Structural and mechanistic insights into RAF kinase regulation by the KSR/CNK/HYP complex

by

Thanashan Rajakulendran

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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2011

Abstract

The RAS/RAF/MEK/ERK pathway is the prototypical cellular signal transduction cascade and has been the focus of intense scrutiny over the last two decades. As a mitogenic pathway, its activation is a potent driver of cellular growth and survival, and its deregulation underlies many cancers. While RAS family GTPases have long been recognized as prolific human oncogenes, a landmark study in 2002 also established the RAF family kinase as a bona fide oncogene (Davies et al., 2002). Indeed, aberrant RAS-RAF signaling underlies nearly one-third of all human cancers (Wellbrock et al., 2004). Notably, mutations in RAF are found with astounding frequency in certain cancers (e.g. 70% of malignant melanomas) (Dhomen and Marais, 2007). These findings have identified intercepting aberrant RAF function as an ideal therapeutic target. RAF is a Ser/Thr protein kinase and its activity is strictly regulated by a core complex of at least three proteins, namely, KSR, CNK and HYP (Claperon and Therrien, 2007). The mechanism by which the KSR/CNK/HYP complex regulates RAF function remains enigmatic. In particular, the function of KSR in regulating RAF activity is highly controversial. The work described in this thesis was conducted with the aim of: i) understanding the interactions that underlie formation of the KSR/CNK/HYP complex, and ii) elucidating the mechanism by which the complex regulates RAF function. I have attempted to accomplish these aims using a combination of structural biology, biochemistry and cell biology approaches. I begin by presenting the structure of the SAM domain mediated interaction between CNK and HYP. I describe a model for how the CNK/HYP interaction in turn serves to recruit KSR to form the higher-order KSR/CNK/HYP complex.
Subsequently, I describe the allostERIC mechanism by which KSR controls RAF activation via the formation of specific side-to-side kinase domain heterodimers of KSR and RAF. Lastly, I describe a potential mechanism by which RAS directly mediates the attainment of the side-to-side dimer configuration of RAF through its own ability to form dimers. The acquisition of the side-to-side dimer configuration is essential for aberrant RAF signaling in cancers, suggesting future RAF inhibition strategies could be aimed at preventing dimer formation.
Acknowledgements

I would like to begin by expressing my deepest gratitude to Dr. Frank Sicheri – whose role as my supervisor, mentor and friend during my graduate school journey have allowed me to realize my full potential as a scientist. Frank’s passion for science is infectious and his ability to share his passion for science added considerably to my graduate school experience. I would also like to thank Dr. Mike Tyers, who was my co-supervisor from 2006-2007. Although my foray into his lab was brief, it was under Mike’s tutelage that I developed a strong sense of appreciation for in vivo studies employing model organisms. In addition, I would like to convey my gratitude to my supervisory committee members, Dr. Jim Dennis and Dr. John Rubinstein, who were instrumental in helping me to hit the ground running as I began my graduate training. They provided invaluable insight and guidance with all aspects of my research.

I thank my collaborator, Dr. Marc Therrien, whose motivation, encouragement and willingness to embark on my wild science hypotheses moved my research forward. I am truly grateful for his wonderful mentorship and friendship. I would also like to thank Dr. Malha Sahmi in the Therrien Lab for her commitment and dedication to the in vivo aspects of my research. Without her expertise, my research would never have got off the ground.

I must acknowledge Dr. Daniel Mao in the Sicheri Lab. He not only played a role in convincing me to join the Sicheri Lab while I was still a ‘roton’, but he also took me under his wing to mentor me in the ways of the research lab. Without his genuine kindness and mentorship, I would have lost my many battles with PCR and cloning. I am greatly indebted to Dan for always finding the time from his own research to mentor, inspire and to motivate. I must also acknowledge numerous members, past and present, from the Sicheri and Tyers Labs for their invaluable guidance, assistance and friendship over the years. In particular, I thank Dr. Derek Ceccarelli for his help with getting me initiated to the world of X-ray crystallography and helping me
to solve my first crystal structure. Appreciation also goes out to Stephen Orlicky for his advice and input on many aspects of my research over the years.

I recognize that my research would not have been possible without the financial support of the Canadian Institutes for Health Research (CIHR), Canadian Cancer Society (CCS) and The Terry Fox Foundation (TFF). Financial support was also provided in part by a University of Toronto Open Fellowship. I also want to acknowledge the invaluable support and assistance provided by the Advanced Photon Source in Argonne, Illinois and the wonderful staff at BioCARS and NE-CAT.

Finally, I would like to thank my parents and my sister for their encouragement and support during my graduate school journey. It was my parents that introduced me as a child to the wonders of science, and I have embraced it with a passion ever since. I doubt that I will ever be able to convey my appreciation fully, but I owe them my eternal gratitude.
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List of Publications

The following publications pertain to the work described in this thesis:

**Primary research:**


**Review articles:**


Amino Acid Symbols

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Table A.1 – The 20 standard amino acids.

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<td>a.a.</td>
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<td>ADP</td>
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<tr>
<td>AGC</td>
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</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) kinase</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mol</td>
<td>Mole</td>
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<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein database bank</td>
</tr>
<tr>
<td>PDB ID</td>
<td>Protein database bank identifier</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP dependent protein kinase</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile α motif</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>Sec (or s)</td>
<td>Second</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phosphine</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinases</td>
</tr>
<tr>
<td>TKL</td>
<td>Tyrosine kinase-like</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v-raf</td>
<td>Viral-rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
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<tr>
<td>w/v</td>
<td>Weight to volume</td>
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<td>wt</td>
<td>Wild type</td>
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Chapter 1

Introduction
1 Introduction

1.1 The protein kinase family

1.1.1 Protein phosphorylation takes centre stage

Protein phosphorylation regulates virtually all aspects of eukaryotic cellular biology in which the covalent attachment of a phosphate moiety from ATP onto Ser, Thr and/or Tyr residues in proteins regulates a myriad of cellular functions (Fig. 1.1). Underscoring the importance of protein phosphorylation, approximately 2-3% of all eukaryotic genes encode proteins containing the conserved catalytic core necessary for carrying out protein phosphorylation, referred to as the protein kinase domain (Manning et al., 2002).

Nearly six decades ago, protein phosphorylation was first recognized. In 1954, Burnett and Kennedy identified the first protein, Casein kinase, whose specific function was to carry out protein phosphorylation (Burnett and Kennedy, 1954). Significant inroads into our understanding of protein phosphorylation followed on the heels of Burnett and Kennedy’s work in the 1950s. In 1955, Fischer and Krebs documented how the inactive from of glycogen phosphorylase transitions to the active form in the presence of ATP, Mg$^{2+}$ and a protein they termed phosphorylase kinase (Fischer and Krebs, 1955; Krebs and Fischer, 1955). Subsequently, in 1959, Fischer described the exact process by which phosphoryl transfer from ATP to a serine residue in glycogen phosphorylase is responsible for the conversion of the enzyme from an inactive to active state (Fischer et al., 1959).

In the years that followed, the pervasiveness of protein phosphorylation in cellular functions became clear. The discovery of protein phosphatases, enzymes whose function is to remove attached phosphates from proteins and thus antagonistic to the role of protein kinases, underscored the dynamic nature of protein phosphorylation (Ingebritsen and Cohen, 1983). Perhaps the most imperative recognition of the pervasiveness of protein phosphorylation was borne out of studies of signal transduction pathways – the process by which cells perceive and respond to external and internal stimuli. The first example of a
protein kinase signaling cascade to be described was the c-AMP dependent protein kinase (PKA) mediated activation of phosphorylase kinase (Walsh et al., 1968). PKA has served as the prototypical protein kinase and has been the focus of much investigation that has trail blazed our understanding of the entire protein kinase superfamily. PKA also offered the first glimpse into the enigmatic structural features of the kinase domain when it was visualized by X-ray crystallography in 1991 (Knighton et al., 1991).

1.1.2 The human kinome

It quickly became evident that protein kinases are vastly abundant in cells and matched by an equally abundant repertoire of cellular functions to which they contribute. Although vastly diverse in the cellular function(s) to which different kinases take part in, all protein kinases essentially carry out the same basic phosphoryl transfer function. The release of the first draft of the human genome sequence made possible for assigning a dendrogram of all human genes that contain a putative protein kinase domain – 518 distinct genes in total (Manning et al., 2002) (Fig. 1.2). Manning and colleagues divided the genes into eight major groups according to their sequence similarities: AGC, CAMK, CK1, CMGC, STE, TK, TKL and aPK (refer to abbreviations section for full names) (Manning et al., 2002). The protein kinase domain comprises the third largest domain class encoded in the human genome. Most notably, nearly 10% of putative protein kinases contain unexpected residues in highly conserved catalytically critical positions within the kinase domain (Boudeau et al., 2006). Termed pseudokinases, they are thought to be incapacitated in their ability to carry out phosphoryl transfer or do so at greatly diminished rates relative to their fully functional protein kinase counterparts.

1.2 The kinase domain: a structural perspective

1.2.1 The kinase fold

As mentioned above, PKA was the first protein kinase to be structurally characterized and serves as a template for the entire kinase superfamily (Knighton et al., 1991; Taylor et al., 1992) (Fig. 1.3). The catalytic kinase domain, found in all protein kinases, comprises a ~300 residue bilobal tertiary structure in which a small N-terminal lobe is connected by a short hinge region to a larger C-terminal lobe. The hinge region, encompassing approximately 10 residues, confers the kinase domain a degree of flexibility in which the two lobes can move about to modulate the conformation of the catalytic cleft – the space between the two lobes. Also referred to as the ligand or ATP binding pocket, the catalytic cleft is lined
Figure 1.2 – Dendrogram of human protein kinases. The 518 genes possessing a protein kinase domain in the human genome were analyzed by Manning et al., 2002. Seven distinct groups of kinases within the kinase superfamily are shown in this human kinome tree. An eighth group of more divergent atypical kinases (aPK group) is not depicted in the dendrogram. Figure adapted from Manning et al., 2002. Shown in red circle is the location of the RAF/KSR family of kinases within the TKL group (see section 1.4).
by a number of highly conserved residues that are critical to the mechanism of phosphoryl transfer (Huse and Kuriyan, 2002). As suggested by its alternate name, the cleft is also the site of ATP binding in which the adenine ring of ATP becomes nestled into a hydrophobic pocket at the back of the cleft to stabilize what is termed the hydrophobic spine of the kinase domain. ATP binding is tightly coupled to the coordination of two divalent metal ions (usually Mg$^{2+}$) that positions the ATP in the correct orientation for phosphoryl transfer. The metal ions are co-ordinated via the catalytic cleft residues as well as the phosphate groups of ATP.

The N-terminal (N-) lobe is primarily comprised of a five-stranded anti-parallel β-sheet interdigitated with a single helix referred to as helix αC (Fig. 1.3). Helix αC is a critical transducer of conformational changes in the kinase domain and often couples these changes to the adoption of the active/inactive state of the kinase (Huse and Kuriyan, 2002). The loop between helix αC and strand β4 acts as a handle for modulating the conformation of helix αC while simultaneously anchoring the N-lobe to the larger C-terminal (C-) lobe (Kannan et al., 2008).

The C-lobe is predominantly helical in structure. A two-stranded β-sheet at one end of the C-lobe along with the loop element flanking this β-sheet imparts a hydrophobic pocket for accommodating the adenine ring of ATP. The C-lobe contains much of the infrastructure necessary for catalysis, metal binding, and substrate protein binding. A large labile element referred to as the activation segment is also found in the C-lobe (Fig. 1.3). As with helix αC in the N-lobe, the activation segment is a critical transducer of conformational changes in the kinase domain and its conformation is tightly coupled to the activation status of the kinase (Huse and Kuriyan, 2002). Post-translational and/or allosteric modulation of the
conformations of both helix $\alpha_C$ and/or the activation segment constitutes a reoccurring mechanism by which many kinases are regulated (discussed in section 1.2.5).

1.2.2 Defining the kinase domain motifs

From extensive analyses of multiple sequence alignments of divergent protein kinases, Hanks and colleagues outlined the most salient parts within the protein kinase domain that underlie phosphoryl transfer function (Hanks et al., 1988). In all, twelve kinase domain motifs or sub-domains have been defined to date. The sub-domains contain highly conserved residues that mediate ATP and substrate binding as well as the phosphoryl transfer reaction during the course of the catalytic cycle. Four of these sub-domains are described in detail below to better highlight the mechanism by which phosphoryl transfer occurs.

Sub-domain I is defined by the glycine-rich loop between strand $\beta_2$ and $\beta_3$ in the N-lobe that is instrumental to engaging the $\alpha/\beta$ phosphate groups of ATP (coloured orange in Fig. 1.3). The loop essentially forms a lid over the bound ATP and the tip of the loop positions the $\gamma$ phosphate of ATP for phosphoryl transfer (Cox et al., 1994; Huse and Kuriyan, 2002). Sub-domain II is defined by the conserved VAVK motif in which the Lys residue is involved in interactions that co-ordinate ATP (Fig. 1.4). Specifically, the Lys locates to strand $\beta_3$ in the N-lobe and bridges a conserved Glu residue in helix $\alpha_C$ via an electrostatic interaction, while concurrently making interactions with the $\alpha/\beta$ phosphate groups of ATP.

The catalytic loop contained within sub-domain VIb contains the conserved HRD motif. The Asp residue within this motif (Asp166 of PKA in Fig. 1.4) is the catalytic base and functions to correctly orient the hydroxyl acceptor group in the peptide substrate for phosphoryl transfer (Huse and Kuriyan, 2002). Among the most important residues for catalysis is an invariant Asp residue (Asp184 of PKA in Fig. 1.4) found in sub-domain VII as part of the DFG motif. Also known as the Mg$^{2+}$ binding loop, this Asp residue forms contacts with all three phosphates of ATP via coordination of divalent cations (usually Mg$^{2+}$). The DFG motif defines the N-terminal start point of the activation segment described previously.
(Fig. 1.3), while the C-terminal portion of the activation segment is defined by the conserved APE motif of sub-domain VIII (Nolen et al., 2004). Typically ~25 residues in length, the activation segment comprises the activation loop and the substrate binding loop in addition to the Mg$^{2+}$ binding loop. In most kinases, the activation loop contains one or more residues that are subject to regulatory phosphorylation by other kinases or via autophosphorylation. This serves to stabilize the activation loop in a conformation permissive for maximal catalytic activity.

1.2.3 Mechanism for phosphoryl transfer

The mechanism for phosphoryl transfer exploited by protein kinases proceeds via one of two possible routes (Ablooglu et al., 2000). In the first mechanism, referred to as the associative $S_N2$ mechanism, the phosphate is transferred to the substrate hydroxyl-group with accompanying inversion of its stereochemistry in a single step. The hallmark of this mechanism is the formation of a transition state comprising a penta-covalent phosphorane moiety with three negative charges (Ablooglu et al., 2000) (Fig. 1.5). In the second possible mechanism, referred to as the dissociative $S_N1$ mechanism, a trigonal-planar metaphosphate transition state is formed in a two-step mechanism (Ablooglu et al., 2000). Currently, evidence exists that lends support to predictions made by both models (Cook et al., 2002; Madhusudan et al., 2002). The approach has been to utilize compounds predicted to resemble transition state intermediates in the reaction but stable enough to be captured by experimental means. For example, crystal structures of PKA bound to Mg$^{2+}$ ADP and AlF$_3$ (a planar compound that is thought to capture the transition state) have been determined and predicted to represent transition state structures (Madhusudan et al., 2002). Despite their differences, the two mechanisms are not mutually exclusive and are thought to overlap in actuality.
1.2.4 The conformational plasticity of kinase domains

A kinase domain transitions between two conformations: an active (ON) state that coincides with maximal catalytic activity, and an inactive (OFF) state that has minimal activity (Fig. 1.6). Because all protein kinases catalyze the same phosphoryl transfer reaction, upon activation they all adopt catalytically active conformations that are structurally very similar (Huse and Kuriyan, 2002).

This structural similarity stems from the juxtaposition of the two lobes in such a manner as to orient specific residues in the catalytic cleft for nucleotide binding and \( \gamma \)-phosphate transfer. In contrast, the inactive states of kinases are not subject to the spatial constraints that the active states must conform to, and so different families of kinases have evolved unique inactive states in which the adoption of the active conformation is hindered in different ways. Elucidating the regulatory mechanism(s) by which different kinases transition from an inactive to an active conformation is an area of topical interest in kinase biology.

Classically, the active and inactive states are often distinguished by the presence of the Glu-Lys salt-bridge in the N-lobe (see section 1.2.2). This salt-bridge is a defining feature of the active state and is uncoupled in the inactive state. A second distinguishing feature is the establishment of a two-stranded \( \beta \)-sheet between strand \( \beta 6 \) in the catalytic loop and strand \( \beta 9 \) in the activation segment in the active state (Nolen et al., 2004).

1.2.5 Higher order modes of kinase regulation

The pervasiveness of protein kinases in cellular functions arises from the vast assortment of catalytic switching mechanisms employed by different kinases as exemplified by the distinct regulatory mechanisms underlying CDK and Src family kinase function (see below). In general, the catalytic activity of a kinase can be regulated by means of direct modulation of structural elements within the
kinase domain (e.g. helix αC and/or activation loop). Modulation of structural elements is regulated in response to: i) intramolecular interactions of the kinase domain with other parts of the protein in which it is found (e.g. in Src family kinases); ii) via intermolecular interactions with regulatory proteins (e.g. in CDK family kinases); or iii) via covalent modification of residues within the kinase domain (e.g. the phosphorylation of site(s) in the activation loop of many kinases) (Huse and Kuriyan, 2002).

Modulation of the conformations of helix αC and/or the activation loop represent two very common mechanisms of kinase regulation. Intramolecular interactions with flanking regions or domains can positively and negatively regulate kinase activity by impinging on the conformation of helix αC and/or the activation loop. The classic example of intramolecular regulation is that of the Src family tyrosine kinases that are shackled to SH2 and SH3 domains (that reside N-terminal to the kinase domain) in their inactive states (Sicheri et al., 1997; Xu et al., 1997) (Fig. 1.7). Specifically, i) an intramolecular interaction between the phosphorylated tail region of the Src kinase domain and the N-terminal SH2 domain, and ii) between the SH3 domain and the linker connecting the SH2 domain to the kinase domain, stabilize inhibitory conformations of both helix αC and the activation loop that is incompatible with catalysis. Release of the SH2 and SH3 domain interactions transitions the kinase domain to an active conformation which is further stabilized by phosphorylation of a residue in the activation loop.

The cyclin-dependent kinase (CDK) family provides a classical example of allosteric kinase regulation via intermolecular interactions with regulatory proteins (Jeffrey et al., 1995) (Fig. 1.8). CDKs are catalytically inactive on their own but convert to the active form upon binding cyclins. In the absence of a bound cyclin, helix αC is rotated away from the orientation necessary to maintain the critical Lys-Glu
ion pair for catalysis as discussed earlier (section 1.2.2). Cyclin binds directly to helix αC and nearby elements in the kinase N-lobe, inducing the reverse rotation that reinstates the Lys-Glu ion pair.

Principles of Src and CDK family kinase regulation exemplify the great diversity in catalytic switching mechanisms employed by different kinases. With over 500 kinases encoded in the human genome, encompassing dozens of unique kinase families (Manning et al., 2002), elucidating the regulatory mechanisms employed by different kinase families represents a topical area of investigation.

1.3  Protein kinases in disease

Because of their involvement in virtually all aspects of cellular function, it’s not surprising that aberrant protein kinase activity is frequently found in many diseases (Greenman et al., 2007). In particular, owing to the central role of protein kinases in signal transduction cascades that modulate cellular growth and survival, mutations in protein kinases occur at impressive frequencies in many cancers. The recent success story of the kinase inhibitor Gleevec in treating patients with chronic myeloid leukemias has identified protein kinases as druggable targets (Schindler et al., 2000). Indeed, presently, drug discovery efforts targeting protein kinases constitute one of the largest investments in the pharmaceutical industry (Janne et al., 2009).

Mutations in protein kinases that convert the enzyme to a constitutively inactive or active state can interfere with normal cellular functions. Mutations in kinases that underlie many diseases continue to be uncovered at unprecedented frequencies. The protein kinase superfamily represents the largest number of genes with somatic mutations in human tumours, suggesting that aberrant kinase activity might contribute to tumourigenesis (Greenman et al., 2007). Many mutations in several kinases have been validated as disease driving mutations that meet the criteria for disease causality rather than bystander mutations. To illustrate the pervasiveness of aberrant protein phosphorylation in disease, Table 1.1 lists protein kinases that are commonly mutated in various cancers (Lahiry et al., 2010).
1.4 RAF kinases enter the limelight

Nearly 30 years ago, Rapp and colleagues cloned C-RAF, the cellular homologue of the v-raf oncogene that was acquired by a murine retrovirus (Rapp et al., 1983). Independently, Bister and co-workers identified a new oncogene, the naturally occurring avian retrovirus Mill-Hill No. 2 (MH2), which they named v-mil after the virus (Jansen et al., 1983). Subsequent sequence comparisons and biochemical studies confirmed that both groups had identified orthologues of the same protein kinase from mice and chickens, respectively. Homologues of C-RAF were found in *Drosophila melanogaster* (D-Raf) and *Caenorhabditis elegans* (lin-45), and two related genes, A-RAF and B-RAF, were later described in vertebrates as Ser/Thr specific kinases (Wellbrock et al., 2004).

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Examples of somatic mutations associated with tumorigenesis</th>
<th>Examples of effects of gene target deletion in transgenic animal models</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>Widespread with greater incidence in ovary, skin, colon and thyroid cancers and glioblastomas</td>
<td>Midgestation lethality with vascular defects due to endothelial apoptosis</td>
</tr>
<tr>
<td>BTK</td>
<td>Lung carcinoma</td>
<td>Missense mutation leads to failure of mature B lymphocyte production and of immunoglobulin heavy chain rearrangement</td>
</tr>
<tr>
<td>CHEK2</td>
<td>Glioblastomas</td>
<td>Embryonic stem cells fails to maintain γ-irradiation-induced arrest in G2 phase</td>
</tr>
<tr>
<td>ERBB3</td>
<td>Prostate, bladder and breast cancers and glioblastomas</td>
<td>Prenatal lethality due to lack of Schwann cells and precursors</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Stem cell leukemia lymphoma (FGFR1–ZNF198 chimerism), pancreatic adenocarcinomas, glioblastoma, breast carcinomas and lung cancers</td>
<td>Embryonic KO is embryonic lethal due to lack of embryonic growth and mesodermal patterning</td>
</tr>
<tr>
<td>FLT4</td>
<td>Increase in metastasis in adenocarcinoma and lymph node cancer, glioblastoma, kidney and ovary carcinoma and melanoma</td>
<td>Missense mutation in the catalytic domain leads to chylous ascites accumulation and limb swelling</td>
</tr>
<tr>
<td>INSR</td>
<td>Stomach and skin cancers and glioblastomas and colorectal cancer</td>
<td>Normal birth but postnatal fatal diabetic ketoacidosis</td>
</tr>
<tr>
<td>IRAK4</td>
<td>Prostate cancer</td>
<td>Severely impaired interleukin 1 and Toll-like receptor signalling</td>
</tr>
<tr>
<td>JAK3</td>
<td>Acute megakaryoblastic leukemia and gastric adenocarcinoma</td>
<td>Knockout of the catalytic domain leads to reduced number of thymocytes and severe B cell and T cell lymphopenia</td>
</tr>
<tr>
<td>RET</td>
<td>Lung, ovarian, bladder, large intestinal carcinomas, pheochromocytoma, thyroid tumours and glioblastomas</td>
<td>Severe defects in kidney and enteric nervous system development</td>
</tr>
<tr>
<td>STK11</td>
<td>Widespread with greater incidence in lung, cervical and pancreatic cancers and melanoma</td>
<td>Midgestation lethality due to neural tube defects, mesenchymal cell death and vascular abnormalities</td>
</tr>
</tbody>
</table>

Table 1.1 – Protein kinases in disease. Table modified from Lahiry et al., 2010.
All RAF proteins share a common architecture, with three conserved regions: N-terminal CR1 and CR2 regions followed by a C-terminal kinase domain (referred to as CR3; detailed in section 1.4.3). A-RAF is the smallest isoform, ~68 kDa, while C-RAF is ~74 kDa. B-RAF undergoes alternative splicing to produce a range of proteins from ~75 to 100 kDa. The three isoforms display distinct and overlapping tissue tropism. C-RAF mRNA is ubiquitously expressed, while A-RAF mRNA is concentrated in urogenital organs and B-RAF mRNA is largely restricted to neuronal tissues. However, subsequent studies have shown that all three isoforms are fairly ubiquitously expressed in all tissues with some enrichment for protein levels in certain tissue types (e.g. B-RAF in neuronal tissues) (Wellbrock et al., 2004).

Studies have shown that the three RAF proteins carry out redundant and non-redundant functions. Knockout mice studies have revealed that a-raf<sup>-/-</sup> survive to birth, but die after 7–21 days from neurological and gastrointestinal defects (Pritchard et al., 1996). b-raf<sup>-/-</sup> or c-raf<sup>-/-</sup> die in utero between 10.5 and 12.5 days post-coitum. The b-raf<sup>-/-</sup> embryos are characterized by growth retardation, and vascular and neuronal defects, while c-raf<sup>-/-</sup> embryos succumb to widespread apoptosis within the developing liver (Huser et al., 2001; Wojnowski et al., 1997).

1.4.1 The ERK/MAPK signaling cascade

As iterated previously, protein kinases often serve as critical transducers in signal transduction cascades. This notion is perhaps best illustrated by the RAF family of protein kinases that sit within the highly conserved ERK/MAPK signaling pathway – the prototypical signal transduction pathway/cascade in eukaryotes (Claperon and Therrien, 2007) (Fig. 1.9). The ERK module consists of the RAS GTPase, which is the upstream regulator of RAF function. Binding of extracellular ligands (e.g. growth factors, cytokines and hormones) to

Figure 1.9 – The ERK pathway comprises a three-tiered kinase cascade involving RAF, MEK and ERK family kinases. Activation of the cascade begins with growth factor stimulated activation of receptor tyrosine kinases (RTKs) which trigger activation of the RAS GTPase, which in turn recruits and and facilitates RAF activation. Activated ERK is a strong driver of cell proliferation and anti-apoptosis.
their cognate cell-surface receptors triggers RAS activation by promoting exchange of GDP for GTP in RAS. Activate RAS-GTP initiates a complex series of events that culminates in RAF activation (see below). Activated RAF phosphorylates the activation loop of the downstream dual-specificity (i.e. capable of phosphorylating both Ser/Thr and Tyr residues) kinases MEK1 and MEK2. Activated MEK1/2 in turn phosphorylate ERK1 and ERK2 on their activation loops. Depending on the cellular context, activated ERKs impinge on a myriad of diverse cellular functions including cell growth, survival and differentiation through phospho-regulation of substrate proteins involved in transcription, metabolism and cytoskeletal rearrangements (Wellbrock et al., 2004).

1.4.2 B-RAF: a potent cancer driver

The ERK module has long been recognized as a potent oncogenic pathway. Historically, this realization was largely due to the presence of RAS in the pathway – the most frequently mutated oncogene in human cancers (Vigil et al., 2010). Indeed, RAS mutations occur in about one-third of all human tumours and intercepting aberrant RAS function has long been considered the ‘holy grail’ of anti-cancer therapeutics. As critical effectors of RAS in the ERK pathway, RAF kinases were initially thought to relay an oncogenic RAS signal. However, in 2002, seminal work from Davies and colleagues uncovered the RAF family member, B-RAF, as mutated in many human cancers (Davies et al., 2002). Further genome wide sequencing of a variety of human tumours identified B-RAF as the most frequently mutated gene within the kinase superfamily (Greenman et al., 2007).

B-RAF is mutated in approximately 8% of all cancers, with the highest mutation frequencies seen in malignant melanoma (~70%), papillary thyroid cancer (30-53%), and serous ovarian cancer (up to 30%) (Dhomen and Marais, 2007; Garnett and Marais, 2004). Importantly, oncogenic B-RAF mutations have been shown to causally link deregulated activation of B-RAF kinase activity and downstream ERK signaling. The most common oncogenic mutations in B-RAF result in a constitutively active kinase. These findings have identified intercepting RAF kinase activity as an ideal therapeutic target in human cancers. Furthermore, as protein kinases are bona fide druggable targets, unlike small G-proteins such as oncogenic RAS, the development of B-RAF kinase inhibitors has garnered unprecedented efforts from the pharmaceutical industry (Arkenau et al., 2011).
1.4.3 RAF kinase regulation: a riddle wrapped up in an enigma

The discovery of B-RAF as an oncogene ushered in a wave of renewed interest in the field to elucidate the molecular basis for RAF kinase regulation. Not surprisingly, such rapid and widespread research efforts have ignited much controversy and disagreement surrounding the details of the mechanisms uncovered to date. What is clear is that RAF kinase regulation is a highly complex process. Minimally, switching RAF from its inactive to active state involves membrane recruitment, binding to regulatory proteins, conformational changes and several phosphorylation events in the CR1-CR3 regions (Udell et al., 2011). The process is largely similar for all three RAF isoforms, but there are also subtle but crucial differences in how each isoform is regulated – the reader is directed to excellent reviews on this topic not discussed here (Claperon and Therrien, 2007; Udell et al., 2011; Wellbrock et al., 2004).

In quiescent cells, RAF exists in a catalytically inactive state in the cytoplasm (see below). GTP-loaded RAS associates with RAF. This interaction contributes to conformational changes whereby the kinase domain of RAF is exposed to further regulatory events leading to a fully active kinase (Claperon and Therrien, 2007). These additional regulatory events include phosphorylation of a series of residues distributed throughout the entire RAF protein. Two of these phosphorylation sites are thought to be in the activation loop that stabilizes the productive conformation of the kinase domain as discussed in section 1.2.2.

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1.4.4 KSR, CNK and HYP form a RAF regulatory complex

Genetic screens in *Drosophila* and *C. elegans* using dominant active *RAS* alleles have long been the gold standard for uncovering novel genes that modulate ERK signaling. Taking advantage of dominant active alleles of both *RAS* and *RAF* has allowed researchers to identify genes that function either upstream or downstream of the steps leading to RAF activation. Such approaches have identified three key regulators of ERK signaling required for RAF activation.

One such novel regulator to be identified was kinase suppressor of *RAS* (KSR) (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). KSR encodes an evolutionarily conserved protein in metazoans comprising a pseudokinase domain that is highly related to the kinase domain of RAF, which together are part of the larger TKL family of eukaryotic protein kinases (Fig. 1.11). The pseudokinase status of KSR has been the centre of controversy since its discovery. While biochemical evidence for residual KSR kinase activity has been described (Brennan et al., 2011; Goettel et al., 2011; Xing et al., 2004), the two KSR orthologues in vertebrates (*KSR1* and *KSR2*) contain a substitution of the VAVK motif in sub-domain II of the kinase domain as discussed in section 1.2.3. The Lys to Arg substitution in the VAVK motif is predicted to significantly attenuate phosphoryl transfer function. Consistent with the lack of a phosphoryl transfer function for KSR, mutation of the kinase sub-domain II in *Drosophila* KSR (which contains an intact sub-domain II in contrast to vertebrae KSRs) has no impact on its ability to support RAF activation and ERK signaling (Claperon and Therrien, 2007). In addition to the pseudokinase domain, KSR contains three other conserved regions of unknown structure and an atypical cystein-rich C1 domain (Fig. 1.12). C1 domains in many other proteins often serve as binding sites for lipids (Claperon and Therrien, 2007).

**Figure 1.12** – The domain architecture of KSR. The C-terminal half of the protein shares similarity to the RAF family of kinases, including a Cystein-rich domain, a Ser/Thr-rich region and a kinase domain.

Connector enhancer of KSR (CNK) is another evolutionarily conserved protein of metazoans that is required for RAF activation (Therrien et al., 1999; Therrien et al., 1998) (Fig. 1.13). Initial characterization of CNK revealed a physical interaction with RAF, and depletion of CNK by RNAi in *Drosophila* S2 cells decreases RAF kinase activity. CNK exerts a complex and multifaceted role in RAF
Figure 1.13 – The domain architecture of CNK.

Activation that is thought to underlie the switch-like behaviour of ERK signaling (Douziech et al., 2003; Laberge et al., 2005). CNK appears to influence the translocation of RAF to the plasma membrane (Anselmo et al., 2002), and thus may serve to enhance the association of RAF with RAS at the membrane. In support of this, CNK contains a number of protein-protein (SAM and PDZ) and protein-lipid (PH) interaction domains, suggesting that it acts as a lipid-regulated scaffold.

A third protein involved in regulating RAF kinase activity is HYP (HYP, also known as Aveugle) (Douziech et al., 2006; Raignant et al., 2006). As with KSR and CNK, HYP is highly conserved amongst metazoans and consists of a single sterile \( \alpha \) motif (SAM) domain. Co-immunoprecipitation of affinity tagged HYP from Drosophila S2 cells showed a robust interaction with KSR and CNK. Immunoprecipitation of endogenous KSR protein from S2 cells results in co-purification of the KSR/CNK/HYP complex, which proves the formation of the complex in vivo (Douziech et al., 2006). This co-purification of the complex can be selectively perturbed by RNAi knockdown of endogenous KSR, CNK or HYP. Consistent with the notion that the KSR/CNK/HYP complex regulates RAF activity, RAF also associates with the complex in co-immunoprecipitation experiments.

Interestingly, the overexpression of KSR alone, but not CNK or HYP alone can stimulate RAF activity (Claperon and Therrien, 2007; Douziech et al., 2006). This suggests that CNK and HYP do not directly mediate RAF activation, but somehow assist KSR, which possesses the intrinsic ability to activate RAF (Fig. 1.14). CNK and HYP are thought to act as organizing centres to position RAF with its activator KSR.

1.4.5 The pseudokinase KSR is a RAF activator

As introduced above, KSR and RAF are closely related proteins and by virtue of the high sequence similarity between the RAF kinase domain and the KSR pseudokinase domain, the two proteins are considered to have arisen from an ancestral gene duplication event (Claperon andFIGURE 1.14 – KSR-dependent RAF activation assay in S2 cells. RAF activation is monitored by the levels of phosphorylated MEK (pMEK). KSR overexpression can drive RAF activation in the absence of CNK or HYP (using dsRNA to knockdown CNK or HYP). Figure adapted from Rajakulendran et al., 2009.
Therrien, 2007) (Fig. 1.11). While the RAF kinase domain possesses catalytic activity that is necessary for signaling in the ERK pathway, the apparent pseudokinase status for KSR led to speculation that KSR functions solely as an organizing center (scaffold) by its ability to mediate protein-protein interactions. In support of this notion, the pseudokinase domain of KSR constitutively and directly associates with MEK, the downstream substrate of RAF in the ERK pathway (Kolch, 2005). This led to the belief that KSR functions mainly as a recruiter of RAF’s substrate (namely MEK).

Consistent with KSR functioning to recruit a substrate, mutations in the pseudokinase domain of KSR that disrupt MEK binding result in a KSR protein unable to facilitate ERK signaling (Claperon and Therrien, 2007). Recent work, however, has revealed that substrate recruitment is secondary to a direct role of KSR in RAF activation. Specifically, an R732H mutation in the pseudokinase domain of KSR completely abolishes ERK signaling (Fig. 1.15), but fully retains the ability to bind MEK (Douziech et al., 2006). This mutation also does not perturb the ability of KSR to interact with CNK and HYP to form the RAF regulatory complex. Together, these results suggest that a surface on KSR encompassing Arg732 is involved in the RAF activation process.

1.4.6  **CNK and HYP provide a platform to juxtapose KSR with RAF**

CNK and HYP can bind each other in the absence of KSR, while KSR cannot bind to CNK alone or HYP alone (Douziech et al., 2006). The CNK/HYP interaction likely serves to regulate the timing of when KSR is brought into proximity with RAF. Once the KSR/CNK/HYP complex is fully formed (in a GTP-loaded RAS signal dependent manner), the entire complex can translocate to the plasma membrane in part by i) the potential ability of CNK to interact with membrane lipids through its PH domain, and ii) the interaction of RAF with RAS in the inner leaflet of the membrane (Fig. 1.16). Upon stable association of the entire complex at the membrane surface, CNK and HYP serve to organize the positioning of KSR with RAF. This serves to accomplish RAF kinase activation mediated by KSR, and...
recruit the RAF substrate MEK (bound to KSR) and its subsequent activation by RAF that ensures signal transmission through the ERK pathway.

1.5 Project Aims

Upon commencing my studies in November of 2005, my eventual collaborator Dr. Marc Therrien at the University of Montreal had a manuscript under review (Douziech et al., 2006) which described: i) the ability of KSR/CNK/HYP to form a complex (as evidenced by Co-IP experiments) that regulates RAF activation in Drosophila S2 cells, and ii) the enigmatic ability of KSR to directly drive RAF activation in S2 cells.

At the time, two pressing questions remained: 1) what are the molecular determinants that mediate formation of the KSR/CNK/HYP complex; and 2) what is the mechanism by which KSR drives RAF activation? I set out to directly address these two mechanistic questions utilizing a structural and biochemical approach, complemented by in vivo studies in S2 cells in collaboration with Dr. Therrien’s lab. The results of my efforts are described in Chapters 3-5 of this thesis.

The specific aspects I will cover are:

i) the structural basis for the interaction between CNK and HYP, which in turn serves to recruit KSR to form the KSR/CNK/HYP complex;
ii) the precise mechanism by which KSR directly drives RAF kinase activation;
iii) a mechanistic model for how RAS functions to modulate RAF kinase activation at the membrane.
Chapter 2

Materials & Methods
2  Materials and Methods

2.1  Common reagents, solutions and buffers

2.1.1  Lysogeny broth (LB)

Preparation of 1 liter of LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 l of ddH2O. Autoclaved at 121 °C for 30 min. Prior to use, 50 μg/ml of Amp or Kan antibiotic was added. For LB agar plates, 15 g agar was added prior to autoclaving.

2.1.2  Agarose gel electrophoresis buffer and DNA sample buffer TAE (Tris-acetate-EDTA)

Preparation (1 l): 48.4 g tris base, 11.4 ml of glacial acetic acid, 100 ml of 0.1 M EDTA (pH 8.0).

2.1.3  SDS-PAGE stock solutions

Separating layer (for 10 mini-gels) (15%):
- 40% acrylamide 26.2 ml
- 1.5 M Tris-HCl, pH 8.6 17.6 ml
- ddH20 25.2 ml
- 10% SDS 0.75 ml
- TEMED 62 μl
- 10% APS 216 μl

Stacking layer (for 10 mini-gels) (~4%):
- 40% acrylamide 3 ml
- 2 M Tris-HCl, pH 6.8 1.86 ml
- ddH20 24 ml
- 10% SDS 300 μl
- TEMED 30 μl
- 10% APS 256 μl

2.1.4  Protein electrophoresis buffer

10× solution of tris-glycine SDS-polyacrylamide gel running buffer: dissolve in 1 l volume: 30.3 g of tris-base, 144 g glycine and 10 g of SDS.
2.1.5  SDS sample buffer

6x SDS sample buffer: 250 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue, and 10% (v/v) DTT.

2.1.6  SDS-PAGE visualisation solution

Proteins analysed by SDS-PAGE were visualized using Coomassie staining solution prepared by dissolving 2.5 g of Coomassie blue in 400 ml of ddH$_2$O, 100 ml of glacial acetic acid and 500 ml of ethanol and mixed by stirring overnight. ddH$_2$O was used was de-staining on a nutator.

2.2  Materials and methods for in vitro studies

2.2.1  Plasmids

The SAM domain of human CNK2 (residues 5-84) was amplified by PCR and subcloned into the pProEx-HTa expression vector (Invitrogen). Full-length dHYP and the minimal SAM domain construct (residues 21-98) were PCR amplified and inserted into the pETM-30 vector (EMBL, Protein Expression Facility). Human B-RAF (residues 448-723) were cloned into pETM-30 vector. Human K-RAS (residues 1-167) was cloned into pProEx-HTa vector. Variant SAM domain and B-RAF kinase domain mutants were generated by two-step PCR-based targeted mutagenesis or using the QuickChange commercial kit (Stratagene).

2.2.2  Protein expression and purification

All SAM domain proteins were expressed in *Escherichia coli* BL21 (DE3) strain (Novagen). Proteins were purified by Ni affinity chromatography using a HiTrap Chelating HP column (Amershalm) and eluted with imidazole. The eluate was treated with TEV to cleave off the 6xHis tag in pProEx-HTa or the 6xHis-GST tag in pETM-30. TEV cleaved proteins were dialyzed into buffer and applied to a HiTrap Chelating HP column to elute untagged proteins. Eluate was concentrated and applied to a Superdex 75 gel filtration column (Amershalm) for final purification to yield a highly homogenous protein prep (Fig. 2.1). To obtain a selenomethionyl derivative of hCNK2$^{SAM}$ and dHYP$^{SAM}$, *E. coli* B834 cells were transformed and grown in minimal medium supplemented with selenomethionine (Rajakulendran et al., 2008). All proteins were purified into buffer containing 10

![Figure 2.1 – SDS-PAGE analysis of dHYP after final purification from Superdex 75 gel filtration. >99% pure dHYP is visualized by Coomassie staining (blue) as shown.](image)
mM HEPES (pH 7.0), 200 mM NaCl and 5 mM β-mercaptoethanol.

All B-RAF proteins were recombinantly in *E. coli* BL21 cells as TEV protease-cleavable 6xHis-GST tagged fusions. To increase the level of soluble protein expression in *E. coli*, 16 specific mutations were introduced in B-RAF as described (Tsai et al., 2008). Expressed proteins were bound to Ni-NTA and eluted with imidazole and subjected to TEV protease treatment. Further purification was performed by subtractive Ni-NTA and size exclusion (Superdex 200) chromatography (Rajakulendran et al., 2009). Proteins were purified into buffer containing 10 mM HEPES (pH 7.0), 200 mM NaCl and 1.5 mM TCEP.

K-RAS proteins were recombinantly in *E. coli* BL21 cells as TEV protease-cleavable 6xHis tagged fusions. Expressed proteins were bound to Ni-NTA and eluted with imidazole and subjected to TEV protease treatment. Further purification was performed by subtractive Ni-NTA and size exclusion (Superdex 200) chromatography. Proteins were purified into buffer containing 10 mM HEPES (pH 7.0), 200 mM NaCl and 1.5 mM TCEP.

2.2.3 Crystallization, data collection, structure determination and structure visualization

A discussion of the theoretical aspects of the applied crystallographic techniques is not presented here as this is beyond the scope of this chapter. The reader referred to the relevant literature on this topic (Cid, 1996; Gilliland and Ladner, 1996; Schmidt and Lamzin, 2007).

Crystals were grown by the sitting and hanging drop vapour diffusion method (Gilliland and Ladner, 1996) (Fig. 2.2). Initial crystal screens using commercially available sparse matrix kits (Hampton Crystal Screen I/II and Emerald Biosciences Wizard I/II) were performed using sitting drops. The automated robotic platform Mosquito (TTP Labs) was utilized to setup sitting drops in 96-well format plates. The sitting drop method consisted of a reservoir with added 70 μl of mother liquor. On the platform, 0.2 μl of protein solutions were pipetted and mixed with an equivalent amount of mother liquor. The wells were sealed using Crystal Clear (Hampton).
sealing tape. The hanging drop setup was used for crystal optimization after initial crystallisation hits were established by the sitting drop method. For this, 24-well VDX plates were filled with 0.5 ml of mother liquor solutions. 1.0 μl of the protein solution and an equivalent volume of the mother liquor were pipetted onto a siliconised glass cover slip, which was mounted inverted over the well. A closed system for vapour diffusion was created by distributing petroleum jelly (Vaseline) grease between the cover slip and the top of the well. Crystal visualization, handling and manipulations were performed manually using an inverted-lens light microscope system from Leica.

hCNK2<sup>SAM</sup>/dHYPS<sup>SAM</sup> co-crystals were grown at 4°C by mixing 1 μl of 4–8 mg ml<sup>-1</sup> of each protein with 1 μl of well buffer (100 mM Tris, pH 7.0-7.5, and 12-18% PEG 2000 MME). Flash-freezing of the crystals was performed using the crystallization buffer supplemented with 25% (v/v) glycerol (Rajakulendran et al., 2008). Data was collected at the Advanced Photon Source (APS) of the Argonne National Laboratory on Beamline 24-ID of NE-CAT and analyzed using the HKL2000 software package (Minor et al., 2006). The SHELX set of programs was used to locate heavy-atom sites, calculate phases and for density modification (Sheldrick and Schneider, 1997). Electron density maps calculated from the phases after density modification were used to build an initial model in ARP/wARP (Morris et al., 2002) and refined using REFMAC5 (Murshudov et al., 1997) in the CCP4 software package. Representative |2Fo - Fc| map of the hCNK2<sup>SAM</sup>/dHYPS<sup>SAM</sup> complex is shown in Fig. 2.3.

dHYPS<sup>SAM</sup> alone crystals were grown at 20°C by mixing 1 μl of 13 mg ml<sup>-1</sup> protein with 1 μl of well buffer (100 mM Cacodylate, pH 6.5 and 1 M Sodium citrate) (Fig. 2.4). Flash-freezing of the crystals was performed using the crystallization buffer supplemented with 25% (v/v) glycerol. Data was
collected under a liquid nitrogen stream at the APS on Beamline 14-BM-C of BioCARS and analyzed using the HKL2000 software package. The program PHASER in the CCP4 package was used to find a molecular replacement solution based on the dHYpSAM structure of the hCNK2SAM/dHYpSAM complex as the search model. REFMAC5 (McCoy et al., 2005) was used for iterative cycles of refinement in between manual refinement using Coot (Emsley and Cowtan, 2004). All ribbons and surface representations were generated using PyMOL (DeLano Scientific).

2.2.4 Homology modeling

A multiple sequence alignment of KSR and RAF kinase domains was used to build a structural model of the kinase domain of Drosophila KSR (residues 670-945) in SWISS-MODEL (Schwede et al., 2003). An initial model with a total energy of -7474.3 KJ/mol was generated using the structure of the kinase domain of B-RAF as a template (chain A of PDB ID 1UWH (Wan et al., 2004)). This model was manually edited in COOT and a poorly modelled loop spanning residues 821-838 was removed. To generate the KSR/RAF side-to-side heterodimer, the modelled structure of KSR was superimposed onto chain A of PDB ID 1UWH. All ribbons and surface representations were generated using PyMOL (DeLano Scientific).

2.2.5 Surface plasmon resonance experiments

Surface plasmon resonance (SPR) experiments for the hCNK2SAM/dHYpSAM interaction were performed at room temperature in 10 mM HEPES, pH 7.0 and 200 mM NaCl. dHYpSAM was immobilized on a Biacore Pioneer CM5 sensor chip (GE Healthcare) according to the manufacturer’s instructions. Free hCNK2SAM protein was injected and the binding data was analyzed using BIAevaluation 3.0 software.

SPR measurements were carried out using a BIACore T100 instrument. Wild type dHYp was immobilized on a CM5 sensor chip using standard amine-coupling chemistry and 10 mM HEPES, pH 7.0 was used as the running buffer. The carboxymethyl dextran surface was activated with a 5 min injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M N-hydroxy succinimide (NHS). dHYp (5-10 μM) was coupled to the surface with a 1 min injection of protein diluted in 10 mM sodium acetate, pH 5.5. maiming activated groups were blocked with a 5 min injection of 1 M ethanolamine, pH 8.5. dHYp was immobilised on three flow-cells of a CM5 chip at densities ~2000 response units (RU) performed, leaving one flow cell as a standard to subtract non-specific binding signals.

Data were fitted to a 1:1 binding site model using BIA evaluation 3.0 software. Kinetic association (k_a) and dissociation rate (k_d) constants were separately determined from the BIACore sensograms and
equilibrium dissociation constants (Kd) were calculated using the equation below that describes the law of mass action at equilibrium: K_{d1} = k_{d1}/k_{a1} and K_{d2} = k_{d2}/k_{a2}

2.2.6 Analytical ultracentrifugation

Equilibrium sedimentation was performed with a Beckman Optima XL-A ultracentrifuge and An60Ti rotor. B-RAF and K-RAS samples were prepared in 20 mM Tris (pH 7.5), 200 mM NaCl, 5% glycerol and 1.5 mM TCEP for analysis. For B-RAF, data was collected at 4°C for three protein concentrations (25 μM, 12.5 μM, and 6.25 μM) at three rotor speeds (13,000 rpm, 18,000 rpm and 23,000 rpm for B-RAF_wt and B-RAF_R481H or 12,000 rpm, 17,000 rpm and 25,000 rpm for B-RAF_L487R and B-RAF-L487R/E558K). For K-RAS, data was collected at 4°C for three protein concentrations (50 μM, 25 μM, and 12.5 μM) at three rotor speeds (12,000 rpm, 17,000 rpm and 23,000 rpm). Model analysis of the data was performed simultaneously in a global curve-fitting procedure (Origin software, Beckman). For this, data collected at 13,000 rpm and 18,000 rpm for B-RAF_wt was analyzed at all three protein concentrations; data collected at 18,000 rpm and 23,000 rpm for B-RAF_R481H was analyzed at all three protein concentrations; data collected at 17,000 rpm and 25,000 rpm for B-RAF_L487R was analyzed at all three protein concentrations; data collected at 17,000 rpm and 25,000 rpm for B-RAF_L487R/E558K at 25 μM and 12.5 μM was analyzed. The term “global” refers to fits across all rotor speeds for a given concentration. The global self association fit yielded an average molecular weight (MW) of 57,978 Da for B-RAF wt, and a MW of 21,704 Da for K-RAS.

For B-RAF, the ratio of the observed average MW to the theoretical MW of the monomer is 1.9:1 suggesting that the sample contains mostly dimers. A single-species dimer model best fit the observed data, indicated by the random distribution of the residuals – a measure of goodness of fit (the residual is the difference between the observed value and the predicted value). For B-RAF_R481H, the global self association fit yielded an average MW of 34,544 Da. The ratio of the observed average MW to the theoretical MW of the monomer is 1.1:1 suggesting that the sample contains mostly monomers. A single-species monomer model best fit the observed data. For B-RAF_L487R, the global self association fit yielded an average MW of 48,636 Da. The ratio of the observed average MW to the theoretical MW of the monomer is 1.5:1 suggesting that the sample contains a mixture of monomers and dimers. Consistent with this, a monomer-dimer model resulted in the best fit to the observed data with a dissociation constant (Kd) of 2 μM (Note: In order to reliably estimate Kd values from an AUC experiment, both species in a monomer-dimer equilibrium need to be sufficiently represented in solution; in my AUC analyses, I observed such a monomer-dimer equilibrium only for the B-RAF_L487R dimer mutant). For B-RAF_L487R/E558K, the global self association fit yielded an average MW of 55,472 Da. The ratio of
the observed average MW to the theoretical MW of the monomer is 1.8:1 suggesting that the sample contains mostly dimers and the data was best fit to a single-species dimer model.

2.2.7 GST pull down assays

A 30 μl sample of 50% slurry of glutathione sepharose 4B beads (Pharmacia) was equilibrated in assay buffer (10 mM HEPES, pH 7.0, 100 mM NaCl and 10 mM β-mercaptoethanol). The slurry was mixed with 30 μl of ~4 mg ml⁻¹ GST-fusion protein and incubated for 15 minutes at 4°C on a nutator. The beads were washed three times with 1 ml of the assay buffer and then nutated with 30 μl of 4 mg ml⁻¹ of 6xHis tagged protein in a final volume of 500 μl assay buffer for 1 hour at 4°C. The beads were subjected to three washes with 1 ml of the assay buffer. SDS loading buffer was added to the samples and heated at 90°C for 10 minutes. Proteins were resolved on pre-cast 4-20% (Invitrogen) SDS-PAGE and visualized by Coomassie (Fig. 2.5). Identical samples were resolved by 17% SDS-PAGE and subjected to immunoblot analysis using antibodies specific for the 6xHis tag (Sigma).

2.2.8 Kinase assays

GST-B-RAF fusion proteins were purified as described in section 2.2.2, excluding the TEV cleavage step to retain the 6xHis-GST tag at the N-terminus. Proteins were concentrated in buffer containing 10 mM HEPES pH 7.5, 200 mM NaCl, and 2 mM DTT. Kinase assays were performed using [γ-³²P]-ATP, 30 μM cold ATP, 20 μM MgCl₂, and 1 μM GST-B-RAF and 2 μM MEK at 30°C. Reactions were resolved by SDS-PAGE and visualized with a PhosphorImager (Molecular Dynamics) (Fig. 2.6).
2.3 Materials and methods for in vivo studies

All in vivo studies involving Drosophila S2 cells were conducted by Dr. Malha Sahmi in Dr. Therrien’s laboratory at the Institute for Research in Immunology and Cancer (IRIC).

2.3.1 S2 cell plasmids

Copper-inducible pMet vectors were used for binding and functional assays conducted in S2 cells. pMet-HA-RAS$^{V12}$, pMet-MYC-MEK, pMet-PYO-RAF, pMet-V5-KSR, pMet-PYO-HYP, HYP dsRNAs and pMet-FLAG-dCNK$^{2-549}$ have been described previously (Douziech et al., 2006; Rajakulendran et al., 2008; Rajakulendran et al., 2009). pMet-GST-HYP was generated in two steps. First, a GST cDNA (pGEX4T; Amersham Biosciences) containing a 6X-His tag and a TEV cleavage site at its C-terminus was amplified by PCR and introduced in pMet vector. Second, a Drosophila Hyphen cDNA (residues 2-106) was amplified by PCR and introduced at the C-terminus of GST-His-TEV to create pMet-GST-HYP. Variant HYP and CNK mutants were generated using the QuickChange procedure (Clonetech). The FRB-RAF$^{K455S}$ fusion construct was assembled by inserting an AseI/NotI PCR fragment encompassing residues 328-738 of RAF into the AseI/NotI site of FRB-KSR (Roy et al., 2002). The KSR-RAF chimera-A corresponds to KSR$^{1-665}$ fused to RAF$^{417-739}$, whereas chimera-B replaced the N-lobe of KSR (a.a. positions 666-757) with the one of RAF (a.a. positions 417-505). In both cases, the RAF N-lobe contained a K455M change to catalytically impair its kinase activity and thereby mimicked kinase-inert KSR. Variant full length Drosophila KSR, RAF or FRB/FKBP fusion mutants were generated by QuickChange mutagenesis (Stratagene). Mutagenized cDNAs were fully sequenced to verify that only the desired mutations had been introduced.

2.3.2 S2 cell assays

S2 cells were grown in serum-free insect cell medium (Sigma) at 27°C. Transfection and induction of protein expression were conducted as previously described (Douziech et al., 2006). 36 hrs post-induction cells were lysed in NP-40 lysis buffer (20mM Tris pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol and 1 mM EDTA). Cells were seeded at a density of 1.75 x 10$^6$ cells/ml 24h prior to transfection). dsRNAs were produced and used in RNAi experiments as described previously (Douziech et al., 2006). Protein expression was induced by adding CuSO$_4$ (0.7 mM) 36h before harvesting the cells. For FRB/FKBP-mediated dimer formation, rapamycin (Sigma) was added (1 µM) to the medium 2h prior to harvesting the cells. Lysates, immunoprecipitations, western blot procedures and antibodies were essentially as previously described (Douziech et al., 2006).
For GST pull-down assays, 50 µl of 50% slurry of glutathione sepharose 4B beads (GE Healthcare) equilibrated in lysis buffer was added to protein lysates and rocked for 4 hrs at 4°C. Beads were then washed three times with 1ml of lysis buffer and proteins were eluted with 50 µl of elution buffer (5 mM L-glutathione, 50 mM Tris pH 8.0). Total protein lysates or eluted proteins were resolved on 8-10% SDS-PAGE, transferred to nitrocellulose membranes and immuno-detected using either rabbit α-GST (Calbiochem), rabbit α-V5 (Invitrogen), rabbit α-pMEK (Cell Signaling) or the following mouse antibodies (α-CNK, α-MYC, α-PYO, α-HA) described previously (Douziech et al., 2006; Rajakulendran et al., 2008; Rajakulendran et al., 2009).
Chapter 3

Structure of the SAM domain complex of CNK/HYP: insights into KSR/CNK/HYP interactions in RAF activation
3 Structure of the SAM domain complex of CNK/HYP: insights into KSR/CNK/HYP interactions in RAF activation

3.1 Preface

In this chapter, I describe studies carried out to gain a better understanding of the determinants that underlie formation of the KSR/CNK/HYP complex in RAF activation. These studies were performed in collaboration with Dr. Malha Sahmi, a postdoctoral fellow in the laboratory of Dr. Marc Therrien at the Institute for Research in Immunology and Cancer (IRIC) in Montreal. All in vitro studies described henceforth were conducted by me. All in vivo studies employing Drosophila S2 cells were conducted by Dr. Sahmi based on mutations suggested by me. Details of the experimental work are described in chapter 2.

3.2 Specific aims

i) Elucidate the minimal protein domain(s) in KSR, CNK and HYP that support formation of the KSR/CNK/HYP complex capable of stimulating RAF activation.

ii) Elucidate the structural basis for the CNK/HYP interaction.
3.3 Protein expression and interaction studies

3.3.1 The SAM domain

The sterile α motif (SAM) domain (Fig. 3.1), present in both CNK and HYP, is crucial for the ability of CNK/HYP/KSR to associate and for signals to transduce through the RAF-MEK-ERK cascade (Douziech et al., 2006; Roignant et al., 2006). SAM domains comprise an abundant class of protein domains that are best known to mediate finite oligomeric and polymeric protein-protein interactions or binding to RNA (Aviv et al., 2003; Green et al., 2003; Kim et al., 2001; Qiao and Bowie, 2005). Some SAM domains can even bind directly to lipids, although the biological relevance of lipid binding remains to be determined (Li et al., 2007). A characteristic constellation of basic residues in the sequence of some SAM domains is diagnostic for RNA binding ability (Green et al., 2003). In contrast, SAM domains that mediate protein-protein interactions cannot be readily recognized from their primary sequence alone.

The structural basis by which SAM domains form polymers is well understood. Polymerization of SAM domains in general arises from the interaction of two distinct surfaces on the SAM domain termed the mid-loop (ML) and end-helix (EH) surfaces (Kim et al., 2001) (Fig. 3.2). Repeating ML/EH interactions of adjacent SAM domains lead to polymer extension. SAM domain mediated polymerization underlies long-range transcriptional repression by the polycomb group proteins (Kim et al., 2002). Similarly, SAM domain mediated polymerization of Shank3 is central to the assembly of the postsynaptic density complex (Baron et
al., 2006). In ERK signaling, it has been proposed that polymerization via the SAM domains of CNK and HYP could underlie the formation of a large scaffolding complex which serve to recruit an activator of RAF (Roignant et al., 2006).

3.3.2 The SAM domains of CNK and HYP form a stable complex

To determine whether the SAM domains of CNK and HYP interact directly and independently of other factors, I expressed minimal SAM domain constructs in bacteria and tested for an interaction using an *in vitro* GST pull-down assay. I screened for suitable expression constructs for the SAM domains of CNK and HYP from different species including *Drosophila*, mouse and human. I successfully identified expression constructs for the SAM domains of human CNK2 (hCNK2\textsuperscript{SAM}) and *Drosophila* HYP (dHYP\textsuperscript{SAM}). Using a GST pull-down assay, I found that GST-dHYP\textsuperscript{SAM} bound to hCNK2\textsuperscript{SAM} (Fig. 3.3). In contrast, GST protein alone or the RNA binding SAM domain of Vts1 (Aviv et al., 2003) fused to GST did not bind to hCNK2\textsuperscript{SAM}. As SAM domain interactions are known to form multiple oligomeric states, I performed size exclusion chromatography experiments to estimate the stoichiometry of the overall hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex. Separately purified hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} elute as monomers, while co-purified hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex elutes at an apparent molecular mass consistent with a dimeric complex (Fig. 3.4). To determine the dissociation constant for dimerization, I employed surface plasmon resonance experiments and found that the K\textsubscript{d} for dimerization is 92.5 nM (Fig. 3.5). Taken together, these results indicate that hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex binds tightly and directly *in vitro* with an apparent 1:1 stoichiometry to the HYP SAM domain.

![Figure 3.3 – Pull-down analysis of GST-dHYP\textsuperscript{SAM} with hCNK2\textsuperscript{SAM}. GST and GST-Vts1\textsuperscript{SAM} served as controls. Figure adapted from Rajakulendran et al., 2008.](image)

![Figure 3.4 – Gel filtration analysis of bacterially purified singly expressed hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} or coexpressed hCNK2\textsuperscript{SAM} + dHYP\textsuperscript{SAM}. Traces are shown of hCNK2\textsuperscript{SAM} alone, dHYP\textsuperscript{SAM} alone, and the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex. The elution positions of free hCNK2\textsuperscript{SAM}, free dHYP\textsuperscript{SAM}, and the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex are indicated by dashed lines. Figure adapted from Rajakulendran et al., 2008.](image)
3.4 Structural studies of the CNK/HYP interaction

3.4.1 Structure of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex

Since the ML/EH binding mode is characteristic of polymeric SAM domains, I questioned whether the discrete dimerization of hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} involves a novel binding mode or a variation of the polymerization binding mode. To distinguish between these two possibilities, I solved the structure of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex by X-ray crystallography. Crystals containing a single copy of the complex in the asymmetric unit, belonging to the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, were obtained and the structure was solved using the selenomethionine single-wavelength anomalous dispersion (SAD) phasing method.

Figure 3.5 – Equilibrium binding measurements of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} interaction by using Surface Plasmon Resonance. The curve shows a plot of equilibrium response units versus hCNK2\textsuperscript{SAM} concentrations of 1 nM, 10 nM, 100 nM, 1 mM, and 10 mM. The measured association ($k_{on}$) and dissociation ($k_{off}$) rate constants were used in the determination of the equilibrium dissociation constant $K_d$. The apparent dissociation constant was found to be 92.5 ± 23.8 nM. The association rate constant was $3.3 \pm 1.3 \times 10^4$ M\textsuperscript{-1} s\textsuperscript{-1}, and the dissociation rate constant was $3.1 \pm 0.1 \times 10^3$ M\textsuperscript{-1} s\textsuperscript{-1}. Figure adapted from Rajakulendran et al., 2008.

Figure 3.6 – Ribbons representation of the crystal structure of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex is shown on the left. Stereoview of dimer interface shown on the right. Salt-bridge interactions are highlighted by dashed lines. Figure adapted from Rajakulendran et al., 2008.

Since the ML/EH binding mode is characteristic of polymeric SAM domains, I questioned whether the discrete dimerization of hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} involves a novel binding mode or a variation of the polymerization binding mode. To distinguish between these two possibilities, I solved the structure of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex by X-ray crystallography. Crystals containing a single copy of the complex in the asymmetric unit, belonging to the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, were obtained and the structure was solved using the selenomethionine single-wavelength anomalous dispersion (SAD) phasing method.
(Rice et al., 2000). The final model was refined to 2.0 Å resolution to an $R_{\text{factor}}/R_{\text{free}}$ of 21.4%/24.0%. I also obtained crystals of the isolated dHYP$^{\text{SAM}}$ containing two monomers in the asymmetric unit belonging to the space group C222$_1$. The isolated dHYP$^{\text{SAM}}$ structure was solved by molecular replacement using the dHYP$^{\text{SAM}}$ structure from the hCNK2$^{\text{SAM}}$/dHYP$^{\text{SAM}}$ complex as a search model. The final isolated dHYP$^{\text{SAM}}$ structure was refined at 1.9 Å resolution to an $R_{\text{factor}}/R_{\text{free}}$ of 21.7%/26.5%. Pertinent structure determination and refinement statistics are presented in Table 3.1.

<table>
<thead>
<tr>
<th>Space group</th>
<th>dHYP$^{\text{SAM}}$ crystal</th>
<th>hCNK2$^{\text{SAM}}$/dHYP$^{\text{SAM}}$ crystal</th>
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</thead>
<tbody>
<tr>
<td>Cell, Å</td>
<td>$a = 56.0$, $b = 88.5$, $c = 73.3$</td>
<td>$a = 48.5$, $b = 56.4$, $c = 84.0$</td>
</tr>
<tr>
<td>$\alpha = \beta = \gamma = 90^\circ$</td>
<td>$\alpha = \beta = \gamma = 90^\circ$</td>
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<tr>
<td>Data collection</td>
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<td>Wavelength, Å: 0.9792</td>
</tr>
<tr>
<td>Resolution, Å</td>
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<td>2.00</td>
</tr>
<tr>
<td>Unique reflections</td>
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<td>15189</td>
</tr>
<tr>
<td>Completeness, %</td>
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<td>99.7 (97.6)</td>
</tr>
<tr>
<td>$&lt;\beta&gt;/180^\circ$</td>
<td>37.0 (18.4)</td>
<td>25.5 (3.6)</td>
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<tr>
<td>Rsym, %</td>
<td>4.5 (9.4)</td>
<td>6.2 (28.8)</td>
</tr>
<tr>
<td>$R_{\text{factor}}$</td>
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<td>0.2136</td>
</tr>
<tr>
<td>$R_{\text{free}}$</td>
<td>0.2650</td>
<td>0.2395</td>
</tr>
<tr>
<td>rms deviation from ideality</td>
<td>Bond lengths, Å: 0.018</td>
<td>0.021</td>
</tr>
<tr>
<td>Bond angles, °</td>
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<td>1.56</td>
</tr>
<tr>
<td>Ramachandran analysis</td>
<td>Most favored, %</td>
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</tr>
<tr>
<td>Additional allowed, %</td>
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<td>2.8</td>
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<tr>
<td>Avg. B factors, Å$^2$</td>
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<td>32.5</td>
</tr>
<tr>
<td>Waters</td>
<td>174</td>
<td>82</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest-resolution shell: 1.95-1.90 Å for dHYP$^{\text{SAM}}$ crystal and 2.06-2.00 Å for hCNK2$^{\text{SAM}}$/dHYP$^{\text{SAM}}$ crystal.

Both hCNK2$^{\text{SAM}}$ and dHYP$^{\text{SAM}}$ adopt the canonical five helix ($\alpha_1$-$\alpha_5$) SAM domain fold in complex (Stapleton et al., 1999; Thanos et al., 1999) (Fig. 3.6). The structure of dHYP$^{\text{SAM}}$ bound to hCNK2$^{\text{SAM}}$ is virtually unchanged from the isolated dHYP$^{\text{SAM}}$ structure with a root mean square deviation (RMSD) of 0.4 Å for 74 C$\alpha$ atoms (Fig. 3.7). Importantly, the structure of the complex reveals hetero-dimerization to be a variation of the polymer theme in which the EH surface of hCNK2$^{\text{SAM}}$ engages with the ML surface of dHYP$^{\text{SAM}}$. Consistent with this observation, a sequence alignment of CNK orthologues and HYP orthologues reveals high conservation of surface residues that map to the EH surface on hCNK2$^{\text{SAM}}$ and the ML surface on dHYP$^{\text{SAM}}$.
Notably, the ML surface of hCNK2\textsuperscript{SAM} and the EH surface of dHYP\textsuperscript{SAM} are not conserved. In contrast, both the ML and EH surfaces of the polymerizing SAM domain of Polyhomeotic (Ph) is conserved allowing for the formation of polymers through repeating ML/EH interactions (Kim et al., 2002) (Fig. 3.9). The inability of hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} to interact via their ML and EH surface, respectively, would explain why the two proteins form discrete heterodimers rather than extended polymers or homodimers.

The hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} interface buries ~580 Å\textsuperscript{2} of surface area on each SAM domain and involves a higher proportion of polar contacts than observed previously in other ML/EH SAM domain complexes (Kim et al., 2002; Kim et al., 2001) (Fig. 3.6). The ML surface of dHYP\textsuperscript{SAM} is comprised by Asp 53, Arg 57, Arg 61 and Arg 69. These charged residues engage in multiple salt bridge interactions with the oppositely charged side chains of Asp 24, Glu 53, His 62, Glu 64, Glu 68 and Asp 71 on the EH surface of hCNK2\textsuperscript{SAM}. Hydrophobic dimer contacts are formed by Ile 54, Ala 58 and Ile 62 on the ML surface of dHYP and by Ile 60, Gly 61 and Leu 65 on the EH surface of hCNK2\textsuperscript{SAM}. The small side chain of Gly 61 allows hCNK2\textsuperscript{SAM} helix \( \alpha5 \) to pack tightly against the ML surface of dHYP\textsuperscript{SAM}.

Figure 3.8 – CNK\textsuperscript{SAM} and HYP\textsuperscript{SAM} sequence alignments are shown with invariant positions indicated in black, or positions showing conservation of certain residue properties (e.g., charge, hydrophobicity) in gray. Secondary structures of hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} are indicated with helices in cyan and green cylinders, respectively. Interface residues of hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} are indicated by orange stars. A.a., Aedes aegypti; A.g., Anopheles gambiae; H.s., Homo sapiens; M.m., Mus musculus; T.n., Tetraodon nigroviridis; X.t., Xenopus tropicalis. Figure adapted from Rajakulendran et al., 2008.
3.4.2 Validation of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} dimer interface: In vitro

In order to identify the essential determinants of dimerization and to confirm that the crystal structure reflects the solution structure of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} complex, I individually substituted surface contact residues on hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} and analyzed the interaction potential of these mutants using a GST pull-down assay (Fig. 3.10). In agreement with predictions from the crystal structure, a R61E charge reversal mutation on the ML surface of dHYP\textsuperscript{SAM} strongly reduced binding to hCNK2\textsuperscript{SAM}. Introduction of a double charge reversal R57E/R61E at the ML surface resulted in no detectable interaction with hCNK2\textsuperscript{SAM}. In contrast, a R57A/R61A double mutant showed a residual ability to interact.

In converse experiments, a R59S/E68G double mutation or the D71A single mutation on the EH surface of hCNK2\textsuperscript{SAM} had no effect on the capacity to interact with GST-dHYP\textsuperscript{SAM}.

The D71R and I60A single mutants of the EH surface showed reduced capacity to bind GST-dHYP\textsuperscript{SAM}. Introduction of the double charge reversal E68R/D71R at the EH surface resulted in no detectable interaction with GST-dHYP\textsuperscript{SAM}. A control mutation Y78A outside the EH surface on hCNK2\textsuperscript{SAM} had no effect on binding to GST-dHYP\textsuperscript{SAM}.

The observation that different mutants had different effects on the ability to dimerize can be reconciled by the crystal structure. The double R57E/R61E dHYP\textsuperscript{SAM} mutant likely destabilizes the SAM-SAM complex most by abrogating four favourable salt-bridge interactions as well as introducing a strong electrostatic repulsion at the interface. The single
R61E dHYP\textsuperscript{SAM} mutant perturbs only two salt-bridge interactions and introduces a weaker electrostatic repulsion than with the R57E/R61E dHYP\textsuperscript{SAM} mutant, thus disrupting the interaction to a lesser extent. The R57A/R61A dHYP\textsuperscript{SAM} mutant perturbs four salt-bridge interactions, but does not introduce any electrostatic repulsion at the interface. Hence the R57A/R61A mutant shows the least effect on perturbing dimerization relative to the charge reversal mutations. A similar rationale explains the more potent effect on dimerization caused by the D71R charge reversal mutant of hCNK2\textsuperscript{SAM} versus the D71A mutant.

3.4.3 Validation of the hCNK2\textsuperscript{SAM}/dHYP\textsuperscript{SAM} dimer interface: In vivo

I next sought verification that the cross-species complex between hCNK2\textsuperscript{SAM} and dHYP\textsuperscript{SAM} in the crystal structure reflects the homo-species complex formed by dCNK/dHYP \textit{in vivo}. For this, I collaborated with Dr. Malha Sahmi in the Therrien lab to conduct experiments in S2 cells. Based on the mutations I had tested \textit{in vitro} (section 3.3.4), Dr. Sahmi prepared similar mutants that targeted SAM domain dimer interface surface residues on dHYP and dCNK constructs. For dCNK, a construct that included the larger SAM-CRIC-PDZ regions (denoted dCNK\textsuperscript{2-549}) was utilized, because the isolated SAM domain was not detectably expressed on its own in S2 cells. Mutations of interface residues equivalent to those on the EH surface of hCNK2\textsuperscript{SAM} were made in dCNK\textsuperscript{2-549}. The mutant constructs were transfected into S2 cells and the overexpressed proteins were tested for binding in a GST pull-down assay (Fig. 3.11). While wild type dHYP bound strongly to wild type dCNK\textsuperscript{2-549}, the single mutation R61D and the double mutation R57A/R61A on dHYP severely reduced binding to wild type dCNK\textsuperscript{2-549}. The double charge reversal mutation R57D/R61D on dHYP caused the strongest reduction on the interaction with dCNK\textsuperscript{2-549}, consistent with the strongest effect seen for the double charge reversal in the \textit{in vitro} pull-down assay.

On the EH surface of dCNK\textsuperscript{2-549}, the double mutation R57S/E66G and the single mutation E69A showed no effect on the ability to interact with dHYP. The single mutations E69R and I58A each showed a
reduced capacity to interact with dHYP, while the double charge reversal E66R/E69R on the EH surface of dCNK<sup>2-549</sup> showed a complete loss of binding, paralleling the effects seen <i>in vitro</i>. The control mutation Y76A on dCNK<sup>2-549</sup> on a surface outside the dimer interface had no effect on binding. These results confirm the dimer interface of the crystal structure mediates the solution interaction between hCNK<sub>2</sub><sup>SAM</sup>/dHYP<sup>SAM</sup> <i>in vitro</i> and between dCNK<sup>2-549</sup>/dHYP <i>in vivo</i>.

### 3.5 CNK/HYP interaction in RAF signaling

#### 3.5.1 CNK/HYP dimerization is essential for RAF signaling <i>in vivo</i>

To test the relevance of the CNK/HYP SAM domain interaction for RAF activation downstream of a constitutively active RAS, Dr. Sahmi carried out RAF activation assays in S2 cells. In this assay, activation of RAF kinase results in phosphorylation of its substrate MEK that can be detected by blotting with an anti-phospho-MEK antibody (Fig. 3.12). Using RNAi, endogenous dHYP was depleted and found to abolish MEK phosphorylation as previously reported. Subsequently, RNAi insensitive variants of wild type or dimerization defective dHYP constructs were introduced and tested for their ability to restore RAF kinase activity as indicated by phospho-MEK levels. While wild type dHYP restored MEK phosphorylation, the R57D/R61D dHYP mutant failed to restore phospho-MEK levels indicating that a direct dCNK<sub>2</sub><sup>SAM</sup>/dHYP<sup>SAM</sup> interaction is essential for RAF signaling. Surprisingly, the R61D and R57A/R61A dHYP mutants fully restore phospho-MEK levels to that of wild type dHYP, despite their reduced binding to dCNK<sup>2-549</sup> in the previous pull-down assay. These results indicate that while the R61D and R57A/R61A dHYP mutants are both impaired in their capacity to bind dCNK<sup>2-549</sup>, only the stronger R57D/R61D dHYP mutant shows both impaired binding and RAF signaling defects.

#### 3.5.2 A CNK/HYP complex recruits KSR

The reduced interaction of the R61D and R57A/R61A dHYP mutants with dCNK<sup>2-549</sup> contrasts the apparently normal function of these mutants in RAF signaling <i>in vivo</i>. I reasoned that this apparent contradiction could be attributed to CNK and HYP acting within a larger protein complex (Douziech et
In this context, secondary interactions could stabilize protein complexes together in the case of weakly perturbing mutations. One simple explanation then for the outwardly normal restoration of phospho-MEK levels in our RAF signaling assay by R61D and R57A/R61A dHYP mutants is that the co-overexpression of several other components in the assay offsets the weaker dimerization defect of R61D and R57A/R61A dHYP mutants.

In order to characterize the component(s) that may be compensating for the R61D and R57A/R61A dHYP mutations, in collaboration with Dr. Sahmi, we first determined if the co-expression of RasV12, RAF, KSR and MEK as in our RAF signaling assay could restore the binding of R61D and R57A/R61A dHYP mutants to dCNK2-549 in the GST pull-down assay. As shown in Fig. 3.13, this is indeed what was observed as the R61D and R57A/R61A dHYP mutants associate with dCNK2-549 as efficiently as wild type dHYP when co-expressed with RasV12, RAF, KSR and MEK. In contrast, the R57D/R61D dHYP mutant is still defective in binding to dCNK2-549.

To identify which of RasV12, RAF, KSR and MEK contribute to the restored binding of R61D and R57A/R61A dHYP mutants to dCNK2-549, I carried out further experiments in close collaboration with the Therrien lab. Since the function of CNK in promoting RAF activation was previously shown to depend on determinants within the kinase domain of KSR (Douziech et al., 2006), I hypothesized that the kinase domain of KSR might compensate for the dimerization defect of R61D and R57A/R61A dHYP mutants. To test this, in collaboration with Dr. Sahmi, we co-overexpressed KSR kinase domain along with dHYP and dCNK2-549 to test for binding in our GST pull-down assay (MEK was also co-expressed with KSR kinase domain as overexpressed KSR kinase domain alone is unstable in the absence of co-overexpressed MEK). As shown in Fig. 3.14, the R61D and R57A/R61A dHYP mutants associate with dCNK2-549 as stably as al., 2006).
wild type dHYP, even though these mutants showed significantly reduced binding to dCNK\textsuperscript{2-549} in the absence of co-overexpressed KSR kinase domain. In contrast, the R57D/R61D dHYP mutant still displays a defect in binding to dCNK\textsuperscript{2-549}. These results are consistent with our RAF signaling results and identify KSR as part of the dCNK\textsuperscript{2-549}/dHYP complex that stabilizes the SAM-SAM interaction. Moreover, the pull-down assays demonstrate a strong association of the minimal KSR kinase domain to dCNK\textsuperscript{2-549}/dHYP. KSR binding depends on dCNK\textsuperscript{2-549}/dHYP dimerization as demonstrated by the strongly reduced binding of KSR kinase domain to the dCNK\textsuperscript{2-549}/dHYP\textsuperscript{R57D/R61D} mutant complex. Taken together, our results suggest that the dimerization of CNK and HYP mediated by their SAM domains is necessary for KSR recruitment via the kinase domain of KSR to form the CNK/HYP/KSR complex.

### 3.6 Concluding remarks

Building on previous biochemical studies, the results discussed in this chapter present a structural link between CNK, HYP and KSR in RAF activation (Rajakulendran et al., 2008). I found that SAM domains mediate direct interaction between CNK and HYP which underlie formation of the KSR/CNK/HYP ternary complex (Fig. 3.15). I posit that CNK exists in an inactive state in which the SAM domain is hidden from HYP binding. Upon upstream RAS activation, an unknown mechanism reconfigures CNK such that its SAM domain is available to bind HYP. This in turn recruits KSR through an interaction with the KSR kinase domain. Figure 3.15 depicts the kinase domain of KSR contacting both HYP and the SAM-CRIC-PDZ domains in CNK. Whether this is true remains to be elucidated.
Chapter 4

Mechanism of action of KSR in RAF kinase activation
4 Mechanism of action of KSR in RAF kinase activation

4.1 Preface

In this chapter, I describe studies carried out in order to elucidate the mechanism by which KSR exerts its activation potential on RAF. These studies were performed in collaboration with Dr. Malha Sahmi, a postdoctoral fellow in the Therrien lab at the Institute for Research in Immunology and Cancer (IRIC) in Montreal. All *in vitro* studies described henceforth were conducted by me. All *in vivo* studies employing *Drosophila* S2 cells were conducted by Dr. Sahmi based on mutations and experimental designs suggested by me. Details of the experimental work are described in chapter 2.

4.2 Specific aims

i) Elucidate the mechanism by which KSR drives RAF activation.

ii) Elucidate the basis by which the oncogenic RAF E558K mutation potentiates kinase activity.
4.3 **KSR possesses an intrinsic capacity to stimulate RAF activation**

4.3.1 **Overexpression of KSR unleashes its RAF activation potential**

Previous studies demonstrated that the capacity of KSR to bind MEK was required for the ability of RAF to phosphorylate MEK (Roy et al., 2002). This result supported the notion that KSR functions in RAF substrate presentation. Interestingly, recent studies from the Therrien lab showed that co-overexpression of KSR with RAF and MEK stimulated RAF-dependent MEK phosphorylation (Douziech et al., 2006). Because KSR is normally in complex with other components that facilitate RAF activation, namely CNK and HYP (Douziech et al., 2006; Rajakulendran et al., 2008) (see chapter 3), overexpression of KSR without co-overexpression of its interacting partner proteins would perturb the optimal stoichiometry of KSR containing complexes with the net effect of decreasing RAF activation. Since there is increased RAF activation, this suggested that KSR might possess an inherent RAF activating capacity that becomes apparent upon overexpression. Consistent with the prediction that KSR might directly stimulate RAF activation, recent work from the Therrien lab demonstrated that overexpression of KSR can drive RAF activation independently of other components normally required for activation (Douziech et al., 2006). Specifically, the co-overexpression of KSR, RAF and its substrate MEK in S2 cells leads to activation of RAF in a KSR concentration-dependent manner in the presence or absence of RNAi-mediated knockdown of *ras* or co-overexpression of a constitutively active RAS(V12) RA F activation was assessed by immunoblotting for phosphorylated MEK (pMEK). The catalytically inactive RAF_K455M mutant served as a negative control. dsRNA, double-stranded RNA. Figure adapted from Rajakulendran et al., 2009.

**Figure 4.1** – Co-overexpression of KSR, RAF and its substrate MEK as indicated in S2 cells leads to activation of RAF in a KSR concentration-dependent manner in the presence or absence of RNAi-mediated knockdown of *ras* or co-overexpression of a constitutively active RAS(V12) RAF activation was assessed by immunoblotting for phosphorylated MEK (pMEK). The catalytically inactive RAF_K455M mutant served as a negative control. dsRNA, double-stranded RNA. Figure adapted from Rajakulendran et al., 2009.
4.3.2 The kinase domain of KSR is minimally sufficient for its RAF activating property

Figure 4.2 – Side-to-side dimer interface residues are conserved in all KSR and RAF proteins. Sequence alignment of the kinase domains of KSR and RAF from divergent organisms highlighting conserved residues (the position of Arg732 is indicated by the red arrow). For comparison, the sequence of the kinase domains of LCK and PKA are co-aligned demonstrating that the side-to-side dimer contact residues are unique to the KSR/RAF family. The sequence of the kinase domain N-lobe is boxed in red and the secondary structural elements are indicated above the sequence. Aligned sequences correspond to those from Drosophila (d), human (h), mouse (m), zebrafish (z), and chicken (c). Only the B-RAF sequence is shown for species where multiple RAF isoforms exist. Figure adapted from Rajakulendran et al., 2009.
In 2006, Dr. Therrien’s group identified a mutation in KSR (R732H) within its kinase domain that completely abolished its RAF activating capacity yet fully retained its ability to bind MEK and RAF (Douziech et al., 2006) (see section 1.4.5). This mutant provided a starting point for unraveling the mechanism by which KSR directly activates the catalytic function of RAF.

Since the kinase domain of KSR is most similar to that of RAF (Manning et al., 2002) (see section 1.4.4), I hypothesized that the previously determined crystal structure of the kinase domain of human B-RAF might provide a good model to discern the mechanism of action of the KSR_R732H mutation. Indeed, Arg732 is not only invariant across all KSR proteins, it is invariant across the larger RAF/KSR family (but not in other closely related kinases) (Fig. 4.2). Intriguingly, the crystal structure of the kinase domain of B-RAF reported in 2004 (Wan et al., 2004), contained two RAF kinase domains that interact in a unique side-to-side fashion involving the N-lobe of their kinase domains (Fig. 4.3). This mode of dimerization, which was not appreciated to date, was observed in all subsequent RAF structure analyses representing distinct crystal lattices (Hatzivassiliou et al., 2010; King et al., 2006; Tsai et al., 2008), suggesting that the mode of dimerization is functionally relevant rather than an artifact of crystal packing. Side-to-side dimerization of the RAF kinase domain buries a large surface area (~1280 Å²) and provocatively involves helix αC, a key structural element whose conformation serves a regulatory function in numerous protein kinases (section 1.2.3). Most notably, a specific mode of dimerization involving helix αC underlies an allosteric mechanism for kinase activation for both PKR (Dar et al., 2005) and EGFR (Zhang et al., 2006) kinase domains (Fig. 4.4). As the structure of the RAF kinase domain adopts a productive conformation in the dimeric crystal configuration, I reasoned that side-to-side dimerization itself might directly modulate the attainment of an active kinase conformation of RAF.
4.3.3 A conserved dimerization interface in RAF/KSR family proteins

Projection of KSR/RAF conserved residues onto the RAF crystal structure revealed that nearly the entire side-to-side dimer contact surface of RAF, but no other surfaces, are conserved across the larger KSR/RAF family (Fig. 4.5). This suggested that KSR might form an analogous dimer structure. Moreover, the position of Arg481 (the equivalent of Arg732 in KSR) at the center of the side-to-side dimer interface (Fig. 4.6) of the B-RAF crystal structure hinted at the basis by which the mutation of Arg732 in KSR might exert a functional effect by perturbing dimerization (for simplicity, I use the Drosophila RAF numbering scheme for discussion of human B-RAF positions; see Table 4.1 on page 48 for list of residue equivalence between B-RAF and Drosophila RAF).

Figure 4.4 – Comparison of the B-RAF side-to-side mode of dimerization with the specific mode of dimerization of PKR (PDB ID = 2A19) and EGFR (PDB ID = 2GS2) kinase domains. Helix αC is a participant in all three modes of dimerization and adopts a productive conformation in the dimeric state. By inference from the EGFR6 and PKR5,7 kinases, where a transition from a dimeric to a monomeric state correlates with a movement of helix αC to a non-productive conformation, dimerization of the RAF kinase domain may modulate catalytic function through an analogous allosteric mechanism influencing the conformation/position of helix αC. Figure adapted from Rajakulendran et al., 2009.

Figure 4.5 – Projection of highly conserved residues across both KSR and RAF orthologues onto the crystal structure of the BRAF kinase domain (top panel; PDB ID 1UWH (Wan et al., 2004)) highlights common side-to-side dimer contact surfaces visualized originally in crystal structures of BRAF (bottom panel). Figure adapted from Rajakulendran et al., 2009.
In order to investigate the potential of the RAF kinase domain to form dimers in solution, I employed analytical ultracentrifugation experiments. Equilibrium sedimentation analysis confirmed that RAF can form dimers under the conditions tested (i.e. micromolar concentrations) (Fig. 4.7). Consistent with the mode of dimerization seen in the crystal structure, mutation of Arg481 in B-RAF converted it to a predominant monomer in solution. This result shows that the side-to-side dimer configuration of RAF visualized in the crystal environments is also sampled in solution. Based on these findings, I reasoned that the R732H mutation in KSR most likely perturbs KSR’s ability to form an analogous side-to-side homodimer or to form a side-to-side heterodimer with RAF. This in turn could explain the mechanism by which the KSR_R732H mutation abolishes RAF activation.

**Figure 4.6** – Crystal structure of B-RAF highlighting the position of Arg481 and its interactions (equivalent to Arg732 in KSR) at the center of the side-to-side dimer interface (PDB ID 1UWH (Wan et al., 2004)). Figure adapted from Rajakulendran et al., 2009.

**Figure 4.7** – AUC analysis reveals that mutation of Arg 481 (Arg481His) in BRAF transitions the protein from a dimer (left panel) to a monomer (right panel) in solution. The red line denotes a fitted curve to the self-association model. The residuals for the fit are shown in the top panels. See chapter 2 for experimental details. Analysis performed by the Dept. of Biochemistry AUC facility. Figure adapted from Rajakulendran et al., 2009.
4.3.4 Perturbing KSR/RAF dimerization impedes RAF activation

If KSR mediates RAF activation by a mechanism involving the formation of a specific side-to-side dimer with itself (i.e. KSR/KSR side-to-side homodimer) or a heterodimer with the kinase domain of RAF (i.e. KSR/RAF side-to-side heterodimer), then mutation of other dimer interface residues on KSR in close vicinity to Arg732 might also impair RAF activation. To test whether mutations of residues at the side-to-side dimer interface impact RAF activation by KSR, I collaborated with Dr. Malha Sahmi in the Therrien lab to carry out RAF activation assays in S2 cells. Using a KSR/RAF/MEK co-overexpression activation assay, Dr. Sahmi found mutations localized to the dimer interface perturbed RAF activation (Fig. 4.8). Specifically, individual mutation of four additional residues (G700W, F739A, M740W and Y790F) on KSR severely impeded its ability to induce RAF activation.

If KSR mediates RAF activation by forming a specific side-to-side heterodimer with the kinase domain of RAF, then mutations of the corresponding positions on RAF should also impair RAF activation. As shown in Fig. 4.8, I also found this to be the case. In contrast, control mutations remote from the side-to-side dimer interface on the kinase domains of both KSR and RAF showed no significant effect on RAF activation. These results confirm that the integrity of the side-to-side dimer interface on KSR and on RAF is essential for RAF activation.

While the results above are consistent with the possibility that KSR and RAF heterodimerize through their kinase domains, it is equally possible that KSR/KSR

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<td>Gln687</td>
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Table 4.1 – List of residue equivalence between human B-RAF and Drosophila RAF.

Figure 4.8 – The individual effect of side-to-side dimer interface mutations on KSR and on RAF and their effect on RAF activation was assessed by monitoring the levels of phosphorylated MEK in S2 cells. Control mutations outside the interface correspond to K460A, E601A and M640A in RAF, and D710A, E859A and V898A in KSR. Figure adapted from Rajakulendran et al., 2009.
side-to-side homodimers might instead contribute to RAF activation. To demonstrate that the formation of side-to-side kinase domain heterodimers by KSR and RAF per se leads to RAF activation, I selected an assay that would allow for specifically enforcing the formation of KSR/RAF heterodimers. The system employed the FRB/FKBP fusion proteins (Muthuswamy et al., 1999) to inducibly promote KSR/RAF side-to-side heterodimer formation by the addition of rapamycin in vivo (Roy et al., 2002).

Towards this end, a region encompassing the minimal kinase domains of KSR and RAF were fused to the FRB and FKBP fragments, respectively (Fig. 4.9). The use of the FRB/FKBP fusion in conjunction with a myristoylation signal on the FKBP fusion construct (to localize it to the membrane) allowed for strict modulation of heterodimerization of the kinase domains in a rapamycin-dependent manner (Roy et al., 2002). Dr. Sahmi tested the constructs in this setup in S2 cells for RAF activation potential (Fig. 4.10).

Promoting the KSR/RAF heterodimer by addition of rapamycin was indeed sufficient to potently activate RAF as evidenced by the elevated levels of phosphorylated MEK. RAF activation was selectively perturbed by specific mutations at the side-to-side dimer interface on both KSR (G700W, R732H, L738R, F739A, M740W, Y790F) and on RAF (G450W, R481H, F488A, F488L, M489W, Y538F), but not by control mutations outside the side-to-side dimer interface of KSR or RAF (D710A, E589A and V898A in KSR; K460A, E601A and M640A in RAF).
together, these results indicate that formation of the side-to-side heterodimer between KSR and RAF kinase domains is both sufficient and necessary for RAF activation under the conditions tested.

### 4.3.5 Dimerization is a pervasive theme in RAF activation

As both RAF and KSR likely form identical side-to-side dimers, by virtue of having near identical dimerization surfaces, it is conceivable that both KSR/RAF heterodimers and RAF/RAF homodimers might equally promote RAF activation, assuming RAF activation and downstream signaling is solely dependent on forming the kinase domain side-to-side dimer. To investigate whether the RAF/RAF homodimers can also lead to RAF activation, I chose to use the FRB/FKBP/rapamycin system to drive side-to-side homodimer formation of RAF kinase domains in vivo to monitor RAF activation. To ensure my interpretation of side-to-side dimer formation induced activation is not confounded by trans autophosphorylation activity within the RAF/RAF homodimer, I introduced a mutation (K455S) in the FRB-RAF fusion to catalytically impair its kinase activity (i.e. to effectively mimic the kinase dead state of KSR). As shown in Fig. 4.11, rapamycin induced formation of RAF/RAF homodimers can indeed drive RAF activation in a manner dependent on the ability to form the side-to-side dimers. To unequivocally demonstrate that the formation of kinase domain side-to-side dimers controls RAF catalytic activity, I employed in vitro kinase assays in which recombinantly expressed RAF kinase domain (purified to homogeneity) was monitored for its ability to phosphorylate purified MEK (Fig. 4.12). I found that the isolated kinase domain has weak activity towards MEK, which is substantially increased in response to enforced dimerization via fusion to the dimer promoting GST moiety. In contrast, introduction of the R481H dimer interface mutation in RAF (equivalent mutation in B-RAF is R509H) ablates kinase activity even when fused to GST. These results demonstrate that the formation of side-to-side kinase domain dimers is indispensible for RAF activation.
Recent studies in mammalian cells, where multiple RAF isoforms exist (see section 1.4), have found that RAF activation can also occur upon the physical juxtaposition of two isoforms of RAF mediated by 14-3-3 proteins (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001). Intriguingly, this activation route is independent of a phosphoryl transfer mechanism as reflected by the fact that in such RAF/RAF heterodimers (e.g. B-RAF/C-RAF dimers), a kinase-dead isoform of RAF can activate a wild-type isoform of RAF in trans (Rushworth et al., 2006). This behaviour is highly reminiscent of how KSR activates RAF. I reasoned that 14-3-
3 proteins, which are intrinsically dimeric (Marais and Marshall, 1995), act to promote the specific side-to-side dimer conformation in the RAF crystal structure in a manner analogous to our forced FRB-RAF/FKBP-RAF system.

Consistent with the possibility that 14-3-3 acts to promote RAF dimers, my modeling studies showed that the binding of dimeric 14-3-3 proteins concurrently to the C-terminal extension of two RAF kinase domains is fully compatible with the adoption of a side-to-side dimer configuration (Fig. 4.13). Interestingly, the 14-3-3 consensus binding site in the RAF kinase domain is highly conserved in both RAF and KSR molecules (Fig. 4.14), suggesting that 14-3-3 could also act to promote RAF homodimers and KSR/RAF heterodimers.

Demonstrating that 14-3-3 is indeed relevant for RAF activation, Dr. Therrien’s group found that depletion of endogenous 14-3-3 proteins perturbed KSR-dependent RAF activation. Consistent with the notion that 14-3-3 mediates dimerization of KSR with RAF, mutation of the consensus 14-3-3 site in both KSR and RAF impaired RAF activation (Fig. 4.15). These results suggest that 14-3-3 proteins might act to promote specific KSR/RAF and RAF/RAF side-to-side kinase domain dimers.

4.3.6 RAF dimerization underlies aberrant RAF activation by oncogenic RAF_E586K

How does my identification of the side-to-side dimer fit into the context of other known activating events in RAF signaling? The mapping of human cancer causing mutations to the activation segment (see section 1.2) of B-RAF proved unequivocally that the activation segment of RAF is also a key modulator of its catalytic function (Wan et al., 2004). Consistent with this, the Therrien lab previously found that a mutation in the activation segment of Drosophila RAF (RAF-AL_E586K) strongly hyperactivated its catalytic activity, suggesting that it likely acts via a similar mechanism as those identified in human
cancers (Douziech et al., 2006). This raises the question of how kinase domain dimerization and the modulation of activation segment conformation are coordinated. Are both events essential for the transmission of a downstream signal or is each event sufficient on its own? If both are essential, then oncogenic activation segment mutants of RAF should still be sensitive to dimer interface mutations. Suggesting that this in fact is the case, introduction of a mutation (R481H) within the side-to-side dimer interface in *Drosophila* RAF effectively nullifies the aberrant signaling properties of RAF-AL \(^{ED}\) (Fig. 4.16).

Intriguingly, while most oncogenic RAF mutations act through modulation of the activation segment, one particular mutation, RAF_E558K (E586K in human B-RAF), is located on the opposite surface of the kinase domain from the activation segment (Wan et al., 2004) and its mechanism of kinase activation remained enigmatic. Most conspicuously, Glu558 lies on the side-to-side dimer interface. If dimerization is indeed critical for RAF activation, I questioned whether RAF_E558K might promote kinase activity by promoting dimerization. I reasoned that mutation of Glu558 to the longer Lys (E558K) could potentially introduce a hydrogen bond with Ser561 (conservative Thr588 in B-RAF) on the second RAF protomer thereby promoting dimer formation. Indeed, I found that the RAF_E558K mutation promoted kinase domain dimerization in solution (Fig. 4.17). Wild type RAF kinase domain is predominantly a dimer in solution (at the micromolar concentrations tested), which prevented

![Figure 4.16](image)

**Figure 4.16** – RAF activation assay using overexpressed full-length RAF and MEK proteins in S2 cells. The dimer interface mutation RAF_R481H abrogates the pronounced activation potential of the activation segment mutation RAF_T571E/T574D. Figure adapted from Rajakulendran et al., 2009.

![Figure 4.17](image)

**Figure 4.17** – AUC analysis reveals that the oncogenic E558K mutation in B-RAF transitions the B-RAF_L487R dimer mutant from weak monomer-dimer equilibrium (left panel) to a dimer (right panel) in solution; residue numbering scheme corresponds to *Drosophila* RAF. Figure adapted from Rajakulendran et al., 2009.
a direct test of the RAF_E558K mutant for enhanced dimerization potential. To circumvent this problem, I employed the RAF_L487R dimer mutant which displayed a weak monomer-dimer binding equilibrium in solution. As predicted, introduction of the E558K mutation (RAF_L487R/E558K double mutant) transitioned RAF_L487R back to a predominantly dimeric state.

To investigate how the RAF_E558K mutation functions to hyperactivate RAF in vivo, I utilized the FRB/FKBP/rapamycin system in S2 cells in collaboration with the Therrien lab. When the E558K mutation was introduced in the kinase-dead (K455S) background (FRB-RAF_K455S/E558K double mutant), it displayed no activity in isolation, but strongly hyperactivated the FKBP-RAF counterpart in a rapamycin dependent manner in the assays performed by Dr. Sahmi (Fig. 4.18). Taken together, the ability of the E558K mutant to act in trans (i.e. in the context of a kinase dead mutant) in vivo, and the ability of the E558K mutation to promote kinase domain dimerization in vitro reveal that the mechanism by which the oncogenic RAF_E558K mutation acts is by promoting side-to-side dimers. These results raise the possibility that small molecules strategies directed at preventing the formation of side-to-side dimers by RAF could serve as a therapeutic for RAF-dependent human tumors, one that would complement conventional strategies currently directed at inhibiting RAF enzymatic activity by blocking the catalytic cleft (Udell et al., 2011).

4.3.7 KSR kinase domain C-lobe recruits MEK for substrate targeting by RAF

Although RAF/RAF homodimers are also competent for activation, the level of activation (based on monitoring phospho-MEK signals as readout for RAF activation status), is not as robust as that resulting from KSR/RAF heterodimers (Fig. 4.10 and Fig. 4.11). If the side-to-side dimer surfaces are in fact functionally equivalent on both KSR and RAF, this observation suggests that the KSR kinase domain may have a second function that is not shared with RAF. Based on the fact that KSR can stably bind MEK while RAF cannot (Roy et al., 2002), specialization of KSR function for MEK binding may be at the root of the difference in activity of KSR/RAF versus RAF/RAF dimers. Since the side-to-side
dimerization surface is comprised mainly by the N-lobe of KSR and RAF kinase domains, and MEK binding function is critically dependent on the C-lobe of KSR (Roy et al., 2002; Stewart et al., 1999), then a RAF N-lobe–KSR C-lobe chimera might possess both essential functions of the KSR kinase domain. If true, one would predict that substitution of the N-lobe of RAF into KSR, but not the whole kinase domain of RAF into KSR, would lead to the maintenance of KSR’s ability to promote RAF mediated phosphorylation of MEK (Fig. 4.19). Experiments in collaboration with the Therrien lab suggest that this indeed is the case.

As shown in Fig. 4.20, overexpression of a form of KSR with a full kinase domain swap with RAF (Chimera-A) poorly activated RAF, while overexpression of a form with just an N-lobe swap (Chimera-B) was as potent as wild type KSR in promoting MEK phosphorylation by RAF. Confirming that MEK binding is indeed found in the C-lobe of KSR, Chimera-B but not Chimera-A bound to MEK as assessed by co-immunoprecipitation (Fig. 4.20). Taken together, these results highlight two distinct functions for the kinase domain of KSR in RAF signaling. Firstly, the kinase domain of KSR mediates RAF substrate targeting by recruiting MEK. Secondly, the kinase domain of KSR forms a side-to-side heterodimer with the kinase domain of RAF that underlies an allosteric mechanism for RAF catalytic activation.

4.4 Concluding remarks

Although dependent on many more components, the KSR mediated mechanism of RAF activation appears analogous in principle, if not execution, to those employed by the eIF2α and EGFR protein kinases. In the case of the eIF2α protein kinase PKR, the attainment of a specific dimer

Figure 4.19 – Schematic of the experimental design for the N-/C-lobe chimeric proteins between KSR and RAF.

As shown in Fig. 4.20, the ability of wild-type KSR, RAF and KSR–RAF chimaeric constructs to drive RAF activation in S2 cells was assessed by monitoring levels of phosphorylated MEK. Assay was performed by Dr. Malha Sahmi (IRIC). Figure adapted from Rajakulendran et al., 2009.
configuration by the kinase domain is regulated by the binding of dsRNA viral by-products to regions N-terminal to the kinase domain (Dar et al., 2005; Dey et al., 2005). In the case of EGFR kinase (Fig. 4.21), adoption of a unique dimer configuration by its kinase domain is regulated by the binding of growth factors to the extracellular ligand binding domain of the receptors (Zhang et al., 2006). Reflecting the importance of self interaction in the function of all three protein kinase families, residues comprising the self interaction surfaces of the kinase domain in addition to the catalytic infrastructure are evolutionarily conserved within each kinase family. In this regard, KSR is essentially equivalent to a RAF molecule. In effect, I reason that RAF and KSR evolved from a single ancestral progenitor, one that possessed protein kinase catalytic activity. Following a gene duplication event, KSR dispensed with phosphoryl transfer function while RAF retained kinase function. However, both maintained the ability to form allosteric dimers and this selective pressure maintained the side-to-side dimer interface and interdependence between KSR and RAF proteins in ERK signaling (Rajakulendran et al., 2009). The evolutionary history between RAF/KSR proteins is highly reminiscent of the EGFR/HER kinase family. HER3, like KSR, has dispensed with kinase activity but retains the ability to form specific heterodimers with its kinase-competent counterparts (EGFR, HER2 and HER4) to allosterically modulate their activity (Jura et al., 2009; Zhang et al., 2006).

**Figure 4.21** – RAF family kinase activation is regulated by the formation of specific hetero- and homodimers between RAF isoforms and KSR family proteins. In an analogous manner, the EGFR family kinase is regulated by the formation of specific dimers between EGFR and HER family proteins.
Chapter 5

Allosteric RAF dimers promoted by a dimeric RAS
In this chapter, I describe studies carried out in order to test if the ability of RAS to form specific dimers plays a role in the RAF dimerization-dependent activation mechanism. These studies were performed in collaboration with Dr. Malha Sahmi, a postdoctoral fellow in the laboratory of Dr. Marc Therrien at the Institute for Research in Immunology and Cancer (IRIC) in Montreal. *In vitro* studies described henceforth were conducted by me, except where indicated otherwise. All *in vivo* studies employing *Drosophila* S2 cells were conducted by Dr. Sahmi based on mutations and experimental designs suggested by me. Details of the experimental work are described in chapter 2.

### 5.2 Specific aims

- *i)* Elucidate whether the specific dimer configuration of RAS in its active state visualized in crystal structures is biologically relevant for RAS function.

- *ii)* Elucidate whether the dimer state of RAS has direct bearing on the attainment of the dimer conformation and activation of RAF.
5.3 **RAS adopts a specific dimer configuration in crystal structures**

5.3.1 **The RAS GTPase: a master switch**

RAS proteins operate as molecular switches for signal transduction pathways originating on cellular membranes (Bar-Sagi and Hall, 2000; Macara et al., 1996). Mammalian cells ubiquitously express three isoforms of RAS, namely, H-RAS, N-RAS and K-RAS. These highly homologous GTPases interact with a common set of regulatory exchange factors and a set of overlapping target effector proteins to transduce signals from growth factor receptors (typically RTKs). In doing so, RAS proteins act as master regulators of cellular proliferation, differentiation and apoptosis. The three mammalian RAS proteins share a common fold that encompasses three main functions: 1) bind guanine nucleotides (i.e. GDP or GTP); 2) the switch I and switch II loops that undergo conformational changes on GTP–GDP exchange to adopt distinct conformations in the apo, GDP-bound or GTP-bound states; and 3) the binding surfaces for effectors, exchange factors (GEFs) and GTPase-activated proteins (GAPs) (Hancock, 2003). The mechanism by which RAS proteins carry out GTP hydrolysis is beyond the scope of this thesis and the reader is directed to several excellent reviews on this topic (Li and Zhang, 2004; Sprang, 1997; Wittinghofer, 2006). The C-terminal ~25 residues is poorly conserved between RAS isoforms. This region comprises the sequences that dictate post-translational processing, membrane anchoring and trafficking of newly synthesized and processed RAS to membranes (Hancock, 2003). The ability of RAS to dynamically transition from a GDP-bound conformation to a GTP-bound conformation underlies its capacity to regulate cellular events by differentially binding to downstream effectors such as the RAF family kinases.

**Figure 5.1** – A ribbons representation (top panel) of the tertiary structure of RAS GTPase (PDB ID 5P21 (Pai et al. 1990)). The nucleotide binding P-loop is coloured blue, and the neighbouring loop (switch) regions that undergo large conformational changes in response to GDP/GTP exchange are highlighted in violet. A bound GTP molecule is coloured red. A secondary structure view of the GTPase domain is shown in the bottom panel.
5.3.2 The α4-α5 dimer configuration of RAS

As I showed in chapter 4, RAF family kinases are catalytically modulated in response to the formation of specific side-to-side dimers (Rajakulendran et al., 2009). As RAF activity must be exquisitely turned ON and OFF in response to various stimuli, one possible means of regulating RAF is by controlling the attainment of the dimeric state of RAF. At a meeting in which I presented the work described in chapter 4 (ICAM Kinase Symposium, December 2008, San Diego, California), Dr. John Kuriyan (Professor, University of California Berkeley) suggested an intriguing possibility for how RAF dimer formation might be regulated by RAS. Given that under physiological conditions, RAF activity is strictly dependent on RAS function, Dr. Kuriyan questioned whether a recurring dimer conformation of RAS he had observed in crystal structures of RAS might in turn directly couple to the promotion of RAF dimers.

The notion that RAS itself has dimerization potential was previously reported by Inouye and colleagues in 2000 in which they described: 1) an ability of RAS to form dimers, and 2) that the ability of RAS to dimerize was important for RAF activation (Inouye et al., 2000). As discussed in chapter 4, the intrinsic activity of the RAF kinase domain is strongly augmented in the presence of a secondary dimer promoting module (see section 4.3.5). This begs the question of whether RAS might play the role of a secondary dimer promoting module in RAF activation by virtue of RAS having dimerization potential itself.

Based on Dr. Kuriyan’s observation that RAS adopts a specific dimer state in several crystal structures, I analyzed 74 individual crystal structures of RAS in various states (e.g. bound to various nucleotides;
various mutants) reported in the Protein Data Bank (Fig. 5.2). From my analysis of 74 RAS crystals structures representing 18 unique crystal forms (i.e. different crystal structures with similar lattice parameters were considered to represent the same crystal form), I found that RAS adopts a specific dimer state, which I term the α4-α5 dimer, in its active form. In addition to GTP-bound RAS, I define the active state of RAS to include mutant RAS-GDP complexes in which the conformation of the switch II region adopts a configuration that more closely resembles that of RAS-GTP (see section 5.3.3). The adoption of an active-like conformation of the switch II region in RAS-GDP is promoted by: 1) point mutations that alter the conformation of switch II in the presence of either GTP or GDP; or 2) interactions with targets/effectors that induce the adoption of an active signaling-competent form (Filchtinski et al., 2010).

Of the 18 unique crystal forms, 7 crystal forms represent RAS in an inactive state (i.e. apo or GDP-bound) and do not adopt the α4-α5 dimer conformation. In contrast, 10 crystal forms represent an active state of RAS in which it adopts the α4-α5 dimer conformation. A single crystal form contains an active-state RAS, but does not adopt the α4-α5 dimer conformation (Fig. 5.2). The absence of the α4-α5 dimer in this active-state crystal form cannot be rationalized. However, the overall striking correlation (17 out of 18 crystal forms) that the adoption of an active state of RAS is coincident with the attainment of the α4-α5 dimer conformation suggests that dimerization is not an artefact of crystal packing, but instead reflects an intrinsic propensity of RAS to form dimers in a regulated manner (Fig. 5.3).

Importantly, if an active RAS manifests as a dimer, it would be predicted that the acquisition of the RAS dimer conformation would not impede the ability of RAS to productively engage its downstream effectors, including RAF family kinases. Indeed, I found that the α4-α5 dimer state is
fully compatible with concurrent binding to effector proteins through their RAS binding domains (RBDs) (Fig. 5.4). The α4-α5 dimer state is also compatible with RAS being anchored in the membrane and orients the effector binding region on both RAS protomers parallel to the plane of the membrane, which would be necessary for productive RAS—effector interactions (Fig. 5.4).

5.3.3 GTP/GDP exchange might regulate RAS dimerization

I reasoned that under normal conditions (i.e. wild type RAS), GDP exchange for GTP (remote from the α4-α5 dimer interface) might allosterically promote dimer formation. GTP binding-induced conformational changes in the critical switch II region of RAS (although not directly involved at the α4-α5 dimer interface), correlates with the adoption of the α4-α5 dimer conformation. Specifically, within the switch II region, Arg68 stabilizes the N-terminus of the switch conformation through an interaction with Ala59/Gly60 in the GTP-loaded state (Hall et al., 2002) (Fig. 5.5); this interaction is disrupted in the presence of GDP. A striking example of the correlation between the switch II conformation and dimerization is revealed by the recent structure of wild type RAS-GDP bound to a mutant RAS binding domain (RBD) of RAF (Filchtinski et al., 2010). This mutant RBD binds RAS-GDP with ~100-fold higher affinity than wild type RBD. Binding of this mutant RBD to RAS-GDP in fact induces a change in the switch II conformation of RAS to more closely resemble that of the GTP-loaded state and the adoption of the α4-α5 dimer configuration (Fig. 5.5).
Dimer formation might be a recurring theme in RAS like small GTPases

Surprisingly, I found that the attainment of the α4-α5 dimer conformation extends to other small GTPases outside of RAS within the RAS superfamily (Fig. 5.6). Specifically, Rab11b of the Rab family adopts a similar α4-α5 dimer conformation in its GTP bound state, but not in its GDP bound state, while Rnd3 of the Rho family which lacks GTPase activity and thus constitutively binds GTP (Fiegen et al., 2002), similarly adopts the α4-α5 dimer conformation (Fig. 5.6). Most strikingly, RAS, Rab11b and Rnd3 share no sequence conservation of dimer interface residues between each other (Fig. 5.7), suggesting that the adoption of the α4-α5 dimer topology, rather than the specific residues that mediate dimer formation, has been selectively conserved through evolution. I reason that the conserved nature of the α4-α5 dimer topology across divergent small GTPases hints an underlying conserved function for the α4-α5 dimer of RAS, Rab11b and Rnd3.
5.3.5 How might RAS function in RAF activation?

Promotion of the side-to-side dimer configuration is necessary and sufficient to drive RAF catalytic activation. In this regard, RAS binding to RAF could serve to unleash the inherent dimerization potential of the RAF kinase domain, for example, by relieving an auto-inhibitory interaction between the RAF kinase domain and the auto-inhibitory N-terminus of RAF (Claperon and Therrien, 2007) (see section 6.3) or to actively promote dimerization by virtue of RAS having dimerization potential itself. Consistent with the notion that the ability of RAS to form dimers might in turn actively promote RAF dimerization, Farrar et al. showed that the function of RAS in RAF activation could be circumvented by means of artificially driving RAF dimerization in which the specific and regulated dimerization potential of a fused external domain (and not simply as a result of its fusion to RAF per se) stimulates RAF activity (Farrar et al., 1996).

My efforts to validate the ability of RAS to form dimers in solution (as opposed to in a crystalline environment) did not yield conclusive results. I found that RAS-GTP in solution behaves as a monomer at concentrations up to ~50μM, the highest concentration I could test by analytical ultracentrifugation (AUC) due to constraints on the maximum concentration suitable with the instrument optics (Fig. 5.8). One possibility is that membrane anchorage of RAS serves to substantially increase the local concentration of RAS by depositing it on a spatially restricted two-dimensional surface which shifts the equilibrium in favour of dimers. Indeed, a previous study had found that RAS in its GTP-bound state exists as a dimer when reconstituted in a membrane environment, but remains a monomer in free solution (Inouye et al., 2000). The protein concentrations used in crystallization likely mimic such high concentrations achieved at the membrane (>>50μM) leading to the formation of the α4-α5 dimer conformation in the crystal environment. Alternatively, as RAS undergoes extensive lipid modifications (Hancock, 2003), one cannot rule of the possibility that such modifications unleash the dimerization potential of RAS.

![Figure 5.8 – AUC analysis of RAS-GTP. The observed molecular weight in solution is consistent with a monomeric RAS species. Analysis performed by the Department of Biochemistry AUC facility.](image-url)
5.4 Concluding remarks

The isolated kinase domain of RAF has relatively weak catalytic activity that is substantially increased by fusion to an external dimerization domain. As RAF function is dependent on RAS under normal conditions, it is tempting to speculate that the α4-α5 dimer conformation of RAS visualized in crystal structures might directly underlie the promotion of RAF dimers in ERK signaling (Fig. 5.9). My attempts to test this model in vivo using S2 cells have been inconclusive (Fig. 5.10). Specifically, Dr. Sahmi in the Therrien lab found that mutations predicted to perturb RAS dimerization had no effect on RAF activation in S2 cells (as evidenced by the levels of phospho-ERK/MAPK). One caveat with this approach is that the assay depends on overexpressed constitutively active (GTP-bound) RAS proteins, which might have a high propensity to dimerize even in the presence of a single or double mutants predicted to perturb the dimer interface. Future experimental strategies employing a more sensitive assay that directly monitors RAS dimerization in cells using BRET is being developed by the Therrien lab.

**Figure 5.9** – Model for RAF activation by a dimeric RAS. Left panel: Inactive RAS-GDP exists as a monomer unable to bind and recruit RAF to the membrane. Right panel: RTK activation by growth factor stimulates GTP loading of RAS to form RAS dimers that in turn recruit a pair of RAF molecules to promote RAF dimerization and activation.

**Figure 5.10** – Monitoring for ERK (MAPK) activation in a RAS-dependent assay in S2 cells. The α4-α5 dimer mutants, G48W, N127F, R131W and K165W do not exhibit a major effect on ERK activation as measured by the levels of phosphorylated ERK (pMAPK). In contrast, a control RAS effector binding mutant (S17N) showed a marked reduction in pMAPK levels. Assay performed by Dr. M. Sahmi (IRIC).
Chapter 6

Discussion and Perspective
6 Discussion and perspective

6.1 Kinases and pseudokinases: master regulators of cellular functions

Protein kinases regulate a plethora of diverse cellular functions. Their highly controlled activation is subject to an equally diverse repertoire of regulatory mechanisms. Pseudokinases – a class of proteins that possess a domain structurally related to a protein kinase domain that lacks phosphoryl transfer function, are emerging as critical yet enigmatic regulators of protein kinases (Boudeau et al., 2006; Rajakulendran and Sicheri, 2010; Zeqiraj and van Aalten, 2010).

Nearly ~10% of kinase domain containing proteins are classified as pseudokinases owing to the presence of substitutions of conserved catalytic residues lining the active site, which are predicted to ablate ATP binding and/or phosphoryl transfer (Boudeau et al., 2006). Pseudokinases were initially thought to function primarily as organizing centres (or scaffolds/adaptors) in the cell. However, recent structural and mechanistic characterization of kinase domains from pseudokinases has revealed some unexpected findings, such as the ability to tightly bind nucleotide in a highly regulated manner despite loss of multiple conserved residues that normally mediate kinase-nucleotide interactions (Zeqiraj et al., 2009). If pseudokinases simply act as inert docking modules as previously thought, it seems paradoxical that they retained certain kinase-specific attributes through evolution, such as the ability to bind ATP. An emerging notion that provides a new framework for re-evaluating the role of pseudokinases is the intriguing finding that some pseudokinases possess the specific ability to allosterically regulate their kinase-competent counterparts (some even retain residual kinase activity, albeit the biological significance of this remains elusive) (Rajakulendran and Sicheri, 2010). The studies described in this thesis pertaining to the mechanism of action of the pseudokinase KSR in RAF activation underscore this emerging notion.

In addition to the mechanism of action of KSR (Rajakulendran et al., 2009), recent studies have revealed that for certain kinases like the EGFR family kinases, which are activated by the formation of specific allosteric dimers, closely related pseudokinase variants can also serve as direct activators by retaining the ability to form allosteric heterodimers, despite having dispensed with phosphoryl transfer function (Jura et al., 2009; Zhang et al., 2006) (Fig. 4.21). Reflecting the importance of self interaction in the function of both EGFR and RAF/KSR protein kinase families, residues comprising the self interaction surfaces of the kinase domain have been selectively conserved through evolution between kinase-active and pseudokinase members. In effect, the kinase-active and pseudokinase member within each family evolved from a single ancestral progenitor, one that possessed both protein kinase catalytic activity and a
specific non-catalytic allosteric function. Following a gene duplication event, one gene dispensed with phosphoryl transfer function to take on the dedicated task of functioning as an allosteric activator of its kinase-active partner (Rajakulendran and Sicheri, 2010).

Pseudokinases appear to have evolved opportunistically. Take for example the case of Ire1 and RNaseL, which comprise a unique subfamily of proteins possessing a protein kinase domain and fused ribonuclease domain (Credle et al., 2005; Dong et al., 1994). The ribonuclease activity of both enzymes is regulated by the adoption of a specific dimer configuration via their kinase domains, and dimerization in turn is potentiated by nucleotide binding to the kinase domain (Lee et al., 2008). Thus, the kinase domain in Ire1 and RNaseL function as regulated dimerization modules that activate the latent ribonuclease function. In this regard, while Ire1 still retains phosphoryl transfer function, RNaseL has completely dispensed with phosphoryl transfer function.

6.2 The CNK and HYP SAM domains in RAF signaling

My structural characterization of the hCNK2\(^{\text{SAM}}\)/dHYP\(^{\text{SAM}}\) complex reveals that discrete SAM domain dimerization can also occur through the ML and EH surfaces previously known to mediate polymerization (Kim et al., 2001). The ML surface on HYP\(^{\text{SAM}}\) has evolved to selectively recognize the EH surface on CNK\(^{\text{SAM}}\) with high affinity. In contrast, the EH and ML surface on HYP\(^{\text{SAM}}\) and CNK\(^{\text{SAM}}\), respectively, are non-functional and show no sequence conservation across species. I presume that there has been no selective pressure for maintaining residues at the non-functional surfaces.

6.2.1 SAM domain dependent dimerization: implications for regulation

How might SAM domain mediated dimerization of CNK/HYP facilitate interaction with the kinase domain of KSR? One possibility is that the minimal SAM domain dimer complex of CNK and HYP itself forms a composite docking site for binding the kinase domain of KSR. If true, a good candidate interaction site within the SAM dimer complex resides within a region encompassing the C-termini of helix \(\alpha5\) in both CNK and HYP SAM domains. This region of
each SAM domain is solvent exposed, well conserved and spatially juxtaposed (Fig. 6.1).

Consistent with the possibility that this region composes an interaction site for KSR kinase domain, a triple mutation D81A/N82A/L83A targeting three conserved residues C-terminal to helix α5 in dCNK\textsuperscript{SAM}, was shown by the Therrien lab to selectively perturb interaction with KSR, but not with dHYP\textsuperscript{SAM} (note that the crystallization construct of hCNK2\textsuperscript{SAM} contained the equivalent of N82 at the C-terminus, but electron density is not visible past Y78 in the final refined model) (Rajakulendran et al., 2008).

My ability to directly test this composite interaction site binding model has been hampered by expression problems. Specifically, I cannot express the isolated SAM domains of dCNK or hHYP or the kinase domain of hKSR, which would allow us to test for direct binding of proteins from a common species. I found that a minimal SAM domain dimer between hCNK and dHYP does not bind to the kinase domain of dKSR but that this was likely due to a cross species effect (Fig. 6.2). Indeed while a larger single species complex between a dCNK\textsuperscript{2-549}/dHYP binds to the kinase domain of dKSR in S2 cells, the equivalent cross species complex of hCNK\textsuperscript{2-485}/dHYP does not (Fig. 6.2).

As such, a second possibility that cannot be ruled out at this time is one in which the CRIC-PDZ regions C-terminal to the SAM domain in CNK comprises the KSR kinase domain binding site (in conjunction with the SAM domain or in isolation in a manner dependent on the SAM domain binding HYP). I posit that this site remains hidden until the SAM domain of HYP engages the SAM domain of CNK. Future efforts in the Sicheri and Therrien labs to resolve how precisely SAM domain dimerization between
CNK and HYP participate in binding to the kinase domain of KSR are directed at solving the structure of a minimal CNK^{SAM-CRIC-PDZ}/HYP/KSR^{kinase domain} complex.

6.3 KSR and RAF: ancestral genes with diversification of function

6.3.1 KSR: specialization of function in RAF substrate presentation

While the physiological RAF activating complex comprises minimally the KSR/CNK/HYP complex, KSR alone possesses the intrinsic capacity to directly stimulate RAF activation (Douziech et al., 2006). CNK and HYP appear to be tuned to regulate when KSR is brought into proximity with RAF to drive its activation by forming KSR/RAF side-to-side heterodimers and ensuing ERK signaling (Rajakulendran et al., 2009). The kinase domain of KSR also has a second critical role in ERK signaling by binding to and recruiting MEK to RAF. Recently, the crystal structure of the KSR/MEK interaction was determined, confirming that MEK binds KSR primarily through determinants in the C-lobe of KSR (Brennan et al., 2011) (Fig. 6.3). Taken together, these results highlight two distinct functions for the kinase domain of KSR in RAF signaling. Firstly, the kinase domain of KSR mediates RAF substrate targeting by recruiting MEK. A recent study reported the intriguing possibility that KSR plays an active role in presenting MEK for phosphorylation by RAF (Brennan et al., 2011). Specifically, KSR is constitutively bound to MEK in which MEK adopts an inactive conformation incapable of being phosphorylated by RAF. Upon KSR/RAF dimerization through the side-to-side interface, MEK is thought to undergo conformational changes that permit access to RAF (Brennan et al., 2011). The second function of KSR, unrelated to its role in MEK recruitment, involves the ability to form side-to-side heterodimers with the kinase domain of RAF that underlies an allosteric mechanism for RAF catalytic activation (Rajakulendran et al., 2009).
6.3.2 RAF catalytic switching: it’s all about dimers

Dimerization of the RAF kinase domain with KSR or with other RAF molecules is central to its activation mechanism. I posit that other regulatory events that impinge on RAF activation may also act by modulating dimerization. In this regard, the large group of auxiliary proteins that act together with RAF and KSR, such as CNK, HYP and 14-3-3 proteins, may serve to spatially and temporally regulate the formation of side-to-side dimers (Udell et al., 2011). In the absence of RTK/RAS activation, a regulatory element in the N-terminus of RAF engages the C-terminal kinase domain to inhibit catalytic activity by an unknown mechanism (Claperon and Therrien, 2007) (Fig. 6.4). I reason that this autoinhibitory interaction may interfere with the ability of the kinase domain to adopt a productive dimer configuration. Consistent with this notion, N-terminally truncated versions of RAF are catalytically hyperactivated (Chong and Guan, 2003). Upon RAS activation, RAF is recruited to the plasma membrane through a direct interaction of its N-terminus with RAS which may displace the N-terminus/kinase domain inhibitory interaction (Claperon and Therrien, 2007), thereby freeing the kinase domain to adopt a productive side-to-side dimer. As RAS has been found to dimerize in membranes (Inouye et al., 2000), it is tempting to speculate that RAS itself directly promotes RAF dimerization. Indeed, RAS in its active GTP-bound state adopts a specific dimeric configuration in crystal structures, while in its inactive GDP-bound state RAS appears monomeric. The only exceptions to this trend that may prove the rule are certain mutants of RAS which adopt a dimer state in crystallographic environments even in the absence of GTP. Clearly this unappreciated structural characteristic of RAS and its affect on RAF activation warrants further investigation.

6.4 Conclusions and future perspectives

Most recently, several groups have demonstrated that ATP-competitive B-RAF inhibitors can induce B-RAF/C-RAF complex formation and the resultant stimulation of C-RAF activity in trans, underscoring the relevance of RAF/RAF interactions in modulating kinase activity in disease (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulakis et al., 2010). Indeed, subsequent studies have confirmed the formation of side-to-side dimers underlies aberrant RAF activation resulting from RAF inhibitor treatment in certain cancers (that is, inhibitor binding to RAF promotes RAF dimerization) (Hatzivassiliou et al., 2010; Poulakis et al., 2010). The results highlight a need for caution when treating patients with B-RAF inhibitors as inhibitor binding can have the counter-intuitive effect of actually promoting kinase domain dimers and RAF activity. Whether this results from the promotion of KSR/RAF heterodimers in addition to RAF homodimers remains an open question.
The allosteric modulation of RAF activity by KSR is a departure from the conventional dogma that KSR is yet another passive scaffold like CNK or HYP in RAF signaling (Kolch, 2005). At the same time, the ability of KSR to allosterically drive RAF activation provides an elegant explanation for the longstanding and controversial issue of whether KSR function in RAF signaling requires bona fide KSR kinase activity (Claperon and Therrien, 2007). However, the debate as to whether KSR is truly a pseudokinase devoid of phosphoryl transfer function is still far from settled. In fact, a number of recent reports suggest KSR does possess residual phosphoryl transfer function in vitro (Brennan et al., 2011; Goettel et al., 2011). The physiological relevance of this residual activity remains
to be determined. The possibility that KSR kinase activity acts in concert with its allosteric function in RAF signaling is certainly intriguing and will be an exciting avenue for future investigations.

The question still remains as to how KSR/RAF dimerization is regulated. As iterated in chapters 4 and 5, two critical upstream regulators, RAS and 14-3-3, appear to directly promote RAF dimerization. What events regulate when RAS and 14-3-3 are mobilized to drive RAF dimerization? RAS is activated by upstream RTK stimulation (Macara et al., 1996). Whether active GTP-loaded RAS itself might form dimers that in turn promote RAF dimerization remains to be determined. 14-3-3 proteins, which are intrinsically dimeric, bind to a phosphorylated motif found C-terminal to the kinase domain in KSR and RAF to promote dimerization (Rajakulendran et al., 2009). The kinases that are responsible for phosphorylating the 14-3-3 site on KSR and RAF remain elusive. These will be topical areas of investigation for future studies. Equally intriguing is the question of how the cell regulates specific dimer formation between the multiple isoforms of RAF and KSR proteins that are present in higher-order metazoa. Given that all isoforms of RAF and KSR share a nearly identical dimer interface, the cell must have a separate mechanism in place for selectively driving dimer formation between any two specific isoforms. What is the relative activity of the different dimer combinations? The heterodimers comprising KSR/RAF are thought to be more active by virtue of KSR’s specific capacity to function as both an allosteric activator of RAF and to simultaneously recruit the RAF substrate MEK (Rajakulendran et al., 2009). If promotion of side-to-side dimers activates RAF, then it follows that the disruption of the dimers must lead to inactivation.

Nearly three decades have passed since the discovery of the RAF family kinases. Despite the tremendous amount of insights gained into their function and regulation in physiology and pathology, many more questions remain unanswered. The discovery of B-RAF as a prolific human oncogene (Davies et al., 2002) has ignited unprecedented efforts into understanding this family of kinases in academia and the pharmaceutical industry alike. Central to understanding RAF function appears to be the role of the key players that impinge on RAF activity. The inroads made into understanding how the KSR/CNK/HYP complex forms and functions provide an exciting framework for future studies.
References


