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Regulation of C-RAF stability by the RanBPM/CTLH complex

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Abstract

RanBPM is an evolutionarily conserved multi-domain protein that has been implicated in the regulation of several cellular process including protein stability, cell migration, gene transcription, and apoptosis. RanBPM is identified as a key member of the CTLH complex, an orthologous complex to a yeast E3 ubiquitin ligase complex, the exact function of which remains unknown. Previously, our laboratory identified RanBPM as an inhibitor of the ERK1/2 pathway through the modulation of C-RAF protein levels. This study shows that RanBPM-mediated degradation of C-RAF occurs through the proteasome and the entire CRA domain of RanBPM is necessary for direct interaction with C-RAF and for effective downregulation of C-RAF in human cells. Finally, the CTLH complex member RMND5A regulates endogenous C-RAF protein levels further implicating the CTLH complex in RanBPM-mediated regulation of C-RAF stability. This study provides further insight into the function of RanBPM and the CTLH complex, and their regulation of C-RAF.

Keywords

RanBPM, C-RAF, ERK1/2 pathway, CTLH complex, CRA domain, protein stability, RMND5A

Co-Authorship Statement

This thesis was written by Christina McTavish and edited by Caroline Schild-Poulter. All experiments and procedures described in this thesis were performed by Christina McTavish, with the exception of the following:

- HeLa RanBPM shRNA and non-targeting control shRNA stable cell lines were generated by Dawn Bryce.
- pCMV-HA-RanBPM-ΔC4 and pCMV-HA-RanBPM-ΔC1, with resistance to shRNA targeting, were generated by Caroline Schild-Poulter, Dawn Bryce, Patricia Christian, and Tung Bai.
- pET28a-ΔN-C-RAF was cloned by Matthew Maitland.
- *RMND5A*$^{-/-}$ HEK-293 CRISPR-Cas9 knockout cell line was generated by Matthew Maitland and Xu Wang.

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List of Abbreviations

AC adenylyl cyclases

ANOVA one-way analysis of variance

AP-1 activator protein 1

APP amyloid precursor protein

AR androgen receptor

ARMc8 armadillo repeat containing 8

Aβ amyloid-beta

BACE1 β-site APP-cleaving enzyme 1

Bax Bcl-2-associated X protein

Bcl-2 B cell lymphoma 2

Bcl-X_L B cell lymphoma extra large

BLT2 leukotriene B4 receptor 2

cAMP cyclic adenosine monophosphate

CDK11^{p46} caspase-processed C-terminal domain of cyclin-dependent kinase 11

CHIP C-terminus of constitutive HSC70-interacting protein

CR conserved regions

CRA CT11-RanBPM

CRISPR clustered regularly interspaced short palindromic repeats

CTLH C-terminal to LisH

DMEM Dubecco's modified eagle medium

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

ERK extracellular signal-regulated kinase

FBPase fructose 1,6-bisphosphatase

FBS fetal bovine serum

FMRP fragile X mental retardation protein

FOX Forkhead box

$GABA_A$ γ-aminobutyric acid

Gid glucose induced degradation deficient

GOLF gemcitabine, oxaliplatin, leucovrin, and 5-Fluorouracil

GR glucocorticoid receptor

GST glutathione-S-transferase

GTP guanosine triphosphate

GTPase guanosine triphosphatase

HA human influenza hemagglutinin

HDAC6 histone deacetylase 6

HEK human embryonic kidney

HEPES hydroxyethyl piperazineethanesulfonic acid

HIPK2 homeodomain-interacting protein kinase

HMG High Mobility Group

HOXA5 homeobox A5

HRS hepatocyte growth factor-regulated tyrosine kinase substrate

HSC constitutive heat shock protein

HSP heat shock protein

IPTG Isopropyl ß-D-1-thiogalactopyranoside

IR ionizing radiation

JNK Jun N-terminal kinase

LB Luria Bertani

LFA-1 lymphocyte function-associated antigen-1

LisH lissencephaly type-1-like homology

LRP low-density lipoprotein receptor-related protein

MAEA macrophage erythroblast attacher

MAPK mitogen-activated protein kinase

MDPK myotonic dystrophy protein kinase

MEK mitogen-activated protein kinase kinase

MET mesenchymal epithelial transition factor

Mirk minibrain-related kinase

MUSCLE multiple sequence comparison by log-expectation

NEM N-ethylmaleimide

NF-κB nuclear factor kappa B

NP-40 Nonidet P-40

OD_{600} optical density at 600 nm

p75NTR p75 neurotrophin receptor

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PCR polymerase chain reaction

PKA protein kinase A

PMSF phenylmethylsulfonyl fluoride

PVDF polyvinylidene fluoride

RAF rapidly accelerated fibrosarcoma

RanBP9 Ran-binding protein 9

RanBPM Ran-binding protein in the microtubule-organizing center

RAS rat sarcoma

Rb retinoblastoma tumour suppressor protein

RING really interesting new gene

RIPA radioimmunoprecipitation assay

RMND5A required for meiotic nuclear division 5 homolog A

Rok Rho-binding kinase

RTK receptor tyrosine kinases

shRNA short hairpin RNA

Src sarcoma

SDS sodium dodecyl sulphate

sgRNA single-guide RNA

SH3 Src homology 3

SIE3 Symbiosis receptor-like kinase-interacting E3 ubiquitin ligase

SplA spore lysis A

SPRY spore lysis A and ryanodine receptor

TAF4 TATA box binding protein-associated 4

TBS-T Tris-buffered saline with Tween-20

TFIID transcription factor II D

TR thyroid hormone receptor

TRAF6 tumour necrosis factor receptor-associated factor 6

Tris Tris-hydroxymethyl amino methane

TUBE tandem ubiquitin binding entities

TWA1 two hybrid-associated protein 1 with RanBPM

TX100 TritonX-100

TβRI transforming growth factor beta receptor type I

USP11 ubiquitin carboxyl-terminal hydrolase 11

WDR26 WD repeat-containing protein 26

XIAP X-linked inhibitor of apoptosis proteins

Chapter 1

1 Introduction

1.1 Cancer

Following several decades of rapid advancements in the scientific field, cancer research has developed significantly. With cancer as the leading cause of death in Canadian citizens (1), a wealth of knowledge has been gathered and has revealed cancer to be a complex disease involving many changing factors. As of 2017, *Canadian Cancer Statistics* estimates that 1 in 2 Canadians will develop cancer in their lifetime, and 1 in 4 will die from the disease (1). Therefore, uncovering the complexities of this disease and discovering new avenues for prevention and treatment are imperative to our health and longevity.

Numerous studies indicate that cancer pathogenesis is a multistep process that requires various genetic modifications to occur to cause the progression of a normal human cell, which is strictly monitored during growth, replication, and death, into a malignant body that loses these regulations. These lost regulations can be categorized into ten hallmarks, these being evasion of growth suppression, sustained proliferative signals, resistance to cell death, sustained angiogenesis, replicative immortality, activation of metastasis, deregulation of cellular energetics, avoiding immune destruction, inflammation, and genomic instability (2).

Understanding the underlying molecular mechanisms leading to malignancy can provide the necessary information to develop new strategies for treatment or possibly even its prevention. This study investigates the role of Ran-binding protein in the microtubule organizing center (RanBPM) in the inhibition of the extracellular signal-regulated kinase (ERK) 1/2 pathway, a pathway commonly found to be deregulated in cancers, through its regulation of rapidly accelerating fibrosarcoma (C-RAF) protein stability.

1.2 RanBPM

1.2.1 General overview

RanBPM, also referred to as RanBP9, was the ninth Ran binding partner to be identified by yeast two-hybrid method in search of Ran binding proteins (3). It was first identified as a 55 kDa protein that interacts with Ran, however RanBPM did not contain the consensus Ran-binding domain (3), and was later shown to have no interaction with Ran *in vivo* (4). RanBPM is a ubiquitously expressed, non-enzymatic protein with nuclear and cytoplasmic localization owing to its two nuclear localizing signals and one nuclear export signal (5). RanBPM has also been identified as an evolutionarily conserved protein which shares close homology to a wide variety of species, including lower eukaryotes, plants, and fungi (6).

The initial findings of RanBPM being a 55 kDa protein were later dismissed, as the full length form of RanBPM was found to be a 90 kDa protein. Additionally, the full length RanBPM was recovered by gel filtration methods in a large protein complex of 670 kDa (4). RanBPM has since been implicated in several cellular processes including cellular growth, migration and adhesion, as well as the regulation of gene transcription and apoptosis (7-15). Several studies have identified an array of proteins that interact with RanBPM, which leads most to believe RanBPM to be a scaffold protein (16).

1.2.2 Conserved domains and structure

RanBPM contains several conserved domains, each with the potential to provide a protein-protein interaction site (Figure 1.1). RanBPM consists of five domains, the N-terminal proline/glutamine rich domain, the spore lysis A and ryanodine receptor domain (SPRY), the lissencephaly type-1-like homology (LisH) domain, the C-terminal to LisH (CTLH) domain, and the C-terminal CT11-RanBPM (CRA) domain (3, 4, 17, 18). The N-terminal is a highly disordered, proline and glutamine rich domain (4) containing six predicted Src homology 3 (SH3)-binding domains (19). The SPRY domain is a conserved structural domain that mediates protein-protein interactions (18, 20). This domain was first identified in *Dictyostelium discoideum* dual specificity kinase spore lysis A (SplA) and ryanodine receptors (21), and has been since shown to be a domain present in many

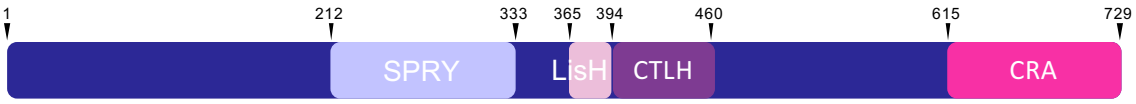

Figure 1.1 Schematic diagram of full-length RanBPM. Conserved domains are shown: the SPRY (spore lysis A and ryanodine receptor) domain; the LisH (lissencephaly type-1-like homology) domain; the CTLH (C-terminal to the LisH) domain; and the CRA (CT11-RanBPM) domain. Figure adapted from (16).

proteins in higher eukaryotes. Little information is known of the role and function this domain plays in many proteins, however it is interesting to note that the majority of SPRY domain containing proteins also possess a RING (really interesting new gene) – type zinc-finger domain (20), and over one-third of proteins with both a SPRY and RING domain that were investigated exhibit E3 ubiquitin ligase activity (22). Recently, the crystal structure of the RanBPM SPRY domain was elucidated, it being the only one of the four conserved domains with its structure solved. The RanBPM SPRY domain forms a β-sandwich fold that consists of two seven-stranded anti-parallel β-sheets with two α-helices, one at each terminal (23). C-terminal to the SPRY domain are the LisH and CTLH domains. Together, these two domains have been shown to contribute to the regulation of microtubule dynamics (17) and to mediate dimerization and oligomerization (24-26). Lastly, the C-terminal CRA domain has been identified as a novel protein-protein interaction domain containing a predicted six α-helices reminiscent of the death domain superfamily (18).

Since its discovery, RanBPM has been shown to associate with several proteins through its interaction domains. To date, there are over 75 protein interactions that have been documented, with many of these interactions resulting in an alteration in cellular function (16). As RanBPM does not possess enzymatic activity, it is unclear as to how these alterations in cellular functions occur.

1.2.3 CTLH complex

When the full-length form of RanBPM was discovered, it was recovered in a 670 kDa protein complex (4). This complex was later identified as a seven-member complex termed the CTLH complex as the majority of the complex members featured both a LisH and a CTLH domain (27). The CTLH complex is comprised of RanBPM along with RMND5A (required for meiotic nuclear division 5 homolog A), MAEA (macrophage erythroblast attacher), Muskelin, TWA1 (two hybrid-associated protein 1 with RanBPM), and ARMc8 (armadillo repeat containing 8) (27, 28) (Figure 1.2). Each member of the CTLH complex, with the exception of Muskelin, possess a *Saccharomyces cerevisiae*

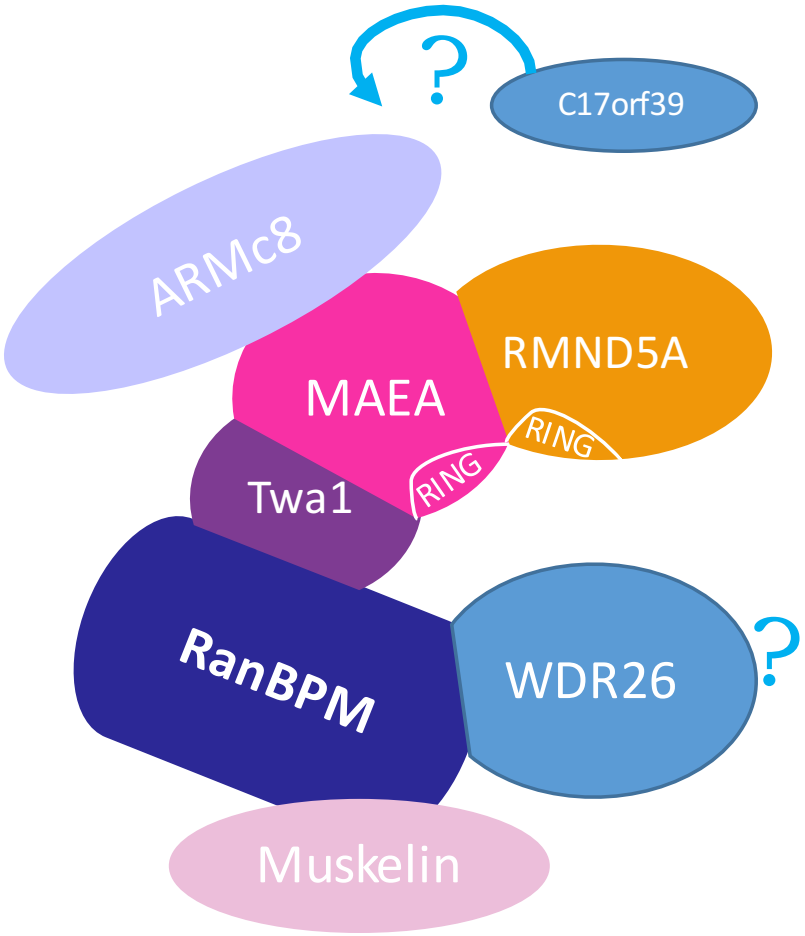

Figure 1.2 Proposed model of the mammalian CTLH complex. The CTLH complex is comprised of RanBPM (Ran binding protein microtubule organizing center) along with RMND5A (required for meiotic nuclear division 5 homolog A), MAEA (macrophage erythroblast attacher), Muskelin, TWA1 (two hybrid-associated protein 1 with RanBPM) and ARMc8 (armadillo repeat containing 8). WDR26 (WD repeat 26) remains to be recognized as a CTLH complex member. C17orf39 has not been identified in the mammalian CTLH complex, however it is proposed to identify specific target substrates similar to its yeast orthologue GID4 (16). Figure adapted from (16).

orthologue which compose a large protein complex called the glucose induced degradation deficient (Gid) complex (6, 29).

The Gid complex is comprised of seven GID proteins that are required for the proteasome-dependent degradation of fructose 1,6-bisphosphatase (FBPase). The gluconeogenic enzyme FBPase is rapidly degraded by the Gid complex when *S. cerevisiae* cells are switched from a nonfermentable carbon source to glucose (30). There are in fact two pathways that exist for FBPase degradation, one is dependent on the ubiquitin-proteasome system, while the other is dependent on the vacuolar proteolysis system, however only three members of the Gid complex participate in the vacuolar proteolysis system, one being GID1, the orthologue to RanBPM (30). GID1 functions primarily as the scaffold to the Gid complex mediating its interactions with the remainder of the complex through its LisH and CTLH domains (29).

Two other members of the Gid complex, GID2 and GID9, each contain a RING-type zinc-finger motif, which are indicative of E3 ubiquitin ligase activity. RING domains interact with and function in conjunction with E2s, ubiquitin conjugating enzymes, to mediate the ubiquitination of targeted substrates (31, 32). Although GID9 does possess the RING motif necessary for E3 ubiquitin ligase activity, it did not demonstrate E3 ligase activity *in vitro* (31). GID2, however, has been shown to polyubiquitinate proteins *in vitro*, and mutation to the RING motif was shown to abolish the polyubiquitination of FBPase (33). The orthologue of GID2, RMND5A, also possesses the RING motif, and the *Xenopus laevis* orthologue has been shown to have E3 ubiquitin ligase activity *in vitro*, however this activity has not been demonstrated in mammals (34).

Although the Gid complex is a proven E3 ubiquitin ligase complex in yeast, the mammalian CTLH complex has yet to be shown to exhibit the same activity (6). Other orthologous E3 ubiquitin complexes exist, such as the symbiosis receptor-like kinase (SymRK)-interacting E3 ubiquitin ligase (SIE3), a plant-specific E3 ligase that contains the conserved CTLH, CRA, and RING domains, and has been shown to bind and ubiquitinate SymRK *in vitro* and in *Lotus japonicas* (35). A similar complex has also been identified to exist in *Arabidopsis thaliana* (36). Although no data has yet surfaced to

suggest that the mammalian CTLH complex has E3 ubiquitin ligase activity, there are data that link members of the CTLH complex to the degradation pathway. Overexpression of ARMc8, the orthologue of GID5, has been shown to result in the degradation of α-catenin, and ARMc8 has also been shown to associate with β-catenin (37). ARMc8A has also been shown to associate with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), an essential factor to endosomal and lysosomal dependent degradation of receptor proteins through endocytosis (38). Muskelin is a key factor in the internalization of α1 subunit-containing γ-aminobutyric acid ($GABA_A$) receptors in neurons (39). WD repeat domain 26 (WDR26), which is the orthologue to GID7 and a putative member of the CTLH complex, was also shown to promote the downregulation of β-catenin (40).

Evidence suggests that members of the CTLH complex have an effect on various protein levels and functions. However, further insights into these studies are still needed to understand how the CTLH complex functions, as currently no functional data exist for the complex as a whole. Only individual subunits have been studied in isolation, with RanBPM as the most studied subunit.

1.2.4 Role of RanBPM in apoptosis

Substantial evidence suggests that RanBPM has a role in the activation of apoptosis. We have demonstrated that overexpression of RanBPM results in the activation of caspase-3 leading to cell death by apoptosis in HeLa cells (41). Exposure to ionizing radiation (IR) causes DNA damage and triggers the activation of the apoptotic pathway primarily through the mitochondrial pathway regulated by the B cell lymphoma 2 (Bcl-2) protein family (42). Short hairpin RNA (shRNA) knockdown of RanBPM in the presence of IR led to reduced mitochondria-associated Bcl-2-associated X protein (Bax), a pro-apoptotic Bcl-2 family protein, and protein levels of anti-apoptotic proteins Bcl-2 and B cell lymphoma extra large ($Bcl\text{-}X_L$) were significantly increased leading to reduced caspase-3 activation and apoptosis (13, 41). These studies were further substantiated, as RanBPM overexpression was linked to decreased Bcl-2 protein levels, and increased Bax protein levels and oligomerization, causing mitochondrial fragmentation and the release of

cytochrome *c* ultimately leading to increased apoptotic activity (10, 16). Moreover, downregulation of RanBPM in gastric cancer cells also proved to effectively render the cells insensitive to apoptosis-inducing agents (8). Also, the overexpression of RanBPM in transgenic mice was shown to lead to mitochondrial instability and promoted amyloid-beta (Aβ)-induced apoptosis in neuronal cells (43).

Through its protein-protein interaction domains, RanBPM has been shown to interact with proteins that function to regulate the apoptotic pathway. Interactions between RanBPM and caspase-processed C-terminal domain of cyclin-dependent kinase 11, ($CDK11^{p46}$), a protein involved in apoptotic signaling and a promoter of apoptosis when ectopically expressed, have been documented (44). Interactions of RanBPM and homeodomain-interacting protein kinase (HIPK2), an activator of the tumour suppressor protein p53, and the p75 neurotrophin receptor (p75NTR) have also been shown to occur (11, 45). RanBPM interacts with the tumour suppressor protein p73, enhancing its pro-apoptotic activity through inhibition of its ubiquitination and its subsequent degradation (9), while overexpression of RanBPM causes the upregulation of endogenous p73 mRNA and protein levels (10). Overall, these studies support that RanBPM has a pro-apoptotic effect; however, the mechanisms by which RanBPM regulates its interacting proteins still remain largely unknown.

1.2.5 Role of RanBPM in transcriptional regulation

RanBPM has also been suggested to be involved in the regulation of gene transcription. Microarray analysis comparing cells stably expressing shRNA for RanBPM to shRNA controls showed changes in gene expression, influencing transcriptional pathways associated with development and tumourigenesis (47). Downregulation of RanBPM revealed over-representation of transcription factor binding sites within the promoters of the differentially expressed genes, specifically of homeobox A5 (HOXA5), the Forkhead box (FOX) family of transcription factors, and the High Mobility Group (HMG) family of transcription factors, suggesting that RanBPM functions in the regulation of the transcriptome through these factors (47). Furthermore, RanBPM was also shown to interact with the TATA box binding protein-associated 4 (TAF4) subunit of the transcription factor II D (TFIID), a transcriptional co-activator for nuclear receptors (48).

Nuclear receptors thyroid hormone receptor (TR), androgen receptor (AR), and glucocorticoid receptor (GR), were also shown to interact with RanBPM, enhancing their transcriptional activity (49-51). Interestingly, proteomics studies have implicated the CTLH complex as a co-regulator complex for nuclear receptors (52). RanBPM has been shown to associate with, and negatively regulate, tumour necrosis factor receptor-associated factor 6 (TRAF6) E3 ubiquitin ligase activity causing reduced activation of the nuclear factor kappa B (NF-κB) signaling pathway (53). Through these studies, it can be surmised that RanBPM is capable of regulating gene transcription through direct and indirect modulation of transcription factors.

1.2.6 Role of RanBPM in adhesion, morphology, migration

Associations between RanBPM and proteins responsible for the regulation of adhesion, morphology and migration have also been reported. Integrins play a significant role in the regulation of cell survival, proliferation, adhesion and migration, as integrins mediate cell-to-cell, and cell-to-extracellular matrix interactions, as well as participate in signal transduction (55). RanBPM has been reported to interact directly with the β2-integrin, LFA-1, and β1-integrin, with the latter interaction resulting in accelerated endocytosis of β1-integrin, disrupting cell adhesion and focal adhesion signaling and assembly (43, 54). Interestingly, some data reveals that RanBPM and Muskelin function to regulate cell morphology, as knockdown of either proteins resulted in enlarged cell perimeters of human lung carcinoma cells (56). Additionally, RanBPM was shown to interact with the PlexinA1 receptor, and through the overexpression of RanBPM cell spreading and outgrowth were reduced (57).

RanBPM has been shown to negatively regulate cell migration, as it inhibits leukotriene B4 receptor 2 (BLT2)-mediated motility and its downregulation has been linked to increased cellular motility in human embryonic kidney (HEK) cells (13, 14). Metastatic gastric tumours were also shown to harbour low expression levels of RanBPM transcripts compared to non-metastatic tumours (8). Interestingly, RanBPM has also been shown to upregulate migration as its interaction with mesenchymal epithelial transition factor (MET) receptor tyrosine kinase, caused the activation of ERK1/2 thereby increasing

cellular migration (11). As RanBPM displays divergent roles in cell migration, this may suggest that RanBPM also plays a role in normal development and tumour suppression.

1.2.7 Role of RanBPM in cancer

Tumorigenesis is viewed as dysregulation of several cellular processes, including, but not limited to sustained proliferative signals, replicative immortality, tissue invasion, and evasion of apoptosis and immune destruction (2). As described above, RanBPM has been suggested to participate in the regulation of these cellular processes (7-15). When comparing a human gene expression array with RanBPM downregulated cells to normal cells, 167 genes were identified to have altered expression (47). Of these genes, over one-third were associated with cancer. Previous works in our laboratory have shown RanBPM to play an inhibitory role in cancer development. RanBPM has been shown to have an inhibitory effect on histone deacetylase 6 (HDAC6), a cytoplasmic deacetylase necessary in the formation of aggresomes (58), which, when overexpressed, plays a key role in cellular transformation and metastasis (59). Our laboratory has also linked RanBPM to the regulation of the intrinsic apoptotic pathway through inhibition of the ERK1/2 signaling pathway. RanBPM was shown to downregulate C-RAF protein levels and disrupts the heat shock protein (HSP) 90-C-RAF complex, thereby preventing the downstream phosphorylation and activation of ERK1/2 (13). RanBPM proved necessary to the activation of the intrinsic apoptotic pathway, as its knockdown revealed a marked increase in anti-apoptotic Bcl-2 and Bcl-X_L mRNA levels, and decreased mitochondrial-associated Bax (13, 41). Downregulation of RanBPM effectively resulted in the evasion of apoptosis and the loss of growth factor dependence, as growth serum deprivation enhanced cell growth and proliferation (13, 41) demonstrating that RanBPM does exhibit tumour suppressive properties.

Similarly, downregulation of RanBPM in gastric cancer cells has been shown to cause the reduction in cell adhesion, promoting cell motility and survival (8). Furthermore, distant metastatic masses were shown to exhibit reduced levels of mRNA and RanBPM protein levels (8). RanBPM has also been shown to stabilize p73 mRNA and protein levels during DNA damage, allowing p73 to act as a growth-suppressor and inducing apoptosis

(9, 10). These findings suggest that RanBPM demonstrates a tumour suppressive role, and therefore should be regarded as a potential therapeutic of cancer inhibition.

1.3 Mitogen-activated protein kinases

Mitogen-activated protein kinase (MAPK) pathways are an important link between extracellular signals and eliciting a response from the cell. Currently, there are six discrete groups of MAPK pathways that have been identified and characterized in mammalian cells, namely ERK1/2, ERK3/4, ERK5, ERK7/8, p38, and Jun N-terminal kinase (JNK) (Figure 1.3) (60-63). These pathways are responsible for the strict regulation of cellular processes essential to the successful survival of cells, providing the important controls needed in cellular growth, proliferation, differentiation, migration, and apoptosis (64). Dysregulation of these pathways alter cellular metabolism and result in developmental defects and cancer development (2).

1.4 C-RAF

1.4.1 ERK1/2 signaling pathway

The ERK1/2 signaling pathway is a topic of great importance as its deregulation has been documented in approximately one-third of all human cancers and has been linked to many aspects of the tumour phenotype (64). In this pathway, receptor tyrosine kinases (RTK) are activated by extracellular ligands, leading to the loading of guanosine triphosphate (GTP) to rat sarcoma (RAS) GTPase, and the recruitment of RAF kinases (C-RAF, B-RAF, and A-RAF) to the plasma membrane for activation (Figure 1.4). RAF then activates mitogen-activated protein kinase kinase (MEK)1/2 by phosphorylation, and MEK1/2 subsequently phosphorylates ERK1/2, activating numerous cellular processes. Overexpression of RTK or RTK activating ligands, or activating mutations in *rtk*, *ras*, or *b-raf* would lead to the constitutive activation of the ERK1/2 signaling pathway (65).

The ERK1/2 pathway possesses the ability to affect a diversity of substrates with divergent functions: within the nucleus, targeting transcription factors and kinases; in the cytoplasm, targeting kinases and structural proteins; and at the plasma membrane, regulating adhesion, cell-to-cell communication and survival (66). Therefore, ERK1/2

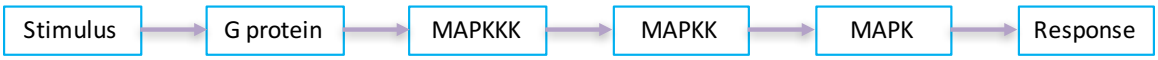

Figure 1.3 Simplified overview of MAPK pathways.

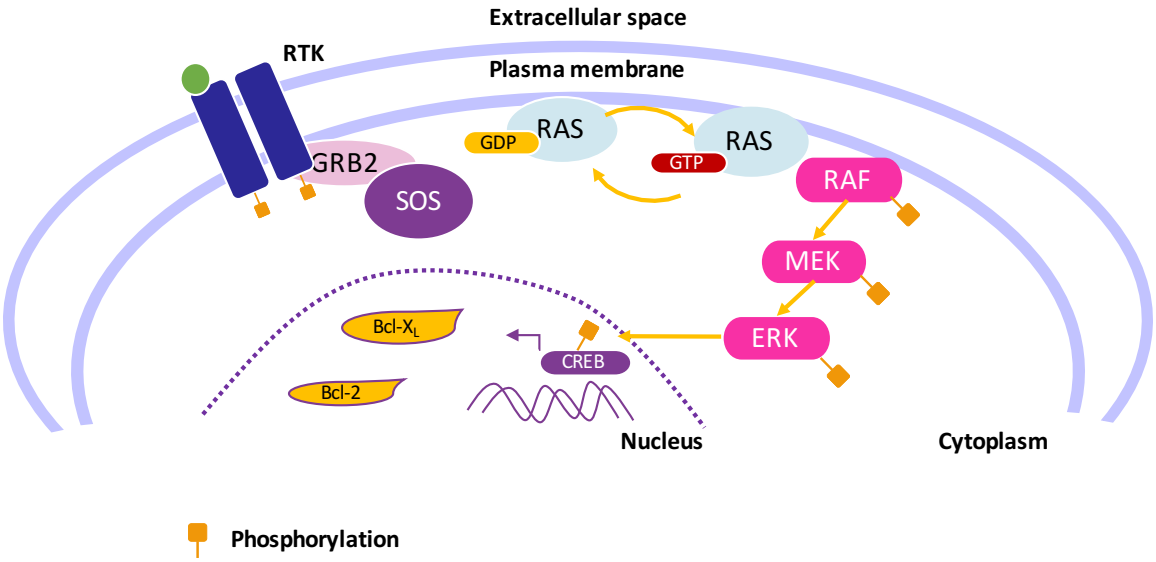

Figure 1.4 ERK1/2 signaling pathway. Extracellular ligands bind to and activate receptor tyrosine kinases (RTK) causing autophophorylation, which promotes association of activated RTK to intracellular GRB2 and SOS and the loading of membrane-bound RAS with GTP. Activated RAS then initiates a cascade of phosphorylation leading to the activation of RAF, MEK, and ERK, respectively. ERK activation leads to phosphorylation of targets within the cytoplasm and the nucleus. Specifically, the activation of transcription factor CREB by activated ERK leads to the transcription of anti-apoptotic factors Bcl-2 and Bcl-X_L.

signaling is strictly monitored within cells.

1.4.2 RAF family kinases

Three RAF isoforms exist in mammals (Figure 1.5A). C-RAF, the first RAF isoform identified in 1985 (67), B-RAF, and A-RAF, each share a common structure. Each isoform consists of three conserved regions (CR), each possessing their own distinct function necessary to the activity and regulation of the RAF kinases. CR1 contains the RAS binding domain necessary for RAS binding and subsequent attachment to the plasma membrane for activation, as well as a secondary RAS binding site, a cysteine-rich domain, which is required for RAF autoinhibition of the kinase domain (68). CR2 contains activating and inhibitory phosphorylation sites regulating RAS binding and RAF activation (69). Most importantly, CR3 contains the kinase domain, which is activated upon phosphorylation of the activating segment, also contained in the CR3 (70). The regulatory domains CR1 and CR2 are necessary for the regulation of RAF kinase activity, as upon their removal, constitutive oncogenic activation is achieved (71). As RAF kinases activate MEK1/2, RAF kinases indirectly activate ERK1/2, therefore RAF kinases are subjected to extensive regulation.

1.4.3 Activation cycle of C-RAF

Since C-RAF was the first identified RAF isoform, a wealth of knowledge was gathered about this isoform. However, much focus was later shifted to B-RAF, as several mutations were documented in this isoform which were determined to lead to oncogenesis (72). During the inactive state, C-RAF is held in a closed conformation by the N-terminal regulatory region folding over the C-terminal catalytic domain, with the 14-3-3 dimer stabilizing the conformation by binding phosphorylated S259 (pS259) of the N-terminal, and pS621 of the C-terminal (73). The activation of C-RAF was studied intensively (Figure 1.6) however, it is not completely understood. Briefly, pS259 is dephosphorylated (74) releasing 14-3-3 from the N-terminal and revealing the RAS binding sites allowing for RAS binding and recruitment to the plasma membrane (75, 76). The activation segment of CR3 is then phosphorylated, specifically S338, to achieve full kinase activation with RAF homo- or heterodimerization (77), leading to its

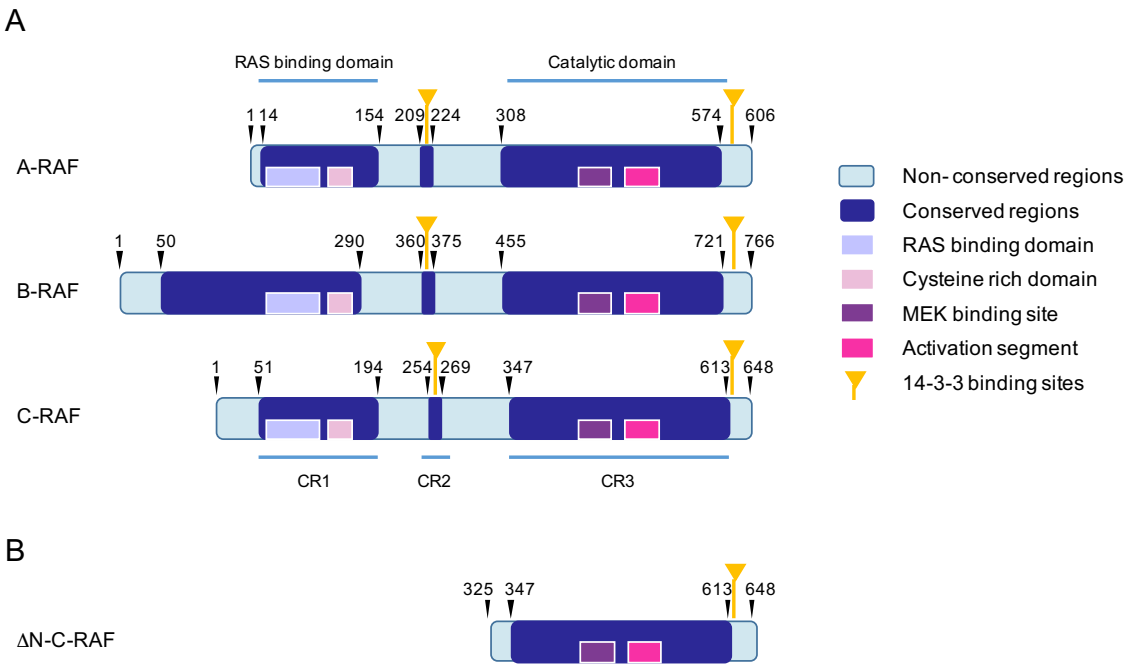

Figure 1.5 Schematics of RAF protein kinases. (A) Human A-, B -, and C-RAF kinases share a common structure. CR1, CR2, and CR3 are conserved regions between all human RAF isoforms. The RAS binding domain and catalytic domain are indicated. **(B)** ΔN-C-RAF corresponds to the catalytic region, or CR3, of C-RAF. Figure adapted from (72).

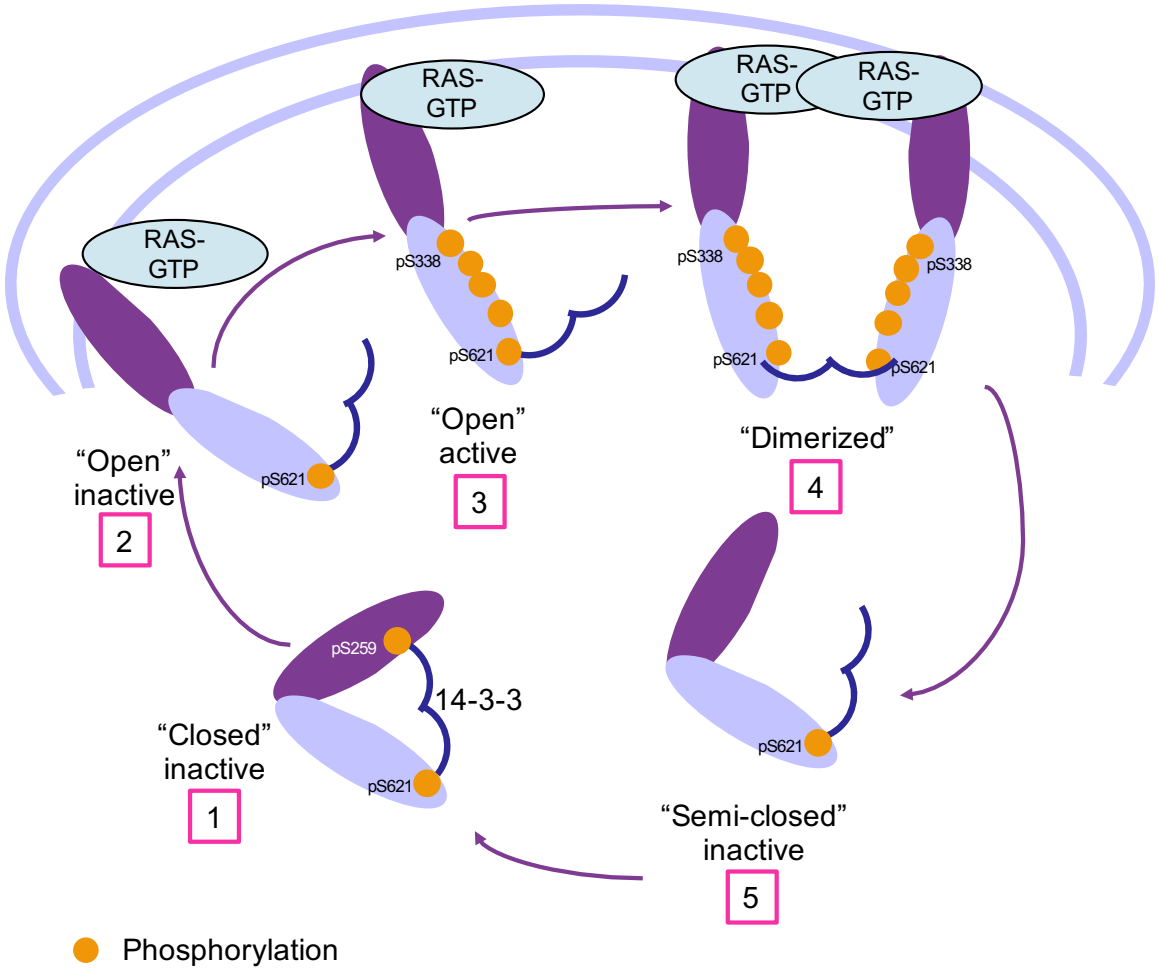

Figure 1.6 C-RAF activation cycle. (1) C-RAF is held in a "closed" inactive conformation by the N-terminal regulatory region (dark purple) folding over the catalytic C-terminal region (light purple), and is stabilized by the 14-3-3 dimer which binds pS259 of the N-terminal region and pS621 of the C-terminal region. **(2)** C-RAF progresses to the "open" inactive state when pS259 is dephosphorylated releasing 14-3-3 from the N-terminal, revealing the RAS binding domain allowing RAS to bind and recruit C-RAF to the plasma membrane. **(3)** The CR3 activation segment is phosphorylated, specifically pS338, to achieve a full "open" active conformation **(4)** and full kinase activation with RAF heterodimerization. **(5)** Deactivation is initiated by the dephosphorylation of pS338, deactivating the activation segment, and the phosphorylation of S259, allowing 14-3-3 to rebind the N-terminal and reforming the closed inactive conformation. Figure adapted from (72).

subsequent interaction with MEK (78, 79). Deactivation is initiated by the dephosphorylation of pS338, deactivating the activation segment, and the phosphorylation of S259, causing 14-3-3 to rebind the N-terminal and reforming the closed inactive conformation (80). In addition to 14-3-3 regulation, C-RAF has also been shown to be regulated through direct feedback phosphorylation by ERK1/2, inhibiting RAS interaction and promoting phosphorylation at inhibitory sites (81).

1.4.4 Activation by heterodimerization

Dimerization is a common feature of kinase activation. Heterodimerization of C-RAF and B-RAF was demonstrated to occur in multiple cell lines following mitogen activation (77). C-RAF and B-RAF were found to be cross-linked by the 14-3-3 dimer via their C-terminal domains. ERK1/2 feedback phosphorylation was also found to regulate heterodimerization by limiting the lifetime of the heterodimer (81).

Approximately 66% of melanomas, and 2% of all human tumours harbour a B-RAF mutation (82). When B-RAF mutations in cancers were first being identified, V600E stood out as the most frequent mutation, as well as the strongest stimulating mutation of B-RAF kinase activity (82). Less frequent mutations exhibited very low or no activity and still were able to hyperstimulate the ERK1/2 pathway (83). These low-to-no activity B-RAF kinases mutants were achieving stimulation of the ERK1/2 pathway through activation of C-RAF by forming heterodimers (77, 84). Regulation of heterodimerization by RAS is abolished in these cases, and constitutive dimerization occurs leading to aberrant activation of MEK1/2 (84).

1.4.5 MEK-independent signaling

Only one substrate has been identified and shared across all RAF kinases, that being MEK1/2. However, B-RAF possesses the strongest activity for MEK1/2 activation, rendering C-RAF and A-RAF as seemingly unnecessary MEK kinases. C-RAF and A-RAF are likely involved in subtler processes independent of MEK (85). Alternative phosphorylation substrates for C-RAF have been described, such as: adenylyl cyclases (AC) 2, 5, and 6, AC generates cyclic adenosine monophosphate (cAMP), which

activates protein kinase A (PKA), a negative regulator of C-RAF (86); retinoblastoma tumour suppressor protein (Rb), causing inactivation of Rb leading to cell cycle progression (87); Rho-binding kinase (Rok)-α and myotonic dystrophy protein kinase (MDPK), both regulators of myosin contractility (88, 89). Through gene knockout mice, distinct functional roles of RAF kinases were found: A-RAF plays a role in neurological and intestinal development (90); B-RAF functions in vascular development (91); and finally C-RAF functions in liver development (92). Each isoform possesses its own distinct function within cells essential to embryonic development.

1.4.6 C-RAF protein stability

Extensive controls are in place to insure that RAF kinase activity is strictly regulated. Protein stability and levels within the cell are also important regulators of kinase signaling pathways. The chaperone protein HSP90 is a well-researched regulator of RAF kinases. HSP90 is highly conserved and abundant, and is found to associate with, and chaperone, a wide array of client proteins (93). HSP90 supports the proper folding of proteins involved in normal cellular activities with the help of more than 20 co-chaperones to guide and modulate proteins and their activities (93). The interaction between HSP90 and RAF is essential to RAF stability and its activity as a signal transducer within the ERK1/2 signaling pathway (94), as the treatment with geldanamycin, an inhibitor of HSP90, caused the rapid dissociation of the HSP90-RAF and RAS-RAF complexes, and caused subsequent reduction in RAF half-life and plasma membrane association (95).

Although HSP90 is generally regarded to stabilize client proteins, it also plays a role in their degradation. C-terminus of constitutive heat shock protein (HSC) 70-interacting protein (CHIP), a highly conserved E3 ubiquintin ligase across species, associates with the molecular chaperone proteins HSC70-HSP70 and HSP90 causing client proteins to be ubiquitinated and subsequently degraded via the proteasome (96). C-RAF is one of these client proteins that is targeted by CHIP for ubiquitination (97), and degradation by the proteasome (98). X-linked inhibitor of apoptosis proteins (XIAP), another E3 ubiquitin

ligase, has also been found to interfere with C-RAF stability, promoting ubiquitination through HSP90-mediated CHIP, independent of its own E3 ligase activity (99).

Interestingly, incidences of CHIP-independent modes of ubiquitination of C-RAF have also been documented. For successful activation of C-RAF, S621 must be autophosphorylated in order to allow for correct folding and stability, as pS621 is necessary to bind 14-3-3 to its C-terminal (73). Without the phosphorylation of S621, C-RAF is effectively kinase-dead and is degraded by the proteasome. However, degradation is not exclusively regulated by CHIP, as siRNA knockdown of CHIP did not yield altered levels of kinase-dead C-RAF (100). Treatment with the oxidative glucose metabolite methylglyoxal and abolishing extracellular adhesion has been shown to cause degradation of C-RAF through the ubiquitin-proteasome system (101, 102). However, the E3 ubiquitin ligase was not identified in either case.

A lesser-known stability regulator of C-RAF is RanBPM, which was identified to bind the C-RAF kinase domain in a yeast two-hybrid analysis (103). Studies conducted in our laboratory have shown that RanBPM is capable of regulating C-RAF protein levels. In shRNA RanBPM cells, RanBPM was co-expressed with a constitutively active form of C-RAF causing a significant decrease in ectopic C-RAF protein levels, as well as a decrease in C-RAF-dependent activation of ERK1/2 (13). Endogenous C-RAF protein levels were also observed to be increased in shRNA RanBPM cells (13). Upon re-expression of RanBPM, the effect was rescued. Co-immunoprecipitation experiments also confirmed that C-RAF and RanBPM form a complex in mammalian cells, as endogenous RanBPM forms a complex with the ectopically expressed C-RAF kinase domain, and that endogenous C-RAF also forms a complex with ectopically expressed RanBPM (13). Finally, evidence was also provided to show that RanBPM is able to cause a decrease in HSP90-C-RAF complex formation, and the destabilization of endogenous C-RAF (13). However, the mechanism underlying this regulation still remains unknown, as further insights into RanBPM's regulation of C-RAF protein stability still remain to be elucidated.

1.5 Interaction between RanBPM and C-RAF

Unpublished work conducted in our laboratory by Wesley Bérubé-Janzen has made strides to the elucidation of RanBPM regulation of C-RAF protein stability. Proximity ligation assay data has visualized the endogenous complex formations of C-RAF with both RanBPM, and MAEA, the CTLH complex member featuring the degenerate RING-type zinc-finger domain. Ectopic expression of RanBPM with deletions of its conserved domains co-expressed with ΔN-C-RAF, a constitutively active construct of C-RAF featuring only its kinase domain (Figure 1.5B) (79), revealed that the LisH and CTLH domains, the disordered N-terminal, and the C-terminal CRA domain were required in the regulation of ΔN-C-RAF protein stability, as their deletion resulted in upregulated ΔN-C-RAF protein levels. However, glutathione-S-transferase (GST) pull down of ectopically expressed GST-ΔN-C-RAF showed loss of interaction with co-expressed RanBPM only when the CRA domain was deleted. *In vitro* GST pull down of bacterially expressed CRA domain demonstrated that the loss of interaction between RanBPM and ΔN-C-RAF was due to the direct interaction mediated by the CRA domain.

Taking all these findings together, we can surmise that through the CRA domain RanBPM is able to directly interact with C-RAF and promote its interaction with the remaining components of the mammalian CTLH complex. RanBPM recruits C-RAF to the CTLH complex through its LisH and CTLH domains imparting regulation on C-RAF protein stability (Figure 1.7).

1.6 Hypothesis and objectives

The mechanism by which RanBPM imparts regulation on C-RAF, due to its apparent lack of enzymatic activity, remains unclear. Therefore, it is hypothesized that RanBPM interacts with and promotes the degradation of C-RAF by targeting C-RAF for ubiquitination by the mammalian CTLH complex. The work presented herein proposes to address the following objectives:

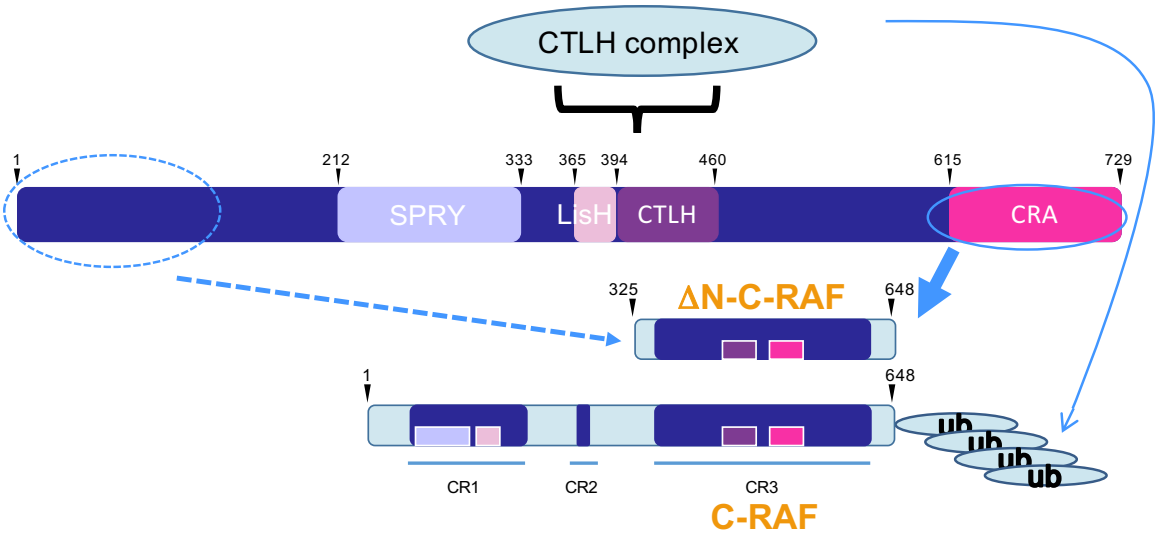

Figure 1.7 Working model of C-RAF protein stability regulation by RanBPM. Three RanBPM regions, N-terminal (1-102), LisH/CTLH (360-460) and C-terminal CRA (615-729) are necessary to regulate C-RAF protein stability, but only the CRA domain is able to interact with C-RAF. Our working hypothesis is that RanBPM interacts with C-RAF and recruits it to the CTLH complex associated through interaction with the LisH and CTLH domains to promote C-RAF ubiquitination and degradation. The role of the N-terminal domain is unclear, but it maybe involved in RanBPM stability and folding (5).

(1) *Determine whether the effect of RanBPM on C-RAF protein stability is dependent on the proteasome;*
(2) *Define the region of interaction between RanBPM and C-RAF and investigate the effects on C-RAF regulation in vitro; and*
(3) *Investigate the effect on C-RAF regulation by other members of the CTLH complex.*

Over the past several decades it has become increasingly clear that tight regulation of the ERK1/2 signaling pathway, if through kinase activity or protein stability, is a critical part to the prevention of unregulated cellular proliferation, differentiation, apoptosis, and migration. With the identification of RanBPM having associations with the ERK1/2 signaling pathway, a pathway commonly deregulated in cancer, elucidating the tumour-suppressive role of the RanBPM/CTLH complex will provide some relief to the increasing need to find new MAPK-targeted cancer therapies. As a whole, the research presented herein will examine the regulatory role that the RanBPM/CTLH complex imparts on C-RAF protein stability.

Chapter 2

2 Materials and methods

2.1 Chemicals and reagents

2.1.1 Cell culture

Dubecco's modified eagle medium (DMEM), L-glutatmine, sodium pyruvate, trypsin, fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Wisent Inc. (St. Bruno, QC, CA). Dimethyl sulfoxide (DMSO), N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal (MG-132), and G418 sulphate was purchased from BioShop Inc. (Burlington, ON, CA). JetPRIME® Transfection Reagent was purchased from PolyPlus Transfection (Illkirch, France).

2.1.2 *In vitro* assays

Sodium deoxycholate, hydrochloric acid (HCl), Tween-20, sodium dodecyl sulphate (SDS), sodium chloride (NaCl), Tris-hydroxymethyl amino methane (Tris), ethylenediaminetetraacetic acid (EDTA), hydroxyethyl piperazineethanesulfonic acid (HEPES), and potassium chloride (KCl) were purchased from Wisent Inc. (St. Bruno, QC, CA). Glycerol was purchased from Caledon Laboratory Chemicals Ltd. (Georgetown, ON, CA). TritonX-100 (TX100), Nonidet P-40 (NP-40), dithiothreitol (DTT), aprotinin, leupeptin, pepstatin, sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), and pheylmethylsulfonyl fluoride (PMSF) were purchased from BioShop Inc. (Burlington, ON, CA). Glutathione-agarose beads were purchased from Sigma-Aldrich (Oakville, ON, CA). Anti-Ubiquitin TUBE (non-selective) Agarose-TUBE 1 by LifeSensors (Malvern, PA, USA) and N-ethylmalinide (NEM) by Sigma-Aldrich (Oakville, ON, CA) were provided by Dr. J. Rylett (University of Western Ontario, London, ON, CA). Polyvinylidene fluoride (PVDF) membranes, Bio-Rad Protein Assay Dye Reagent Concentrate, and Clarity Western ECL substrate were purchased from Bio-Rad (Mississauga, ON, CA). No Name-Skim Milk Powder was purchased from Loblaw Companies Ltd. (Brampton, ON, CA).

2.1.3 Cloning and bacterial expression

KOD Hot Start DNA Polymerase was purchased from EMD Millipore Corporation (Billerica, MA, USA). Primers were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). Restriction enzymes were purchased from NEW England Biolabs Inc. (Ipswich, MA, USA). Isopropyl ß-D-1-thiogalactopyranoside (IPTG), Yeast Extract, and Bio-Tryptone were purchased from BioShop Inc. (Burlington, ON, CA).

2.2 Antibodies

2.2.1 Primary antibodies

C-RAF (C-12; sc133), ß-actin (I-9; sc-1616-R), and GST (B-14; sc138) antibodies were purchased from Santa Cruz Biotechniology Inc. (Santa Cruz, CA, USA). RanBPM (71-001) antibody was purchased from BioAcademia (Suita, Osaka, Japan). Human influenza hemagglutinin (HA; H9658) antibody was purchased from Sigma-Aldrich (Oakville, ON, CA). RMND5A (NBP1-92337) antibody was purchased from Novus Biologicals (Oakville, ON, CA). Table 2.1 summarizes primary antibody dilutions used in this work.

2.2.2 Secondary antibodies

Peroxidase-conjugated AffiniPure Goat anti-Mouse IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), and Blotting Grade Goat anti-Rabbit IgG (H+L) (Human IgG Adsorbed) Horseradish Peroxidase Conjugate was purchased from Bio-Rad Laboratories Inc. (Mississauga, ON, CA). Both secondary antibodies were used at 1:5000 dilutions.

2.3 Cloning and plasmids

pCMV-HA-RanBPM was provided by Dr. Mark Nelson (University of Arizona, Tucson, AZ, USA) and previously mutagenized as described in (41) to be resistant to shRNA targeting. pCMV-HA-RanBPM-ΔC4 was previously generated as described in (58) pEBG-GST-ΔN-C-RAF was provided by Dr. Zhijun Luo (Boston University, Boston, MA, USA).

Table 2.1 Concentrations of primary antibodies used in Western blot analysis.

Antibody	Species	Dilution
C-RAF (C-12)	Rabbit	1:1000
ß-actin (I-19)	Rabbit	1:2000
GST (B-14)	Mouse	1:500
RanBPM	Rabbit	1:2000
HA	Mouse	1:10000
RMND5A	Rabbit	1:300

pET28a-ΔN-C-RAF was generated by Matthew Maitland through the isolation of the ΔN-C-RAF fragment in pEBG-GST-ΔN-C-RAF using BamHI and NotI, followed by ligation with T4 DNA ligase into the bacterial expression vector pET28a (EMD Millipore, Billerica, MA, USA). pGEX-4T-1-GST-C1 was generated by Wesley Bérubé-Janzen through polymerase chain reaction (PCR) amplification of RanBPM amino acids 649-729 from pCMV-HA-RanBPM. PCR was performed using the KOD Hot Start DNA Polymerase kit according to manufacturer's protocol. The PCR amplicon was digested with BamHI and SalI-HF, and was ligated with T4 DNA ligase into the bacterial expression vector pGEX-4T-1 (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

pGEX-4T-1-GST-C1-1, pGEX-4T-1-GST-C1-2, and pGEX-4T-1-GST-C4 were generated through PCR amplification of RanBPM amino acids 615-669, RanBPM amino acids 663-729, or RanBPM amino acids 471-729, respectively, from pCMV-HA-RanBPM. pGEX-4T-1-GST-C4A, pGEX-4T-1-GST-C4B, and pGEX-4T-1-GST-C4C were generated through PCR amplification of RanBPM amino acids 471-639, RanBPM amino acids 471-669, or RanBPM amino acids 471-692, respectively, from pGEX-4T-1-GST-C4. PCR amplicons were digested with EcoRI-HF and XhoI, and were subsequently ligated into the bacterial expression vector pGEX-4T-1. pGEX-4T-1-GST-C4D was generated through PCR amplification of RanBPM amino acids 471-639 and 671-729, from pGEX-4T-1-GST-C4. Blunt end ligation was performed using T4 Polynucleotide Kinase. pGEX-4T-1-GST-C4-Q703L T750L, and pGEX-4T-1-GST-C4-R625L E626L were generated by site-directed mutagenesis of pGEX-4T-1-GST-C4, performed using KOD Hot Start DNA Polymerase. pCMV-HA-RanBPM-R625L E626L was generated by site-directed mutagenesis of pCMV-HA-RanBPM, performed using KOD Hot Start DNA Polymerase. Restriction enzymes were used as directed in the manufacturers' protocols.

2.4 Cell lines and cell culture

2.4.1 Stable shRNA RanBPM cell lines

HeLa, HCT116, and HEK-293 cells were purchased from the American Type Culture Collection. Cells were grown in high-glucose DMEM supplemented with 8% FBS, 1%

sodium pyruvate, 1% L-glutamine, and 4.5 g/L glucose at 37°C and 5% CO_2. Cell lines stably expressing either RanBPM shRNA or non-targeting control shRNA were previously generated in (13, 41). To maintain stable expression of the shRNA in HeLa, HCT116, and HEK-293 cells, G418 sulphate was used at 0.35 g/L, 0.35 g/L, and 0.45 g/L, respectively.

2.4.2 CRISPR-Cas9 R*MND5A*$^{-/-}$ HEK-293 cell line

HEK-293 cells were purchased from the American Type Culture Collection. Cells were grown in high-glucose DMEM supplemented with 8% FBS, 1% sodium pyruvate, 1% L-glutamine, and 4.5 g/L glucose at 37°C and 5% CO_2. Generation of *RMND5A* $^{-/-}$ HEK-293 cells was completed by Schild-Poulter laboratory members Matthew Maitland and Xu Wang. Briefly, sgRNA (single-guide RNA) was designed against exon 3 of *rmnd5a* using www.benchling.com (On-target score = 65.7, Off-target score = 86.0). Top and bottom oligomers (5' – CACCGTGGAGCACTTCTTTCGACA – 3'; 5' – AAACTGTCGAAAGAAGTGCTCCAC – 3') were annealed together and ligated with pSpCas9(BB)-2A-Puro (PX459) V2.0 that was digested with BpiI. Early passage wild-type HEK-293 cells were seeded on 12-well dishes and transfected with 800 ng of plasmid DNA. Forty-eight hours post-transfection, cells were put under puromycin selection for seven days. Afterwards, remaining single cells were transferred to a 48-well dish and expanded. Loss of RMND5A protein was confirmed by Western blot and DNA mutation was checked by sequencing. The top two predicted off-target sequences were also checked and revealed no changes.

2.5 Transfections and treatments

JetPRIME Transfection Reagent was used according to manufacturer's protocols for transfection of HeLa and HEK-293 cells. The amount of transfection DNA used was determined by comparative protein expression 24 h post-transfection. pBS-SK (Agilent Technologies, Santa Clara, CA, USA) was used to supplement transfected DNA to the minimum DNA amount requirements. Transfected cells were grown at 37°C and 5% CO_2 for 24-30 h. MG-132 or DMSO (vehicle) was used at 10 μM for 6-24 h.

2.6 Cell extracts

2.6.1 Preparation of mammalian cell extracts

HeLa, HCT116, and HEK-293 cells were scraped in cold PBS, and centrifuged for 3 minutes at 8000 rpm. Cell pellets were lysed on ice for 25 minutes in either whole cell extract buffer (150 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 7.4, 10% glycerol, 0.5% NP-40, and 0.5% TX100), radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, 1% TX100, 0.1% sodium deoxycholate, and 0.1% SDS), or tandem ubiquitin binding entities (TUBE) cell lysis buffer (0.15 M NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.5, 10% glycerol, 0.5% NP-40 1% TX100, 50 μM MG-132, and 1.25 mg/mL NEM) and supplemented with 10 μg/mL aprotinin, 2 μg/mL leupeptin, 2.5 μg/mL pepstatin, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, and 0.1 mM PMSF. The cell lysates were clarified at 4 °C for 20 minutes at 13 000 rpm.

2.6.2 Bacterial protein expression and extracts

Bacterial expression plasmids were transformed into *Escherichia coli* strain BL21-DE3. Single tranformant colonies were selected and grown in Luria Bertani (LB) liquid culture media at 37 °C overnight. Cultures were diluted 1:100 into fresh growth media and grown to an optical density at 600 nm (OD_{600}) of 0.4 – 0.5. Protein expression was induced using 0.1 mM IPTG and grown overnight at 10 °C. Bacterial cultures were pelleted at 4 °C for 15 minutes at 4000 rpm. Bacterial cell pellets were lysed in GST protein lysis buffer (25 mM HEPES pH 7.4, 100 mM KCl, 2 mM EDTA, and 20% glycerol) supplemented with 0.1% NP-40, 10 μg/mL aprotinin, 2 μg/mL leupeptin, 2.5 μg/mL pepstatin, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, and 0.1 mM PMSF. Bacterial lysates were sonicated on ice with the Sonic Dismembrator Model 100 (Thermo Fisher Scientific Inc.) and clarified at 13 000 rpm for 10 minutes at 4 °C.

2.7 Western blot analyses

Protein samples were resolved by SDS polyacrylamide gel electrophoresis (PAGE) on variable percentages of acrylamide gels. Gels were transferred to PVDF membranes by semi-dry transfer at 25V for 25 minutes. Membranes were blocked with 5% skim milk in

Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.05% Tween-20) at room temperature for 1h with agitation. Primary antibodies were hybridized to membranes either overnight at 4 °C, or 2h at room temperature, with agitation. Secondary antibodies were hybridized to the membranes at room temperature for 1h, with agitation. Membranes were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories Inc.), images were taken using a ChemiDoc MP (Bio-Rad Laboratories Inc.), and images were analyzed by Image Lab software (Bio-Rad Laboratories Inc.).

2.8 *In vitro* assays

2.8.1 Bacterial *in vitro* GST pull-down assay

GST-tagged bacterial protein extracts were quantified, and approximately 400 µg total protein was bound to 25 µL of Glutathione-agarose beads. GST-tagged protein samples were diluted to 200 µL with GST protein lysis buffer and an additional 600 µL of GST binding buffer (15 mM HEPES pH 7.4, 60 mM KCl, 1.2 mM EDTA, and 12 % glycerol) was added to the protein sample. Each sample received a final concentration of 0.6% NP-40, 0.8% TX100, 10 µg/mL aprotinin, 2 µg/mL leupeptin, 2.5 µg/mL pepstatin, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, and 0.1 mM PMSF. Samples were incubated with Glutathione-agarose beads at 4 °C for 2h with rotation. Beads were subsequently washed three times with binding buffer supplemented with 0.6% NP-40, 0.8% TX100, 1 mM DTT, and 0.1 mM PMSF. Each sample was incubated with 600 µg of ΔN-C-RAF bacterial extract with a final volume of 800 µL of binding buffer and a final concentration of 0.6% NP-40, 0.8% TX100, 10 µg/mL aprotinin, 2 µg/mL leupeptin, 2.5 µg/mL pepstatin, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, and 0.1 mM PMSF. Samples were incubated with rotation at 4 °C for 2h. Beads were washed three times with binding buffer supplemented with 0.6% NP-40, 0.8% TX100, 1 mM DTT, and 0.1 mM PMSF. Beads were resuspended in SDS loading dye (0.105 g/mL SDS, 0.093 g/mL DTT, 0.35 M Tris-HCl pH 6.8, and 30% glycerol), boiled for 10 minutes, and centrifuged at 10 000 rpm for 10 seconds. The resulting supernatant was analyzed by Western blot. Relative

ΔN-C-RAF protein levels were quantified by normalizing ΔN-C-RAF to the uppermost GST-fusion protein band, and comparing values to GST when set to a value of 1.0.

2.9 Statistical analyses

Statistical analyses were performed using GraphPad PRISM (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) was performed to compare multiple groups and two-sample t-test assuming equal variance was performed to compare pairs of groups. Graphed data are presented as mean ± standard deviation (SD) and are determined to be significant when $p < 0.05$.

Chapter 3

3 Results

3.1 C-RAF is regulated by the proteasome through RanBPM

The Gid complex is the key regulator of the gluconeogenesis pathway in *S. cerevisiae*, where it is responsible for the degradation of FBPase through the ubiquitin proteasome system (30). The Gid complex functions as the E3 ubiquintin ligase that polyubiquinates FBPase targeting it for degradation through the proteasome. Components of the mammalian CTLH complex share a striking resemblance to their Gid complex counterparts, and exhibit evolutionary conservation in a vast variety of species (6), suggesting that the mammalian complex may exhibit similar activity. GID1, the orthologue to RanBPM (30), is a central component of the E3 ubiquitin ligase and functions primarily as the scaffold to the Gid complex mediating its interactions with the remainder of the complex through its LisH and CTLH domains (29). Deletion of the SPRY, the LisH, or the CTLH domain resulted in impaired FBPase degradation owing to lack of ubiquitination suggesting that any alterations to GID1 affects E3 ubiquitin ligase activity of the complex (29).

Previous studies showed that C-RAF protein levels are increased in RanBPM shRNA cells, suggesting that RanBPM may function to target C-RAF for degradation (13). To determine whether RanBPM regulates C-RAF protein stability through the proteasomal degradation pathway, similar to the regulation of FBPase by GID1 through the GID complex, RanBPM-targeting or non-targeting control shRNA HeLa, HEK-293, and HCT116 cells previously described in (13, 41), were used to assess C-RAF protein levels in conditions of proteasomal impairment in order to determine whether the RanBPM loss of function would affect C-RAF degradation. All cell lines were treated with either 10 μM of the proteasome inhibitor, MG132, or with the vehicle, DMSO. MG132 inhibits the proteasome, preventing the degradation of ubiquitin-conjugated proteins, thus resulting in their accumulation within cells. However, cells harbouring impaired E3 ligase activity

because of the loss of RanBPM would lack this accumulation. Cells were harvested 24 h post-treatment and analyzed by Western blot.

HeLa, HEK-293, and HCT116 cells expressing RanBPM shRNA showed minimal accumulation of C-RAF protein levels during proteasomal inhibition, whereas control shRNA cells exhibited a 1.85 ± 0.55 – fold (SD), 1.59 ± 0.19 – fold (SD), and 2.36 ± 0.50 – fold (SD) increase accumulation of C-RAF, respectively, when compared to their respective vehicle-treated controls (Figure 3.1). This suggests that the downregulation of RanBPM impairs proteasome-dependent degradation of C-RAF.

3.2 The CRA domain of RanBPM is highly conserved throughout evolutionary time

The C-terminal CRA domain was shown to function as a protein-protein interaction domain and was predicted to have six α-helices, reminiscent of the death domain superfamily (18). Previous co-immunoprecipitation and *in vitro* pull down assays performed in our laboratory showed that RanBPM and C-RAF complex formation is dependent on the RanBPM CRA domain suggesting that the CRA domain functions to mediate an interaction between RanBPM and C-RAF (13, 104). In order to map the region of interaction between the CRA domain and C-RAF, sequence alignment of CRA domain homologues was performed (Figure 3.2) using the multiple sequence alignment online server MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (105-108). Through multiple sequence alignment of RanBPM CRA domain homologous sequences, residues that have been conserved through evolution would be inferred as important to the function of the CRA domain as a protein interaction surface. However, identifying which residue may be important for this interaction proved difficult, as sequence alignment of the RanBPM CRA domain revealed that 77.4% of residues were fully conserved, 11.3% held conservation of strongly similar properties, 1.7% of residues had conservation of weakly similar properties, and 9.6% were not conserved. Therefore, alternative measures must be taken to define the region of the CRA domain responsible for C-RAF binding.

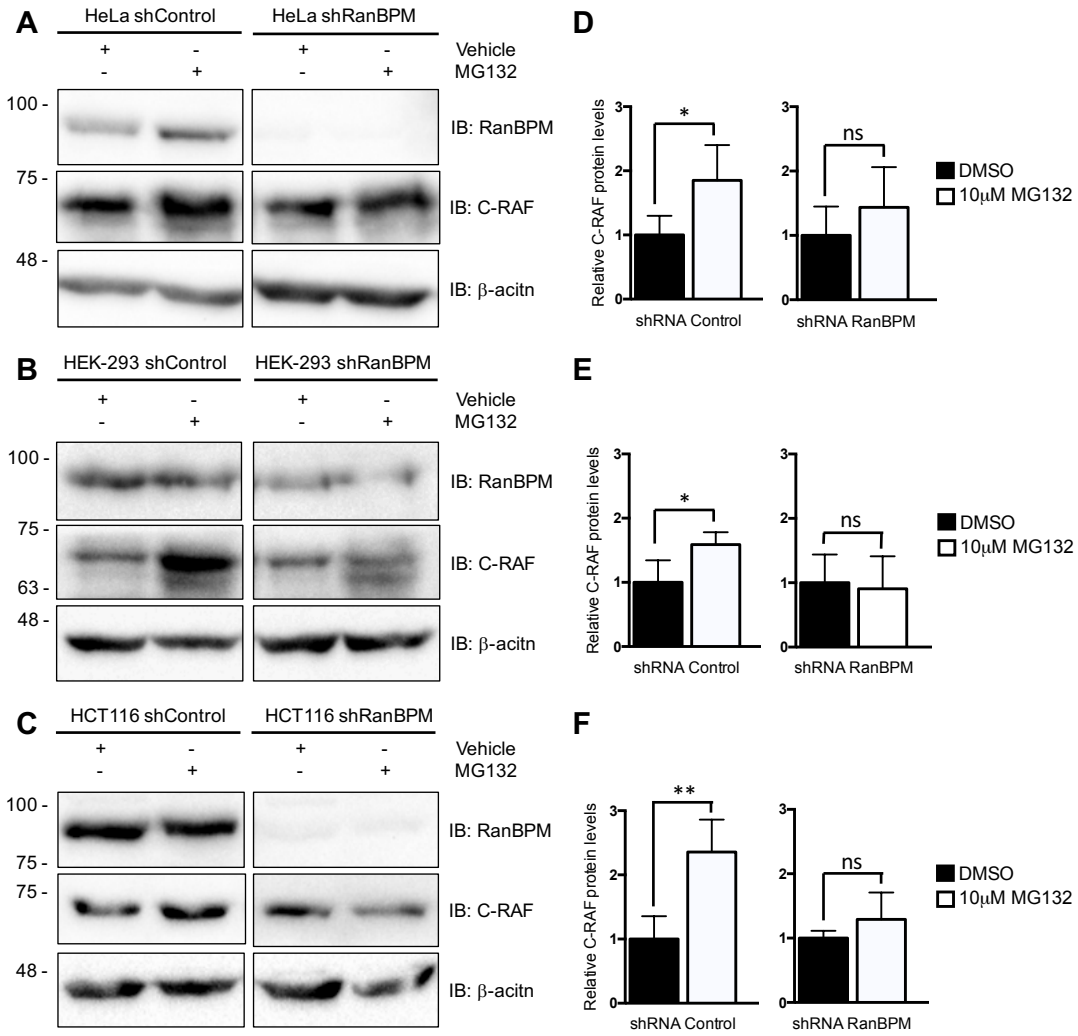

Figure 3.1 C-RAF is regulated by the proteasome through RanBPM. Stable non-targeting shRNA control and shRNA RanBPM cells were treated with 10μM MG132 or DMSO, as vehicle, for 24h. RIPA buffered whole cell extracts of **(A)** HeLa, **(B)** HEK-293, and **(C)** HCT116 were analyzed by Western blot with RanBPM, C-RAF and β-actin antibodies to detect RanBPM, C-RAF and β-actin proteins, respectively. C-RAF protein levels were normalized to β-actin levels. Quantifications of relative C-RAF protein levels for **(D)** HeLa, **(E)** HEK-293, and **(F)** HCT116 are shown with error bars indicating SD (n=4). A two-sample t-test, assuming equal variance, was used to determine significance. ** $p<0.05$; ** $p<0.01$; ns* not significant.

```
X. laevis    AAVERMICFGRELQAMSEQLRRERGKNATNKNMLKDAFSLLAYSDPWNSPVGYQLDPIQR
H. sapiens   AAIERMIHFGRELQAMSEQLRRDCGKNTANKKMLKDAFSLLAYSDPWNSPVGNQLDPIQR
M. musculus AAIERMIHFGRELQAMSEQLRRECGKNTANKKMLKDAFSLLAYSDPWNSPVGNQLDPIQR
D. rerio     AAIERMIQFGRELQSMSEHLRRERGKNSANKKMLKDAFSLLAYSDPWNSPVGYQLDSIQR
             **:**** ******:***:***: ***::**:********************* ***.***

X. laevis    EHVCSSLNSAILDIHNLPKQPPLSLALEQASQCLEMMAQCGIGSCAFARVADYLH
H. sapiens   EPVCSALNSAILETHNLPKQPPLALAMGQATQCLGLMARSGIGSCAFATVEDYLH
M. musculus EPVCSALNSAILETHNLPKQPPLALAMGQATQCLGLMARSGVGSCAFATVEDYLH
D. rerio     EPVCSTLNSAILETHNLPKQPPLALAMGQAAQCLSLMARTGSGSCAFASVDDYLH
             * ***:******: *********:**: **:*** :**. * ****** * ****
```

Figure 3.2 Sequence alignment of the conserved RanBPM CRA domain. RanBPM CRA domain sequences of *Homo sapiens*, *Mus musculus*, *Xenopus laevis* and *Danio rerio*, were aligned using the multiple sequence alignment online server MUSCLE (105-108) * *fully conserved*; : *conservation of strongly similar properties*; . *conservation of weakly similar properties.*

3.3 The CRA domain of RanBPM interacts directly with ΔN-C-RAF

As an alternative to single point mutations, we thought to design partial deletions of the CRA domain to disrupt or remove the site of interaction with C-RAF. As there is no crystal structure of RanBPM or the CRA domain currently available, a predicted tertiary structure was elucidated by the RAPTORX online server (Figure 3.3A) (16, 109). The predicted tertiary structure corroborated the previous assumption that the CRA domain has a high propensity for α-helices, with 6 α-helices spanning the entirety of the domain (Figure 3.3B). We, therefore, generated various deletion mutants of the RanBPM CRA domain to evaluate the effect of these deletions on the association of C-RAF. Deletions were guided by the predicted tertiary structure and data previously generated by Wesley Bérubé-Janzen which showed amino acids 649 – 729 of RanBPM, denoted C1, bound directly to ΔN-C-RAF, a constitutively active construct of C-RAF featuring only its kinase domain, and inversely that their deletion significantly decreased this interaction (104).

To assess interaction between the CRA domain and ΔN-C-RAF, deletion mutants of the CRA domain were cloned into the bacterial expression vector pGEX-4T-1, downstream of GST and GST pull down assays were performed (Figure 3.4). Initial CRA domain constructs were modelled after C1 and guided by the predicted tertiary structure. The CRA domain is mapped to amino acids 615 – 729 of RanBPM, therefore only a portion of the CRA domain is represented by C1, which contains helices IV, V and VI, and a small portion of helix III. We, therefore, derived two GST-fusion CRA constructs which featured amino acids 615 – 669 spanning helices I, II, and III, denoted C1-1, and amino acids 663 – 729 containing helices IV, V, and VI, denoted C1-2. These constructs were purified using glutathione-agarose beads and incubated with crude *E. coli* cell lysate of ΔN-C-RAF. The GST-fusion constructs were pulled down and analyzed by Western blot to evaluate relative levels of bound ΔN-C-RAF protein. All GST pull down assays were normalized to the negative control, GST, and compared to the positive ΔN-C-RAF binding control, GST-C4, which contains amino acids 471 – 729 of RanBPM, and therefore includes sequences C-terminal to the CTLH domain, namely the CRA domain.

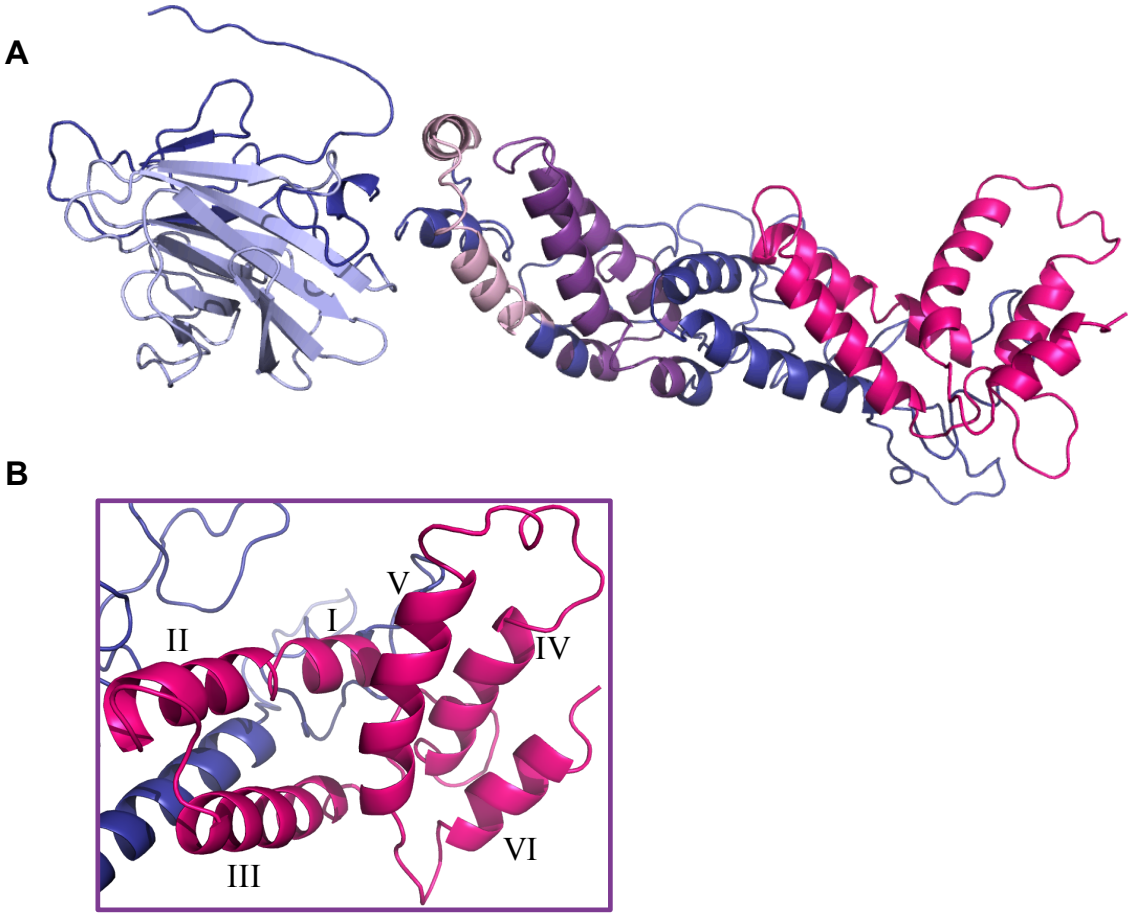

Figure 3.3 Predicted tertiary structure of RanBPM. (A) Full-length RanBPM with the omission of the unstructured N-terminal region, amino acids 1-153 and **(B)** an expanded image of the RanBPM CRA domain with α-helices labelled I through VI are shown. SPRY, LisH, CTLH, and CRA domains are shown in light-blue, light-pink, violet, and hot-pink, respectively. Linker regions are shown in dark blue, and unstructured regions are omitted. The structure prediction of RanBPM was elucidated by RAPTORX software (109) and adapted from (16).

GST-C1 displayed significant interaction with ΔN-C-RAF, although quantifications showed reduced binding compared to GST-C4, by 2.6 ± 0.6 –fold (SD), which confirmed previous findings (Figure 3.5A; Bérubé-Janzen, unpublished). Constructs GST-C1-1 and GST-C1-2 were constructed to examine the binding of helices I – III and helices IV – VI to ΔN-C-RAF, respectively. However, both constructs appeared to lack protein stability *in vitro*, as they bound less ΔN-C-RAF than that of the negative control. Both constructs displayed a drastic decrease in ΔN-C-RAF binding by 4.6 ± 0.5 –fold (SD), and 4.5 ± 0.5 –fold (SD), respectively.

To increase protein stability, CRA domain constructs were expanded to include the portion of RanBPM C-terminal to the CTLH domain, in other terms, modelling the constructs after C4 to include RanBPM sequences N-terminal to the CRA domain (Figure 3.4). Similar to the strategy used for the two previous constructs, CRA deletion mutations were guided by the predicted tertiary structure. We derived an additional four GST-fusion CRA constructs: C4A which spans amino acids 471 – 639 and helices I and II; C4B contains amino acids 471 – 669 and helices I – III; C4C is comprised of amino acids 471 – 692 and helices I – IV; and finally, C4D which includes amino acids 471 – 639 and 671 – 729, and helices I – II and IV – VI. ΔN-C-RAF binding was again tested by GST pull down assay and the mutants' binding to ΔN-C-RAF was quantified and compared to that of GST-C4: GST-C4A showed decreased binding by 3.6 ± 0.5 –fold (SD); GST-C4B decreased by 3.1 ± 0.6 –fold (SD); GST-C4C decreased by 2.7 ± 0.5 –fold (SD); and GST-C4D decreased by 2.1 ± 0.6 –fold (SD) (Figure 3.5B).

Overall, these data suggest that each helix of the CRA domain has a role to play in the binding of ΔN-C-RAF. As more helices were deleted from the CRA domain, less binding of ΔN-C-RAF was observed. However, two portions of the CRA domain seemed worth investigating, helices I and II, and helices V and VI, demonstrated by C1 and C4C, respectively. Deletion of helices I and II, and helices V and VI resulted in a sharp decrease in ΔN-C-RAF binding with the least amount of the CRA domain deleted.

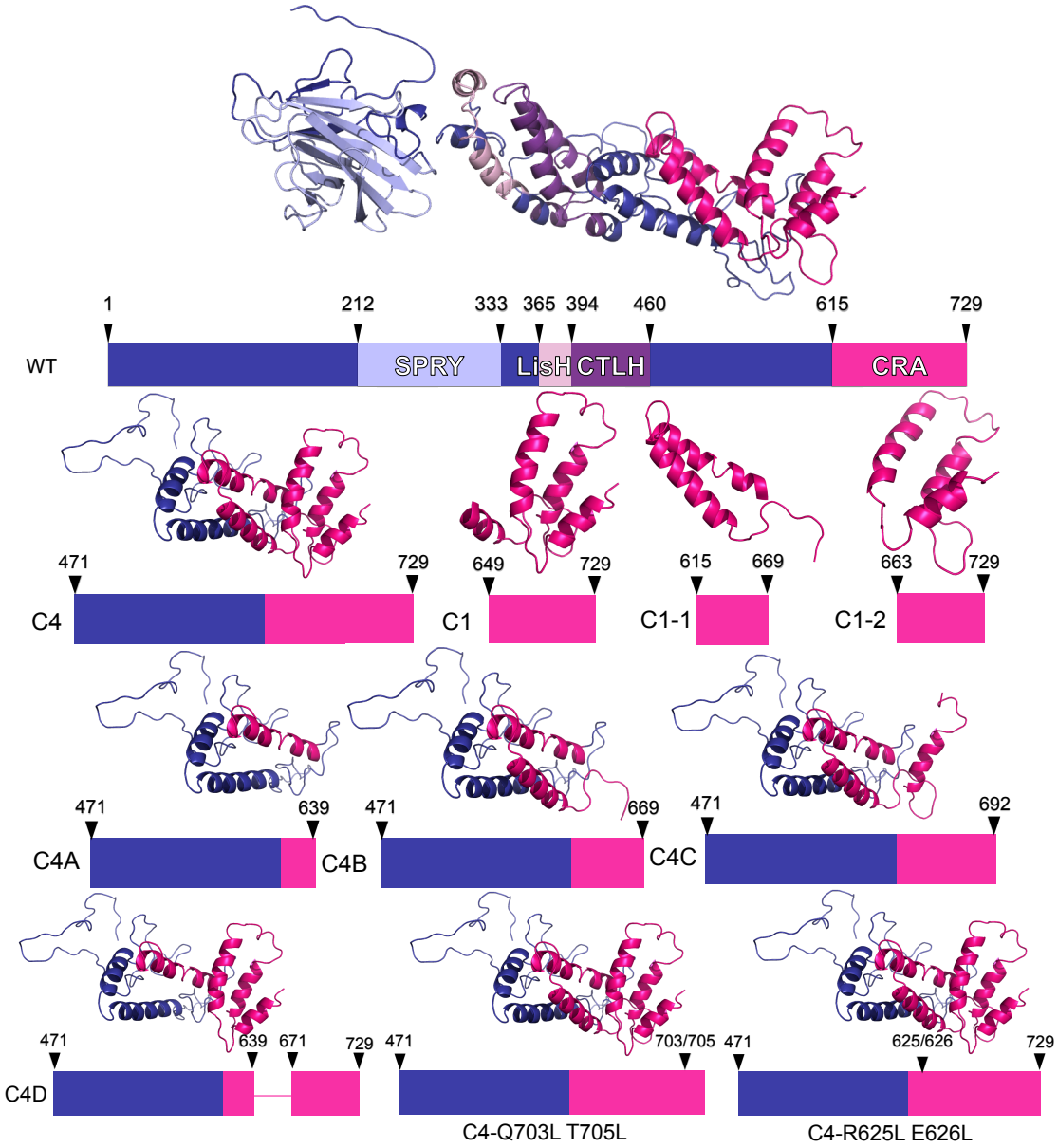

Figure 3.4 Schematics and predicted structures of the C-terminal CRA domain mutants of RanBPM. C-terminal CRA domain mutants were cloned into the bacterial expression vector pGEX-4T-1, downstream of GST.

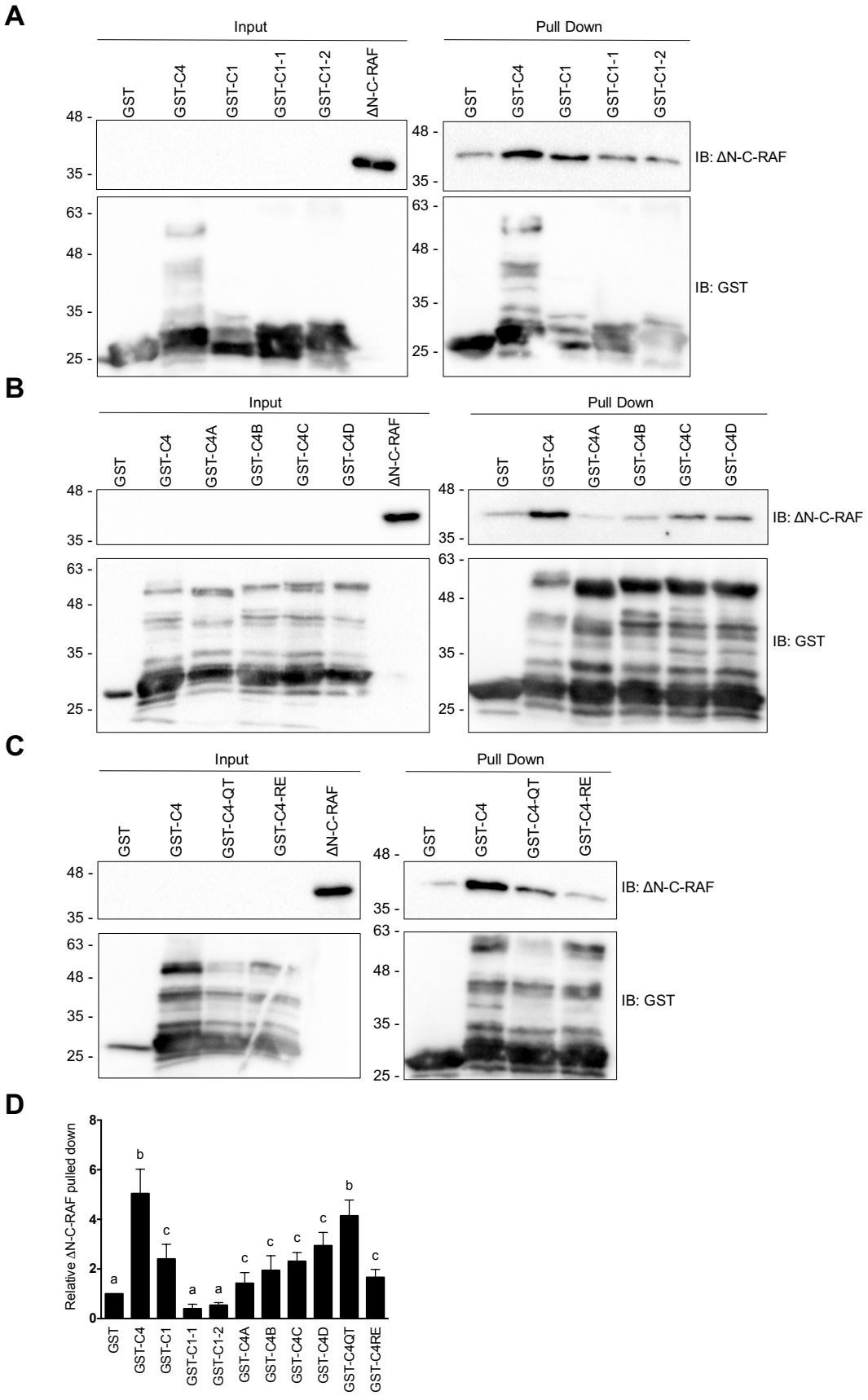
A
Input
Pull Down
GST
GST-C4
GST-C1
GST-C1-1
GST-C1-2
ΔN-C-RAF
48 -
35 -
63 -
48 -
35 -
25 -
IB: ΔN-C-RAF
IB: GST
B
Input
Pull Down
GST
GST-C4
GST-C4A
GST-C4B
GST-C4C
GST-C4D
ΔN-C-RAF
IB: ΔN-C-RAF
IB: GST
C
Input
Pull Down
GST
GST-C4
GST-C4-QT
GST-C4-RE
ΔN-C-RAF
IB: ΔN-C-RAF
IB: GST
D
Relative ΔN-C-RAF pulled down
0
2
4
6
8
a
b
c
a
a
c
c
c
c
b
c
GST
GST-C4
GST-C1
GST-C1-1
GST-C1-2
GST-C4A
GST-C4B
GST-C4C
GST-C4D
GST-C4QT
GST-C4RE

Figure 3.5 The CRA domain of RanBPM interacts directly with ΔN-C-RAF. GST pull-down assays were performed using GST, GST-C4, **(A)** GST-C1, GST-C1-1 and GST-C1-2; **(B)** GST-C4A, GST-C4B, GST-C4C, and GST-C4D; and **(C)** GST-C4-Q703L T705L, denoted GST-C4-QT, and GST-C4-R625L E626L, denoted GST-C4-RE, *E. coli* extracts and crude ΔN-C-RAF *E. coli* extracts. Pull-downs were analyzed by Western blot with C-RAF and GST antibodies to detect ΔN-C-RAF and GST-fusion proteins, respectively**. (D)** Relative ΔN-C-RAF protein levels were quantified by normalizing ΔN-C-RAF to the uppermost GST-fusion protein band, and comparing values to GST when set to a value of 1. Quantifications are shown with error bars indicating SD (*n = 3-5)*. Statistical analysis was performed using ANOVA.

Therefore, point mutations were designed to target the center of helices I and II, or helix V, in efforts to disrupt the helical structures within the CRA domain and the binding interface between CRA and ΔN-C-RAF. Site-directed mutagenesis was used to create C4 with point mutations R625L and E626L within helix I and II, denoted C4 – R625L E626L, or with point mutations Q703L and T705L within helix V, denoted C4 – Q703L T705L. ΔN-C-RAF binding was tested by GST pull down assay: GST-C4- R625L E626L showed a significant loss in ΔN-C-RAF binding with a 3.4 ± 0.5 –fold (SD) decrease, whereas GST-C4- Q703L T705L did not disrupt the interaction, showing only a slight decrease in ΔN-C-RAF binding (Figure 3.5C). These results suggest that the mutation R625L E626L made to helix I and II of the CRA domain was sufficient to disrupt the interaction between the CRA domain and ΔN-C-RAF *in vitro*.

3.4 The CRA domain of RanBPM is necessary for the regulation of ΔN-C-RAF in human cells

Previous studies conducted in our laboratory have shown that both endogenous C-RAF and ectopically expressed ΔN-C-RAF are downregulated by RanBPM, as the downregulation of RanBPM resulted in an increase of both endogenous and ectopic C-RAF protein levels and the re-expression of RanBPM rescued these effects (13). To validate our *in vitro* binding data (Figure 3.5D), mutant RanBPM CRA domains were cloned into the mammalian expression vector pCMV downstream of the HA-tag (Figure 3.6), and co-transfected with pEBG-GST-ΔN-C-RAF into HeLa RanBPM shRNA cells. We chose to evaluate the regulation of ectopically expressed ΔN-C-RAF as the effects of RanBPM regulation are more prominent compared to the regulation of endogenous C-RAF (13). pCMV-HA-RanBPM- ΔC1 containing a deletion of amino acids 649 – 729 of RanBPM, and pCMV-HA-RanBPM- R625L E626L were chosen to investigate their effect on ΔN-C-RAF regulation. pCMV, pCMV-HA-RanBPM-WT, the full-length form of RanBPM, or pCMV-HA-RanBPM-ΔC4 containing a deletion of amino acids 471 – 729 of RanBPM, were also transfected into HeLa shRNA cells to serve as a transfection control, a positive control for ΔN-C-RAF regulation, or a negative control for ΔN-C-RAF regulation, respectively. Given that our *in vitro* binding data showed a significant reduction in ΔN-C-RAF binding of constructs C1 and C4 – R625L E626L, we expected

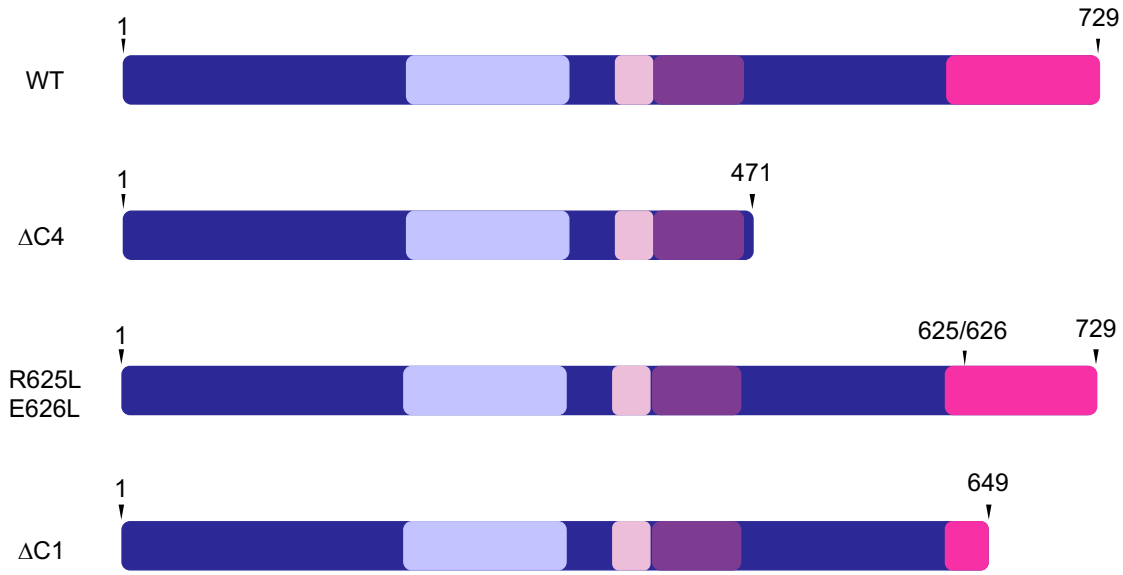

Figure 3.6 Schematics of RanBPM and mutants used in transfection. WT, ΔC4, R625L E262L, and ΔC1 RanBPM mutant constructs were cloned into the pCMV-HA mammalian expression vector.

to see an equivalent loss in ΔN-C-RAF regulation *ex vivo*. These mutations should theoretically cause impaired targeting of ΔN-C-RAF to the CTLH complex resulting in decreased ubiquitination and subsequent degradation of ΔN-C-RAF.

HA-RanBPM-ΔC1 showed significant loss of ΔN-C-RAF regulation, resulting in a 2.9 ± 0.3 –fold increase (SD) in ΔN-C-RAF protein levels compared to HA-RanBPM-WT confirming our *in vitro* binding data and previous results reported by Wesley Bérubé-Janzen (Figure 3.7A, C; unpublished). However, evaluation of the R625L E626L point mutations in this system did not confirm the *in vitro* binding data (Figure 3.7B, C) as the ectopic expression of HA-RanBPM- R625L E626L maintained the regulation of ΔN-C-RAF protein levels to a level comparable to HA-RanBPM-WT.

3.5 RMND5A regulates endogenous C-RAF protein levels

The Gid complex functions as an E3 ubiquitin ligase to regulate key enzymes of the gluconeogenesis pathway through polyubiquitination and subsequent degradation (30). GID2, the orthologue to the mammalian RMND5A, is essential for glucose-induced ubiquitination of FBPase (30). GID2's E3 ligase activity is conferred by its degenerate RING-type zinc-finger domain as its activity to polyubiquintinate proteins has been shown to occur *in vitro*, and mutation to its RING domain abolishes FBPase polyubiquitination and degradation *in vivo* (33). The mammalian orthologue of GID2, RMND5A, also possess the RING motif and it is hypothesized to function as the E3 ligase to the CTLH complex (6), although this activity has yet to be confirmed.

RMND5A was knocked out of HEK-293 cells via CRISPR-Cas9 (*RMND5A* $^{-/-}$) in order to investigate its role in endogenous C-RAF protein regulation. This knockout would theoretically cause impaired degradation of C-RAF, as the removal of RMND5A from the CTLH complex would abolish the presumed E3 ubiquitin ligase activity of the complex. Western blot analysis of *RMND5A* $^{-/-}$ cell extracts showed a 6.7 ± 1.4 –fold (SD) increase of endogenous C-RAF protein levels compared to wild-type HEK-293 (Figure 3.8). Endogenous C-RAF regulation was rescued by ectopically expressing HA-RMND5A in *RMND5A* $^{-/-}$ cells for 24 h, decreasing endogenous C-RAF

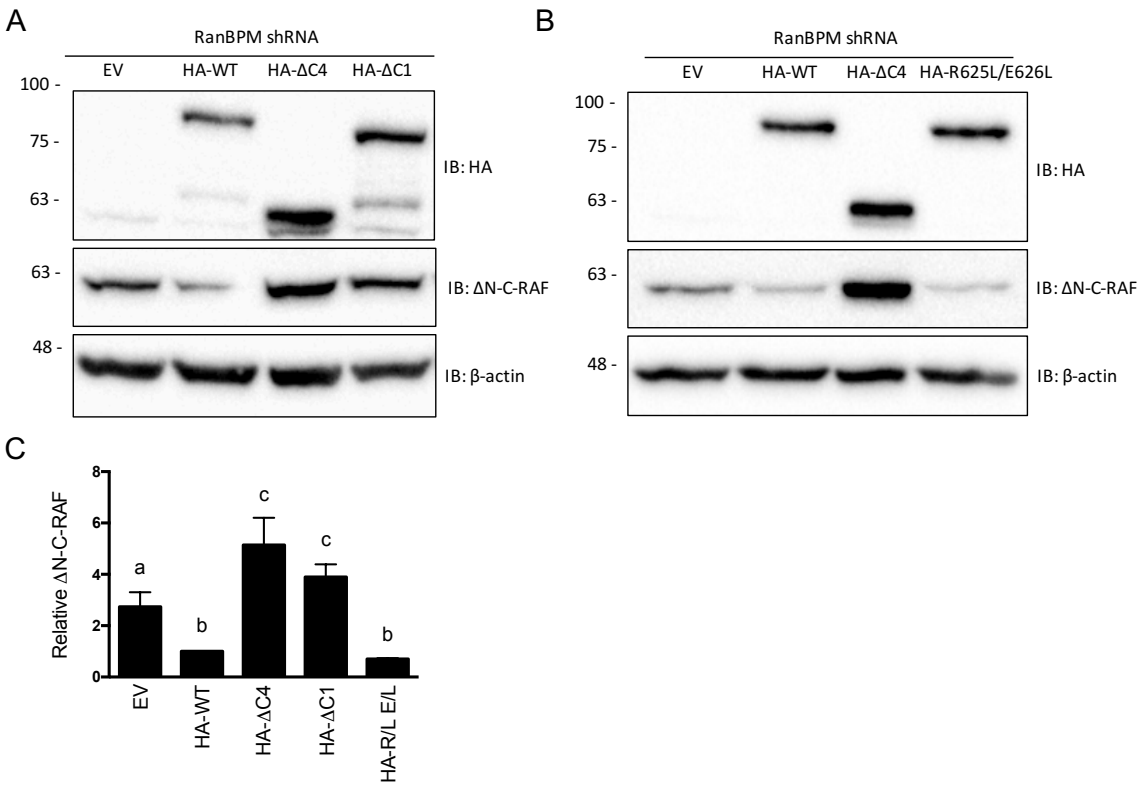

Figure 3.7 The CRA domain of RanBPM is necessary for the regulation of ΔN-C-RAF in cells. HeLa RanBPM shRNA cells were transfected with pEBG-GST-ΔN-C-RAF and either pCMV empty vector, pCMV-HA-RanBPM-ΔC4, **(A)** pCMV-HA-RanBPM-ΔC1, or **(B)** pCMV-HA-RanBPM-R625L E626L, and whole cell extracts were prepared 24 h post-transfection and analyzed by Western blot. HA, C-RAF and β-actin antibodies were used to detect HA-RanBPM constructs, ΔN-C-RAF and β-actin proteins, respectively. **(C)** Relative ΔN-C-RAF protein levels were quantified by normalizing ΔN-C-RAF to β-actin, and comparing values to HA-WT when set to a value of 1. Statistical analysis was performed with ANOVA. Error bars indicate SD *(n=4)*.

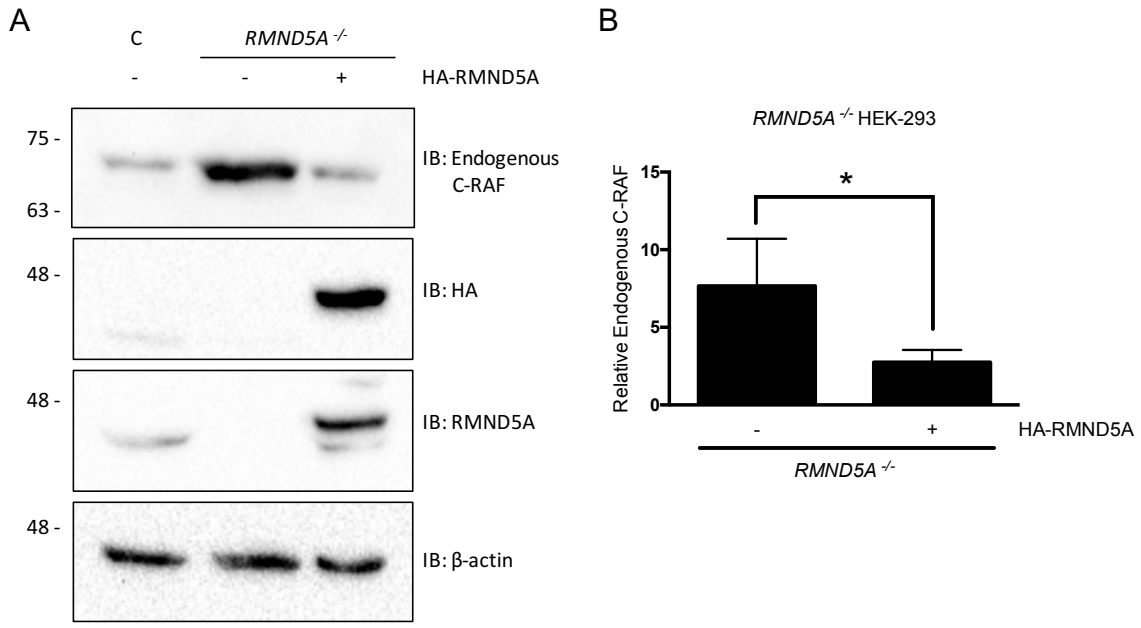

Figure 3.8 RMND5A regulates endogenous C-RAF protein levels. (A) RIPA-buffered whole cell extracts of wild-type HEK-293 cells and CRISPR-Cas9 *RMND5A*$^{-/-}$ HEK-293 cells were prepared and analyzed by Western blot. pCGN-HA-RMND5A was transfected into *RMND5A*$^{-/-}$ HEK-293 cells to rescue the effect on endogenous C-RAF protein levels. C-RAF, HA, RMND5A, and β-actin antibodies were used to detect endogenous C-RAF, exogenous HA-RMND5A, endogenous RMND5A, and β-actin, respectively. **(B)** Relative endogenous C-RAF protein levels were quantified by normalizing C-RAF to β-actin, and comparing values to wild-type HEK-293 when set to a value of 1. Quantifications are shown with error bars indicating SD (*n=4)*. A two-sample t-test, assuming equal variance, was used to determine significance. * $p < 0.05$

protein levels by 4.9 ± 1.6 –fold (SD) compared to mock-transfected *RMND5A* $^{-/-}$ cells. These results demonstrate that endogenous C-RAF protein levels are affected by RMND5A expression, and suggest that RMND5A plays a key role in the regulation of endogenous C-RAF protein levels.

Chapter 4

4 Discussion

4.1 Summary of findings

The aim of this study was to characterize the regulation of C-RAF stability by RanBPM and the CTLH complex. We hypothesized that RanBPM interacts with and promotes the degradation of C-RAF by targeting it for ubiquitination by the CTLH complex. Specifically, we aimed to determine whether the effect of RanBPM on C-RAF protein stability is dependent on the proteasome, define the region of interaction between RanBPM and ΔN-C-RAF, and to investigate the effect on C-RAF regulation by other members of the CTLH complex. In summary, we found that RanBPM regulates C-RAF degradation through the proteasome, and the entire CRA domain of RanBPM is necessary for direct interaction with ΔN-C-RAF and for its effective downregulation in human cells. Finally, the CTLH complex member RMND5A regulates endogenous C-RAF protein levels further implicating the CTLH complex in RanBPM-mediated regulation of C-RAF stability.

4.2 RanBPM and the CTLH complex promote the regulation of C-RAF protein levels in a proteasome-dependent manner

In addition to regulation through phosphorylation, C-RAF has been shown to be regulated through protein-protein interactions (69, 110, 111). Interactions with RAS and 14-3-3 proteins, homo- and heterodimerization, and association with chaperone proteins HSP90 and HSP/HSC70, all participate in the regulation of C-RAF stability and activity (112). Although C-RAF has been studied for more than 30 years, very little information has come to light on C-RAF degradation. Current literature proposes C-RAF degradation to occur in a proteasome-dependent manner (112). XIAP and the HSP90 inhibitor geldanamycin were both shown to be responsible for the direct destabilization of the HSP90-C-RAF complex resulting in enhanced proteasome-dependent degradation of C-RAF (98, 99).

In Chapter 3.1, we investigated whether the loss of RanBPM function would affect C-RAF degradation under conditions of proteasomal impairment. In wild-type cells expressing RanBPM, treatment with the proteasome inhibitor MG132 was shown to augment C-RAF protein levels. In contrast, cells with the stable downregulation of RanBPM did not show accumulation of C-RAF. The downregulation of RanBPM likely resulted in a reduction in C-RAF ubiquitination which prevented its accumulation in proteasome-impaired conditions. This would strongly suggest that RanBPM functions to mediate the recruitment of a protein complex to C-RAF, causing the destabilization of HSP90 and C-RAF, leading to the ubiquitination of C-RAF and its degradation by the proteasome.

Previous experiments were performed in our laboratory to identify the E3 ligase responsible for RanBPM-enhanced degradation of C-RAF. CHIP was investigated as a possible candidate, as previous studies have shown C-RAF to be a CHIP substrate (97, 99). However, CHIP was not identified in complex with C-RAF and RanBPM (113), suggesting CHIP may not be the E3 ligase responsible for RanBPM-mediated C-RAF ubiquitination. As RanBPM is the orthologue to the yeast GID1 protein (6), an essential component to the E3 ligase Gid complex (29), we hypothesized that the mammalian CTLH complex fills this mechanistic void. RanBPM would function to recruit C-RAF to the CTLH complex to promote C-RAF degradation. RanBPM is likely to function independently of CHIP, as there have been other modes of C-RAF ubiquitination documented. For example, siRNA knockdown of CHIP did not prevent the degradation of the S621A C-RAF mutant by the proteasome (100). Treatment with the oxidative glucose metabolite methylglyoxal, abolishing extracellular adhesion, and the use of the chemotherapy regimen GOLF (gemcitabine, oxaliplatin, leucovrin, and 5-Fluorouracil) have also been shown to cause degradation of C-RAF through the ubiquitin-proteasome system independently of CHIP (101, 102, 114). This leads us to believe that RanBPM interaction with C-RAF may serve to recruit the CTLH complex, thereby destabilizing the HSP90-C-RAF complex and promoting C-RAF ubiquitination by the CTLH complex, and enhancing proteasomal degradation of C-RAF. Although there is currently no evidence that demonstrates E3 ubiquitin ligase activity of the CTLH complex as a whole, there is plenty of evidence to suggest that members of the CTLH complex are involved in

the process of ubiquitination and degradation. For example, WDR26 was demonstrated to promote the downregulation of β-catenin (40), and ARMc8α has been shown to promote the association of HRS with ubiquitinated proteins, as well as promote the degradation of α-catenin when overexpressed (37, 38). Also, the CTLH complex was shown to bind Axin, a scaffold protein which has a role in the regulation of the ubiquitination and degradation of β-catenin (115). RanBPM has also been shown to be involved in the process of ubiquitination. For example, RanBPM has been shown to interact with transforming growth factor beta receptor type I (TβRI), reducing auto-ubiquination of TRAF6, an E3 ubiquitin ligase (7). However, there still remains research to be done in order to implicate RanBPM and the CTLH complex, in the process of ubiquitination.

Previous data collected by Wesley Bérubé-Janzen demonstrated that endogenous C-RAF and MAEA form a complex *in situ* (104), implicating an interaction between C-RAF and the CTLH complex. In Chapter 3.4 we showed that the CTLH complex member RMND5A also regulates endogenous C-RAF protein levels. CRISPR-Cas9 knockout of RMND5A in HEK-293 cells *(RMND5A$^{-/-}$)* showed a drastic increase in endogenous C-RAF protein levels, while re-expression of RMND5A reversed the effect. As RMND5A is the mammalian orthologue to GID2, the Gid complex member responsible for the E3 ligase activity of the complex (27, 28), these observations further support the role of the CTLH complex as the E3 ligase responsible for RanBPM-mediated C-RAF degradation.

4.3 The evolutionarily conserved CRA domain of RanBPM is required for direct interaction with, and regulation of, C-RAF

Numerous studies have shown that RanBPM is a promiscuous protein, interacting with over 75 proteins (16). Only a handful of studies have implicated specific conserved domains of RanBPM to be responsible for its interaction. The RanBPM SPRY domain appears to mediate the majority of these interactions. For example, the RanBPM SPRY domain has been shown to associate with CDK11^{p46} (44), ubiquitin carboxyl-terminal hydrolase 11 (USP11) (116), steroid receptors AR and GR (51), MET (11) BLT2 (14), and many others (16). The LisH and CTLH domains have also been shown to mediate the interaction between RanBPM and HDAC6 (58), RanBP10 (117), and Alzheimer's

disease related proteins amyloid precursor protein (APP), low-density lipoprotein receptor-related protein (LRP), and β-site APP-cleaving enzyme 1 (BACE1) (118). However, protein-protein interactions involving the RanBPM CRA domain are limited to the fragile X mental retardation protein (FMRP) (18) the only protein reported as an interacting protein through this domain, and with C-RAF which was identified by previous MSc student Wesley Bérubé-Janzen (104).

In Chapter 3.2, we showed that the RanBPM CRA domain is highly conserved between four homologous sequences. Additionally, in Chapter 3.3 we showed that any alteration made to the CRA domain structure resulted in a significant decrease in ΔN-C-RAF binding *in vitro*, while in Chapter 3.4 we showed a loss in regulation of ΔN-C-RAF by RanBPM when the CRA domain was impaired. Overall, this suggests that the RanBPM CRA domain sequence and its structure are essential to the proper binding, and regulation of C-RAF by RanBPM.

In Chapter 3.3, *in vitro* deletion mutants C1 and C4C showed a significant decrease in ΔN-C-RAF binding. This suggests that helices I, II, V and VI are important to the interaction between the CRA domain and ΔN-C-RAF. Site-directed mutagenesis was used to disrupt the secondary structure of helices I and II or helix V in hopes of disrupting the interaction between the CRA domain and C-RAF. *In vitro* binding data suggested that the disruption of helices I and II was effective in disrupting this interaction. However, ectopically expressed RanBPM – R625L E626L retained its regulation of ΔN-C-RAF comparable to wild-type RanBPM suggesting that the point mutation did not disrupt the interaction between the CRA domain and ΔN-C-RAF *ex vivo*.

In vitro binding of the RanBPM CRA domain to ΔN-C-RAF was studied in isolation, whereas the regulation of ΔN-C-RAF by RanBPM – R625L E626L was studied *ex vivo* allowing the RanBPM mutant to interact with the CTLH complex. As the CRA domain is not required for the association of RanBPM to the CTLH complex (29) it is possible that the CRA domain structure was stabilized by the formation of the CTLH complex thereby preserving the binding interface between the CRA domain and C-RAF, while *in vitro*, the

CRA domain structure may have been compromised when studied in isolation. This stabilizing effect can also be observed in the formation of the Gid complex, as the stability of the members of the Gid complex is dependent on their interactions within the complex (29). The R625L E626L mutation of the CRA domain may have also enhanced the affinity of the bond between the CRA domain and C-RAF. Work by Haling and colleagues determined that the molecular basis of the interaction between the B-RAF kinase domain and MEK-1 was the hydrophobic interface that functions to shield the flexible activation segments of both proteins within the B-RAF activation site (119). With the R625L E626L mutation in the CRA domain, it is possible that this mutation had increased the hydrophobicity of the CRA domain, thereby increasing C-RAF binding affinity. In the future, it will be important to determine whether reducing the hydrophobicity of the CRA domain results in reduced affinity for C-RAF, thus reducing its regulation in human cells.

How RanBPM is able to interact with C-RAF and mediate its downregulation is important to determining how this interaction impacts ERK1/2 signaling. Our laboratory has already demonstrated that without RanBPM-mediated downregulation of C-RAF, phosphorylation of MEK1/2 and ERK1/2 is increased, subsequently causing the increased transcription of anti-apoptosis regulators Bcl-2 and Bcl-X_L (13). However, this is only a fraction of what occurs upon dysregulation of the ERK1/2 signaling pathway. A recent phospho-proteomic study has implicated the ERK1/2 signaling pathway in over 2500 phosphorylation events with approximately one-quarter of these events as a result of direct ERK1/2 phosphorylation (120). Efforts thus far have focused on determining the effects of activated ERK1/2 on the regulation of transcription factors as transcription factors govern essential cellular processes. For example, transcription factors ELK1 and c-FOS were identified as substrates of activated ERK1/2 (66, 121), and as a result of their increased expression due to ERK1/2 activation, cell-cycle regulatory proteins are expressed enabling the progression of the cell through Gap 1 phase of the cell cycle (122). These phosphorylation events resulting from deregulated ERK1/2 activation lead to unrestrained differentiation and proliferation, and deregulated motility and apoptosis, ultimately leading to tumourigenesis (2, 66, 72). Therefore, the regulation of C-RAF mediated by RanBPM and the CTLH complex presents as a significant topic of research.

4.4 Limitations of the study and future work

Although there are currently two proteins to have been identified to interact with the RanBPM CRA domain, it is improbable that all the RanBPM CRA domain interacting proteins have been identified. The kinase domains of A-RAF and B-RAF share a strong resemblance to that of the C-RAF kinase domain, with a high value of 71.8% sequence identity (Figure 4.1), therefore it is probable that the RanBPM-mediated and RMND5A-mediated degradation of C-RAF presented in this study is not exclusive to C-RAF. Preliminary studies conducted in our laboratory have shown that *RMND5A*$^{-/-}$ cells also contain higher protein levels of A-RAF and B-RAF (123). This suggests that RMND5A, the CTLH complex member assumed to possess E3 ligase activity, functions to regulate A-RAF and B-RAF in addition to C-RAF. Future studies should focus on implicating the remaining CTLH complex members by investigating the effects of knocking out each member of the complex on the regulation of human RAF kinases.

As each RAF isoform has distinct functions within cells, RanBPM and the CTLH complex may function to mediate A-RAF function in the developmental stages of neurological and intestinal growth, and apoptosis during epithelial differentiation (90, 124). More importantly, RanBPM and the CTLH complex may be further implicated in the regulation of the ERK1/2 signaling pathway and the prevention of its dysregulation as the B-RAF isoform is commonly mutated in a significant number of cancers (72), with higher incidence of B-RAF mutations in melanoma and thyroid cancers (125, 126).

Although the data presented in Chapter 3.4 does strongly implicate RMND5A in the regulation of C-RAF protein levels, it still remains unclear as to whether RMND5A does so through E3 ubiquitin ligase activity. During this study, several attempts were made to show changes in the ubiquitination state of C-RAF. However, this proved to be difficult. Previous to the establishment of the *RMND5A*$^{-/-}$ HEK-293 cell line, HeLa cells expressing RanBPM-targeting shRNA and non-targeting shRNA were used to investigate C-RAF ubiquitination. Early attempts were focused on showing ectopically expressed

```
A-RAF    SEVQLLKRIGTGSFGTVFRGRWHGDVAVKVLKVSQPTAEQAQAFKNEMQVLRKTRHVNIL
B-RAF    GQITVGQRIGSGSFGTVYKGKWHGDVAVKMLNVTAPTPQQLQAFKNEVGVLRKTRHVNIL
C-RAF    SEVMLSTRIGSGSFGTVYKGKWHGDVAVKILKVVDPTPEQFQAFRNEVAVLRKTRHVNIL
         .:: :  ***:******:.*.*********:*:*  **.:* ***.**: **********

A-RAF    LFMGFMTRPGFAIITQWCEGSSLYHHLHVADTRFDMVQLIDVARQTAQGMDYLHAKNIIH
B-RAF    LFMGYSTKPQLAIVTQWCEGSSLYHHLHIIETKFEMIKLIDIARQTAQGMDYLHAKSIIH
C-RAF    LFMGYMTKDNLAIVTQWCEGSSLYKHLHVQETKFQMFQLIDIARQTAQGMDYLHAKNIIH
         ****: *.  :**:**********:***: :*.*:*.:***:**************.***

A-RAF    RDLKSNNIFLHEGLTVKIGDFGLATVKTRWSGAQPLEQPSGSVLWMAAEVIRMQDPNPYS
B-RAF    RDLKSNNIFLHEDLTVKIGDFGLATVKSRWSGSHQFEQLSGSILWMAPEVIRMQDKNPYS
C-RAF    RDMKSNNIFLHEGLTVKIGDFGLATVKSRWSGSQQVEQPTGSVLWMAPEVIRMQDNNPFS
         **:*********.**************:****:: .** :**:****.******* **:*

A-RAF    FQSDVYAYGVVLYELMTGSLPYSHIGCRDQIIFMVGRGYLSPDLSKISSNCPKAMRRLLS
B-RAF    FQSDVYAFGIVLYELMTGQLPYSNINNRDQIIFMVGRGYLSPDLSKVRSNCPKAMKRLMA
C-RAF    FQSDVYSYGIVLYELMTGELPYSHINNRDQIIFMVGRGYASPDLSKLYKNCPKAMKRLVA
         ******::*:********.****:*. ************ ******: .******.**::

A-RAF    DCLKFQREERPLFPQILATIELLQRSL
B-RAF    ECLKKKRDERPLFPQILASIELLARSL
C-RAF    DCVKKVKEERPLFPQILSSIELLQHSL
         :*:*  .:**********::**** .**
```

Figure 4.1 Sequence alignment of RAF protein kinase domains. Sequences of human RAF protein kinase domains were aligned using the multiple sequence alignment online server MUSCLE (105-108). ***** *fully conserved*; **:** *conservation of strongly similar properties*; **.** *conservation of weakly similar properties.*

HA-ubiquitination of endogenous C-RAF, where antibodies targeting HA or C-RAF were used to immunoprecipitate HA-ubiquitin-tagged endogenous C-RAF under proteasome and deubiquitinase inhibition. However, the antibodies were unable to precipitate significant levels of C-RAF protein (data not shown). As RanBPM has been shown to preferentially bind to the active form of C-RAF (13), it would be improbable that we would be able to discern a change in endogenous C-RAF ubiquitination as both active and inactive forms of C-RAF exist within human cells.

Next, we investigated the ubiquitination of ectopically expressed ΔN-C-RAF, however we were again unsuccessful. GST-ΔN-C-RAF and HA-ubiquitin were co-expressed in HeLa RanBPM-targeting shRNA and non-targeting shRNA cells under conditions of proteasome and deubiquitinase inhibition. GST pull-down revealed that ΔN-C-RAF was degraded, suggesting a low protein half-life of ΔN-C-RAF when bound to glutathione-agarose beads. Also, no HA-ubiquitin-tagged ΔN-C-RAF was detected within the sample (data not shown).

Finally, we attempted to show endogenous ubiquitination of ectopically expressed ΔN-C-RAF in HeLa RanBPM-targeting shRNA and non-targeting shRNA cells, and HEK-293 wild-type and *RMND5A*$^{-/-}$ cells by tandem ubiquitin binding entities (TUBE) assay. TUBE functions by preferentially isolating polyubiquitinated proteins and provides protection from deubiquitination and degradation from the proteasome. ΔN-C-RAF was ectopically expressed in each cell line and treated with proteasome and deubiquitinase inhibitors. The resultant cellular lysate was incubated with TUBE. No visible change in amounts of ubiquitinated ΔN-C-RAF protein was observed by Western blot analysis in either HeLa or HEK-293 cell types (data not shown).

A recurring issue has been observed with the analysis of C-RAF ubiquitination. Previous studies have been able to show polyubiquitination of human C-RAF (97-102, 114, 127), however showing a decrease or increase to the level of endogenous ubiquitination of C-RAF due to the loss or the addition of an E3 ligase in human cells, respectively, has not been documented. The TUBE assay does present itself as a potentially valuable tool to show variations in C-RAF ubiquitination, however further optimization is still required.

Efforts should be concurrently focused on demonstrating E3 ligase activity of human RMND5A and demonstrating C-RAF as a RMND5A E3 substrate, thereby implicating the CTLH complex as an E3 ubiquitin ligase complex.

4.5 Conclusion

Overall, this study aimed to investigate the regulation of C-RAF stability by RanBPM and the CTLH complex. Although this study has added to our knowledge of RanBPM-mediated regulation of C-RAF and further implicates the CTLH complex in this process, the exact function of both still remains unclear. Further exploration into the function of RanBPM and the CTLH complex is essential to understanding the role of this protein complex in the regulation of ERK1/2 signaling, and the prevention of human tumourigenesis.

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Abstracts:

McTavish, C., Bérubé-Janzen, W. and Schild-Poulter. Regulation of C-RAF stability by the RanBPM/CTLH complex.
4th annual Robarts Research Retreat
London, ON
June 13th 2016

McTavish, C., Bérubé-Janzen, W. and Schild-Poulter. Regulation of C-RAF stability by the RanBPM/CTLH complex.
13th annual Oncology Research and Education Day
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June 17th 2016

McTavish, C., Bérubé-Janzen, W. and Schild-Poulter. Regulation of C-RAF stability by the RanBPM/CTLH complex.
14th annual Oncology Research and Education Day
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