 1 hour. The proteins were renatured by incubating the gel in 50 ml of renaturation buffer [50 mM Tris pH 8.0, 5 mM β–mercaptoethanol, 0.04% Tween-20 (v/v)] for 60 minutes at 4°C 2X. The renaturation buffer was replaced with fresh renaturation buffer and left at 4°C agitated overnight. The following day, the gel was washed with 40 mM HEPES pH 8.0, 10 mM $MgCl_2$ with 2 mM DTT for 1 hour at room temperature. Phosphorylation reaction was carried out by incubating the gel in 10 ml of 40 mM HEPES pH 8.0, 10 mM $MgCl_2$, 0.5 mM EGTA, 50 µM ATP and 25 µCi [$\gamma$-$^{32}$P]ATP for 2 hours at room temperature. The gel was washed 10X to stop the reaction with a solution containing 5% trichloroacetic acid (w/v) and 1% sodium

pyrophosphate (w/v). Finally, the gel was dried onto Whatmann 3MM and analyzed using a phosphorimager.

## 2.12 Fluorescence Activated Cell Sorting (FACS)

Cells were grown, transfected and treated as required. The cell culture medium containing detached cells was collected in a 15 ml conical tube. Adherent cells were trypsinised [0.05% trypsin (v/v), 0.02% EDTA v/v), in DMEM, obtained from Gibco] and resuspended in fresh growth medium and combined with the medium from the same sample. A small aliquot of this cell suspension was run in a CASY 1 Cell Counter to determine relative cell number on each sample. The remaining cell suspension was centrifuged (1000 rpm for 7 minutes and washed once in ice-cold PBS and resuspended.in 200 µl PBT [(PBS pH 7.4, 0.5%BSA (v/v), 0.1% Tween-20 (v/v)]. Cells were fixed with the addition of 1.8 ml of 4°C 70% ethanol while slowly vortexing and were incubated for at least 1 hour or overnight at 4°C. After fixing, cells were centrifuged and supernatant were discarded. Cells were incubated with 300 µl of staining solution containing 250 µg/ml of RNAse (Qiagen) and 10 µg/ml of propidium iodide (Molecular Probes) for 30 minutes at room temperature in the dark. After incubation, cells were pelleted and resuspended with PBT. Cells were wrapped in aluminium foil to protect from light. Analysis was performed on a Becton Dickinson FACScan.

## 2.13 Caspase Assay

The caspase assay was employed to measure caspase activity or apoptotic cells using a Caspase-Glo 3/7 assay from Promega that uses a luminogenic substrate containing the DEVD sequence. Cells were grown and transfected in a six well plate. Detached and undetached cells were collected and washed once with cold PBS. Cells were resuspended with PBS and aliquot of cell suspension were counted. 100 µl 1.5 X $10^4$ of the cell suspension and PBS (blank) was transferred to a white-walled 96-well plate. 100 µl of Caspase-Glo 3/7 reagent was added and incubated at room temperature for 2 hours.

The luminescence of the samples was measured using a luminometer. The luminescent signal is directly proportional to the caspase activity of the cells.

## 2.14 Data and Statistical Analysis

Data are presented as mean +/- standard error of the mean (SEM) unless otherwise indicated. Student's test was used for comparisons. Data analysis was performed using Microsoft Office Excel. To quantify the bands obtained from Western blot analysis, ImageJ software based analysis (http://rsb.info.nih.gov/ij/) was used.

# CHAPTER 3

# Scaffold property of CNK1 in the Hippo Tumour Suppressor Pathway

## 3.1 Introduction

The assembly of signalling molecules, proteins and kinases into a complex by a scaffold protein guides an array of cellular decisions. One of the classical properties of a scaffold protein is the ability to confer combinatorial inhibition. A simpler model of this phenomenon is schematically described in figure 3.A. As described in the previous chapter, this phenomenon is also called the prozone effect. This phenomenon stems from the precipitin reaction test widely used by immunologists, where increasing amounts of antigens added into a series of equal amounts of antibodies results in a maximum precipitate when an optimum antigen-antibody concentration is achieved, and then declines at higher antigen titre (Bray and Lay, 1997). Because of the three-dimensional lattice of antigen and antibody molecules, it has been posited that a similar effect may occur in multimeric protein complexes such as scaffold proteins in cells (Ferrell, 2000; Levchenko et al., 2000).

Indeed, insights from computational modelling have provided indispensable conceptual recommendations for experimentalists (Endy and Brent, 2001). However, although it is difficult to test most of the concepts, progress has been made over the past decade and one of the first and best studied examples of the complexity of scaffold protein is Ste5p of the yeast *Saccharomyces cerevisiae;* which is also the most characterized proto-typical scaffold (Flatauer et al., 2005; Park et al., 2003; Harris et al., 2001). Studies of Ste5p established scaffold proteins as physiologically important regulators that bind multiple signalling components of the three-tiered mitogen-activated protein kinase (MAPK) cascade in yeast (Elion et al., 2005; Morrison and Davis, 2003). Of note, the term scaffold protein and adaptor protein are sometimes interchanged to describe proteins that function to bind proteins together. In the literature however, adaptor proteins are defined as proteins that have a rather limited function, that is to bind partners together and localized in specific cellular space (Buday and Tompa, 2010). Scaffold proteins, on the other hand, are larger multidomain proteins that assemble several components of a signalling cascade such as the MAPK modules for efficient transit of signals for proper responses to extra- and intracellular stimuli (Burack and Shaw, 2002).

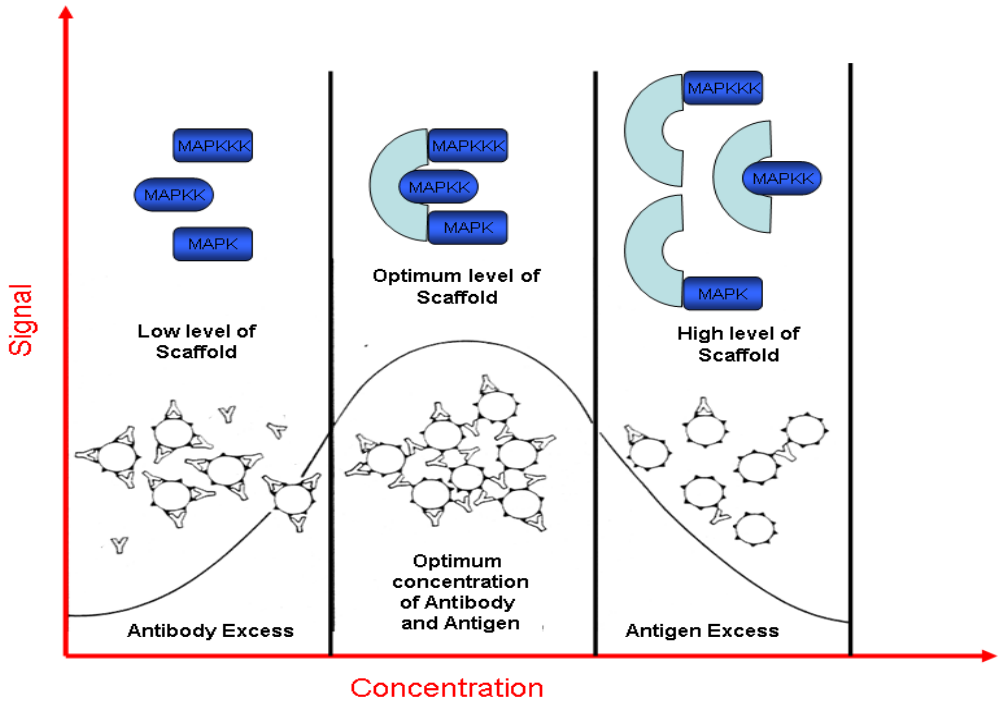



**Figure 3.A**. **Prozone effect in comparison to combinatorial inhibition.** The interaction of antigen and antibody results in lattice formation and production of clumps or agglutination thus precipitates as there is equivalence or optimum level of antigen and antibody concentration. From the precipitin curve, antigen or antibody excess results in lesser or absence of precipitate. This occurs as the Ag-Ab complex is unable to link and form large aggregates that are visible as precipitates. The lack of agglutination at high concentrations of antibodies is referred to as the prozone effect (Bray and Lay, 1997). Meanwhile, combinatorial inhibition refers to weakening of signal transmission due to dilution of pathway components. Given that the function of scaffold protein is to assemble interacting proteins together, such those of the MAPK cascades, the amount of scaffold requires it be titred at optimum levels of the components to which it binds. When scaffold concentration exceeds that of the interacting proteins it coordinates, the output of the signalling cascade decreases (Levchenko et al., 2000; Ferrell, 2000). It is this biphasic effect of scaffold protein via combinatorial inhibition that is analogous to the prozone effect of antigen-antibody reaction, hence the appearance of the signature "bell-shaped" curve.

As the MAPK signalling pathway is conserved from yeast to humans, several data suggest that scaffolding proteins similar to Ste5 are likely to play an important role in organizing MAPK modules in mammalian cells as well (Dard and Peter, 2006). Indeed, mammalian cells appear to employ, at least in part, a similar mechanism to ensure efficient complex modes of activation of mammalian MAPK cascades including several other non-MAPK-based signalling pathways to execute crucial and diverse cellular functions including proliferation, differentiation and apoptosis (Morrison and Davis, 2003; Yoshioka, 2004; Zeke et al., 2009). However, the number of scaffolds and interacting partners that are emerging will likely impose further complexity to the regulation of MAPK signalling. In addition, the complexities of scaffold proteins are further increased by the functioning of super-scaffolds such as MORG1, which connect different scaffolding complexes forming supra-scaffolded protein complexes (Vomastek et al., 2004; Kolch, 2005). Therefore, complex mechanisms involving pathway integration may also be attributed to scaffold proteins as well. Indeed, cross-talk between downstream components of opposing EGFR signalling and the Hippo pathway has been demonstrated (O'Neill et al., 2004; Mattalanas et al., 2007). As discussed in detail in chapter 1, Raf-1 a main component of the EGFR signalling pathway, and MST2 of the apoptotic Hippo pathway was suggested to confer a crosstalk model. The Hippo pathway originating from the RASSF1A tumour suppressor in association with MST2 activates its substrate LATS1, consequently activating downstream components leading to p73-mediated transcription of *puma* that result in apoptosis (Mattalanas et al., 2007). However, MST2 which is at the core of this pathway was shown to be negatively regulated by the proto-oncogene Raf-1.

Common to this central hub of the pathway, that is Raf-1, and the tumour suppressors RASSF1A and MST2 kinase, is their association with CNK1. As previously discussed in Chapter 1, CNK1 modulates the tyrosine kinase Src-dependent activation of Raf-1 in a concentration dependent manner typical of a scaffold protein and that B-Raf also binds CNK1, although the significance of this interaction is unknown (Ziogas et al., 2005). CNK1 was also reported to support Ras-mediated apoptosis by its ability to bind the tumour suppressor RASSF1A and the pro-apoptotic kinases MST1/2 (Rabizadeh et al., 2004). As aforementioned, upon serum-starvation Raf-1 associates with MST2 to inhibit its activity and its association with RASSF1A independent of Raf-1's kinase activity

(O'Neill et al., 2004; Mattalanas et al., 2007). Recently, it has been demonstrated that Akt inhibits MST2 activity and promotes its interaction with Raf-1, while RASSF1A overexpression disrupts their interaction (Romano et al., 2010). In addition, other RASSF family proteins such as RASSF5/6 regulate MST kinases differently as well (Ikeda et al., 2009; Pfeifer et al., 2010). MST2 cleavage and activation are inhibited by IGF1 through the PI3K/Akt pathway (Kim et al., 2010). Alternatively, an unexpected positive role of MST2 in the Raf-1/ ERK pathway has been described (Kilili and Kyriakis, 2010). Collectively however, the underlying mechanism of Raf-1:MST2 complex is not yet clear. Indeed, the upstream regulation of these two opposing kinases or in-parallel of the Hippo pathway is quite complex and the integration and inducers of the Raf-1:MST2 complex in a paradoxical function has yet to be investigated. The experiments described in this chapter were carried out in an attempt to examine the dynamics of the Raf-1:MST2 complex. Herein, I identified the scaffold protein CNK1 as a mediator in the formation and dissociation of this complex and demonstrated a possible role of CNK1 in the Hippo pathway apoptotic signalling by its association and regulation of its core components.

## 3.2 Results

### 3.2.1 CNK1 mediates MST2 and Raf-1 interaction upon serum starvation in a concentration-dependent manner

To demonstrate the proto-typical "bell-shaped" signature of a scaffold protein, it is usually accomplished by perturbing endogenous levels by overexpression of the particular scaffold and observing a specific pathway output or association of relevant signal transduction cascade component. Therefore, a Myc-tagged CNK1 expression plasmid was used to transiently increase basal cellular levels of CNK1 into the human cervical cancer cell line, HeLa cells, obtain expression of low and high levels of CNK1 and determine whether ectopically expressed CNK1 can modulate Raf-1:MST2 complex. Serum deprivation of several cancer cell lines including HeLa cells enhances formation of the Raf-1:MST2 complex, preventing MST2 activation and pro-apoptotic signalling (O'Neill et al., 2004; Mattalanas et al., 2007; Romano et al., 2010). As previously mentioned CNK1 has been implicated to interact with MST2 and Raf-1 in separate studies and demonstrated in several human carcinoma cell lines (Rabizadeh et al., 2004; Ziogas et al., 2005). However, the requirements for this trimeric interaction have not yet been characterised.

In the light of these observations, it is possible that protein scaffold CNK1 is involved in this mechanism. Therefore, I assessed whether CNK1 has the ability to manifest scaffold behaviour when complexed with its interacting proteins in a dose-dependent fashion under this condition. To obtain different expression levels, we transiently overexpressed Myc-tagged CNK1 expression plasmid in an increasing amount (0.1-1.5 μg). As a control, a corresponding empty vector was used. HeLa cells were grown overnight in serum-containing medium prior to 16-20 hours of serum-deprived culture medium. Following serum starvation of 24h post-transfected HeLa cells, endogenous Raf-1 and MST2 proteins were immunoprecipitated and examined by western blotting for their ability to interact and form a complex when different levels of Myc-CNK1 are expressed. To monitor the different levels of Myc-CNK1 expressed in the cells, total

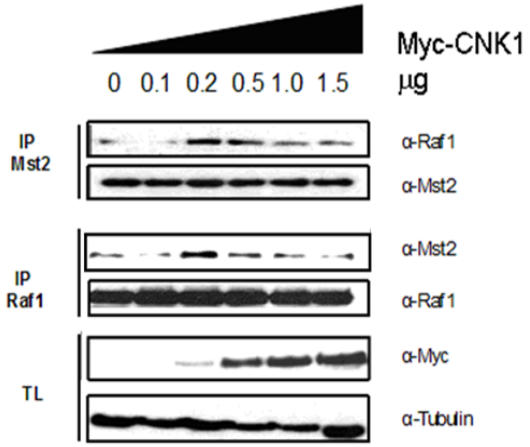
cell lysates were probed with Anti-Myc tag antibody. Equal loading of lysates was confirmed by stripping and reprobing membrane blots using an anti-tubulin antibody. Western blot analysis under this condition shown in Figure 3.B.I indicates that compared to control cells, transfected cells at a low concentration (0.2 μg) of Myc-CNK1 constructs markedly enhanced the amount of Raf-1 co-immunoprecipitated with MST2. However, cells that were transfected with much higher concentration of Myc-CNK1 constructs (1.5 μg) dissipates this interaction (first panel). This observation was confirmed by performing a reciprocal co-immunoprecipitation wherein Raf-1 protein is immunoprecipitated instead, and immunoblotted with Anti-MST2 antibody (third panel). Consistently, the amount of MST2 co-immunoprecipitated with Raf-1 in the Myc-CNK1 transfected cells exhibits a concentration-dependent behaviour. Moreover, because this change of MST2:Raf-1 complex exhibits a similar pattern dependent on the concentration of transfected Myc-CNK1 plasmid regardless from which it has precipitated, either IP MST2 or IP Raf-1, I measured the density of the protein bands detected on the immunoblots. Figure 3.B.II shows the relative quantification of the band intensities immunoprecipitated MST2: Raf-1 interaction from six independent co-immunoprecipitation experiments. MST2 co-precipitated with Raf-1 immunoprecipitates and Raf-1 co-precipitated with MST2 immunoprecipitates from cells transfected with increasing amount of Myc-CNK1 plasmid were quantified by densitometry, normalised to the empty vector control. A similar dose-dependent profile was obtained when Myc-tagged CNK1 expression plasmid was transfected into the human breast cancer cell line MCF-7 as shown in Figure 3.B.III.

Taken together, these observations suggest that when cells are deprived of growth factors, the scaffold protein CNK1 at a certain optimum level may possibly regulate a specific response by forming a discrete signalling component such as a Raf-1:MST2 complex, thereby facilitating the kinase-independent Raf-1 inhibitory role towards MST2. Raf-1, when it complexes with MST2, prevents its dimerization and impending pro-apoptotic activation (Mattalanas et al., 2007). In contrast, higher expression of CNK1 dissociates this complex. As a consequence, upon release from Raf-1, free MST2 may then be available for activation upon stress signals or pro-apoptotic stimuli such as serum starvation. Indeed, in response to Fas and serum starvation, Raf-1 -/- mouse embryo fibroblasts are sensitive to apoptosis with an increase in MST2 activity (O'Neill

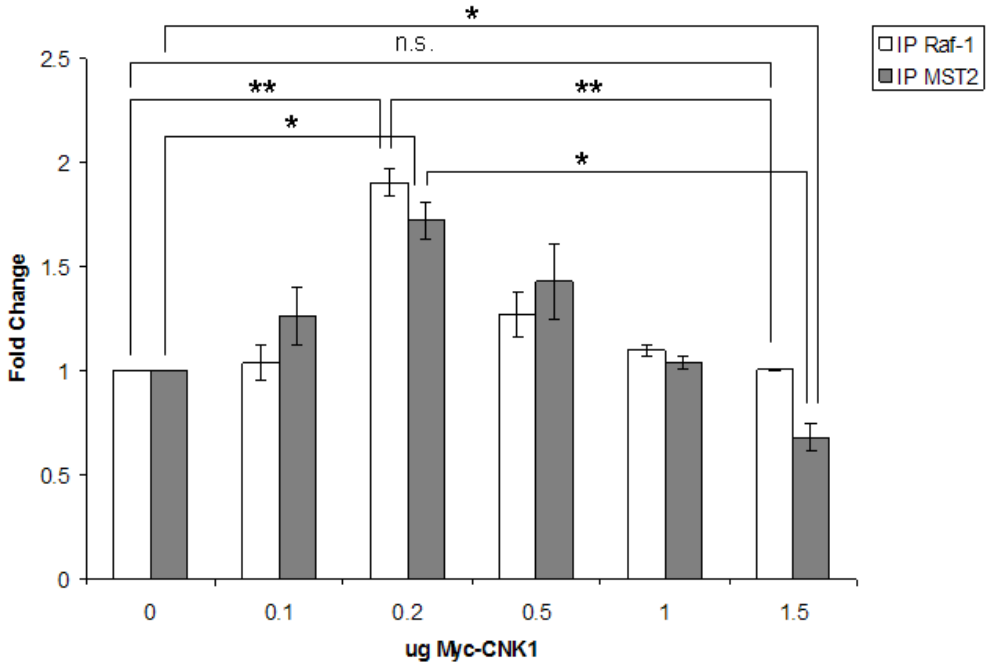
et al., 2004). MAPK scaffold proteins might either facilitate or inhibit signalling depending on their concentration, thus producing a bell-shaped concentration response curve (Yoshioka, 2004; Kolch 2005). The graph in Figure 3.B.II shows the relative quantitation of band intensities of either MST2 or Raf-1 immunoprecipitates suggests a proto-typical scaffold property of a 'bell-shaped' signature. Consistent with the idea of multimeric protein complexes are formed through scaffold proteins, the results indicate that CNK1's ability to exhibit a bimodal regulation is possibly by assembling an inhibitory Raf-1:MST2 complex conferring negative signalling at an optimal expression, but when expressed at higher levels, it disperses this complex by titrating the components freeing MST2 for activation, therefore promoting positive signalling.

**Figure 3.B CNK1 mediates MST2 and Raf-1 interaction upon serum-starvation in a concentration-dependent manner.**

**I**




**II**



**III**

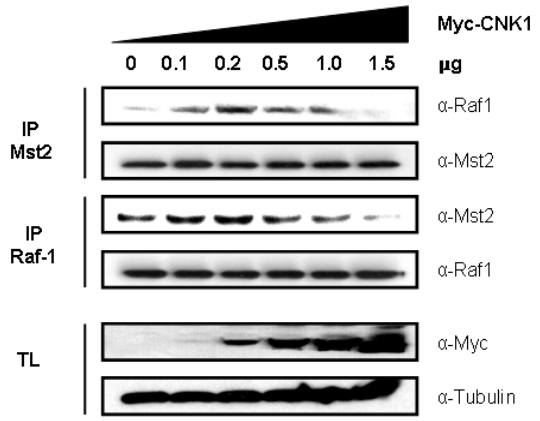



**Figure 3.B. CNK1 mediates MST2 and Raf-1 interaction upon serum-starvation in a concentration-dependent manner. (I)** HeLa cells were transiently transfected using an increasing amount of Myc-tagged CNK1 expression plasmids (0.1-1.5 µg Myc-CNK1 DNA concentration) and empty vector. After 16-20hr serum-starvation, endogenous MST2 and Raf-1 immunoprecipitates were prepared, subjected to SDS-PAGE followed by western blotting and probed with indicated antibodies. Anti-Myc was used to monitor the amount of expressed Myc-CNK1, and anti-tubulin for loading control of proteins is shown in the lower panel. (**II**) The band intensities of Raf-1:MST2 interaction from the immunoblots of six dose-dependent Myc-CNK1 overexpression co-IP experiments (three Raf-1 immunoprecipitates from MST2 co-IP experiments and three MST2 immunoprecipitates from Raf-1 co-IP experiments) were quantified by densitometry and were normalised to the control. Results are expressed as mean +/- SEM, n=3, *P=<0.05, ** P=<0.005, n.s. P= >0.05. Densitometry of immunoblot band intensity was performed using Image J software from National Institutes of Health. (**III**) Transfection of MCF-7 cells was performed in a similar manner as the HeLa cells. Following serum starvation, cells were lysed and MST2 and Raf-1 immune complexes were analysed by western blotting and probed with indicated antibodies. Cell lysates were probed with antibody recognising the Myc tag to determine Myc-CNK1 expression and with antibody directed against tubulin as control for loading of proteins.

### 3.2.2 CNK1 affects interaction of MST2 kinase and its substrate LATS1

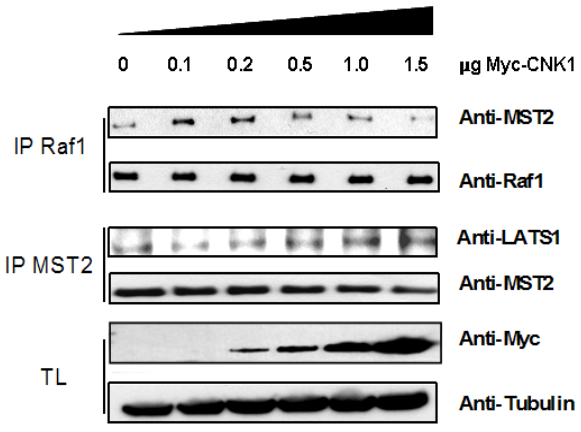
As discussed in Chapter 1, components of the Hippo pathway that include MST1/2, LATS1/2, and YAP are highly conserved in mammals. These are Hpo, Wts and Yki *Drosophila* homologs respectively (Edgar, 2006; Avruch et al., 2011). Pioneering work in Drosophila has established the Hippo pathway as a key regulator of the cell cycle, apoptosis and development; and importantly, in human cells a very similar signalling module exists (Harvey et al., 2003; Jia et al., 2003; Udan et al., 2003; Wu et al., 2003). Although it is now firmly established that the Hippo pathway is a tumour suppressor pathway that restricts cell cycle progression and proliferation and promotes apoptosis; new components and new molecular interactions are continuously being added to the pathway (Harvey and Tapon, 2007; Bao et al., 2011). An emerging theme is the regulation of the core components by different upstream protein complexes (Grusche et al., 2010). A mammalian model proposed, as described in chapter 1, is the induction of apoptosis via activation of LATS1 by MST2 mediated by RASSF1A upon apoptotic stimuli (Mattalanas et al., 2007) intriguingly all are tumour suppressors.

In light of the above findings that the Raf-1:MST2 complex may possibly be mediated by CNK1 in a concentration-dependent fashion and because LATS1 is a direct substrate of MST2, further examination of the MST2 and LATS1 interaction is warranted. Therefore, I set-out to perform similar experiments as described above, i.e. to overexpress myc-CNK1 in increasing amount (0.1-1.5 μg Myc-CNK1 DNA concentration) in HeLa cells including empty vector (pcDNA3) as a control and to determine how the interaction between LATS1 and MST2 is altered. Figure 3.C.I (upper panel) shows overexpression of Myc-CNK1 mediates the interaction of Raf-1:MST2 complex in HeLa cells deprived of growth factors in a dose-dependent fashion, consistent with the previous experiment (Figure 3.B.I). Interestingly, MST2 precipitates subjected to western blotting using an anti-LATS1 antibody indicate that the MST2 and LATS1 interaction was decreased in an intermediate CNK1 expression but increases as higher concentrations of Myc-CNK1 protein is expressed compared to control (Figure 3.C.1 third panel). Reciprocal co-immunoprecipitation shows that when LATS1

precipitates are probed with anti-MST2, the amount of MST2 detected is altered dependent on the amount of CNK1 expressed, in a manner consistent with the MST2 IP. Here, the results indicate that release of MST2 from Raf-1 at a higher CNK1 expression corresponds to the increased association of MST2 and its substrate LATS1, while recruitment of MST2 to Raf-1 mediated by an intermediate CNK1 expression corresponds to the decrease in binding of MST2 to LATS1. Further supporting this idea, previous reports showed that MST2 when released from Raf-1, promotes LATS1 and MST2 association via RASSF1A, allowing LATS1 to phosphorylate YAP1. YAP1 is then translocated to the nucleus, resulting in apoptosis of Hela and MCF-7 cells (Mattalanas et al., 2007; Kawahara et al., 2008). Together, these data suggest that CNK1 perturbs the MST2-LATS1 interaction, corresponding to a dose-dependent modulation of the Raf-1: MST2 complex. This may show an important role of the scaffold protein CNK1 in the regulation of the two core components of the Hippo pathway in apoptotic signalling.

**FIGURE 3.C. CNK1 affects interaction of MST2 kinase and its substrate LATS1**

**I.**



**II.**

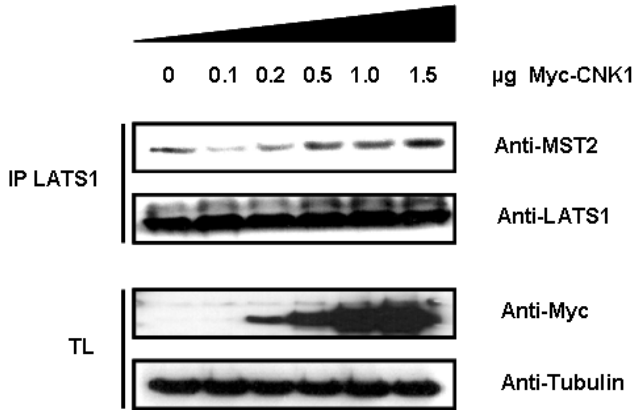



**FIGURE 3.C. CNK1 affects interaction of MST2 kinase and its substrate LATS1.** (**I**) HeLa cells were transfected with Myc-tagged CNK1 expression plasmid at indicated DNA concentrations (0.1-1.5 µg Myc-CNK1) including an empty vector as a control. Following 16-20hr serum starvation, endogenous Raf-1 and MST2 immunoprecipitates were performed in accordance with the co-immunoprecipitation assay as described in the experimental procedures (Chapter 2). Protein interactions were determined by western blot with indicated antibodies. Western blot image is a representative of two independent experiments. (**II**) Reciprocal co-immunoprecipitation wherein LATS precipitates immunoblotted with anti-MST2 shows consistently the interaction of LATS1 and MST2 is affected by CNK1 levels in HeLa cells. Aliquots of cellular lysates were examined to monitor increasing expression of CNK1 levels using an anti-Myc tag antibody and by using anti-tubulin antibody as a loading control.

### 3.2.3 Levels of CNK1 affect MST2 phosphorylation

Kinase activity is a key biochemical property of protein kinases that are involved in all fundamental properties in the cell (Johnson and Hunter, 2005). Kinases can be turned on or off by phosphorylation by binding of activator proteins, inhibitor proteins, small molecules, or linker proteins such as scaffold proteins, or by controlling their location in the cell relative to their substrates (Enslen and Davis, 2001; Bardwell et al., 2001; Manning et al., 2002). Phosphorylation by protein kinase is one of the most common mechanisms in eukaryotic cells; hence it forms the basis of cell signalling networks (Yaffe and Elia, 2001). The protein kinase MST1/2, similar to other kinases, is complex and subjected to post –translational modifications. Although, the MST1/2 kinases have been shown to be regulated by several mechanisms, including phosphorylation, dimerization, proteolysis and protein-protein interaction, the integration of these mechanisms of action is still unclear (Creasy et al., 1995; Callus et al., 2006; Praskova et al., 2008; Zhao et al., 2008). Moreover the role of interacting partners such as the scaffold CNK1 in the regulation of its phosphorylation and activity has not been investigated. Furthermore, Raf-1 when complexed with MST2 inhibits its phosphorylation and kinase activity (O'Neill et al., 2004), and in the light of our finding that CNK1 may play a role in the MST2: Raf-1 complex formation, it is conceivable that CNK1 may also be involved in this mechanism.

Thus, I next investigated whether the phosphorylation state of MST2 is influenced by the perturbation of the MST2: Raf-1 complex dependent on Myc-CNK1 expression levels. As before, HeLa cells were transfected with increasing amounts of Myc-tagged CNK1 expression plasmids (0.02-1.5 μg Myc-CNK1) including empty vector as control. Following serum starvation, endogenous MST2 was immunoprecipitated and then subjected to western blotting. MST kinase is phosphorylated at multiple sites, including key threonine residues in the activation loop which are critical role in its activation; hence, phosphorylated MST2 was detected with an antibody to phosphothreonine (Glantschnig et al., 2002, Deng et al., 2003; O'Neill et al., 2004). As shown in Figure 3.D, Western blotting of MST2 immunoprecipitates in lysates of control cells showed that at endogenous CNK1 levels, a modest amount of Raf-1:MST2 complex forms (third

panel), which corresponds to a basal MST2 kinase phosphorylation (fifth panel). However, in cells transfected at low concentration of myc-CNK-1 plasmid (0.05-0.5 μg DNA) enhanced Raf-1:MST2 formation and corresponded to attenuated MST2 kinase phosphorylation. Whereas, in cells transfected at a higher concentration of myc-CNK1 plasmid (1.5 μg DNA) which dissociates the Raf-1:MST2 complex corresponded to elevated levels of phosphorylated MST2. As a control, reciprocal Raf-1 immunoprecipitates were also analysed and probed for anti-MST2 (first panel) to show the Myc-CNK1-concentration mediated perturbation of the Raf-1:MST2 complex, as previously shown (Figure 3.B.I). Also as predicted, smaller increment titration of Myc-CNK1 plasmid levels has flattened the 'bell-shaped' curve obtained using myc-CNK1 to scaffold the formation of the Raf-1:MST2 complex as illustrated in Figure 3.D. This suggests that the Raf-1:MST2 complex is sensitive to expression levels of CNK1. This is consistent with previous observations that phosphorylation of MST2 is dampened when complexed with Raf-1 (O'Neill, et al., 2004; Mattalanas et al., 2007; Romano et al., 2010). Together, this data indicates that CNK1 may play a role in MST2 regulation, as CNK1 could influence the Raf-1: MST2 complex dependent of its expression levels. Therefore, it will be important to confirm this finding by analysing MST2 kinase activity under this condition.

**FIGURE 3.D. Levels of CNK1 affect MST2 phosphorylation**

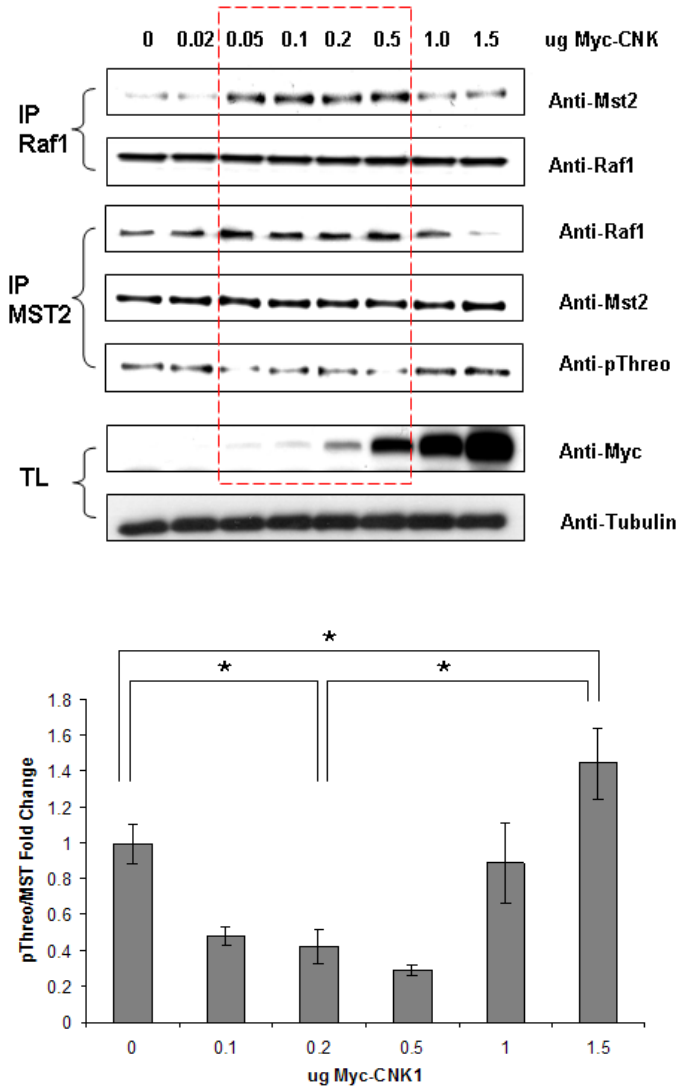



**FIGURE 3.D. Levels of CNK1 affect MST2 phophorylation.** HeLa cells were transfected with the Myc-CNK1 expression plasmid at the indicated DNA concentrations including an empty vector as a control. Following serum starvation for 24 hours, the cells were then lysed and equal amounts of proteins were subjected to immunoprecipitation (IP MST2 and IP Raf-1) and immunoblotted with indicated antibodies. Cellular extracts were examined for the increasing expression of CNK1 levels using anti-Myc, and anti-tubulin as a loading control. The ratio of pThreo to total MST2 was quantified by densitometric evaluation of Western blots. The data represent the means ± the SEM of three independent experiments,*$P < 0.05$.

### 3.2.4 Levels of CNK1 affect serum-starvation induced apoptosis

Components of the Hippo pathway are deregulated in cancer due mostly to epigenetic inactivation of tumour suppressors such as RASSF1A in hepatocellular carcinoma, MST1/2 kinases in soft tissue sarcomas, and LATS1/2 kinases in breast cancers (Calvisi et al., 2006; Macheiner et al., 2006; Seidel et al., 2007; Takahashi et al, 2005). However, at this moment the extent to which the Hippo pathway components vary in terms of tissue specificity and frequency, including alterations in expression and specific roles in cancer, is yet to be clarified. For example, suppression of Hippo/ MST2-mediated apoptosis can be elicited by either inhibition of MST2 phosphorylation by AKT kinase or interaction of MST2 with the proto-oncogene Raf-1 in several cancer cell lines (O'Neill et al., 2004, Jang et al., 2007). More importantly, the biochemical mechanism of the two core Hippo components MST1/2 and LATS1/2 activation by upstream effectors to mediate apoptosis in response to cellular environmental stress in several cell lines is less well defined, and thus requires further investigation (Avruch et al., 2011).

Hippo/MST-mediated apoptosis is brought about via RASSF1A's activation of MST2 upon release from Raf-1, promoting MST2's downstream phosphorylation of LATS1. In turn, YAP1 becomes phosphorylated by LATS1, dissociates from LATS1 and then translocates to the nucleus to form a complex with p73, ultimately leading to transcription of PUMA which results in apoptosis (Mattalanas et al., 2007). Interestingly, p73's activation of PUMA, independent of p53, has been shown to be induced by serum starvation in several colon cancer cell lines, triggering apoptosis (Ming et al., 2008). It is unclear, however, how specific phosphorylation responses and interactions of opposing MST2 and Raf-1 kinases are controlled, that trigger eventual activation of downstream signalling and ultimately promote apoptosis. Data demonstrated so far suggests a possible upstream mechanism which coincides with this model; that is, CNK1 concentration-dependent assembly or disassembly of a Raf-1:MST2 complex corresponds to MST2 activity and downstream formation of a MST2:LATS1 complex. Thus, the immediate question to ask was if this regulation of the Raf-1:MST2 complex, MST2 phosphorylation and MST2 interaction with its

substrate LATS1 have functional consequences in Hippo pathway-mediated, serum-starvation induced apoptosis.

To determine this, HeLa cells that were subjected to different levels of overexpression of CNK1 in similar experiments to those described above (Figure 3.D) were set-up and analysed for apoptotic activity. This was done by measuring the sub-genomic DNA content of apoptotic cells using the FACS (Fluorescence activated cell sorting) technique, as the presence of fragmented DNA in cell is a key hallmark of apoptotic cell death (Kitazawa et al., 2002). HeLa cells were transfected with increasing amounts of Myc-CNK1 as indicated. Serum-starvation was extended to 36 hours post-transfection to stimulate apoptosis. Figure 3.E. shows that apoptosis of cells which were minimally transfected with Myc-CNK1 plasmid (0.1 μg Myc-CNK1 DNA) compared to basal cells was decreased that shows a baseline of 5-7% apoptosis, while apoptosis in cells transfected with higher levels of Myc-CNK1 plasmid (1.5 μg Myc-CNK1 DNA) increased compared to cells transfected at lower levels, but to a modest increase in cells at endogenous basal levels under this condition. The data indicate that expression levels of CNK1 affects serum-starvation induced apoptosis of cells. Although perturbation of CNK1 expression levels may also trigger other non-hippo/MST2 signalling pathways that are involved in regulating apoptosis, This data suggests that following stress stimuli such as growth factor withdrawal of cells by serum starvation, CNK1 when expressed at low level may be able to rescue cells from undergoing apoptosis by binding Raf-1 to MST2 in order to sequester MST2 from activation. However, titration of the Raf-1:MST2 complex caused by elevated levels of CNK1 expression may result in the disruption of the complex, liberating MST2, which can then be activated and interact with its substrate LATS1 to promote apoptosis.

**FIGURE 3.E. Levels of CNK1 affect serum-induced apoptosis.**

**I**

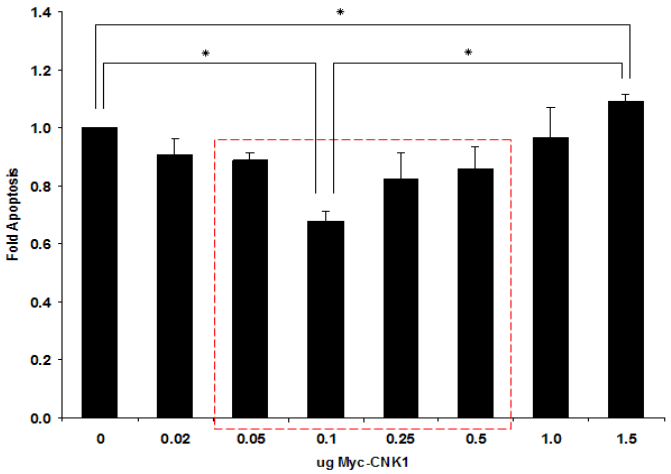



**FIGURE 3.E. Levels of CNK1 affect serum-induced apoptosis.** Hela cells were transfected with increasing amounts of Myc-CNK1 as previously described. To induce apoptosis, cells were serum-starved for 36 hours post-transfection. Both detached and attached cells were collected and an aliquot of cells was stained with hypotonic PI (Propidium Iodide)-stain solution. Percent apoptotic cells were assessed using propidium iodide incorporation into fragmented DNA and quantified with FACSCAN flow cytometer. Graph shows fold changes of apoptosis relative to control. Statistical analysis were carried-out using Student's t test; n=3, error bars indicate mean ± SEM, *$p<0.05$.

### 3.2.5 CNK1 links MST2 and Raf-1 to modulate Hippo pathway-mediated apoptosis

As described in Chapter 1, scaffold proteins function as modulators of signal transduction by linking proteins into close spatial proximity to facilitate functional protein-protein interactions. Therefore, it is plausible that reducing endogenous CNK1 levels under similar conditions used in previous experiments should disrupt the Raf-1: MST2 complex. The disruption of this complex will allow free MST2 to signal downstream of the Hippo pathway and elicit apoptosis. To determine this, we employed siRNA-mediated knock-down of endogenous CNK1 expression. HeLa cells were also transfected with a scrambled, non-targeting siRNA to serve as a negative control and an siRNA pool specific to CNK1 at two different concentrations (120 and 140 pM) by lipofectamine as described in Chapter 2. Cells were serum-starved for 24 hours following transfection similar to previous experimental conditions. These cells were lysed and subjected to immunoprecipitation using Raf-1 or MST2 antibodies, as described before.

As demonstrated in Figure 3.F.I, introduction of siRNAs targeting the CNK1 gene, unlike control siRNA, reduced the expression levels of endogenous CNK1 (bottom panel), more so at an siRNA concentration of 140 pM. The depletion of endogenous CNK1 levels in HeLa cells disrupted the Raf-1 and MST2 interaction (first panel) from Raf-1 immunoprecipitates. Reciprocal co-immunoprecipitation confirms the disruption of the Raf-1:MST2 interaction (third panel). These data suggest that the scaffold protein CNK1 is necessary to link Raf-1 to MST2. As the dissociation of Raf-1:MST2 complex results in MST2 phosphorylation (Figure 3.D) and activation (O'Neill, et al, 2004; Mattalanas et al., 2007), the direct consequence of CNK1 knockdown to this complex, i.e. whether it results in MST2 activation, was therefore tested.

Indeed, knockdown of endogenous CNK1 by siRNA augmented MST2 activation as indicated by an in-gel kinase assay of MST2 immunoprecipitates (Figure 3.F II, first panel). These data suggest that specific knockdown of CNK1 expression by RNAi in

HeLa cells results in Raf-1's dissociation from MST2, which enables free MST2 to be activated. Thus, these data indicate that the CNK1 protein levels affect HeLa cells response to serum-starvation. Because downregulation of endogenous CNK1 expression increases the MST2 kinase activity in response to serum starvation, they also suggest that the endogenous levels of CNK1 protein in HeLa cells functions as an inhibitor of this response, since its depletion results in increased activation. MST2 kinase when activated, dissociates from Raf-1 (O'Neill et al., 2004), then binds (Figure 3.C) and phosphorylates its substrate LATS1 for downstream Hippo apoptotic signalling (Mattalanas et al., 2007). Further MST2 co-immunoprecipitation experiments in cells in which CNK1 expression had been knocked down are shown in Figure 3.F.II (third panel). These reveal that dissociation of MST2 from Raf-1, enhanced LATS1 and MST2 interaction, which corresponds to its increased kinase activity. Collectively, these data suggest that Raf-1 requires CNK1 for its inhibitory function towards MST2 activation, and may prevent its association to its substrate LATS1, and possibly its activation. Hence, it may be important to analyse LATS1 kinase activity under this condition to confirm this finding.

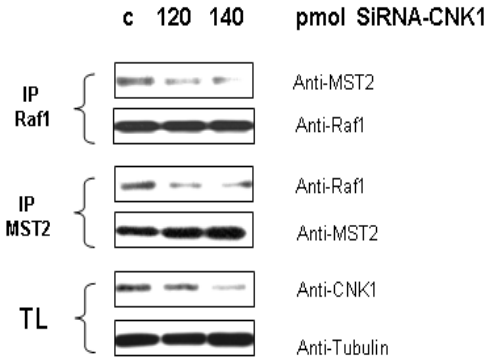
As it is well documented that when the Hippo/MST2 pathway is activated via stress stimuli it triggers apoptosis of cells (Radu and Chernoff, 2009; Bao et al., 2011) and together with the above observations, I sought to measure the apoptotic activity in serum-starved HeLa cells in which levels of endogenous CNK1 had been reduced by RNAi. Apoptosis was determined by flow cytometric analysis of cell populations with sub-G1 DNA content, as previously described. As shown in Figure 3.G (upper graph), depletion of CNK1 by RNAi of either concentration (120 pmol/ 140 pmol siRNA-CNK1) led to significantly enhanced serum-starvation induced apoptosis compared to siRNA control cells. Complementary to this, CNK1 knockdown results in the decline of cell numbers due to continual cell death by apoptosis as compared to siRNA control cells (lower graph). This result suggests that the endogenous level of CNK1 can regulate the sensitivity of HeLa cells in response to serum-starvation induced apoptosis.

This model predicts that CNK1 knockdown induction of apoptosis in HeLa cells may be dependent on the pro-apoptotic components of the hippo pathway. Therefore, if knockdown of associated components, in contrast to CNK1's effect, will influence this response was analysed. A screening RNAi experiment to analyze the efficiency of non-

targeting siRNAs to MST2 and RASSF1A protein by western blot is shown in Figure 3.H.I. Next, a caspase-activity –based apoptosis assay was performed as described in Chapter 2 (Materials and Methods), to determine the effect of co-knockdown of these two proteins in HeLa cells on serum-starvation induced apoptosis. As increased caspase activity is a hallmark of apoptosis, the measurement of their levels report directly on apoptosis (Degterev et al., 2003; Elmore, 2007). Figure 3.H.II shows that the depletion of endogenous CNK1 promoted apoptosis compared to the siRNA control, consistent with the previous apoptosis result by FACS analysis in Figure 3.G. In contrast, depletion of endogenous RASSF1A and MST2 by siRNA reduced serum starvation-induced apoptosis, suggests that both proteins are necessary components of the hippo-pro-apoptotic pathway and their depletion therefore causes Hela cells to become resistant to serum-starvation induced apoptosis. As expected, depletion of Raf-1 promoted apoptosis in HeLa cells compared to siRNA control, suggesting that MST2 is free from Raf-1's kinase-independent inhibitory function, therefore sensitizing cells to serum-starvation induced apoptosis. Interestingly, co-depletion of CNK1 and MST2 prevented cells from apoptosis as there was no significant change compared to control cells, suggesting that siRNA-CNK1 induced apoptosis is dependent on the presence of MST2 expression in cells. In contrast, co-depletion of CNK1 and RASSF1A still sensitize cells to apoptosis suggesting that RASSF1A is negligible in MST2 activation. Meanwhile, co-depletion of CNK1 and Raf-1 markedly induced apoptosis compared to control cells including cells depleted with Raf-1 alone suggests that both CNK1 and Raf-1 is necessary for MST2 inhibition and that Raf-1 requires CNK1 to link with MST2 to fully exert its inhibitory role. Interestingly, depletion of CNK1 alone compared to Raf-1 seems to be more efficient in inhibiting apoptosis. However, Raf-1 siRNA efficiency screening by western blot was not performed, therefore confirmation of these results is warranted. Together, these finding indicate that CNK1 may play a role in the regulation of the Hippo/MST2 pro-apoptotic pathway by linking Raf-1 to MST2, facilitating Raf-1's inhibitory function towards MST2, thereby preventing its activation upon apoptotic stimuli.

**FIGURE 3.F. Depletion of CNK1 impairs Raf-1 and MST2 interaction**

**I.**




**II.**

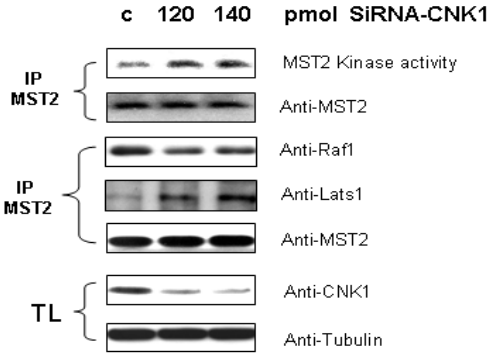



**FIGURE 3.F. Depletion of CNK1 impairs Raf-1/MST2 interaction. (I)** HeLa cells were transfected with a pool of CNK1-specific siRNAs at different concentrations as indicated, or with scrambled control siRNA. Following serum starvation and lysis of cells, immunoprecipitations of Raf-1 and MST2 proteins were carried out and examined by immunoblotting using antibodies as indicated. Cell extracts (TL) were used to confirm depletion of CNK1, and anti-tubulin antibody was used as a loading control. **(II)** Similar to the experiments above, following serum starvation for 24 hours, the cells were then lysed and equal amounts of proteins were subjected to immunoprecipitation and immunoblotted with indicated antibodies. Cellular extracts were examined for the increasing expression of CNK1 levels using anti-CNK1, and anti-tubulin as a loading control. For the kinase assay, MST2 immunoprecipitates were separated by SDS-PAGE, and in-gel kinase assays were carried out with MBP (myelin basic protein, as substrate) embedded in the gel as described in Chapter 2 (Materials and Methods). Western blot image (I and II) is a representative of two independent experiments.

## FIGURE 3.G. Depletion of CNK1 induces apoptosis in HeLa cells

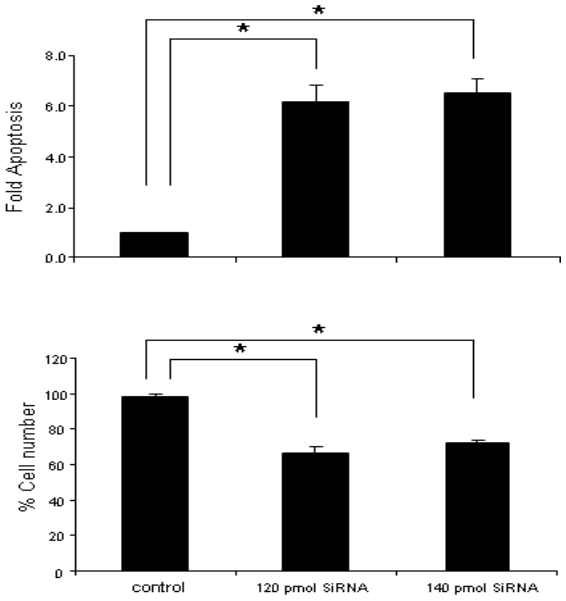
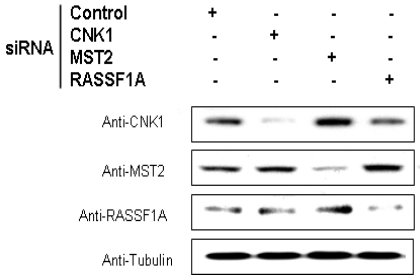



**FIGURE 3.G. Depletion of CNK1 induces apoptosis in HeLa cells.** CNK1 siRNA-induced apoptosis of serum-starved HeLa cells at indicated concentrations was detected by measuring the population of cells containing sub-genomic DNA fragments stained with propidium iodide using a flow cytometer (upper graph). Results are expressed as mean +/- SEM, n=3, *P=<0.05, Student's t test. Cell counting was performed and calculated electronically using the CASY-1 cell counter and analyzed from the same HeLa cells prior to fixation and staining for the flow cytometry apoptosis assay. Results are expressed as a percent of the control value: mean +/- SEM, n=3, *P=<0.05, Student's t test.

**FIGURE 3.H. CNK1 induces apoptosis via Hippo/MST2 components**

**I.**




**II.**

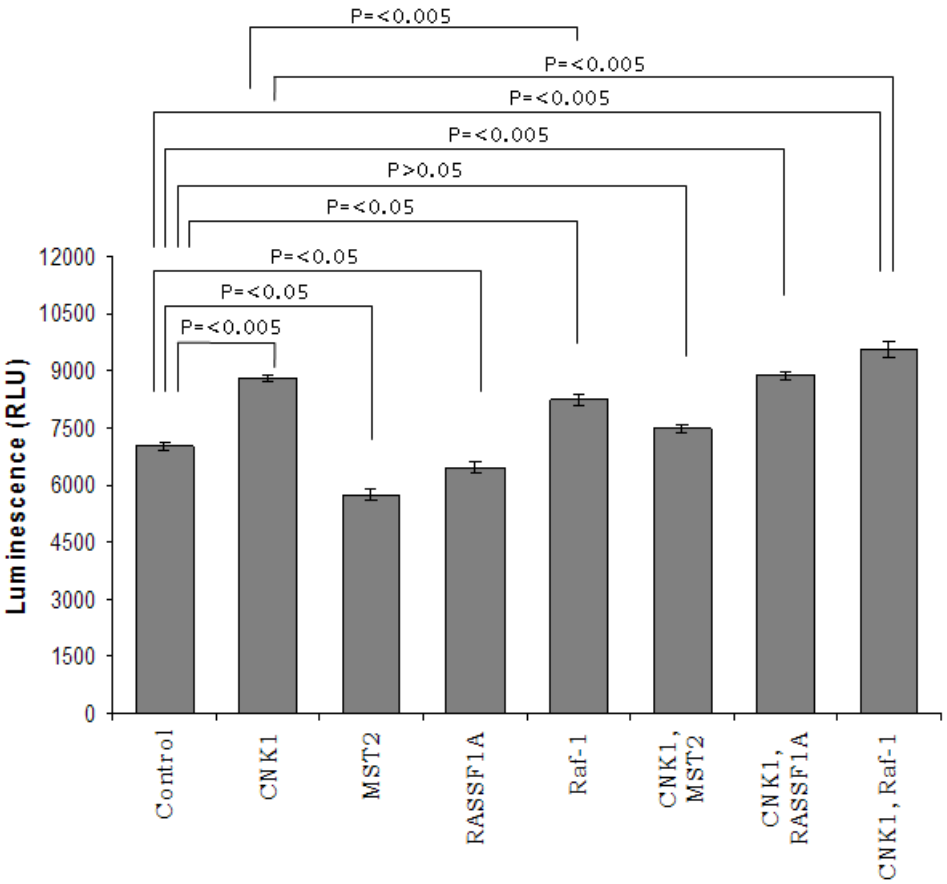


**FIGURE 3.H. CNK1 induces apoptosis via Hippo/MST2 pathway components. (I)** HeLa cells were transfected with siRNA specific to CNK1, MST2, RASSF1A and scrambled siRNA control. Whole-cell extracts were prepared, equal amounts of protein were separated by SDS-PAGE, and blots were probed with the indicated antibodies to show depletion of corresponding protein, or tubulin as a loading control. The data above is representative of two independent experiments. **(II)** As before, HeLa cells were transfected with control siRNA or siRNA against individual proteins as indicated, or co-transfected with CNK1. A caspase 3/7 luminescence assay was used to determine the level of caspase activated-based apoptosis as described in Chapter 2 (Materials and Methods). The relative luminescent intensity is proportional to the caspase activity present in apoptotic cells. Results are expressed as mean +/- SEM, n=5, P values were calculated using Student's t test.

## 3.3 Discussion

To better understand the signalling dynamics of protein scaffold CNK1 functions, I began the study by investigating the interaction partners of CNK1 involved in the Hippo pathway. Previous studies have shown direct interaction between CNK1 and Raf-1, and it was demonstrated that Src-mediated activation of Raf-1 is regulated dependent on CNK1 concentration in HEK293T cells (Ziogas et al., 2004). It was also reported to support Ras-mediated apoptosis by CNK1's ability to bind the tumour suppressor RASSF1A and the pro-apoptotic kinases MST1/2 (Rabizadeh et al., 2004). Meanwhile, it was documented that Raf-1 and MST2 bind upon serum starvation, and MST2 kinase is inhibited via a mechanism independent of Raf-1's kinase activity (O'Neill et al., 2004). As Raf-1 and MST2 are well established binding partners of CNK1, I examined whether the physical interaction of endogenous Raf-1 and MST2 upon serum starvation is regulated by CNK1.

The results presented here suggest that the scaffold protein CNK1 may play a role in the Hippo/MST2 pathway by regulating the interaction of Raf-1 and MST2. When CNK1 is expressed at a concentration close to endogenous levels, the Raf-1:MST2 complex is increased. However at higher concentrations, the Raf-1:MST2 complex dissipates. Although these findings initially seem counterintuitive, they actually support a typical behaviour of a scaffold protein as demonstrated among other MAPK scaffolds (Kolch, 2005; Yoshioka, 2004). Insights from computer modelling studies referred to this dose-dependent phenomenon as combinatorial inhibition, which refers to the weakening of signal due to dilution of pathway components as scaffold concentration exceeds the optimum level to bind client proteins for it to interact functionally, hence the appearance of the "bell-shaped" curve (Levchenko et al., 2000; Ferrell, 2000). CNK1 modulation of Raf-1 and MST2 interaction seems to reveal a similar dose-dependent behaviour (Figure 3.B). Indeed, functional analyses support this overexpression property of CNK1 in context of the regulation of the Raf-1:MST2 complex. Pro-apoptotic MST2 when complexed with Raf-1 is prevented from phosphorylation and activation, but when liberated, it can be activated and binds to its substrate LATS1 to signal downstream of

the pathway and elicit apoptosis of cells (O'Neill et al., 2004; Mattallanas et al., 2007). Consistent with this model, the level of CNK1 expression that maximally increased the Raf-1:MST2 complex, led to a decreased MST2 and LATS1 interaction (Figure 3.C), decreased MST2 kinase phosphorylation (Figure 3.D) and consequently decreased proportion of apoptotic cells (Figure 3.E). However, overexpression of CNK1 at a higher level that liberates MST2 from Raf-1, resulted in increased MST2 interaction with LATS1 (Figure 3.C), restoration of MST2 phosphorylation (Figure 3.D), as well rescue of cells from serum-induced apoptosis (Figure 3.E). Consistently, a previous CNK1 overexpression study using comparatively higher concentration promoted marked apoptosis through RASSF1A and MST1/2 in HEK293 cells (Rabizadeh et al., 2004). Employing siRNA-mediated experiments further suggests that CNK1 scaffolds MST2 and Raf-1 to regulate the Hippo/MST pathway dependent on its expression levels. Depletion of endogenous CNK1 levels led to disruption of the Raf-1:MST2 complex (Figure 3.F) accompanied by enhanced MST2 activity (Figure 3.G), and sensitised HeLa cells to serum withdrawal-induced apoptosis (Figure 3.H).

Taken together, these results suggest that CNK1 expression levels may dictate the sensitivity of cells towards apoptosis. CNK1 function as an inhibitor and an inducer, CNK1 at endogenous and at intermediate expressions act as an inhibitor and at higher expressions it acts as an inducer. Consistent with this are other scaffold-like properties such that of the scaffold JIP1 which binds protein kinases of the JNK cascade that enhances JNK activation, and inhibits the pathway when overexpressed (Whitmarsh et al., 1998, 2001). Similarly, KSR1 interacts with Raf-1, MEK and ERK, and depending on its expression levels it can either inhibit or enhance ERK activity (Kortum and Lewis, 2004). Interestingly, CNK1 was demonstrated to bind Raf-1 and Src in HEK293 cells, and when CNK1 is overexpressed in low amounts Src-dependent activation of Raf-1 via Ras is increased. At a higher level of CNK1 overexpression, this led to loss of Raf-1 activation (Ziogas et al., 2005). A distinction between the findings of CNK1 as a scaffold protein presented here and from the examples of typical scaffolds described above is that, dose-dependent assembly of Raf-1: MST2 complex mediated by CNK1 results in negative signalling as opposed to positive signalling. Scaffold proteins may regulate cell signalling by assembling both positive and negative signalling components to confer a specific response (Kolch, 2005; Dhanasekaran et al., 2007).

In this context, CNK1 may be partly reminiscent of the even more complex and diverse functions of scaffolds like JIP1, where negative and positive proteins modulate its signal transmission. JIP1 assembles JNK, MKK7, and mixed lineage protein kinase (MLK) proteins to promote the JNK signalling pathway (Whitmarsh et al., 1998, 2001). However, a JNK negative regulator, MAPK phosphatase-7, also binds to JIP1 and inhibits JNK activation by dephosphorylating JNK (Willoughby et al., 2003). Interestingly, CNK1 has also been demonstrated to bind RASSF1A and MST1/2 to promote apoptosis (Rabizadeh et al., 2004). Similar to the scaffold JIP, it is plausible that such negative CNK1/Raf-1/MST2/RASSF1A complex or CNK1/MST2/RASFF1A positive and/or other independent set of components may exist with CNK1. Indeed, scaffold protein functions such that of CNK1 increases the complexity of revealing important roles that may be involved in the regulation of Hippo/MST2 pathway. Moreover, the mammalian Hippo/MST2 pathway is still a relatively young field, and the general mechanisms are still being elucidated (Pan, 2010; Radu and Chernoff, 2009). Therefore further studies understanding the role of scaffolding proteins, specifically determining interaction sites between relevant proteins and stepwise mechanism of the complex formation and dissociation with corresponding biological consequences, is necessary.

Nevertheless, our results are consistent with the dose-dependent model effects of a prototypical scaffold protein. This model is summarized and illustrated in Figure 3.I., whereby relatively small changes in the amount of CNK1 in cells will result in a significant enhancement in the Raf-1:MST2 complex, thereby inhibiting the hippo pro-apoptotic signal. When in excess, CNK1 dilutes-out Raf-1 and MST2 components, thereby releasing MST2 to be activated, binding to its substrate LATS1 to relay apoptotic signals and eventually triggering apoptosis of cells. Although the signal response varies the bell-shaped signature appears to be correlated as the peak falls at relatively similar concentration close to basal levels of CNK1, further supporting that CNK1 is scaffolding Raf-1 and MST2 and regulating pro-apoptotic signalling via the hippo pathway.

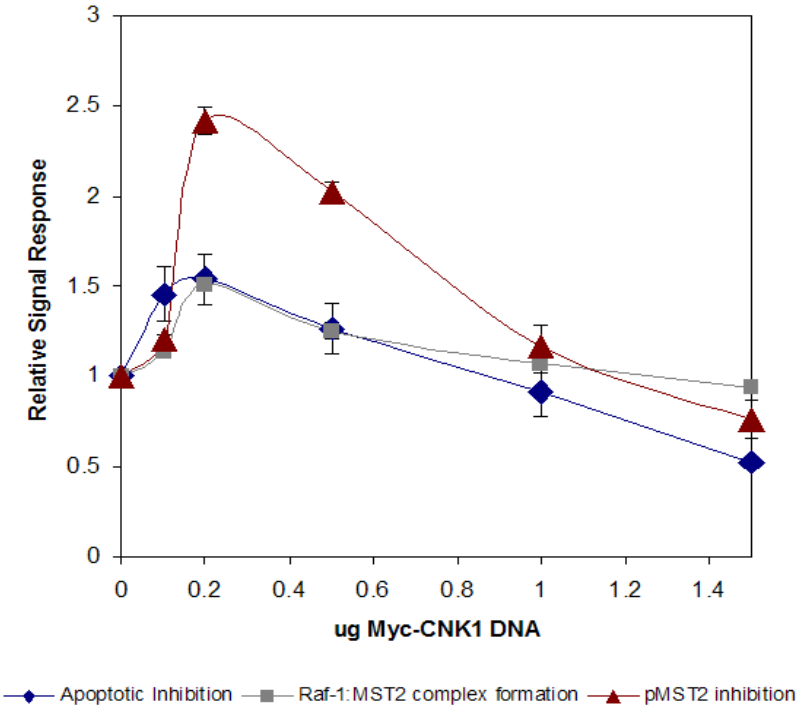



**FIGURE 3.I. CNK1 modulation of Raf-1:MST2 complex formation in a concentration-dependent manner.** CNK1 modulation of Raf-1:MST2 complex formation in a concentration-dependent manner corresponds with the inhibition of MST2 kinase activity and apoptotic signalling. Results used are derived from data presented as follows: The apoptotic inhibition (blue diamond, Figure 3.E, Results are expressed as mean +/- SEM, n=3); phosphoMST2 inhibition (dark red triangle, Figure 3.D, Results are expressed as mean +/- SEM, n=3); and Raf-1:MST2 complex (gray square, Figure 3.B.II, Results are expressed as mean +/- SEM, n=6) were plotted as relative signal response (fold change) versus the relative CNK1 expression levels. All data were normalised to that obtained for control cells.

# CHAPTER 4

# The Temporal feature and Selectivity of Signalling by CNK1

## 4.1 Introduction

The ability of a tumour cells to grow exponentially is linked to imbalance between cell proliferation and cell apoptosis. Therefore, regulation of programmed cell death or apoptosis is usually exploited by tumour cells to get advantage, progress and proliferate. Apoptotic evasion represents one of the true hallmarks of cancer and a crucial characteristic in the resistance of the most aggressive of human cancers to chemotherapy (Hanahan and Weinberg, 2000). Clues on understanding the mechanism integrating proliferative and apoptotic signals in cells is therefore crucial.

As discussed in Chapter 1, there are two well-characterised apoptotic pathways. Apoptosis on cells can be triggered by various stimuli either externally (Extrinsic pathway), e.g. by activation and ligation of cell surface death receptors by specific pro-apoptotic ligands, or internally (Intrinsic pathway), e.g. by DNA damage as a result of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, loss of growth factors, defective cell cycle signalling or other types of severe cell stress (Hengartner, 2000; Danial and Korsmeyer, 2004). Hence, chemical stressors such as staurosporine and exposure of cells to antibodies against their death receptor CD95/Fas induces cell death, and thus are used extensively as a general tool for exploring the molecular regulation of apoptosis (Huang et al., 1999; Chae et al., 2000; Belmokhtar et al., 2001; Zhang et al., 2004; O'Brien et al., 2005; Mollinedo and Gajate, 2006). However, crosstalk between the extrinsic and intrinsic pathways as well as caspase dependent and independent mechanisms may exist (Lockshin and Zakeri, 2002; Fulda and Debatin, 2006; Elmore, 2007). The pro-apoptotic MST1/2 kinase can be activated via caspase and caspase-independent mechanisms in response to cellular apoptotic agents such as staurosporine and Fas ligand (Taylor et al., 1996; Lee et al., 1998; Deng et al., 2003; O'Neill et al., 2004; Mattalanas et al., 2007; Zhang et al., 2009). In line with this, I sought to determine if CNK1 may play a role in mediating Raf-1:MST2 complex under these similar severe apoptotic conditions such as CD95/Fas and staurosporine induced apoptosis compared to previous chapter wherein challenging the dynamics of the complex mediated by CNK1 using growth-withdrawal/ serum-starvation cellular stress and to extend the findings in the Fas death receptor signalling pathway.

Conversely, activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) by mitogenic stimulation leads to activation of Ras-Raf-MEK-ERK/MAPK pathway (Yarden and Schlessinger, 1987; Yarden and Sliwkowski, 2001). Ras activation of Raf kinase directly phosphorylates and activates MEK, which in turn directly phosphorylates and activates ERK (Kyriakis et al., 1992; Howe et al., 1992; Vojtek et al., 1993; Wellbrock et al., 2004). Consequently, activation of ERK is critical for numerous Ras-induced cellular responses including cell growth and proliferation (Kolch, 2000).

Interestingly, mitogenic stimulation and oncogenic Ras also dissociate Raf-1 and MST2 complex. A recent report suggested that in mitogenic conditions, AKT phosphorylates MST2 thereby shifting MST2 affinity away from RASSF1A towards Raf-1 (Romano et al., 2010). However, in contrast, a study demonstrated that mitogenic stimulation enhanced the interaction of Raf-1 and MST2, including an unexpected positive role for MST2 in Raf-1/ERK pathway (Kilili and Kyriakis, 2010). The regulation of Raf-1 and MST2 is quite complex and many of the exact mechanisms involved are currently under intense investigation. However, in recent years, mechanisms involved in the integration between the prosurvival signalling pathway and pro-apoptosis signalling pathways implicate scaffold proteins as they have the ability to assemble diverse proteins into complexes to foster crosstalk between signalling pathways which provides a mechanism to integrate specific responses to diverse array of stimuli in cells (Ebisuya et al., 2005; Good et al., 2011).

As CNK1 is a scaffold protein that contains multidomain architecture to enable binding of several proteins to its interacting domains, and given the fact that CNK1 binds both opposing proto-oncogene Raf-1 and pro-apoptotic MST2 as confirmed by peptide array it is therefore conceivable that CNK1 may play a role in the coordination of proliferative signals and apoptotic signals that impact on both opposing kinases. Therefore, to gain further insight as to the scaffold role of CNK1, I examined the binding dynamics of its partners under these different conditions. The data presented here suggests that CNK1 may potentially provide a level of specificity and temporal regulation by coordinating the opposing kinases Raf-1 and MST2 for efficient and appropriate response to a variety of stimuli.

## 4.2 Results

### 4.2.1 CNK1 is involved in Fas receptor and Staurosporine death signalling

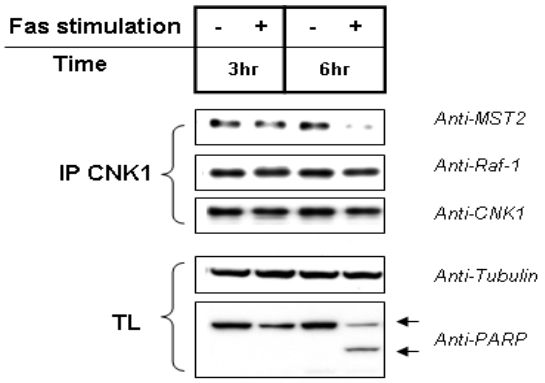
In the same way as the Raf-1:MST2 complex is modulated by CNK1 upon serum-starvation induced apoptosis as suggested by the data in Chapter 3, I therefore examined whether CNK1's regulation is extended in response to these apoptotic agents. As stress signals and apoptotic stimuli dissociate the Raf-1:MST2 complex and CNK1 binds both opposing kinases, I first analyzed the endogenous association of these components before and after treatment of cells to determine whether a dynamic change of complexes occurs in these conditions. HeLa cells were grown overnight in serum-containing medium prior to 16-20 hours of serum-deprived culture medium. After serum starvation, cells were stimulated with either Fas antibody or staurosporine for 3-6 hours and 30-120 minutes, respectively. Cells were lysed and immunoprecipitated with anti-CNK1, followed by immunoblotting with anti-MST2, anti-Raf-1 and anti-CNK1. The protein expression of Poly (ADP-ribose) polymerase-1 (PARP) was also examined by western blotting on the cell lysates. PARP is involved in DNA repair, and is cleaved by caspases during the execution phase of the apoptotic program which upon this decisive event facilitates cellular disassembly. Hence, it is used extensively as a marker of cells undergoing apoptosis (Oliver et al., 1998; Boulares et al., 1999; D'Amours et al., 2001).

Figure 4.A shows that endogenous MST2 and Raf-1 interact with CNK1 consistent with previous studies (Rabizadeh et al., 2004; Ziogas et al., 2005). Surprisingly however, MST2 started to dissociate at 3 hr Fas stimulation from CNK1 and significantly at 6 hr stimulation of cells (first lane) compared to control untreated cells. Conversely, Raf-1 is retained in complex with CNK1 (second lane). Consistently, Staurosporine induced dissociation of MST2 from CNK1 at 30 min and at 120 min, in contrast to Raf-1 (Figure B, lanes 1, 2). MST1/2 are fully activated upon caspase cleavage, or can be initially activated independent of caspase upon apoptotic stimuli at an early time point of treatment prior to cleavage for further full activation (Lee et al., 2001; O'Neill et al.,

2005; Mattalanas et al., 2007). Interestingly, these events seem to be observable prior to the detection of cleaved PARP in these cells. At 2 hr Staurosporine treated cells, MST2 has started to dissociate, while the intact form of PARP is still observable. Subsequently the cells treated with Fas at 6 hr, showed a significant decrease in MST2's interaction with CNK1, along with a concomitant appearance of the 89 kDa cleaved fragment of PARP compared to untreated cells. Although PARP cleavage was not observed in Staurosporine treated cells probably due to shorter exposure time, a clear dissociation of MST2 was evident compared to untreated cells. These data indicate that CNK1 preassembles a CNK1/MST2/Raf-1 signalling module in non-stimulated cells. Taken together, these data suggest that CNK1 may also possibly facilitate the release of pro-apoptotic MST2 kinase in response to a potent apoptotic stimulus such as Fas antibody for activation, while restraining Raf-1 in the complex.

**Figure 4.A and 4.B. CNK1 is involved in Fas receptor and Staurosporine death signalling**

**A.**




**B.**

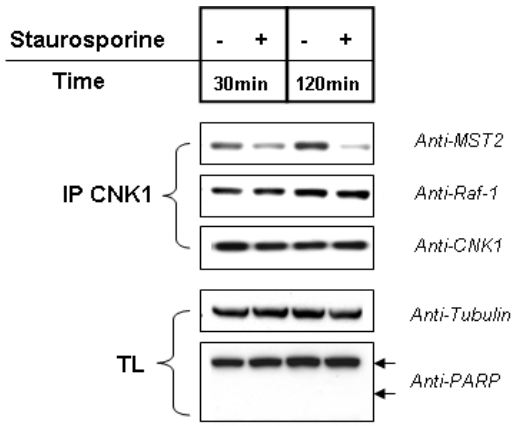



**Figure 4.A and 4.B. CNK1 is involved in Fas and Staurosporine death signalling.** After 16-20 hr serum-starvation, HeLa cells were untreated or treated with either Fas agonistic antibody CH-11 at 100 ng/ml for 3 hr and 6 hr or Staurosporine at 1μM for 30 and 120 min. Cells were lysed and immunoprecipitated (IP) with Anti-CNK1. Endogenous MST2, RASSF1A and Raf-1 co-immunoprecipitated with CNK1 from cells treated or untreated with apoptotic agents were analysed by western blotting with the indicated antibodies. Cells lysates were examined for the intact and cleaved forms of Poly (ADP-ribose) polymerase-1 (PARP) using anti-PARP. Tubulin immunoblot was used as a loading control. Full-length PARP and the cleaved fragment are indicated with arrows. Western blot image is a representative of two independent experiments.

### 4.2.2 CNK1 delays Fas-induced disruption of MST2: Raf-1 interaction.

Reversible protein-protein interactions are common in most biochemical pathways in cells. Indeed, the cellular decision to undergo apoptosis is determined by the integration of multiple survival and death signalling proteins (Degterev et al., 2003; Andersen et al., 2005; Guicciardi and Gores, 2009). Most often, scaffold proteins are dependent on the integration and formation of signalling components to carry out such vital cellular functions (Houtman et al., 2005; Alexa et al., 2010).

As Fas stimulation of cells suggests a time-dependent disruption of MST2 from the CNK1/Raf-1 complex (Figure 4.A), through protein–protein interactions, CNK1 may be able to rescue the dismantling of the complex by increasing local concentration of Raf-1 and preventing release of MST2. To determine this, first a time-course experiment examining the dissociation of the Raf-1:MST2 complex in cells treated with an agonist Fas antibody at different time points (0, 2, 4, 6 hr) but transfected with an intermediate CNK1 plasmid concentration (0.2 μg DNA) that promotes the highest interaction of Raf-1 and MST2 was performed. Hela cells were then transfected with 0.2 μg of Myc-CNK1 plasmid or control empty vector as before. Following serum starvation, cells were treated with an agonist Fas antibody at the indicated time and reciprocal immunoprecipitations of Raf-1 and MST2 proteins were analysed by western blot to investigate the dynamic change of the complex when exposed to Fas mediated by CNK1. Figure 4.C shows that in untransfected control cells, dissociation of Raf-1 and MST2 is already evident at 2 hr Fas stimulation and persists up to 6 hr. Both Raf-1 (first lane) and MST2 (third lane) immunoprecipitates blotted with associated antibodies exhibited similar results. However, cells transfected with Myc-CNK1 plasmid (0.2 μg) display an elevated Raf-1 and MST2 interaction at 2-4 hr time point, and this complex dissipates by 6 hr Fas stimulation. These findings indicate that at a definite exposure time of cells to apoptotic stimuli, CNK1 at a certain expression level may rescue the Fas-induced disruption of Raf-1:MST2 complex.

**Figure 4.C. CNK1 delays Fas-induced disruption of Raf-1:MST2 interaction**

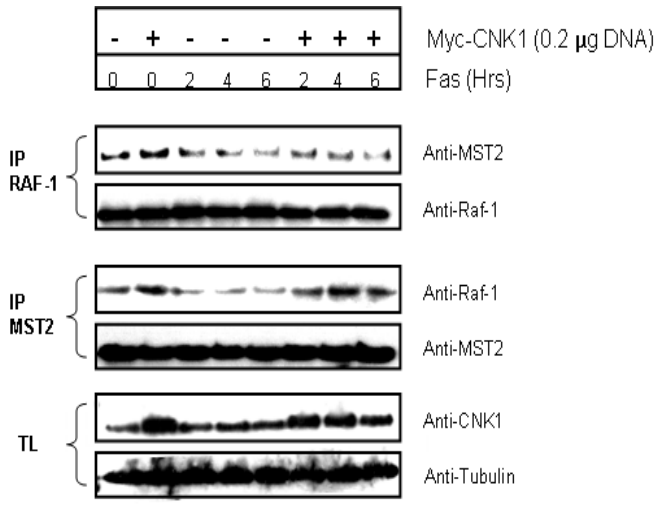



**Figure 4.C. CNK1 delay Fas-induced disruption of Raf-1:MST2 interaction.** After 16 hr serum-starvation of transiently transfected cells either with 0.2 µg Myc-CNK1 expression plasmids or empty vector, cells were treated with 100 ng/ml Fas agonistic antibody CH-11 at indicated time points. Cells were lysed and reciprocal immunoprecipitation of Raf-1 and MST2 were analysed by western blotting with indicated antibodies. Extracts were monitored for CNK1 expression with ani-CNK1 and tubulin for loading control. Western blot image is a representative of two independent experiments.

### 4.2.3 Levels of CNK1 affect MST2 phosphorylation in Fas-stimulated cells.

Raf-1 can interfere with Fas signalling by binding and inhibiting MST2 activation (O'Neill et al., 2004; Mattalanas et al., 2007). In light of the findings that CNK1 may delay the Fas-mediated disruption of Raf-1:MST2 hence may also delay apoptosis, I next wanted to extend these studies and determine whether CNK1 can still mediate the MST2: Raf-1 complex in a concentration-dependent manner during Fas death receptor signalling. I therefore carried out overexpression experiments similar to those in Chapter 3. As before, HeLa cells were transiently transfected with control empty vector or Myc-tagged CNK1 expression plasmids in increasing amounts (0-1.5 μg Myc-CNK1 DNA). Following serum starvation, cells were treated with Fas antibody for 4 hr, lysed and MST2 proteins were immunoprecipitated. Endogenous Raf-1 proteins that co-immunoprecipitated with MST2 were analysed by western blotting. As MST2 is activated via apoptotic agents such as Fas stimulation, phosphorylation status of MST2 was examined using anti-phophoMST2 antibody.

The CNK1-mediated interaction of Raf-1 and MST2 shows a different pattern when cells are treated with Fas at 4 hr stimulation in comparison to serum-starvation studies. Figure 4.D shows that in cells transfected with intermediate CNK1 plasmid concentration (0.05-0.25 μg Myc-CNK1), the Raf-1 and MST2 interaction compared to control (empty vector) was only slightly elevated. However, in cells transfected at higher concentrations (1.5 μg Myc-CNK1 DNA), the Raf-1:MST2 complex was significantly disrupted consistent with serum-starvation studies when cells are transfected with high CNK1 plasmid concentration. This observation also correlated to the phosphorylation status of MST2, i.e. in cells transfected at intermediate CNK1 plasmid concentration, the phosphorylation of MST2 was decreased while at high concentrations it was enhanced (second lane). Cell lysates were also probed with Anti-CNK1 to monitor CNK1 expression levels (fourth lane) and tubulin for loading control (last lane). Although, the results suggest that CNK1 may rescue cells due to inhibition of MST2 phosphorylation

in a concentration dependent manner under a potent apoptotic stimulus, whether the pool of MST2 sequestered by CNK1 to interact with Raf-1 is sufficient to represent the total amount of MST2 activated to induce apoptosis in these cells needs to be clarified by an apoptosis assay. Nevertheless, the results suggest that under a severe apoptotic stimulus, CNK1-mediated assembly of an apoptotic Raf-1:MST2 inhibitory complex may possibly delay or rescue cells from apoptosis via Fas stimulation by its ability to recruit inhibitory Raf-1 pools and simultaneously sequester and protect MST2 from phosphorylation which is crucial for its activation in a concentration dependent manner.

**Figure 4.D. Levels of CNK1 affect MST2 phosphorylation in Fas stimulated cells**

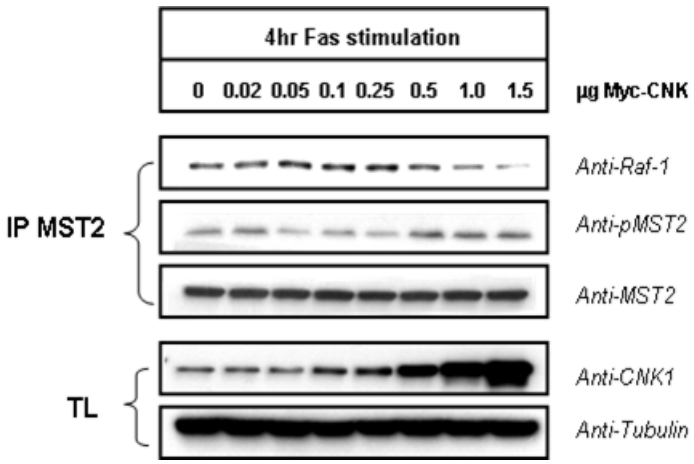



**Figure 4.D. Levels of CNK1 affect MST2 phosphorylation in Fas stimulated cells.** Cells were transfected with empty vector or increasing amounts of Myc-CNK1 plasmid as indicated. Following serum starvation, Cells were treated with 100 ng/ml Fas agonistic antibody CH-11 for 4 hours. Immunoprecipitated MST2 proteins were analysed by western blotting. Phosphorylation level of MST2 was determined using anti-phosphoMST2. Cell lysates were probed with anti-CNK1 to verify CNK1 expression and anti-tubulin for loading control. Western blot image is a representative of two independent experiments.

### 4.2.4 CNK1 reassembles its components in response to mitogenic stimulus

Raf-1 kinase relays the signals from EGFR-Ras to MEK and ERK kinases to confer different biological consequences which are regulated by protein-protein interactions, complex phosphorylation events, and adding to this complexity, implicated to be regulated by a growing number of scaffold proteins (Anselmo et al., 2002; Wellbrock et al., 2004; Kolch, 2005). Formation of appropriate complexes by scaffold proteins plays an important part in the specificity of the cell response to various stimuli (Vondriska et al., 2004; Marinissen and Gutkind, 2005). In line with this idea, I therefore examined if the Raf-1:MST2 complex mediated by CNK1 responds to mitogenic stimuli as Raf-1, a component of the CNK1 complex, is activated upon mitogenic epidermal growth factor (EGF) stimulus. I also investigated whether this complex is involved in the ERK/MAPK signalling pathway. EGF is a ligand that binds and activates EGFR, and has a potent growth-promoting effects in cells, stimulating cellular proliferation and differentiation; hence EGFR is usually hypermutated in cancer (Yarden and Sliwkowski, 2001; Schlessinger, 2002; Breuhahn et al., 2006).

Consistent with previous studies (Rabizadeh et al., 2004; Ziogas et al., 2005) and data presented here (Figure 4.A and 4.B), endogenous Raf-1 and MST2 bind to CNK1 in Hela cells, as shown in Figure 4.E. Surprisingly, analysis of CNK1 immunoprecipitates from cells treated with EGF showed that in contrast to Fas stimulation, MST2 is retained in complex with CNK1, while Raf-1 is dissociated from CNK1 in a time-dependent manner (lanes 1 and 2). This observation is in parallel with the analysis of MST2 immunoprecipitates of cells treated with EGF, i.e. Raf-1 dissociated from MST2 similarly in a time-dependent manner (lane 4). Because the change in CNK1/MST2/Raf-1 complex may be a result of activated Raf-1, cells lysates were examined for the activation of direct downstream kinases of Raf-1. As expected, in cells treated with EGF that dissociated Raf-1 from CNK1/MST2 complex, resulted in the activation of MEK and ERK as revealed by using anti-phosphorylation antibodies (lanes 6 and 7). The study was extended using the growth promoting supplement foetal bovine serum (10% FBS) over different periods of time (30 and 120 min). Consistently, compared to untreated

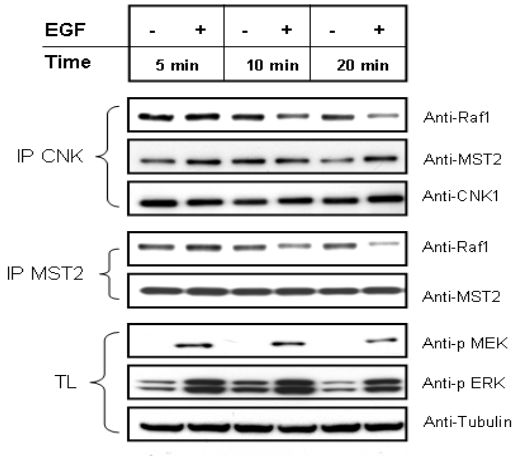
cells Raf-1 readily dissociated from the CNK1/MST2 complex. Raf-1 immunoprecipitates also confirmed the disruption of the Raf-1:MST2 complex in a time-dependent manner upon FBS stimulation of cells. Raf-1 dephosphorylation at S259 of Raf-1 immunoprecipitates suggests activation of Raf-1 kinase, which also confirms direct downstream ERK activity that was observed upon mitogenic stimuli of cells. Consistently, cells treated with insulin growth factor (IGF) and platelet-derived growth factor (PDGF) exhibit similar behaviour (Figure 4.G and 4.H) to EGF and 10% FBS in dissociating Raf-1 from the CNK1/MST2 complex, albeit there is difference in the duration of treatment. Interestingly, in cells treated with either of the mitogens, wherein phospho-ERK is transiently enhanced, Raf-1 dissociates from the CNK1/MST2 complex more readily. However, at longer and sustained stimulation wherein phosphor-ERK is back at basal levels, Raf-1 seems to be retained back to the complex. This observation is especially pronounced in cells treated with the growth factor PDGF. This observation may suggest that CNK1 may respond differently, depending on the type and duration of mitogenic stimulants.

The finding of the Raf-1:MST2 complex dissociation promoted by mitogenic stimuli presented here confirms previously observed reports (O'Neill et al., 2004; Romano et al., 2004). However, it was shown for the first time in this chapter that Raf-1 and MST2 are preassembled with CNK1 prior to a variety of mitogenic stimuli. Taken together, the data suggest that MAPK signal-transduction pathway may influence the ability of CNK1 to reassemble its components, i.e. Raf-1 and MST2 in response to the duration and type-specific mitogenic stimulation.

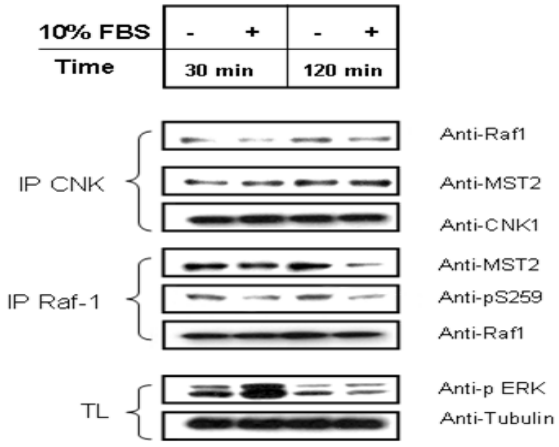
Preliminary studies of ectopic overexpression of CNK1 at different concentrations suggests a possible EGF duration-dependent effects on the Raf-1:MST2 complex (data not shown), however further confirmatory experiments need to be performed to get conclusive data, as I have I focused my attention on mapping the interaction domains of CNK1.

**Figure 4.E and 4.F. CNK1 reassembles its components in response to mitogenic stimulus**

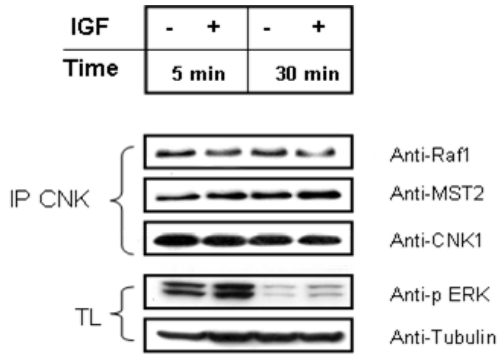
**E.**




**F.**



**G.**




**H.**

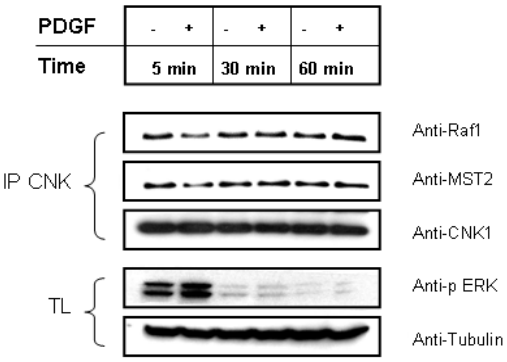



**Figure 4.E and 4.F. CNK1 reassembles its components in response to mitogenic stimulus. (4.E)** Following serum starvation, HeLa cells were either untreated or treated with 200 ng/ml of EGF for the indicated durations. CNK1 immunoprecipitates were analysed for endogenous co-immunoprecipitated MST2 and Raf-1 by immunoblot with indicated antibodies. MST2 immunoprecipitates were also analysed for co-immunoprecipitated Raf-1. MEK and ERK activity were examined using anti-phospho antobodies. **(4.F)** Following serum starvation of HeLa cells, cells were untreated or treated with 10% FBS at indicated durations. Endogenous Raf-1 and MST2 co-precipitated with CNK1 was analysed by immunoblot. Raf-1 immunoprecipitates were also analysed for co-immunoprecipitated MST2, as well as its activity using anti-phospho S259 antibody. Cell lysates were monitored for ERK/MAPK activity. **(4.G, 4.H)** Similar to above, but serum starved cells were untreated or treated with either 100 ng/ml of IGF or 20 ng/ml of PDGF, respectively. Endogenous Raf-1 and MST2 co-precipitated with CNK1 were analysed by immunoblot. Cell lysates were monitored for ERK/MAPK activity. Western blot image (Figure 4.E-4.H) is a representative of two independent experiments.

### 4.2.5 Mapping the interaction domains of CNK1

Because regulated protein–protein interactions play an important role in the transmission of cellular signals, molecular scaffolds have emerged as key modulators of the signalling process (Kolch, 2005). Therefore, to gain further insight as to the role of the CNK1 protein in signal transduction, in particular its role in the regulation of its associated proteins Raf-1 and MST2, a peptide array approach was used to map and determine the binding sites of these proteins to the CNK1 scaffold.

As discussed in Chapter 1, CNK proteins are evolutionarily conserved scaffold proteins crucial to a variety of signalling pathways. It is considered a scaffold protein as its architecture contains several interaction domains (Figure 4.J). The N-terminal part of CNK1 includes a sterile alpha motif (SAM), a conserved region in CNK (CRIC) and a PSD-95/DLG-1/ZO-1 (PDZ) domain. The C-terminal portion of CNK consists of a pleckstrin homology (PH) domain and polyproline sequences. CNK1 also contains a potential coiled-coil region, a unique proline-rich region that spans about 90 amino acids sequences and a conserved region among chordate (CRAC) domain that are common to mammalian CNK isoforms but are absent in *Drosophila* and *C. Elegans* (Claperon and Therrien, 2007; Fritz and Radziwill; 2005)

As indicated in this chapter, Raf-1, MST2 and RASSF1A were indentified to interact with CNK1 using two-hybrid, pull-down and co-immunoprecipitation methods (Rabizadeh et al., 2004; Ziogas et al., 2005). In this study, I explored this interaction further by using peptide array screening analysis that permits highly parallel synthesis of huge numbers of peptides spotted on nitrocellulose. Peptide library formats by use of SPOT-peptide arrays have been successfully used in carrying-out general protein–protein/peptide interaction studies including mapping and analysis of linear binding sites of several receptors, chaperones, cell-skeleton proteins and signalling molecules (Frank, 2002; Hilpert et al., 2007; Wu and Li, 2009).

To do this, synthesised libraries of overlapping 25-mer peptides, each shifted by five amino acids, spanning the complete sequence of CNK1 was immobilised on cellulose membranes and probed with [$^{35}$S] Raf-1, MST2 and RASSF1A labelled proteins using a coupled in vitro transcription/translation kit as described in Chapter 2 Materials and Methods. The radioactive signals on arrays were autoradiographed using a phosphoimager.

As shown in Figure 4.I, the possible binding regions of Raf-1, MST2 and RASSF1A within CNK1 are categorised as strong and medium, and boxed as yellow and red respectively. A detailed analysis of the positive regions on the array indicates that the three proteins involve several sites of contact; dark spots signified positive interactions (Figure 4.J). *In vitro* co-overexpression of both tagged-Raf-1 and truncated CNK1 was shown to interact. In particular, it was shown that full-length CNK1 was able to bind Raf-1, including its C-terminal region (starting at PDZ domain) but not its N-terminal region (SAM and CRIC region). Therefore, it was suggested that Raf-1 binds to the C-terminal of CNK1 (Rabizadeh et al., 2004). Consistently, the results indicated that Raf-1 binds mostly on residues comprising the C-terminal that contains the proline-rich region and PH domain with strong affinity (Figure 4.J, first panel). However, the findings indicate that Raf-1 also binds to CNK1 at several sites in the amino terminal region particularly in the SAM to PDZ domain region.

MST1/2 kinases were found to bind CNK1 through their SARAH domain located on their C-terminal. However, no previous studies reported on the mapping of their binding regions on CNK1 (Rabizadeh et al., 2004). Herein, peptide arrays indicate that MST2 binds to CNK1 mostly at residues 254-463 that contains regions of proline-rich region and PH domain showing strong affinity (Figure 4.J, second panel). Also, a few regions at coiled-coil regions and PDZ domains showed possible binding sites.

Similar to MST2, RASSF1A was previously shown to bind to CNK1 through its SARAH domain on its C-terminal. RASSF1A binds mostly on CNK1's amino-terminal region, 1-282 residues of the N-terminal and 77-279 that contains the CRIC and PDZ binds well to RASSF1A, while residues 199-279 exhibited modest binding. Interestingly, residues 279-713 which lacks the PDZ domain did not bind to RASSF1A.

Therefore, it was suggested that PDZ and CRIC was required for binding of CNK1 to RASSF1A (Rabizadeh et al., 2004). Consistent with this previous report, positive binding signals showed at CRIC and PDZ domains of CNK1 (Figure 4.J, third panel). However, peptide screening suggests that RASSF1A binds a wider region that comprises CRIC, PDZ and the PH domain and a few other strong residues at the C-terminal regions particularly at the coiled-coil regions.

Interestingly, further analysis of the possible binding sites of Raf-1, MST2 and RASSF1A indicates overlapping binding residues on CNK1. In particular, RASSF1A and Raf-1 had 10 similar binding residues to CNK1 either categorised as medium or strong affinity mostly on residues that encompass the PDZ, proline-rich, coiled-coil and PDZ domains. However, Raf-1 has 9 unique binding residues found in the SAM, CRIC and PH domains of CNK1. RASSF1A has 5 unique binding residues found at CRIC, proline-rich and at the C-terminal end of CNK1. Intriguingly, MST2 alone has no unique binding residue as most of it is shared with RASSF1A and all three proteins share 6 binding residues that reside mostly on PDZ (2 residues), Proline-rich (1 residue), PH (1 residue) and coiled-coil regions (2 residues).

As described in Chapter 1, interaction domains are crucial for the specificity of signal transduction downstream of cell-surface receptors and serve as binding modules that control many aspects of cellular mechanism and spatial organization of different molecules. Importantly, scaffold proteins utilize their several protein-protein interaction domains to recruit proteins and to mediate assembly of components for signalling (Pawson and Nash, 2000). In line with this, the finding from this peptide array screening study is consistent with the modular architecture of CNK1, as most of the binding residues exhibited by its client proteins lie mostly on its interacting domains.

**Figure 4.I. Mapping the interaction domains of CNK1**

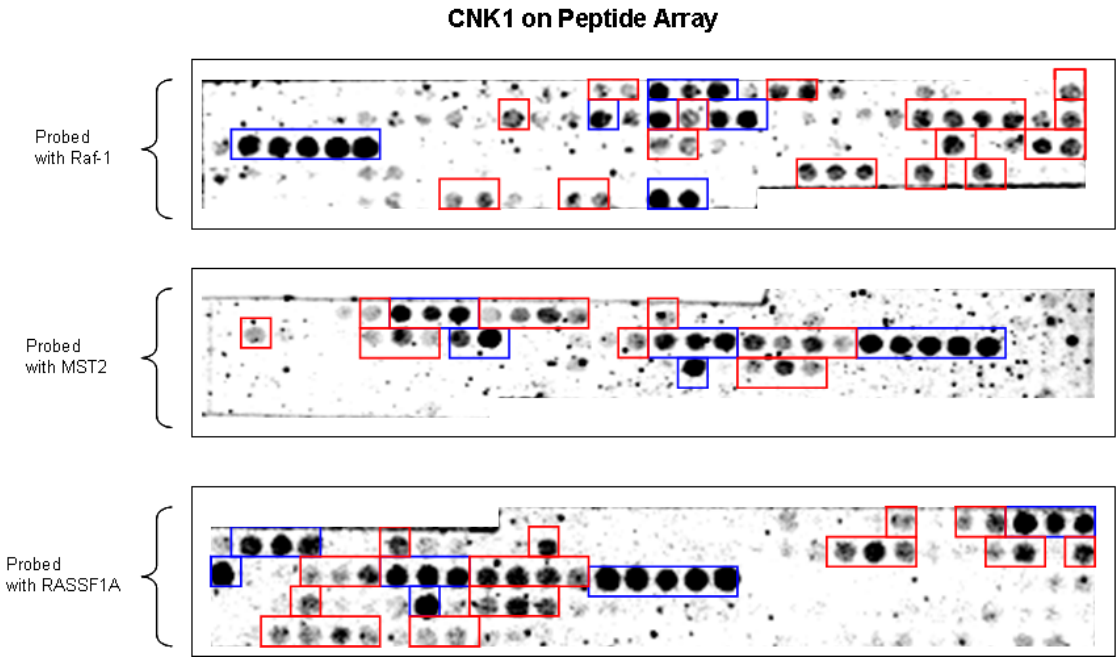



**Figure 4.I. Mapping the interaction domains of CNK1.** Nitrocellulose membranes were spotted with synthetic 25-mer peptides offset by 5 amino acids representing the whole CNK1 sequences. CNK1 on peptide array were probed with [$^{35}$S] Raf-1, MST2 and RASSF1A labelled proteins using a coupled in vitro transcription/translation kit as described in Chapter 2 Materials and Methods. The radioactive signals on arrays were autoradiographed using a phosphoimager. Positive binding sites were categorised as strong (yellow boxes) or medium (red boxes). Peptide array blot image is a representative of two independent experiments.

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**Figure 4.J. Analysis of binding sites from the peptide array**

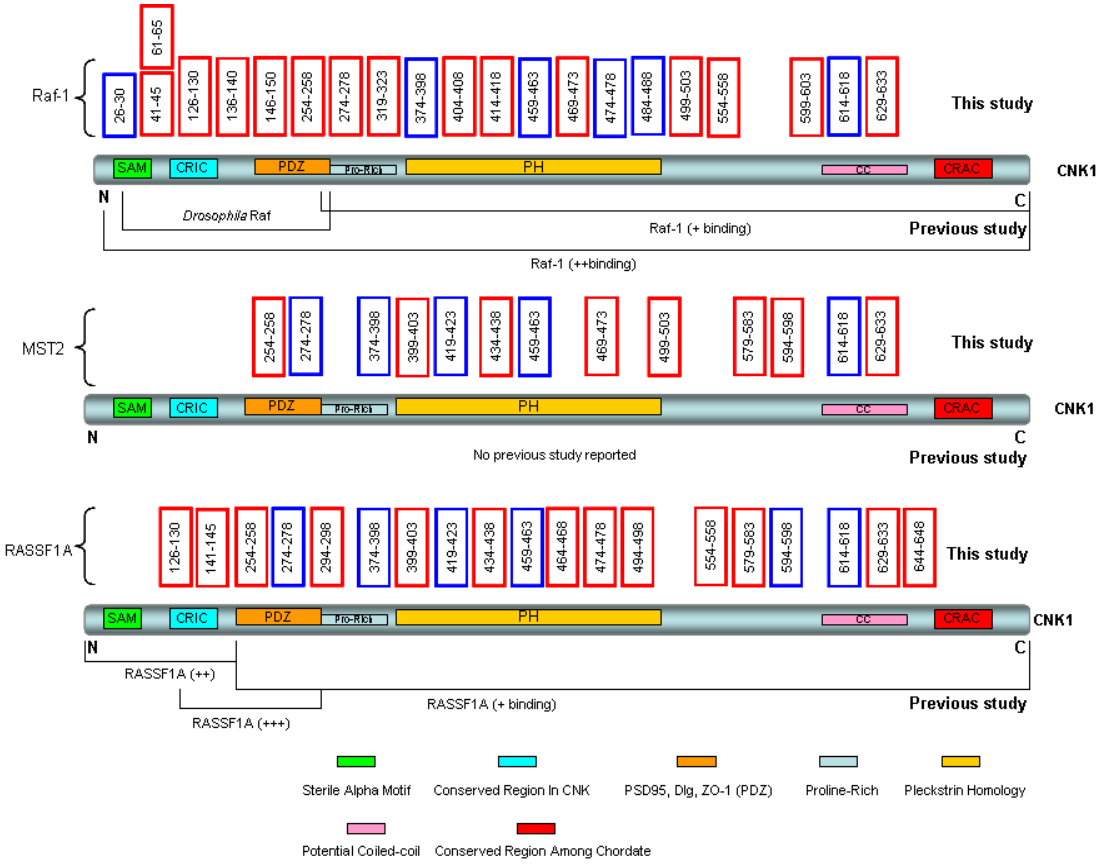



**Figure 4.J. Analysis of binding sites from the peptide array.** CNK1 protein with protein length of 713 amino acids is schematically shown with the following domains and motifs colour coded as indicated. Regions indicated are as follows: 7-70 (SAM domain), 78-164 (CRIC domain), 196-285 (PDZ domain), 285-378 (Pro-rich), 403-503 (PH domain), 616-646 (Coiled-coil). Raf-1, MST2 and RASSF1A used to probe the arrays are bracketed, and the mapped sites of interaction are shown as amino acid numbers and boxed according to binding affinity as indicated by yellow boxes (strong affinity) or red boxes (medium affinity). Indicated above the schematic are the binding sites of Raf-1, MST2 and RASSF1A found in the present study. Interaction sites identified prior to the present study are indicated below.

### 4.2.6 A short motif within CNK1 is necessary in mediating Raf-1:MST2 complex

To further characterise the binding sites mapped from the peptide array screening of Raf-1 and MST2 on CNK1, I therefore set out to generate truncated forms and mutants based on the screening results using site-directed mutagenesis as described in Chapter 2 Materials and Methods. Remarkably, further analysis of the peptide array data revealed a common binding site of Raf-1 and MST2 that stretches along the proline-rich region of CNK1 that exhibited a very strong signal. This was identified as the short motif Arg-Lys-Lys-Ser-Lys (RKKSK) that covers the peptide sequence 374-378, as schematically represented in Figure 4.K.I. This suggests that the short motif region that spans between the proline-rich and PH domain of CNK1 maybe essential for CNK1's function in mediating Raf-1 and MST2 complex.

Unfortunately, inserted tags designed to generate HA-tagged truncated and mutant forms were not verified by sequencing. Because of this technical problem and due to time constraints, I therefore decided to perform an analysis of mutants generated prior to insertion of tags and used to assess the effect on the Raf-1:MST2 complex.

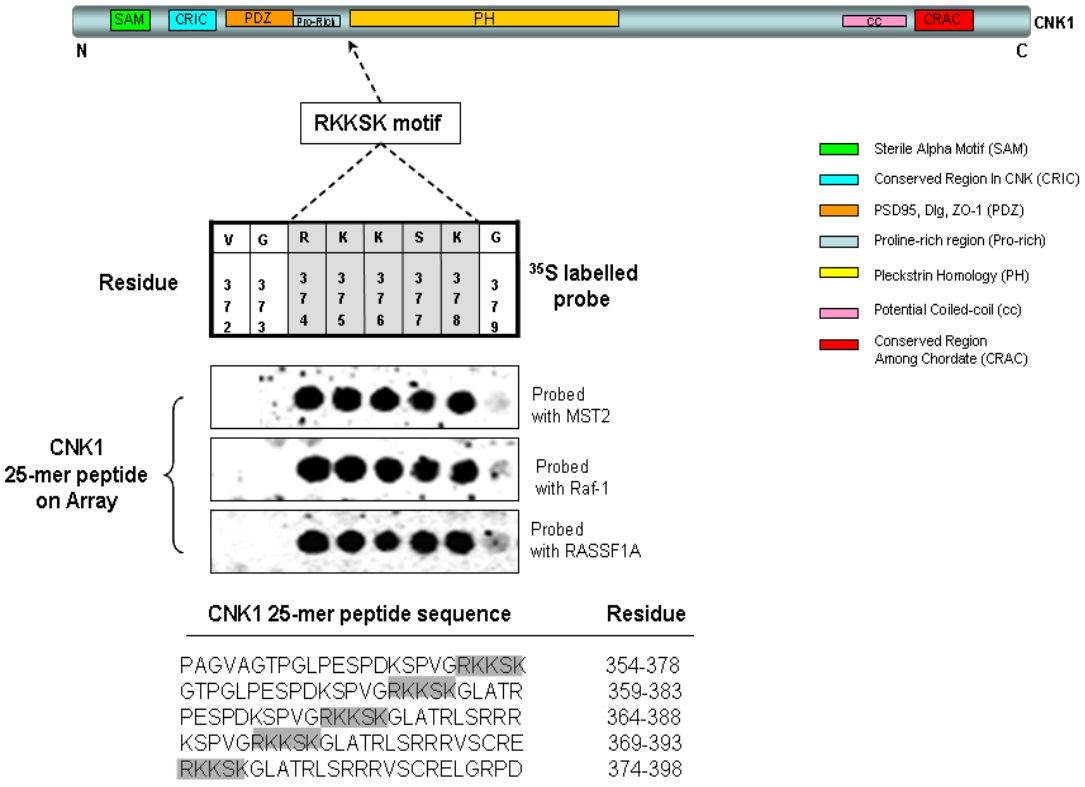
Database searches of CNK1 in the region that spans the short peptide sequence (RKKSK) reveal that the Serine at this residue is a highly predicted Serine phosphorylation site (predicted by Scansite motif Scan; http://scansite.mit.edu/motifscan or NetPhos2.0; http://www.cbs.dtu.dk/services/NetPhos), and interestingly is also predicted as a 14-3-3 binding site. Therefore, site-specific mutagenesis at the Serine residue of the identified short RKKSK motif were generated (i.e. S377A and S377D) and tested for their ability to mediate Raf-1 and MST2 interaction in cells.

Plasmids encoding wild-type CNK1 and the indicated mutants were transiently transfected at increasing amounts into Hela cells i.e. wild-type CNK1 at 0.2, 05, 1.5 μg, and CNK1 mutants at 0.2 and 1.5 μg of plasmid DNA. Following serum starvation, cells were lysed and Raf-1 immunoprecipitates were examined for co-immunoprecipitated

MST2 proteins by immunobloting. As shown in Figure 4.K.II, whereas cells transfected with wild type CNK1 exhibit the usual bell-shaped effect by mediating the interaction of Raf-1 and MST2 in a concentration-dependent manner, CNK1 mutants exhibited a different interaction pattern of Raf-1 and MST2 interaction. Substitution of S377 with Alanine (S377A) mutants showed no change of Raf-1 and MST2 interaction at either intermediate concentration or at high concentrations compared to the wild type control. Thus, replacement of serine 377 of CNK1 with Alanine abrogates scaffold function potentially by changing the binding affinity towards Raf-1 and MST2 as they may no longer be recruited to CNK1. Although, CNK1 transfection protocol has been demonstrated to be highly efficient it should be noted that expression of CNK1 in this western blot experiments was not monitored. Interestingly, substitution of S377 with Aspartic Acid (S377D) mutants showed only an increased interaction of Raf-1 and MST2 at higher concentrations therefore no longer manifest the bell-shaped scaffold effect of mediating Raf-1 and MST2 of wild-type CNK1. A clearer interpretation of this result requires determination of independent binding capacity of these mutants independently of Raf-1 and MST2 by pull-down immunoprecitation using tagged-CNK1 mutant constructs, however these findings suggests that the short RKKSK motif region of CNK1 may play an important role in the scaffolding function in mediating Raf-1 and MST2 interaction.

**Figure 4.K. A short motif within CNK1 is necessary in mediating the Raf-1:MST2 complex**

**I.**




| CNK1 25-mer peptide sequence | Residue |
|---|---|
| PAGVAGTPGLPESPDKSPVGRKKSK | 354-378 |
| GTPGLPESPDKSPVGRKKSKGLATR | 359-383 |
| PESPDKSPVGRKKSKGLATRLSRRR | 364-388 |
| KSPVGRKKSKGLATRLSRRRVSCRE | 369-393 |
| RKKSKGLATRLSRRRVSCRELGRPD | 374-398 |

**II.**

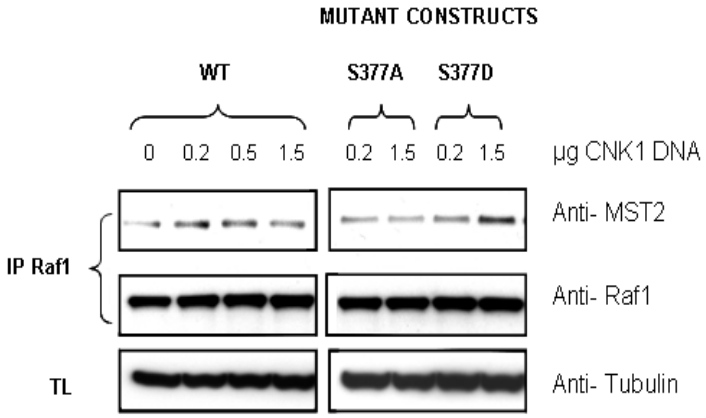



**Figure 4.K. A short motif within CNK1 is necessary in mediating the Raf-1:MST2 complex. (I)** A Schematic of CNK1 showing its multi-domain structure and motifs colour coded as indicated, and locations of CNK1 mutations studied. Amino acid sequence of sequential 25-mer CNK1 peptides offset by 5 amino acids with indicated peptide number identified from the short motif. Positive strong signals on the arrays probed with Raf-1, MST2 and RASSF1A from Figure 4.I are compared to show the common binding motif. **(II)** Analysis of CNK1 mutants and wild-type CNK1 in HeLa cells under serum-starved conditions. Cells were transfected with CNK1 mutants or wild-type CNK at indicated concentrations. Following serum starvation, lysates were subjected to immunoprecipitation and Raf-1 immunoprecipitates were analysed by immunobloting with indicated antibodies. Equal loadings were confirmed using antibodies against tubulin. Western blot image is a representative of two independent experiments.

## 4.3 Discussion

### CNK1 is involved in Fas receptor and Staurosporine death signalling

In Chapter 3, the results presented indicate a scaffold role of CNK1 by mediating the inhibitory Raf-1:MST2 complex in a 'bell-shaped' concentration-dependent manner that results in either inhibiting or promoting a serum-starvation cellular stress-induced MST2 activation and MST2/Hippo apoptosis of cells, typical manifestations of a scaffold protein.

Scaffold proteins are also believed to promote specificity by their ability of binding and coordinating simultaneously to multiple components of a signalling pathway. This specificity is achieved by several mechanisms, including temporal and spatial control of signalling components. In particular, it has been proposed that scaffolds may assemble specific bound components into pathway-dedicated signalling molecules in response to stimuli and prevent components from straying away or protecting them from other signalling intrusions (Murphy and Blenis, 2006; Brown and Sacks, 2009).

The results presented here extended the scaffold functions of CNK1 in the regulation of Raf-1:MST2 complex. In this chapter, I have shown that CNK1 may be involved in Fas death receptor apoptotic signalling through regulation of the tumour suppressor MST2 kinase. First, no previous characterization of the interaction between endogenous MST2, Raf-1 and CNK1 has been reported. Here, it was revealed that in quiescent cells MST2, Raf-1 and CNK1 are preassembled. However, upon Fas stimulation, MST2 dissociates from this trimeric complex (Figure 4.A). These data demonstrate and further support why MST2 and Raf-1 interaction are enhanced in serum-starved cells and dissociate when MST2 is activated (O'Neill et al., 2004), as MST2 is sequestered by CNK1 and links to Raf-1, hence restrains from activation of inevitable loss of growth factors and as

a consequence of this prolonged cellular stress, MST2 is released from the CNK1/Raf-1 complex to be activated, and can therefore promote apoptosis of cells.

## CNK1 delays Fas-induced disruption of MST2: Raf-1 interaction

While Fas agonist antibody or Staurosporine renders a more potent apoptotic stimulus, dissociation of MST2 from CNK1/Raf was relatively rapid, occurring within hours from the start of stimulation (Figure 4.A and 4.B). This finding therefore suggests a time-dependent dissociation of MST2 from the Raf-1/CNK1 complex. To exploit a possible temporal scaffold function of CNK1, an optimal concentration level that promotes reassembly of Raf-1 and MST2 was used to test this possibility. Indeed, the findings revealed that CNK1 may rescue the release and impending activation of MST2 in a time-dependent manner (Figure 4.C and 4.D). MST2 depends on the phosphorylation of Thr180 for kinase activity, which occurs prior to caspase cleavage (Graves et al., 2001; O'Neill et al., 2004). Phosphorylation of full-length MST1/2 can be reversed by protein phosphatases such as PP2A, unlike the cleaved form that translocates to the nucleus where it promotes chromatin condensation during apoptosis (Ura et al., 2001; Deng et al., 2003; O'Neill et al., 2004). Curiously, in a recent mass spectrometry analysis, CNK1 complexes contained peptides from three subunits of the serine/threonine phosphatase PP2A, implicating PP2A as a potential regulator of CNK phosphorylation (Kim et al., 2010). It is therefore plausible that CNK1 at an optimal expression level may provide protein phosphatases such as PP2A by recruiting them to the complex, along with the recruitment of inhibitory Raf-1 function in close proximity to sequester, inactivate and protect MST2 from further activation. Taken together, the time-course Fas stimulation study of the Raf-1:MST2 complex under the regulation of dose-dependent overexpression of CNK1 suggests that CNK1 may manifest a temporal regulation of the Fas death receptor signalling by mediating the phosphorylation and activation of MST2, through linking of Raf-1 and possibly phosphatases to form an inhibitory complex that may delay or restrict Fas death receptor apoptotic signalling in cells.

## Levels of CNK1 affect MST2 phosphorylation in Fas-stimulated cells.

MST2 is also activated independently of caspase cleavage by RASSF1A (Mattalanas et al., 2007; Romano et al., 2010; Guo et al., 2011). RASSF1A phosphorylation of MST2 is specific to full length MST2 as the caspase-cleaved truncated forms do not contain the SARAH domains at the C-terminal of the kinase which are required for RASSF1A binding ((Mattalanas et al., 2007). Intriguingly, the SARAH domain that is present on both proteins binds to the C-terminal of CNK1 (Rabizadeh et al., 2004) which suggests that CNK1-bound MST2 may also be specific to the full length MST2. Moreover, autophosphorylation of MST2 is dependent on abundance on its concentration which appears to have important regulatory significance (Deng et al., 2003). It can be argued that CNK1 may promote such a mechanism, as one function of a scaffold protein is to enhance local concentration of its client proteins and enhance interaction between signalling molecules (Shaw and Filbert, 2009). Incidentally, in the LoVo human colon carcinoma line, the abundance of CNK1 in the MST1 (close homologue of MST2) immunoprecipitate is greatly increased after overnight serum withdrawal, as well as CNK1 and RASSF1A in 293 cells (Rabizadeh et al., 2004). Coincidentally, as reported here, MST2, Raf-1 and CNK1 are preassembled in serum-starved cells further supporting this finding.

Upon apoptotic stimulation, RASSF1A activates and releases MST2 away from Raf-1 and promotes its association to its substrate LATS1 to signal downstream Hippo signalling to promote apoptosis (Mattalanas et al., 2007). More recently, it was reported that RASSF1A activates MST1/2 by preventing dephosphorylation of its activating Thr180 site by protein phosphatase PP2A (Guo et al., 2011). Interestingly, it was also reported by the same group that MST2 and RASSF1A are preassembled in unstimulated cells. In line with these findings and together with the data presented here, it is conceivable that CNK1 scaffold protein may play a central role in regulating this mechanism, given the fact that CNK1 binds both RASSF1A and MST2, and has been demonstrated to promote apoptosis when co-expressed with RASSF1A and MST1/2 (Rabizadeh et al., 2004). In addition, CNK1/RASSF1A/MST2 complexes are

preassembled prior to apoptotic stimulus as supported by previous reports (Rabizadeh et al., 2004; Guo et al., 2011).

Consistent with these findings, it is therefore tempting to speculate the following model that the recruitment of MST2 by RASSF1A to promote MST2 phosphorylation, binding to its substrate LATS1 and inducing apoptosis may be regulated by CNK1. The scaffold protein CNK1 preassembles a CNK1/MST2/RASSF1A sequestered in an inactive complex in quiescent cells, in response to an incoming apoptotic stimulus, CNK1 may then facilitate the activation of MST2 by RASSF1A and release of both proteins to interact that results in the formation of an MST2/LATS1 apoptotic signalling complex.

Nevertheless, the mechanistic steps by which the CNK1/RASSF1A/MST2 complex facilitates a tumour suppressive function and the coordination of an inhibitory Raf-1 into the complex in response to apoptotic stimuli remain unclear. Likewise, the dynamic change that occurs in the CNK1/MST2/Raf-1 complex in response to mitogenic stimuli is still elusive. Although it is not surprising as Raf-1 regulation is highly complex and multifaceted. However, the findings presented here may provide a foundation and a direction in investigating the mechanism of CNK1, in particular the regulation of MST2 and Raf-1 involvement in MAPK signalling.

## CNK1 reassembles its components in response to mitogenic stimulus

MST2 is activated upon stress and apoptotic stimuli leading to dissociation of Raf-1:MST2 complex (Mattalanas et al., 2007). On the other hand, another study has shown that oncogenic Ras and mitogens also disrupt Raf-1:MST2 complex without the activation of MST2 (O'Neill et al., 2004). Immunoprecipitation of CNK1 and analysis of co-precipitated Raf-1 and MST2 here may shed light on to this discrepancy. The findings indicate that prior to mitogenic or apoptotic stimulation, CNK1 preassembles a CNK1/MST2/Raf-1 complex. Upon cellular stimulation, Raf-1 and MST2 dissociates, and whichever signalling molecule may require activation will be released. Alternatively, whichever signalling molecule is not required for signalling is sequestered

to CNK1, dependent on the subsequent specific stimulus. Thus, the dissociation Raf-1 and MST2 in response of either apoptotic or mitogenic stimulation is observed.

However, a recent study by Romano et al. (2010) has suggested an alternative explanation of this conundrum. Because AKT can be activated via activated Ras or by mitogenic stimulation, the authors suggested that AKT phosphorylation of MST2 at T117/T384, which are different from the activating site, may protect MST2 activation during growth factor stimulation by maintaining the dissociation of MST2 from RASSF1A and binding of MST2 to Raf-1 in cancer cells. Interestingly, in a recent report CNK1 was found to be critical for the activation of the downstream PI3/AKT cascade upon IGF stimulation (Lim et al., 2010). Moreover, CNK1 interacts directly with AKT in inhibiting the activation and cytoplasmic sequestration of pro-apoptotic FoxO transcription factors (Fritz et al., 2010). In line with this, MST1/2 promotes apoptosis by activating FoxO in response to oxidative stress (Lehtinen et al, 2006). As AKT binds to the C-terminal and N-terminal of CNK1, it is plausible that CNK1 recruitment of Raf-1 in cooperation with AKT phosphorylation of MST2 induces binding with Raf-1 thereby protecting MST2 and/ or over-all AKT-dependent inhibition of MST2-mediated apoptosis which may further underscore the role of CNK1 in mediating MST2.

However, CNK1-mediated protection of MST2 activation may well be dependent on the ability of CNK1 to recruit Raf-1 and MST2 at an optimal stoichiometric level under apoptotic conditions. In contrast, whether CNK1 is dependent on recruitment of MST2 and Raf-1 to influence Raf-1 kinase activation under mitogenic conditions, and if MST2 has a reciprocal role in Raf-1 regulation remains to be determined. As alluded to earlier, preliminary results indicate that ectopic overexpression of CNK1 at different concentrations suggest a possible EGF duration-dependent effect on the Raf-1:MST2 complex (data not shown), however further confirmatory experiments need to be performed to get a conclusive data as I instead focused my attention on mapping the interaction domains of CNK1 due to time constraints. Interestingly, a recent report demonstrated an unexpected positive role for MST2 in the Raf-1/ERK pathway (Kilili and Kyriakis, 2010). The authors demonstrated that MST2 regulates Raf-1 activation at least in part through the maintenance of optimal expression levels of PP2A-C which in turn reduces Raf-1 Ser-259 inhibitory phosphorylation. As they could not detect

endogenous PP2A-C or PP2A in endogenous MST2 or Raf-1 immunoprecipitates, they therefore suggested that if an endogenous complex exists to stabilize PP2A, the stoichiometry of the endogenous complex may be low, or the complex may be unstable. It is tempting to speculate that this is consistent with the suggested model of CNK1 i.e. the possibility of recruiting PP2A to inhibit MST2 or in this case under mitogenic conditions for Raf-1 kinase regulation, as CNK1 is a possible regulator of protein phosphatases (Kim et al., 2010). It would therefore be interesting to determine if PP2A occurs in complex with CNK1/MST2/Raf-1. Moreover, CNK1 was found to interact with Raf-1 and Src to activate ERK via pro-angiogenic vascular endothelial growth factor (VEGF) but not EGF; however B-Raf also binds CNK1 (Ziogas et al., 2005) which complicates the matter. It is evident that CNK1 involvement with MST2 and Raf-1 regulation is highly complex and has seemingly paradoxical functions. Alternatively, scaffold protein functions have both positive and negative effects on signalling and therefore may fill the gap of some contradictory observations on this opposing Raf-1:MST2 complex.

## Mapping the interaction domains of CNK1

In line with this idea, mapping of the binding sites revealed a complex interaction. However, it is consistent with the scaffold property of CNK1 on its ability to bind proteins in its multidomain structure. Indeed, Raf-1, MST2 and RASSF1A each revealed several possible binding sites with strong affinity towards CNK1's interacting domains. Although, binding regions are consistent with previous studies some previously undiscovered binding sites exhibited strong signals. For example, it was suggested that Raf-1 binds to the C-terminal of CNK1 (Rabizadeh et al., 2004). However, peptide array analysis indicates that Raf-1 also binds to CNK1 at several sites in the amino terminal region particularly in the SAM to PDZ domain region. Interestingly, Rabizadeh et al findings indicate that full-length CNK1 binds more strongly than the C-terminal region alone to Raf-1. Therefore, it could be argued that there may be regions in the amino terminal that may be crucial in Raf-1 binding. Interestingly, in *Drosophila*, the ability of Ras to activate Raf-1 strictly depends on SAM and CRIC domains of CNK (Douziech et al., 2007). It will be interesting to determine if such mechanism exist in mammalian

CNK1 as my results indicate several possible binding sites in these regions. Also, compared to previous reports that RASSF1A binds mostly to CRIC and PDZ of CNK1, my peptide screening data suggests that RASSF1A binds a wider region that comprises CRIC, PDZ and the PH domain and a few other strong residues at the C-terminal regions, particularly at the coiled-coil regions. Incidentally, it was suggested that RASSF1A functions as a scaffold protein as well, as it assembles multiple proteins and contains binding domains that are involved in protein-protein interactions and membrane association (Donninger et al., 2007). Therefore, it is not surprising that the data from peptide arrays indicate a strong affinity and wider span of binding sites compared to previous studies.

Meanwhile, MST2 revealed for the first time in this study binding sites with a strong affinity at the proline-rich and PH domains of CNKI. Interestingly, proline-rich regions are often involved in complex protein association because of the rapid and non-specific nature of their interaction. Also, PH domains are involved in signal transduction particularly in recruiting proteins to specific cellular compartments (Williamson, 1994, Pawson, 1995). Of note, it is conceivable that MST2 binds to wide regions of CNK1 to enable protection from activation or cleavage of its N-terminal upon caspase activation which is supported from the peptide array data.

## A short motif within CNK1 is necessary in mediating Raf-1:MST2 complex

Upon further analysis of these peptide arrays, a short binding motif that is common to Raf-1, MST2 and RASSF1A was found and located at the regions spanning the Proline-rich region and start of the PH domain of CNK1. A mutant construct substituting the S377 to Alanine or Aspartic Acid of the RKKSK motif of CNK1, provides evidence for a probable functional role of the proline-rich region of CNK1 in mediating the Raf-1:MST2 complex. Of note, Akt phosphorylates Serine or Threonine residues in the motif RxRxxS/T (R= arginine, S = serine, T = threonine and x = any amino acid) containing proteins (Yaffe et al., 2001). The phosphorylation of its substrate will in many cases result in subsequent binding of 14-3-3 which also binds to matching consensus motif as

Akt. Regulation of this motif induces conformation, localisation and activate or inhibit intrinsic enzymatic of proteins in the complex (Aitken et al., 2002). As CNK1 has been implicated to interact with Akt or probably 14-3-3 through Raf-1 it will be interesting to test if RKKSK motif on CNK1 may be regulated as well as it is a potential phosphorylation or binding site of either proteins. However, a detailed binding characterisation of these mutations independently of Raf-1 and MST2 including RASSF1A will further confirm these findings.

Interestingly, further analysis of the binding sites of Raf-1, MST2 and RASSF1A indicates overlapping binding residues on CNK1. It is conceivable that these proteins compete for binding to CNK1 in similar manner to how Raf-1 competes with RASSF1A to bind MST2 as their binding site was found to be overlapping at the coiled-coil region (SARAH) C-terminal of MST2 (Mattalanas et al., 2007). A report demonstrated that MST2 binds to the hSav scaffold protein that contains coiled-coil domains on their corresponding coiled-coil regions (Callus et al., 2006). In line with this, peptide arrays showed that MST2 and RASSF1A which both have coiled-coil domains bind to residues comprising the coiled-coil region of CNK1. Alternatively, as CNK1 contains several binding domains which suggest the possibility of CNK1 forming into a hetero-oligomeric complex that may accommodate all three proteins, or even several molecules. For example, PDZ domains are known to interact with greater versatility as they can bind with C-terminal motifs, lipids, and other scaffolds, even with its internal peptide sequences therefore enabling dimerization and assembly of supra-molecular complexes (Harris and Lim, 2001; Pawson, 2007; Lee and Zheng, 2010).

Finally, although highly speculative, it may be likely that distinct CNK1/MST2, CNK1/RASSF1A or CNK/Raf-1 complex may exist or perhaps a macromolecular complex of CNK1/MST2/RASSF1A/Raf-1 is also plausible that renders a more sophisticated mechanism. CNK1 contains four domains that function to facilitate multi-protein-protein interactions, giving it the ability to exhibit allosteric, spatial and conformational regulation including formation of hetero-oligomeric complexes (Claperon and Thierren, 2007). Therefore, whether distinct complexes exist or supra-molecular scaffolded complex exist for CNK1, similar to those that exist for the scaffolds MORG (Vomastek et al., 2008) or JIP1 scaffold (Willoughby et al., 2003)

which assembles opposing molecules as discussed in Chapter 3 remains to be determined. Moreover, a specific stepwise mechanism of CNK1-mediated Raf-1:MST2 complex is yet to be determined and existence of other mechanisms yet to be identified cannot be rule out. However, the findings suggest that CNK1 may play a crucial role in the coordination of both opposing kinases in response to proliferative or apoptotic stimuli.

# CHAPTER 5

# Final Discussion

# Final Discussion

Understanding the mechanisms that regulate signals critical for cell growth or apoptosis is essential in controlling diseases like cancer. Cells have the ability to control signals that they come across and, dependent on this environment, this response determines their fate. Maintenance of these signals that promote cell survival or prevent apoptosis is therefore an important aspect in the instigation of tumourigenesis (Hanahan and Weinberg, 2000).

Indeed, numerous evidence has shown that the balance of the intensity and duration between apoptotic and survival signals transmitted by the different signalling molecules such as the MAPK module mediated by scaffold proteins is a determinant in the decision whether a cell survives or undergoes apoptosis (Murphy and Blenis, 2006). It is not surprising, as MAP kinase signalling is heavily involved in mechanisms that regulate activation of key oncogenes and tumour suppressors as well as specificity and amplification of signal transduction, as described in Chapter 1. In addition, the spatial and temporal organisation of different molecules within the cell is an essential step in coordinating the many distinct activities carried out by the cell in response to an array of stimuli (Mor and Phillips, 2006). In an increasing number of biological signalling processes that are elucidated, scaffold proteins have been found to play a central role in physically assembling the relevant molecular components to carry-out these many diverse activities (Morrison and Davis, 2003; Kolch, 2005).

The aim of this thesis was to examine the role of CNK1 in the regulation of proto-oncogene Raf-1 and tumour-suppressor MST2. First, I have shown that CNK1 manifests a prototypical concentration-dependent manner by mediating the interaction of Raf-1 and MST2 under cellular stress conditions by serum starvation of cells. Given the role of scaffold proteins to assemble different proteins, the concentration of a scaffold needs to be titred or at an optimal concentration of the components it binds, therefore high concentration of scaffolds will dilute out the components (Levchenko, 2000). Consistent with this, at intermediate CNK1 concentrations Raf-1 and MST2 form a complex, thereby allowing Raf-1, independent of its kinase activity, to inhibit MST2

phosphorylation. This is crucial for its activation, and therefore MST2 binding to Raf-1 leads to reduced apoptosis of cells under cellular stress conditions. However, at higher expression of CNK, Raf-1 and MST2 dissociate, therefore freeing MST2 from the inhibitory function of Raf-1, which results in its phosphorylation and induction of apoptosis. This dissociation of the CNK1-mediated Raf-1:MST2 complex results in the association of MST2 with its substrate LATS1, suggesting activation of downstream pro-apoptotic Hippo signalling. The requirement of CNK1 in the mediation of Raf-1:MST2 complex formation was demonstrated further by depleting CNK1 using RNAi. Depletion of CNK1 impaired Raf-1 and MST2 interaction, with a concomitant enhancement of MST2 kinase activity and increased MST2/LATS1 interaction, sensitising cells to growth-withdrawal induced apoptosis. Moreover, co-siRNA experiments of MST2 and CNK1 prevented cells from apoptosis indicating that CNK1-mediated apoptosis is dependent on tumour suppressor Hippo/MST2 signalling. Whereas co-siRNA of CNK1 and Raf-1 markedly induced apoptosis compared to control and depletion of Raf-1 alone which suggest that Raf-1 requires CNK1 to link with MST2 to exert its inhibitory role. Therefore, at physiological levels of CNK1 in Hela cells, it may protect the cells from apoptosis however at a very high expression it may render the cells to be more sensitive towards programmed cell death.

Second, this role of CNK1 was extended in Fas death receptor signalling. I have shown for the first time that MST2 and Raf-1 endogenously interact with CNK1 and are preassembled prior to Fas stimulation. This may explain why MST2 and Raf-1 interaction are enhanced in quiescent cells and dissociate upon cellular stress and apoptotic stimulation. Moreover, the rapid dissociation of MST2 from Raf-1 after Fas stimulation may be delayed or rescued by CNK1 in a time-dependent manner which indicates a temporal regulation role of CNK1. The decision for cells to undergo apoptosis is determined by the integration of multiple survival and death signalling molecules (Utz and Anderson, 2000). Therefore, through protein–protein interactions, CNK1 may directly act on a critical component of the cellular proapoptotic signalling machinery by preventing caspase activation or cleavage of MST2 by compartmentalising a Raf-1:MST2 inhibitory complex.

Third, I have shown that CNK1 may play a role in the mediation of Raf-1 and MST2 interaction in mitogenic conditions. Importantly, I have shown for the first time that MST2, Raf-1 and CNK1 are preassembled prior to mitogenic stimulation, which may explain why Raf-1 and MST2 interaction are also disrupted by mitogenic stimulation. These findings indicate a CNK1-mediated interaction of Raf-1:MST2 dependent of the type and duration of mitogenic stimulants.

Fourth, I have mapped possible binding sites of Raf-1, MST2 and RASSF1A on CNK1 by peptide array, which confirms previous reports but also revealed novel binding sites that suggest complex binding mechanisms. Consistent with this, multidomain scaffold proteins have been demonstrated to assemble different molecules and coordinate complex mechanisms such as conformational, kinase activities and forming large hetero-oligomeric complexes, and are themselves target of regulation (Ebisuya et al., 2005; Good et al., 2011).

Lastly, a short common binding motif of Raf-1, MST2 and RASSF1A to CNK1 revealed from the peptide array data, that it may have an important functional role in mediating Raf-1 and MST2 interaction, or RASSF1A which suggests a scaffold role in the Hippo/MST2 apoptotic signalling. Moreover, mutation of a Serine residue alters Raf-1 and MST2 binding to CNK1, further supporting the significance of this motif.

## Conclusion

In conclusion, scaffold proteins are believed to confer specificity in signal transduction by their ability to assemble a set of multiple binding partners into a pathway-dedicated signalling complex dependent to a particular stimulus and duration, including regulation of spatial and temporal cell signalling processes (Kolch, 2005; Bhattacharyya et al., 2006; Saito, 2010; Malleshaiah et al., 2010). CNK1 protein is characterised as a scaffold protein due to its intrinsic multidomain structure and has been demonstrated to function as a scaffold protein by binding to different proteins involved in several signalling pathways (Fritz and Radziwill, 2011). This thesis has provided evidence in support of

the direct role of CNK1 as a scaffold protein by its ability to mediate the Raf-1:MST2 complex. Moreover, the work presented here contributes to the current understanding of the mechanisms underlying the regulation of the opposing proto-oncogene Raf-1 and tumour suppressor MST2 interaction via apoptotic signals as well as growth-promoting signals. Further study of CNK1's scaffold function is likely to prove fruitful for understanding how cells coordinate and shape the response to their survival or own demise, further contributing to the understanding of cancer cell expansion and progression.

## CNK1 Model

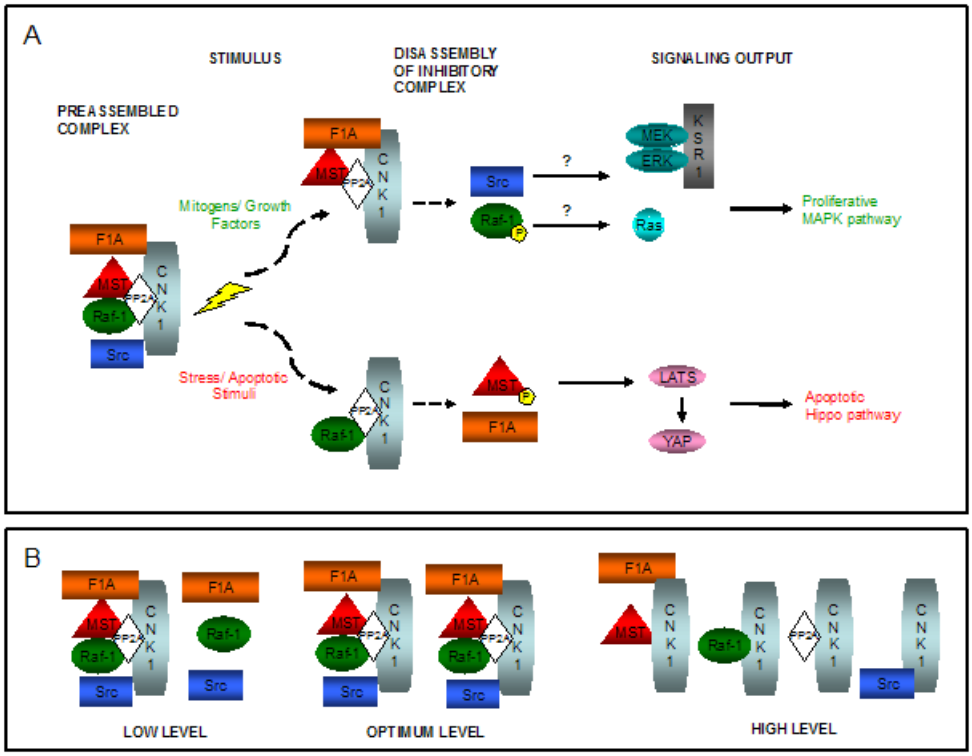



**Figure 4.L. CNK1 Model. A.** CNK1 preassembles a complex of interacting proteins in quiescent cells. Upon stimulus, CNK1 facilitates the disassembly of the complex and induces conformation of appropriate interacting signaling modules to carry-out their function specific to the stimulus. **B.** At low levels of CNK1, regulation of its client proteins is limiting. At optimum levels of CNK1, an efficient control of the complex can be facilitated, however at higher levels of CNK1, client proteins are diluted and therefore regulation of their signaling output is dissipated.

## Future Directions

The cellular outcomes of CNK1 scaffold proteins are as divergent and complex. The challenges ahead include understanding at systems level, of how growth factor signalling and apoptotic signalling makes use of a scaffold protein, possibly such as those of CNK1's circuitry, which is may be crucial for cellular decisions that determine cell fate. Hence, the scaffold features of proteins in cell signalling are now starting to be appreciated. As specific compounds that target a single molecular cellular target in many cases will likely fail for complex diseases like cancer, it has been proposed that molecular networks associated with a disease may be an alternative target for intervention (Pawson and Linding, 2008). This strategy involves a synthetic biology approach that aims to engineer or rewire (i.e. changing assembly of protein kinases) signalling to change the response of cellular pathways, for example by manipulating multidomain proteins to change the response of cells from mitogenic signals to cell death signals (Howard et al., 2003). Nevertheless, accumulating evidence indicates that the properties of multidomain structures such as that of scaffold proteins may prove valuable in understanding important mechanisms for controlling signal transduction pathways and may be exploited for diverse therapeutic and biotechnological applications.

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