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Characterizing the interaction between RanBPM and c-Raf

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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CHARACTERIZING THE INTERACTION BETWEEN RANBPM AND C-RAF

(Thesis format: Monograph)

by

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
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Abstract

RanBPM/RanBP9 is a multi-domain nucleocytoplasmic protein which has been linked to numerous cellular processes including cell adhesion, migration, transcription and apoptosis. Although RanBPM is a member of the mammalian CTLH complex, the counterpart of a conserved yeast E3 ubiquitin ligase complex, its exact function remains unknown. Previous work in our laboratory has shown that RanBPM inhibits the ERK pathway by interacting with the kinase c-Raf and downregulating c-Raf levels. Here, we show that the N-terminus, LisH/CTLH and CRA domains of RanBPM are required for downregulation of c-Raf and that RanBPM interacts directly with c-Raf through its CRA domain. We also provide evidence that MAEA, another CTLH complex member, associates with c-Raf. Therefore, we propose a mechanism by which RanBPM downregulates c-Raf in a CTLH complex-dependent manner. This work contributes to our knowledge of the function of RanBPM and clarifies the relationship between RanBPM and c-Raf, two important proteins in oncogenesis.

Keywords

RanBPM, c-Raf, ERK pathway, CTLH complex, CRA domain, protein stability, protein interactions, oncogenesis

Co-Authorship Statement

This thesis was written by Wesley Bérubé-Janzen and edited by Caroline Schild-Poulter, Victoria Fell and Louisa Salemi. All experiments and procedures described in this thesis were performed by Wesley Bérubé-Janzen, with the exception of the following:

- HeLa control and HeLa 2-7 stable cell lines were generated by Dawn Bryce.
- pCMV-HA-RanBPM- Δ N2, pCMV-HA-RanBPM- Δ C4, pCMV-HA-RanBPM- Δ C1, pCMV-HA-RanBPM- Δ 212, and pCMV-HA-RanBPM- Δ 360, with mutations conferring resistance to shRNA, were generated by Caroline Schild-Poulter, Dawn Bryce, Patricia Christian and Tung Bai.
- pET28a- Δ N-c-Raf was cloned by Matthew Maitland.

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List of Abbreviations

ACs	adenylyl cyclases
AD	Alzheimer's Disease
ANOVA	analysis of variance
APP	amyloid precursor protein
AR	androgen receptor
ARMc8	armadillo repeat containing 8
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
A β	amyloid β
BACE1	β -secretase 1
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
Bcl-XL	B cell lymphoma extra large
BioGRID	Biological General Repository for Interaction Datasets
BLT2	leukotriene B4 receptor 2
BRCA1	breast cancer type 1 susceptibility protein
CDK11	cyclin-dependent kinase 11
c-Fos	cellular FBJ murine osteosarcoma viral oncogene homolog
CHIP	C-terminus of Hsp70-interacting protein
CK2	casein kinase 2
CO ₂	carbon dioxide
CRA	CT11-RanBPM
CREB	cAMP response element-binding protein
CTLH	C-terminal to LisH
DAPK	death associated protein kinase
DD	death domain
DMEM	Dulbecco's modified eagle medium
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
Elk1	ETS domain-containing protein

ERK	extracellular signal-regulated kinase
FBPase	fructose-1,6-bisphosphatase
FBS	fetal bovine serum
FMRP	fragile X mental retardation protein
GDP	guanosine diphosphate
GFP	green fluorescent protein
Gid	glucose induced degradation deficient
GR	glucocorticoid receptor
Grb2	growth factor receptor-bound protein 2
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 2
HMG	high mobility group
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
IAP	inhibitor of apoptosis
IPTG	isopropyl β -D-1-thiogalactopyranoside
IQGAP	IQ motif containing GTPase-activating protein
IR	ionizing radiation
JAK2	Janus kinase 2
KCl	potassium chloride
KSR1	kinase suppressor of Ras 1
LB	Luria Bertani
LisH	lissencephaly type-1-like homology
LRP	low-density lipoprotein receptor-related protein
MAEA	macrophage erythroblast attacher
MEK1/2	mitogen-activated protein kinase kinase 1/2

MET	mesenchymal epithelial transition factor
MSK1/2	mitogen- and stress-activated protein kinases 1/2
MST2	mammalian Ste20-like kinase 2
N/A	not available
NaCl	sodium chloride
NaF	sodium fluoride
NaVO ₄	sodium orthovanadate
ND	not determined
NES	nuclear export signal
NFAT	nuclear factor of activated T cells
NLS	nuclear localization signal
NP40	nonidet P-40
N-region	negative-charge regulatory region
OD ₆₀₀	optical density at 600nm
p75NTR	p75 neurotrophin receptor
PAK1	p21-activated kinase 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKA	protein kinase A
PLA	proximity ligation assay
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP5	protein phosphatase 5
PVDF	polyvinylidene fluoride
Raf	rapidly accelerated fibrosarcoma
Ran	Ras-related nuclear protein
RanBP10	Ran-binding protein 10
RanBPM	Ran-binding protein in the microtubule-organizing center
Ras	rat sarcoma
Rb	retinoblastoma tumour suppressor protein
RKIP	Raf kinase inhibitor protein

Rmnd5a	required for meiotic nuclear division 5 homolog A
Rok- α	Rho-binding kinase α
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH3	Src homology 3
SNP	single nucleotide polymorphism
SOS	son of sevenless
SPRY	dual-specificity kinase splA and ryanodine receptor
Src	sarcoma
TAF4	transcription initiation factor TFIID
TFIID	transcription factor II D
TGF- β	transforming growth factor β
TR	thyroid receptor
TRAF6	tumor necrosis factor-receptor-associated factor 6
Tris	Tris-hydroxymethyl amino methane
TrkA	tropomyosin-related kinase A
TrkB	tropomyosin-related kinase B
Twa1	two hybrid-associated protein 1 with RanBPM
WCE	whole cell extract
WT	wild-type
XIAP	X-linked inhibitor of apoptosis

Chapter 1 – Introduction

1.1. Cancer

According to the Canadian Cancer Society, it is estimated that approximately 267,400 Canadians will be diagnosed with cancer in 2014, with the most common types being prostate, breast, lung, colorectal and non-melanoma skin cancer (1). Cancer is the leading cause of death in Canada, with an estimated 1 in 4 Canadians expected to die from the disease (1). Therefore, it is evident that cancer research focused on understanding the disease and developing new treatments to combat tumour growth are of utmost importance to preserve the health of our population.

Although the average one gram malignant tumour is estimated to contain 10^8 – 10^9 cancer cells, such tumour masses can start with the defiant behaviour of a single cell (2). Cells are programmed to grow, replicate and die when they have reached the end of their lifespan, however a cell can break free from these restraints in the event of genomic mutation and proliferate uncontrollably, resulting in cellular transformation and tumourigenesis. Cancer cells exhibit certain characteristics, termed the hallmarks of cancer, which are acquired during cellular transformation and are predominantly responsible for the progression of the disease (3). Namely, they sustain proliferative signaling, evade signaling from growth suppressors, resist cell death, replicate infinitely, induce angiogenesis and invade surrounding tissues (3).

Understanding the cellular and molecular processes behind each of these hallmarks is key in understanding how a healthy cell transforms into a malignant cell, and ultimately contributes valuable knowledge that can be used to generate novel therapies to fight cancer. This thesis aims to contribute to this pool of knowledge by studying the relationship between RanBPM (Ran-binding protein M) and c-Raf (rapidly accelerating fibrosarcoma), two proteins known to play roles in critical cellular processes which, when perturbed, can lead to the development of the hallmarks of cancer.

1.2. RanBPM

1.2.1. Overview of RanBPM

RanBPM, also known as RanBP9, was initially discovered in a yeast two-hybrid assay as a 55kDa interacting partner for the small guanosine triphosphatase (GTPase), Ran (Ras-related nuclear protein) (4). However, subsequent studies soon uncovered that RanBPM is in fact a 90kDa protein, only weakly interacts with Ran and does not localize to the centrosome, as initially thought (4,5). Since then, RanBPM has generated significant interest and numerous studies have been conducted on the protein to attempt to characterize its function.

RanBPM is widely conserved across mammals, with over 96% similarity between human and mouse RanBPM, although homologs are also present in many other species (6). In mammals, RanBPM has been shown to be ubiquitously expressed, with higher expression in heart, muscle, brain and reproductive tissues (7,8). RanBPM was initially recovered in a 670kDa complex and, since then, its involvement in complex formation has been described extensively (5,9). It has been shown to interact with countless proteins and be implicated in a variety of cellular processes including, but not limited to, transcription, cell adhesion, cell migration and apoptosis (9). Although a considerable number of studies have identified proteins that interact with RanBPM (Table 1.1), a large portion of them lack insight on the functional significance of the interactions (9). For these reasons, RanBPM has widely been hypothesized to be a scaffolding protein, however, its exact function still remains unknown (9).

Table 1.1. Comprehensive list of proteins that have been shown to interact with RanBPM. Methods used to demonstrate the interactions are indicated as yeast two-hybrid (Y2H), mammalian two-hybrid (M2H), pull-down (PD), immunoprecipitation (IP), proximity ligation assay (PLA), confocal microscopy (CM) and/or fluorescence resonance energy transfer (FRET).

Interacting proteins	Methods	Domains of RanBPM required	References
RanBPM	IP	ND	(13)
p73	Y2H, PD, IP	ND	(17)
CDK11^{p46}	Y2H, PD, IP, CM	SPRY	(20)
p75NTR	Y2H, IP	ND	(21)
HIPK2	Y2H, IP, CM	ND	(22)
c-Raf	PD, IP, PLA	CRA	(24), present study
L1	Y2H, PD, IP	SPRY	(25)
MET	Y2H, M2H, PD, IP	SPRY	(8)
TrkB	IP, CM	ND	(26)
APP	IP	SPRY/LisH	(29)
BACE1	IP	SPRY/LisH	(29)
LRP	IP	SPRY/LisH	(29)
FMRP	Y2H, PD, IP, CM	CRA	(14)
plexin-A	Y2H, IP	ND	(34)
TrkA	Y2H, PD, IP	SPRY	(35)
TAF4	Y2H, IP, CM	ND	(36)
AR	Y2H, PD, IP	SPRY	(7)
TR	Y2H, PD, IP	ND	(37)
LFA-1	Y2H, PD, IP	ND	(39)
β1 integrin	Y2H, IP	ND	(39)
BLT-2	Y2H, PD, IP, CM	ND	(43)
BRCA1	Y2H	ND	(44)
Muskelin	Y2H, IP	ND	(42,46)
Twa1	Y2H, IP, PD	ND	(42,46)
Rmnd5a	IP	ND	(46)
MAEA	IP	ND	(46)
ARMc8	IP	ND	(46)
HDAC6	IP, CM	LisH/CTLH	(103)
TRAF6	Y2H, PD, IP, CM, FRET	ND	(107)
USP11	Y2H, IP	SPRY	(109)

1.2.2. Conserved domains

Four conserved functional domains have been identified within the sequence of RanBPM: the SplA and Ryanodine receptor (SPRY) domain, the Lissencephaly type-1-like homology (LisH) domain, the C-terminal to LisH (CTLH) domain and the CT11-RanBPM (CRA) domain (Figure 1.1) (10). The SPRY domain is known to be involved in protein-protein interactions (11). The LisH domain is known to mediate protein dimerization, and is in fact predicted to moderate the dimerization and oligomerization of RanBPM (12,13). Additionally, the LisH domain, together with the CTLH domain, is thought to regulate microtubule dynamics and cell migration (12). The CRA domain, which is predicted to contain six α -helices and resembles a death domain (DD) superfamily domain, has also been shown to function as a protein interaction surface (14). In addition to these four conserved domains, RanBPM also contains a proline- and glutamine-rich N-terminus predicted to contain six Src homology 3 (SH3) binding domains (5,15).

1.2.3. Cellular localization

Although RanBPM was initially thought to be localized to the centrosome, further studies determined its localization to actually be predominantly nucleocytoplasmic (4,5). Recent studies in our laboratory have identified a primary nuclear localization signal (NLS) spanning amino acids 1–25, a secondary NLS spanning amino acids 635–649 and a nuclear export signal (NES) comprising amino acids 140–155, which together govern the subcellular localization of RanBPM (16). The SPRY and LisH/CLTH domains were also shown to be important for cytoplasmic retention of RanBPM, potentially through interactions with cytoplasmic proteins (16).

Indeed, the subcellular localization of RanBPM has previously been shown to be influenced by interactions with other proteins. For example, overexpression of p73 was shown to promote the translocation of RanBPM from



Figure 1.1. Full-length RanBPM with conserved domains indicated. RanBPM contains a SPRY domain (amino acids 212–333), a LisH/CTLH domain (amino acids 367–460) and a CRA domain (amino acids 615–729).

the cytoplasm to the nucleus (17). Given that these two proteins physically interact and p73 is exclusively nuclear, it was hypothesized that an overabundance of p73 could sequester a high proportion of cellular RanBPM in the nucleus (17). Furthermore, under certain cellular conditions, RanBPM localization has been reported to be altered. For example, in response to ionizing radiation (IR), a DNA damage-inducing agent, RanBPM has been shown to shuttle from the nucleus to the cytoplasm (18). This change in localization could occur to allow RanBPM to interact with cytoplasmic apoptotic or DNA damage response proteins, although this speculation has yet to be confirmed (18). Altogether, such evidence suggests that RanBPM localization is important in dictating its function, as it allows RanBPM to interact with various specifically compartmentalized proteins and participate in different signaling pathways.

1.2.4. Role in apoptosis

Although the specific function of RanBPM has yet to be elucidated, there is substantial evidence that it plays an important role in the activation of apoptosis. RanBPM has been shown to interact with the tumour-suppressor protein p73 and enhance its apoptotic activity (17). The interaction between these two proteins was demonstrated to be required for the ability of RanBPM to activate apoptosis, induce mitochondrial membrane permeability, decrease levels of the anti-apoptotic protein Bcl-2 (B cell lymphoma 2), increase levels of the pro-apoptotic protein Bax and enhance Bax oligomerization (19). It has also been demonstrated that, through its SPRY domain, RanBPM interacts with and is phosphorylated by CDK11^{p46} (cyclin-dependent kinase 11), a caspase-cleaved C-terminal kinase segment of the larger CDK11^{p110} isoform (20). Caspase-cleavage of CDK11^{p110} occurs during apoptosis and cleaved CDK11^{p46} continues to propagate apoptotic signals, potentially through a RanBPM-dependent mechanism (20). RanBPM has also been shown to interact with the pro-apoptotic neurotrophin receptor p75NTR (p75 neurotrophin receptor) through its intracellular DD and to interact with the nuclear protein kinase HIPK2

(homeodomain-interacting protein kinase 2), which has been shown to activate and stabilize the tumour-suppressor protein p53 (21-23).

Important studies in our laboratory have demonstrated that RanBPM activates apoptosis in response to DNA damage caused by IR (18). Subsequent studies showed that RanBPM is in fact an inhibitor of the ERK (extracellular signal-regulated kinase) pathway and specifically downregulates the crucial ERK pathway kinase c-Raf at the protein level (24). This resulted in decreased downstream ERK pathway signaling, culminating in decreased levels of the anti-apoptotic protein Bcl-2 and its family member Bcl-XL (B cell lymphoma extra large) (24). This is consistent with previous findings that RanBPM regulates the intrinsic cell death pathway (19). In addition, it was found that downregulation of RanBPM leads to increased cell proliferation, an important hallmark of cellular transformation and cancer (24).

Other studies have also shown that RanBPM restricts ERK pathway signaling. For example, through its SPRY domain, it was observed that an N-terminal fragment of RanBPM interacts with the neural adhesion molecule L1 to inhibit downstream ERK signaling (25). Although it appears that RanBPM is a pro-apoptotic protein that inhibits the ERK pathway, there is some opposing evidence that RanBPM activates the ERK pathway through interactions with the receptor tyrosine kinases TrkB (tropomyosin-related kinase B) and MET (mesenchymal epithelial transition factor), although the latter was shown using a green fluorescent protein (GFP) tagged RanBPM construct (8,26). Therefore, some of the contradiction regarding RanBPM regulation of the ERK pathway could potentially be attributed to the different constructs used in each study and the unknown effects of large tags or truncations on the overall function of RanBPM.

1.2.5. Functions in the reproductive and nervous systems

Numerous studies have demonstrated roles for RanBPM in the reproductive and nervous systems, but nowhere is this more evident than in the

characterization of RanBPM knockout mice. It is well documented that RanBPM knockout mice generally die neonatally, although a small number of newborn pups have been reported to survive into adulthood (27,28). The cause of this neonatal fatality remains unclear, although it has been suggested that these pups are unable to suckle milk, suggesting defects in brain function (28). RanBPM knockout mice suffer pronounced gonadal atrophy, severely compromised spermatogenesis and oogenesis as well as infertility, provided they reach adulthood (27). Furthermore, they display growth retardation and their brains are dramatically reduced in size, especially in the hippocampal and cortical regions, compared to wild-type (WT) mice (28).

A number of important studies have also implicated RanBPM in the development of the neurodegenerative disorder Alzheimer's disease (AD). Interestingly, a truncated form of RanBPM has been shown to be expressed over six times higher in the brains of AD patients compared to those of healthy individuals (13). One of the defining pathological hallmarks of AD is the accumulation of A β (amyloid β) peptides in the brain and RanBPM has been shown to promote A β generation from its precursor APP (amyloid precursor protein) (29). RanBPM accelerates endocytosis of APP and acts as a scaffold for APP, BACE1 (β -secretase 1) and the endocytosis receptor LRP (low-density lipoprotein receptor-related protein) to facilitate BACE1 cleavage of APP into A β (29). Consistent with previously mentioned evidence regarding RanBPM involvement in apoptosis, these studies have shown that RanBPM overexpression causes apoptosis and also potentiates A β toxicity in the brain (30). In addition, RanBPM transgenic mice suffered neurodegeneration, spatial memory loss and a decreased number of neuronal synapses (31).

RanBPM has been shown to interact with proteins involved in other neurodegenerative diseases as well. RanBPM interacts with FMRP, a protein whose loss of expression leads to the most common form of hereditary mental retardation, fragile X syndrome (32). FMRP is an RNA-binding protein predominantly found in neurons and spermatogonia that regulates translation and transport of mRNA (32,33). Through its CRA domain, RanBPM directly binds

and sequesters the RNA-binding region of FMRP, rendering it unable to execute its RNA-binding function (14). RanBPM has also been reported to interact with the neural adhesion molecule L1, which can lead to various X-linked disorders if mutated (9). Inhibition of ERK signaling by an N-terminal fragment of RanBPM suppresses L1-mediated neurite outgrowth and branching in primary neurons (25).

Further functions for RanBPM in the nervous system include interaction with the plexin-A receptor to inhibit axonal outgrowth and induce neuronal contractility (34). RanBPM also been shown to interact with the receptor tyrosine kinases TrkB and TrkA, which both serve as neurotrophin receptors in the brain (26,35). Through its interaction with TrkB, RanBPM was shown to enhance neuronal morphogenesis, and through its interaction with TrkA, RanBPM was shown to reduce downstream expression of the transcription factor NFAT (nuclear factor of activated T cells), which is known to play a role in axon outgrowth and synaptic plasticity (26,35).

1.2.6. Regulation of transcriptional activity

RanBPM has further been suggested to regulate transcriptional activity in the cell. RanBPM has been shown to interact with TAF4 (transcription initiation factor TFIID subunit 4), a subunit of the general transcription factor TFIID (transcription factor II D) (36). TFIID is a member of the RNA polymerase II preinitiation complex that binds the TATA box during the initial steps of gene transcription. The interaction between RanBPM and TAF4 was demonstrated to initiate primary neurite branching in neuronal stem cells, although the transcriptional model by which this occurs has yet to be elucidated (36). Furthermore, RanBPM has been reported to interact with the ligand-dependent transcription factors AR (androgen receptor) and TR (thyroid hormone receptor) and enhance their transcriptional activities (7,37). RanBPM can also augment the transcriptional activity of GR (glucocorticoid receptor), although an interaction between the two proteins has not explicitly been shown (7).

Microarray analyses in our laboratory have shown that RanBPM influences transcriptional pathways primarily associated with cell, tissue and organ development as well as tumorigenesis and cancer (38). Upon RanBPM downregulation, global gene expression changes occurred and over-represented transcription factor binding sites were identified among the upregulated or downregulated genes (38). Among the most over-represented were binding sites for the Forkhead, homeodomain and HMG (high mobility group) transcription factors, providing further evidence that RanBPM regulates transcription by modulating transcription factor activity (38).

1.2.7. Implications in cell morphology, adhesion and migration

There is evidence of RanBPM involvement in cell morphology and polarity, based on its reported interactions with known regulators of these processes. It has been reported that RanBPM interacts with β 1 integrin and the β 2 integrin LFA-1 (lymphocyte function-associated antigen-1) (39,40). Integrins are transmembrane receptors that are well-known for mediating cell-cell and cell-extracellular matrix interactions through focal adhesions, however, they also participate in many signaling pathways within the cell (39). RanBPM has been shown to accelerate endocytosis of β 1 integrin to disrupt integrin-dependent cell adhesion, focal adhesion assembly and focal adhesion signaling (40). Some data also suggests that RanBPM acts in conjunction with Muskelin to regulate cell morphology and cell spreading, as they are found together in a complex and knockdown of either Muskelin or RanBPM in lung epithelial cells led to the same phenotype of increased cell perimeter and disrupted actin distribution (41,42).

Studies in our laboratory have found that RanBPM also inhibits cell migration, as downregulation of RanBPM increased cell migration in human embryonic kidney (HEK) cells (24). These findings are compatible with previous studies showing that RanBPM interacts with the G protein coupled receptor BLT2 (leukotriene B4 receptor 2) and reduces BLT2-mediated cell migration (43).

1.2.8. RanBPM in cancer

Evasion of apoptosis, sustained proliferative signal and tissue invasion mark three of the six primary hallmarks of cancer demonstrated by malignant cells (3). Given its prominent roles in apoptosis as well as restricting cell growth and cell migration, it has been suggested that RanBPM might be playing a role in the prevention of tumour development and oncogenesis. As previously mentioned, RanBPM has been shown to interact with many pro-apoptotic tumour suppressors, inhibit proliferative cell pathways and directly induce apoptosis in response to DNA damage (17-25). It has also been shown to interact with proteins involved in cell motility and directly inhibit cell migration (24,43). Interestingly, RanBPM expression has been found to be altered in many human tumours, including lung, kidney and breast cancer samples (39). In most cases, expression was lost or greatly reduced, validating its characterization as a tumour suppressor protein (39).

RanBPM has also been identified in a high-throughput yeast two-hybrid screen searching for proteins that interact with the C-terminal region of BRCA1 (breast cancer type 1 susceptibility protein), a DNA damage repair tumour suppressor primarily expressed in breast and ovarian tissues (44). Individuals with mutations in BRCA1 are undoubtedly considered to be at high risk to develop breast cancer. Although the functional significance behind the interaction between RanBPM and BRCA1 was not elucidated, the interaction itself further suggests roles for RanBPM in DNA damage control and tumour suppressive activity. Additionally, a breast cancer single nucleotide polymorphism (SNP) was identified a short distance upstream of the RanBPM gene at nucleotide position 13830502 on chromosome 6 (45). Though the specific impact of this SNP on RanBPM expression or function has not been determined, this finding implies that RanBPM may be involved in cancer development and specifically in breast cancer development.

1.2.9. CTLH complex

RanBPM is a known member of the mammalian CTLH complex, along with Muskelin, Twa1 (two hybrid-associated protein 1 with RanBPM), Rmnd5a (required for meiotic nuclear division 5 homolog A), MAEA (macrophage erythroblast attacher) and ARMc8 (armadillo repeat containing 8) (42,46). Each of these proteins, with the exception of Muskelin, have orthologs in *Saccharomyces cerevisiae* which are part of the yeast Gid (glucose induced degradation deficient) complex (Figure 1.2) (6). Gid1, the ortholog of RanBPM, acts as a crucial scaffold in this complex and primarily mediates interactions with other Gid proteins through its LisH and CTLH domains (Figure 1.3) (47). The Gid complex has been shown to function as an E3 ubiquitin ligase (48). In general, E3 ubiquitin ligases are key components of the ubiquitin-protease system (UPS) and act in concert with E1 activating enzymes and E2 ubiquitin conjugating enzymes to ubiquitinate target proteins and send them for degradation through the proteasome (49). The Gid complex specifically targets FBPase (fructose-1,6-bisphosphatase) for proteasome-mediated degradation when glucose becomes available and FBPase is no longer needed for yeast to perform gluconeogenesis (48). Although the Gid complex is a proven E3 ubiquitin ligase in yeast, it is unknown if the CTLH complex performs a similar function in mammalian cells (6). However, there has been some evidence of members of the CTLH complex playing a role in the ubiquitination and degradation of proteins. ARMc8 has been shown to bind HRS (Hepatocyte growth factor-regulated tyrosine kinase substrate) and promote its association with ubiquitinated proteins (50). ARMc8 has also been implicated in the proteasome-dependent degradation of α -catenin, although this was shown to occur independently of ubiquitination (51). Furthermore, both Rmnd5a and MAEA possess a Really Interesting New Gene (RING) domain, which is a defining characteristic of many E3 ubiquitin ligases (6). In fact, evidence suggests that Rmnd5a and its paralog, Rmnd5b, have E3 ubiquitin ligase activity as both have been shown to associate with E2 ubiquitin

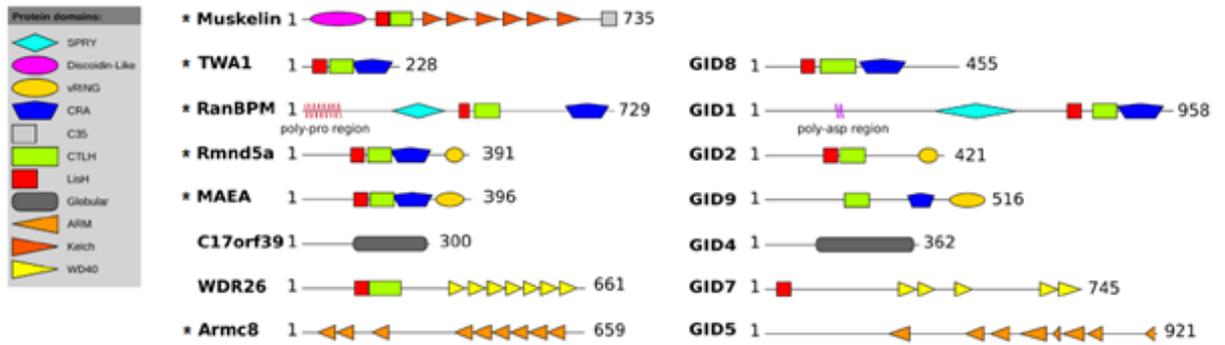


Figure 1.2. Mammalian orthologs of the members of the *S. cerevisiae* Gid complex. Proteins found within the Gid complex are represented on the right, along with their respective mammalian orthologues represented on the left. Conserved domains are indicated and members of the mammalian CTLH complex are denoted with an asterisk. Adapted from (6).

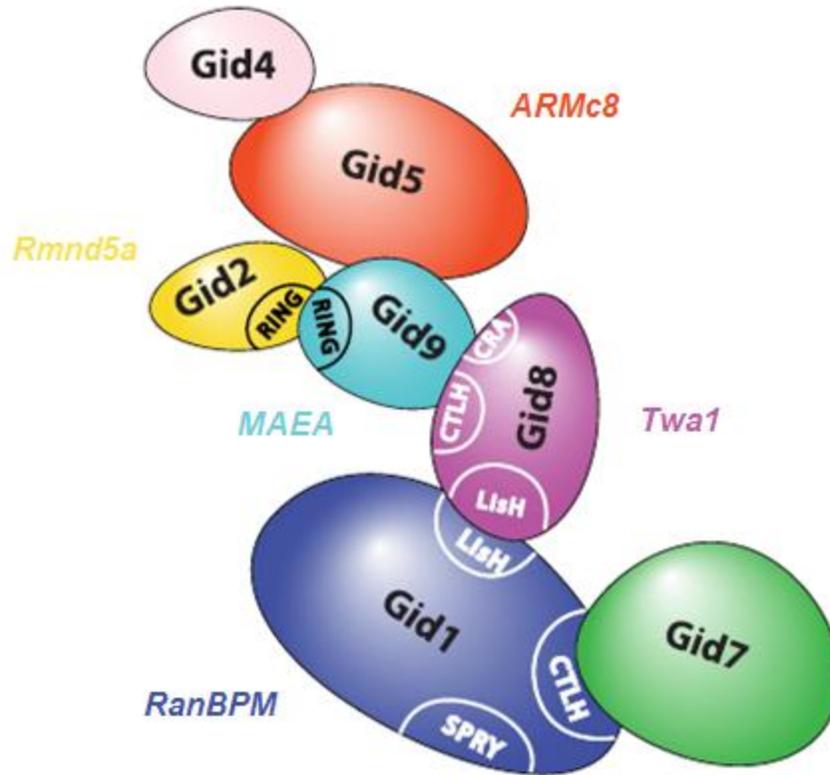


Figure 1.3. Model of the interactions between members of the *S. cerevisiae* Gid complex. Gid1, Gid2, Gid4, Gid5, Gid7, Gid8 and Gid9 interact to form an E3 ubiquitin ligase, with the ortholog of RanBPM, Gid1, serving as a central scaffold in the complex. Orthologs of Gid proteins that are also found in the mammalian CTLH complex are indicated in italics. Adapted from (47).

conjugating enzymes and promote the ubiquitination of the prostatic tumour suppressor NKX3.1 (52).

1.3. c-Raf

1.3.1. Overview of Raf family kinases

The Raf family of serine/threonine kinases have been a hot topic of research since the discovery of the first *raf* gene, retroviral oncogene *v-raf*, in 1983 (53). Mammalian isoforms A-Raf, B-Raf and c-Raf soon generated interest due to their crucial role as signaling molecules in the ERK pathway, a pathway known to play an important role in many crucial cellular processes and whose loss of regulation can be devastating to an organism (54-56). Given that the ERK pathway is upregulated in approximately one-third of all human cancers (56), it has become clear that understanding Raf protein function is critical in understanding the ERK pathway as a whole and its role in cancer development.

The structure of all three mammalian Raf kinases (Figure 1.4) can be divided into a regulatory N-terminal region and a catalytic C-terminal region. The N-terminus contains a primary Ras (rat sarcoma) binding site and a cysteine-rich secondary Ras binding site (54). The C-terminus contains a negative-charge regulatory region (N-region) and an activation segment, both containing multiple phosphorylation sites required for Raf activation (54,57), as well as an adenosine triphosphate (ATP) binding domain. Numerous regulatory phosphorylation sites, both activating and inhibitory, are also found throughout Raf (54).

A-Raf, B-Raf and c-Raf are all ubiquitously expressed in mammals (58), although A-Raf and B-Raf levels have been found to be higher in urogenital organs and neuronal tissues, respectively (59-61). Although all three kinases have been shown to participate in ERK signaling (54), evidence suggests that the isoforms also perform additional non-redundant functions. A-Raf knockout mice tend to die 7–21 days after birth due to neurological and gastrointestinal deficiencies (62), whereas c-Raf and B-Raf knockout mice die *in utero* from a

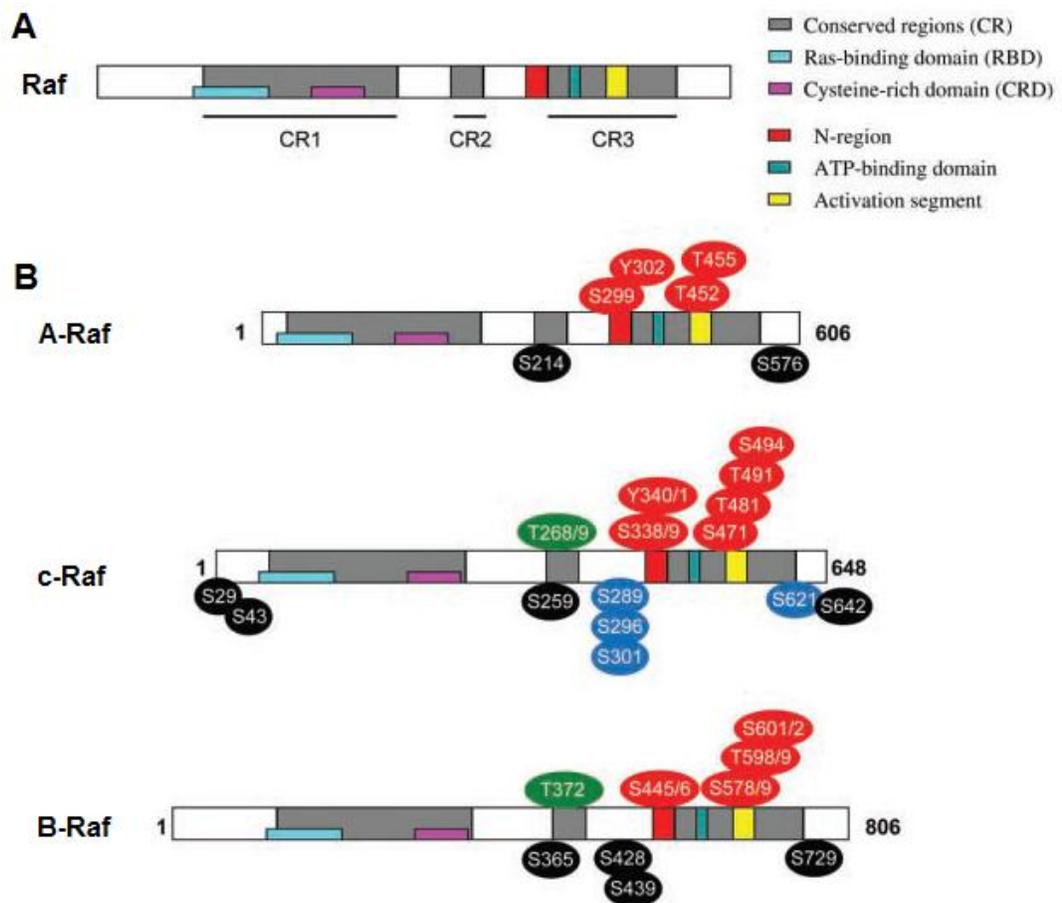


Figure 1.4. Structure, conserved regions and regulatory phosphorylation sites of Raf family kinases. (A) General structure of the Raf kinases with conserved regions indicated. **(B)** Specific structures of A-Raf, c-Raf and B-Raf with activating phosphorylation sites (red), inhibitory phosphorylation sites (black), phosphorylation sites defined as both activating and inhibitory (blue) and autophosphorylation sites (green) indicated. Adapted from (54).

different set of complications (63,64). Although both types of mice demonstrate growth retardation, c-Raf knockout mice exhibit liver defects while B-Raf knockout mice exhibit vascular and neuronal deficiencies (63,64). Given the different phenotypes observed in these knockout mice and the apparent lack of compensation between Raf isoforms, and it is clear that A-Raf, B-Raf and c-Raf function differently despite their relatively conserved structure.

B-Raf, which is the strongest ERK pathway activator of the Raf family, has most recently generated interest due to the discovery of common oncogenic mutations in tumours, such as V600E (54). This mutation mimics phosphorylation of an activating site within the protein, resulting in a constitutively active form of B-Raf and persistent ERK pathway signaling (65). Prior to this discovery, however, c-Raf, the 70kDa isoform also known as Raf-1, was the primary isoform under investigation and thus still remains one of the best characterized Raf kinases (54).

1.3.2. ERK signaling pathway

As previously mentioned, Raf is an important component of the ERK signaling pathway (Figure 1.5). Overall, the ERK pathway has been reported to regulate numerous cellular processes, including cell survival, differentiation, proliferation, motility, transcription and metabolism (54,55). To summarize signaling within the pathway, cell membrane embedded receptor tyrosine kinases (RTKs) are first activated by extracellular ligands and autophosphorylation occurs on the intracellular domains of the receptors (54). The guanine nucleotide exchange factor SOS (son of sevenless) and the adaptor protein Grb2 (adaptor protein growth factor receptor-bound protein 2) are subsequently recruited to the phosphorylated intracellular domains of the receptor (54). SOS activates the cell membrane-linked protein Ras by exchanging its guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (54). Activated Ras initiates a cascade of phosphorylation events where Ras activates Raf, which promotes the activation of MEK1/2 (mitogen-activated protein kinase kinase 1/2), which finally activates

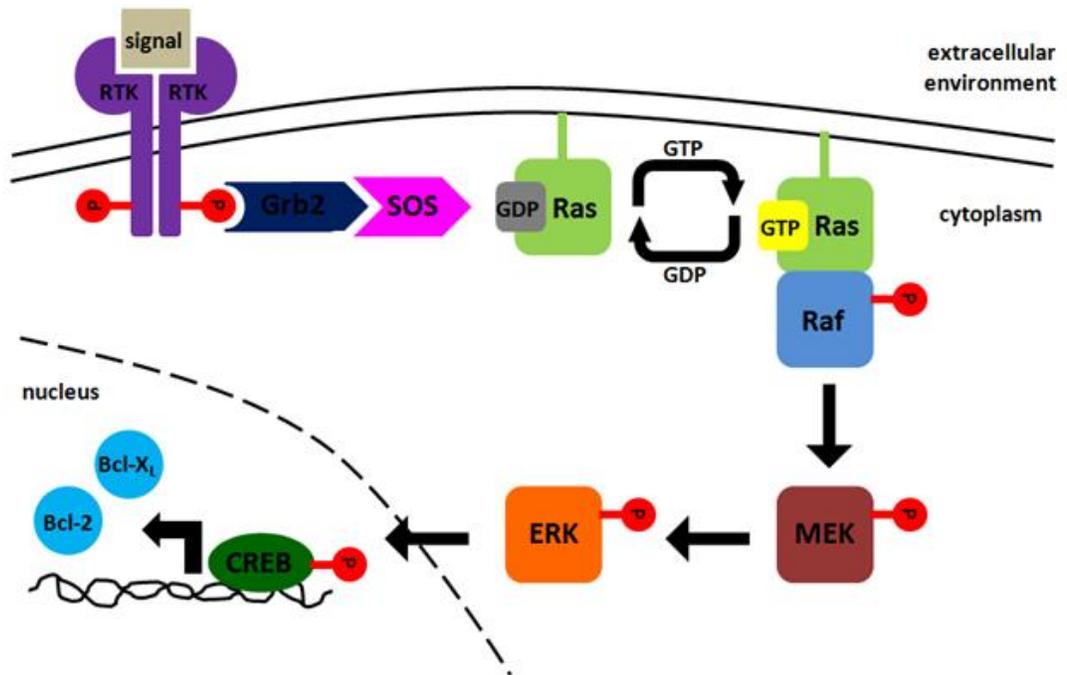


Figure 1.5. Summary of ERK pathway signaling. Extracellular signals promote RTK activation and autophosphorylation. Intracellular Grb2 and SOS are recruited to the phosphorylated receptor, and subsequently promote the exchange of GDP for GTP on membrane-bound Ras. Activated Ras initiates a cascade of activating phosphorylation events involving Raf, MEK and ERK, respectively. Activated ERK has countless substrates in both the cytoplasm and nucleus, including the transcription factor CREB which, when activated, induces transcription of anti-apoptotic factors Bcl-2 and Bcl-XL.

ERK1/2 (54). Phosphorylated active ERK1/2 has over 150 reported targets in the cell, both nuclear and cytoplasmic (54,66).

For example, one of the outcomes of ERK1/2 phosphorylation is the activation of the transcription factor CREB (cAMP response element-binding protein) and the subsequent increase in transcription of certain anti-apoptotic factors, such as Bcl-2 and Bcl-XL (67). Other well-known targets of ERK1/2 include the transcription factors Elk1 (ETS domain-containing protein) and c-Fos (cellular FBJ murine osteosarcoma viral oncogene homolog), the kinases DAPK (death associated protein kinase) and MSK1/2 (mitogen- and stress-activated protein kinases 1/2) and the cytoskeletal element paxillin (66).

Furthermore, a number of scaffolding proteins have been shown to interact with components of the ERK pathway to facilitate signaling. The best-characterized scaffolds include KSR1 (kinase suppressor of Ras 1) and the IQGAP (IQ motif containing GTPase-activating protein) family of proteins, although there are many other scaffolds that have been reported to localize ERK pathway signaling to various compartments within the cell (55).

1.3.3. MEK1/2-independent signaling by c-Raf

Although it has been argued that MEK1/2 is the only Raf substrate (54,58), there is also emerging evidence that Raf is able to regulate a number of signaling molecules independent of the ERK pathway. Given that B-Raf is the primary Raf isoform involved in MEK1/2 activation, it has been suggested that c-Raf and A-Raf have evolved other functions (54,68). For example, adenylyl cyclases (ACs) 2, 5 and 6 have been reported to be phosphorylated and activated by c-Raf (69,70). Given that PKA deactivates c-Raf, and PKA is indirectly activated by ACs, activation of ACs by c-Raf would appear to contribute to negative feedback regulation of c-Raf. Rb (retinoblastoma tumour suppressor protein) has also been shown to be a phosphorylation target of c-Raf, an event which leads to the inactivation of Rb and consequential cell cycle progression (71).

Although c-Raf is a well-characterized kinase, it also affects signaling of some proteins in a kinase-independent manner. For instance, the pro-apoptotic proteins ASK1 (apoptosis signal-regulating kinase 1) and MST2 (mammalian Ste20-like kinase 2) are negatively regulated through direct binding with c-Raf (72,73). Rok- α (Rho-binding kinase α) is also inhibited solely by c-Raf binding, a phenomenon that regulates cell motility and protects against apoptosis (74,75).

1.3.4. c-Raf activation and deactivation

Due to the aforementioned implications of deregulation of the ERK pathway, c-Raf activity is tightly controlled. Regulation of c-Raf activity is a complex process that involves a number of proteins and many phosphorylation and dephosphorylation events. In an inactive state, the N-terminus of c-Raf is folded over and stabilized by a 14-3-3 protein dimer in a conformation that masks the C-terminus (Figure 1.6A) (76). 14-3-3 specifically interacts with c-Raf on two phosphorylated residues, S259 and S621 (77). To activate c-Raf, S259 is dephosphorylated by phosphatases PP2A (protein phosphatase 2A) and PP1 (protein phosphatase 1) and 14-3-3 is released from this binding site (Figure 1.6B) (78). Conformational changes occur which displace the N-terminal regulatory domain of c-Raf from the C-terminal catalytic domain, thus revealing the primary and secondary Ras binding sites previously sequestered within c-Raf (Figure 1.6C) (79). Ras is allowed to bind c-Raf and kinases are recruited to phosphorylate activating sites on c-Raf (Figure 1.6D). These sites include several sites in the activation segment as well as S338, S339, Y340 and Y341 in the N-region (80,81). Phosphorylation of amino acids 338-341 is essential for full c-Raf activation and also for interaction with its substrate MEK1/2 (82). PAK1 (p21-activated kinase 1), JAK2 (Janus kinase 2), Src (sarcoma) and CK2 (casein kinase 2) have each been reported to phosphorylate a subset of these residues (76,83,84), although there are likely other kinases involved that have yet to be identified (54). A number of other phosphorylation sites have been reported to enhance c-Raf activity (54) and c-Raf heterodimerization with B-Raf has also

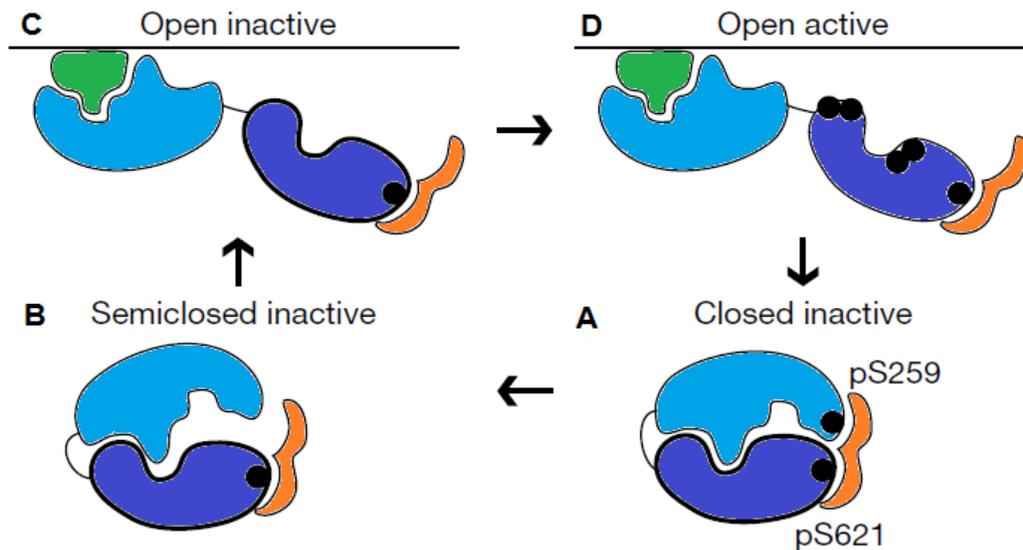


Figure 1.6. Summary of the activation cycle of c-Raf. (A) The N-terminal regulatory region of c-Raf (light blue) sequesters the C-terminal catalytic region (dark blue) in a closed inactive conformation stabilized by 14-3-3 (orange). The interaction between c-Raf and 14-3-3 is stabilized by the phosphorylated residues (black) S259 and S621 on c-Raf. (B) S259 is dephosphorylated and 14-3-3 is released from the c-Raf N-terminus, creating a semiclosed inactive conformation. (C) c-Raf adapts an open inactive conformation, where the N-terminus unmask the C-terminus and binds membrane-bound Ras (green). (D) The C-terminus of c-Raf is phosphorylated on a number of residues, leading to an open active form of the protein. Adapted from (79).

been proposed to increase kinase activity compared to monomeric or homodimeric versions of either protein (85).

During c-Raf deactivation, the phosphorylated N-region serves as a binding site for RKIP (Raf kinase inhibitor protein) (86), which dissociates MEK1/2 from c-Raf (87). PP5 (protein phosphatase 5) binds c-Raf and promotes the dephosphorylation of S338 (88) while PP2A dephosphorylates other activating sites (89). PKA (protein kinase A) has also been reported to contribute to c-Raf deactivation, phosphorylating S43 and S233, which interfere with Ras binding, as well as S259, which interferes with Ras binding and contributes to 14-3-3 binding (90,91). Altogether, these events return c-Raf to its inactive state, stabilized in a closed conformation by 14-3-3.

1.3.5. Regulation of c-Raf stability

In addition to the abundance of phosphorylation and dephosphorylation events that regulate c-Raf activity, there are also some systems known to regulate c-Raf stability and overall c-Raf levels within the cell. One well-known regulator of c-Raf stability is the chaperone protein Hsp90 (heat shock protein 90). Hsp90 is a highly conserved molecular chaperone that mediates the folding of newly synthesized or misfolded client proteins, assembles and disassembles molecular complexes and prevents protein aggregation (92). Hsp90 does not perform these tasks alone, however, as it has been shown to form complexes with over 20 co-chaperones (92).

Although Hsp90 generally functions to help rescue client proteins, it has also been shown to form a complex with Hsp70 (heat shock protein 70), another molecular chaperone, and CHIP (C-terminus of Hsp70-interacting protein), an E3 ubiquitin ligase, to target terminally misfolded proteins for ubiquitination and degradation by the proteasome (92,93). Hsp90 has been shown to bind and stabilize the tertiary structure of c-Raf, allowing it to localize to the membrane, interact with Ras and properly engage in ERK pathway signaling (94-96). Disruption of binding between the two proteins results in proteasomal

degradation of c-Raf (97). There has been evidence that CHIP is able to ubiquitinate c-Raf, suggesting that CHIP is an E3 ubiquitin ligase responsible for proteasomal degradation of the kinase (98,99). XIAP (X-linked inhibitor of apoptosis), a member of the IAP (inhibitor of apoptosis) family of proteins, has been shown to be a modulator of CHIP-mediated c-Raf degradation. Although XIAP itself is an E3 ubiquitin ligase, evidence suggests that XIAP interferes with c-Raf stability and promotes recruitment of CHIP to Hsp90 and c-Raf, independently of its ubiquitin ligase activity (99).

There has also been evidence of c-Raf degradation by mechanisms that do not rely on CHIP. It has been reported that autophosphorylation of S621 is necessary for c-Raf stabilization, as kinase-dead mutants were ubiquitinated and targeted to the proteasome (100). This occurred even when CHIP levels were knocked-down by siRNA, suggesting that other E3 ubiquitin ligases may also play a role in c-Raf downregulation (100). c-Raf has also been shown to be ubiquitinated and degraded by the proteasome in response to disruption of cell adhesion and treatment with the oxidative glucose metabolite methylglyoxal, however the mechanisms by which these events occurred were not determined (101,102).

Studies conducted in our laboratory have shown that c-Raf is downregulated by RanBPM, as shRNA-mediated RanBPM knock-down led to increased c-Raf protein levels and re-expression of RanBPM reversed this effect (24). Downregulation was observed for both endogenous c-Raf and transfected constitutively active c-Raf, but the effect was more prominent on the latter (24). RanBPM shRNA knock-down also led to increased levels of Bcl-2, Bcl-XL, phosphorylated ERK1/2 and phosphorylated MEK1/2 and re-expression of RanBPM reversed these effects in multiple cell lines (24). This suggests that, through its effect on c-Raf, RanBPM is an inhibitor of the ERK pathway. These studies further demonstrated, via immunoprecipitation and pull-down assays, that RanBPM is found in a complex with endogenous c-Raf and can also form a complex with constitutively active c-Raf, consisting only of the catalytic region of the protein (24). RanBPM was also shown to disrupt c-Raf association with

Hsp90, providing insight on a potential mechanism by which c-Raf could be destabilized by RanBPM (24). However, further studies exploring this concept have yet to be conducted and other mechanisms could also contribute to c-Raf downregulation by RanBPM.

1.4. Hypothesis and objectives

Work in our laboratory has shown that RanBPM and c-Raf are found together in a complex and that RanBPM downregulates c-Raf at the protein level (24). However, how the two proteins interact and the mechanism by which RanBPM downregulates c-Raf remains unknown. Therefore, it is hypothesized that specific domains of RanBPM are required for direct interaction with c-Raf and regulation of c-Raf stability by a mechanism that could involve the CTLH complex. The work presented in this thesis aims to specifically address the following objectives:

- (1) Determine which domain(s) of RanBPM are required for regulation of c-Raf stability.
- (2) Identify which domain(s) of RanBPM are required for interaction with c-Raf.
- (3) Investigate the possibility of CTLH complex involvement in c-Raf downregulation.

It has become evident that investigating the key mechanisms that tightly regulate the activity and stability of the components of the ERK pathway, namely c-Raf, is critical in understanding the devastating consequences associated with the loss of regulation of this pathway and can contribute to the development of new therapies to combat cancer. Furthermore, in light of the recent identification of RanBPM as an activator of apoptosis, a better understanding of its effect on c-Raf will help elucidate its role as a critical tumour suppressor. Overall, the work presented in this thesis clarifies the relationship between two important proteins

that play critical roles in cell growth, differentiation, apoptosis and cancer development.

Chapter 2 – Materials and Methods

2.1. Chemicals and reagents

All enzymes and buffers used for cloning were obtained from either New England Biolabs Inc. (Ipswich, MA, USA) or Fermentas Thermo Fisher Scientific Inc. (Waltham, MA, USA) and were used according to the manufacturer's protocol. Hydrochloric acid (HCl) was also acquired from Thermo Fisher Scientific Inc. Fetal bovine serum (FBS), trypsin, L-glutamine, sodium pyruvate, paraformaldehyde (PFA), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), hydroxyethyl piperazineethanesulfonic acid (HEPES), sodium dodecyl sulfate (SDS), Tris-hydroxymethyl amino methane (Tris) and potassium chloride (KCl) were purchased from Wisent Inc. (St. Bruno, QC, Canada), while Dulbecco's modified eagle medium (DMEM) and phosphate buffered saline (PBS) were purchased from both Gibco by Life Technologies Inc. (Burlington, ON, Canada) and Wisent Inc. G418 sulphate, Triton X-100, Nonidet P-40 (NP40), aprotinin, leupeptin, pepstatin, dithiothreitol (DTT), sodium fluoride (NaF), sodium orthovanadate (NaVO₄), pheylmethylsulfonyl fluoride (PMSF) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from BioShop Inc. (Burlington, ON, Canada) while glycerol was acquired from Caledon Laboratory Chemicals Ltd. (Georgetown, ON, Canada).

2.2. Antibodies

Primary antibodies used were RanBPM (K-12, sc-46253 and F-1, sc-271727, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), c-Raf (E-10, sc-7267 and C-12, sc-133, Santa Cruz Biotechnology Inc.), β-actin (I-19, sc-1616-R, Santa Cruz Biotechnology Inc.), human influenza hemagglutinin (HA) (HA-7, H3663, Sigma-Aldrich Inc., Oakville, ON, Canada), glutathione-S-transferase (GST) (B-14, sc-138, Santa Cruz Biotechnology Inc.), Hsp90 α/β (H-114, sc-7947, Santa Cruz Biotechnology Inc.) and MAEA (ab65239, Abcam Inc.,

Cambridge, MA, USA). For each application, primary antibodies were used in the concentrations indicated in Table 2.1.

Secondary antibodies used for Western blot analyses were Peroxidase-conjugated AffiniPure Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and Blotting Grade Goat anti-Rabbit IgG (H+L) (Human IgG Adsorbed) Horseradish Peroxidase Conjugate (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Both were used at a concentration of 1:5000.

2.3. Plasmid constructs

pCMV-HA-RanBPM was a gift from Dr. Mark Nelson (University of Arizona, Tucson, AZ, USA) and was rendered resistant to shRNA degradation via the introduction of two silent point mutations as described in (18). RanBPM deletion mutants pCMV-HA-RanBPM- Δ N2, pCMV-HA-RanBPM- Δ C4, pCMV-HA-RanBPM- Δ C1, pCMV-HA-RanBPM- Δ 212, and pCMV-HA-RanBPM- Δ 360 were generated as described in (18,103). pEBG-GST- Δ N-c-Raf was a gift from Dr. Zhijun Luo (Boston University, Boston, MA, USA).

pET28a- Δ N-c-Raf was generated by isolating a fragment encoding Δ N c-Raf from pEBG-GST- Δ N-c-Raf using BamHI and NotI, and ligating into the bacterial expression vector pET28a (EMD Millipore, Billerica, MA, USA) using T4 DNA ligase. pGEX4T1-GST-WT-RanBPM, pGEX4T1-GST-N2-domain and pGEX4T1-GST-C1-domain were generated by polymerase chain reaction (PCR) amplifying full-length WT RanBPM, RanBPM amino acids 1–102 or RanBPM amino acids 649–729, respectively, from pCMV-HA-RanBPM. PCR was performed using KOD Hot Start Polymerase PCR kit (EMD Millipore) according to the manufacturer's protocol. Primers used for PCR are outlined in Table 2.2. PCR products were subsequently digested with BamHI and Sall-HF and were each ligated into the bacterial expression vector pGEX-4T-1 (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) using T4 DNA ligase.

Table 2.1. Concentrations of primary antibodies used for Western blot and *in situ* proximity ligation assay.

Antibody	Species	Western blot concentration	<i>In situ</i> proximity ligation assay concentration
RanBPM (K-12)	goat	N/A	1:400
RanBPM (F-1)	mouse	N/A	1:50
c-Raf (E-10)	mouse	1:500	1:50
c-Raf (C-12)	rabbit	1:500	N/A
β-actin (I-19)	rabbit	1:2000	N/A
HA (HA-7)	mouse	1:1000	N/A
GST (B-14)	mouse	1:500	N/A
Hsp90 α/β (H-114)	rabbit	N/A	1:100
MAEA (ab65239)	rabbit	N/A	1:200

Table 2.2. PCR primer sequences and descriptions.

Primer	T_m	Sequence (5' to 3')	Description
RBPMfwdBamHI	70.1°C	GCTAGGATCCATGTCCGGGCAGCCGCCG	forward PCR primer to amplify WT RanBPM and N2 domain
RBPMrevSall	65.6°C	CGCGGTACGTCGACTAATGTAGGTAGTCTTCC	reverse PCR primer to amplify WT RanBPM and C1 domain
N2domrevSall	68.8°C	GTATGTCGACTACCCGCTGGCGGGGGC	reverse PCR primer to amplify N2 domain
C1domfwdBamHI	64.3°C	CGATGGATCCAAGGATGCATTTCAGTCTACTAGC	forward PCR primer to amplify C1 domain

2.4. Stable shRNA cell lines and cell culture

HeLa cells were obtained from the American Type Culture Collection (Manassus, VA, USA). HeLa cell lines stably expressing either RanBPM shRNA (clone 2-7) or control shRNA were generated as described in (18). HeLa 2-7 cells and HeLa control cells were cultured in DMEM supplemented with 8% FBS, 1% sodium pyruvate, 1% L-glutamine, 4.5g/L glucose and 0.35g/L G418 sulphate at 37°C in 5% CO₂. Cells were washed with PBS and detached with trypsin upon passaging.

2.5. *In situ* proximity ligation assay

To prepare for the Duolink II *in situ* proximity ligation assay (PLA) (Sigma-Aldrich Inc.), cover slips were pre-treated by outlining with the hydrophobic ImmEdge pen (Vector Laboratories Inc., Burlingame, CA, USA). HeLa cells were seeded on these cover slips at approximately 50,000 cells per cover slip, fixed with 4% PFA for 13 minutes at 4°C, permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature, and blocked for 1 hour with 5% FBS in PBS at room temperature. Cover slips were incubated in the appropriate primary antibodies at the concentrations indicated in Table 2.1 overnight at 4°C. Manufacturer's instructions were followed for the *in situ* PLA. Cover slips were mounted onto glass slides with Prolong Gold antifade reagent with DAPI (Molecular Probes by Life Technologies, Burlington, ON, Canada) and were subsequently analyzed at 358nm (nucleus) and either 555nm or 647nm (fluorescent oligonucleotide probe) with an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA, USA) using a 40x objective. Images were captured using Image-Pro Plus v4.5 software (Media Cybernetics Inc., Bethesda, MD, USA).

2.6. Transfection assays

ExGen 500 *in vitro* Transfection Reagent (Fermentas Thermo Fisher Scientific Inc.), TurboFect Transfection Reagent (Thermo Fisher Scientific Inc.) and JetPRIME Transfection Reagent (PolyPlus Transfection, Illkirch, France) have all been used according to the manufacturer's instructions for transfection of HeLa cells. For each pCMV-HA RanBPM deletion mutant, the amount of construct transfected was adjusted to account for variations in stability between the expressed proteins. In all cases, the amount of DNA used was brought up to the manufacturer's recommendations with the vector pBS-SK (Agilent Technologies, Santa Clara, CA, USA). Transfected cells were incubated 24–48 hours at 37°C in 5% carbon dioxide (CO₂).

2.7. Preparation of mammalian cell extracts

HeLa cells were scraped in cold PBS, centrifuged at 8000rpm for 3 minutes, lysed for 40 minutes on ice in whole cell extract (WCE) buffer (150mM NaCl, 1mM EDTA, 50mM HEPES pH 7.4 and 10% glycerol) and supplemented with 0.5% NP40, 0.5% Triton X-100, 10µg/mL aprotinin, 2µg/mL leupeptin, 2.5µg/mL pepstatin, 1mM DTT, 2mM NaF, 2mM NaVO₄, and 0.1mM PMSF. The lysate was centrifuged at 13,000rpm for 20 minutes at 4°C and the resulting supernatant was collected.

2.8. Bacterial protein expression and preparation of *Escherichia coli* extracts

For each bacterial expression construct, plasmids were transformed into *E. coli* strain BL21DE3. Single transformants were selected and grown in Luria Bertani (LB) medium overnight at 37°C. This culture was diluted 1:150 into fresh LB medium and grown to an optical density at 600nm (OD₆₀₀) between 0.4–0.5. Protein expression was induced with 0.1mM IPTG and the culture was incubated

overnight at 16°C. Bacteria was centrifuged at 4000rpm for 20 minutes at 4°C and subsequently resuspended in lysis buffer (25mM HEPES pH 7.4, 10mM KCl, 2mM EDTA and 20% glycerol) supplemented with 0.1% NP40, 10µg/mL aprotinin, 2µg/mL leupeptin, 2.5µg/mL pepstatin, 1mM DTT, 2mM NaF, 2mM NaVO₄, and 0.1mM PMSF. The cell suspension was sonicated three times for 10 seconds on ice using the Sonic Dismembrator Model 100 (Thermo Fisher Scientific Inc.), centrifuged at 13,000rpm for 10 minutes at 4°C and the resulting supernatant was collected.

2.9. Western blot analyses

Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on either 8% or 10% acrylamide gels and subsequently transferred for either for 1 hour at 100V or overnight at 25V onto a polyvinylidene fluoride (PVDF) membrane. Blots were blocked in 5% non-fat dry milk for at least 1 hour at room temperature, then incubated in primary antibody diluted in 5% non-fat dry milk as indicated in Table 2.1 overnight at 4°C or for 1 hour at room temperature. Blots were incubated in secondary antibody diluted in 5% non-fat dry milk for 1 hour at room temperature and developed using either Western Lightning Enhanced Chemiluminescence (ECL) Substrate (Perkin Elmer Inc., Waltham, MA, USA) or Clarity Western ECL Substrate (Bio-Rad Laboratories Inc.). Images were captured using either Kodak X-OMAR LS film (Carestream Health Inc., Rochester, NY, USA) or the ChemiDoc MP (Bio-Rad Laboratories Inc.) and Image Lab software (Bio-Rad Laboratories Inc.).

2.10. GST pull-down assays

2.10.1. Using HeLa cell extracts

Extracts were quantified and 1800µg total protein was aliquoted for each pull-down sample. Extracts were brought up to 1mL with WCE buffer to a final

concentration of 0.4% NP40, 0.4% Triton X-100, 20µg/mL aprotinin, 4µg/mL leupeptin, 5µg/mL pepstatin, 2mM DTT, 4mM NaF, 4mM NaVO₄, and 0.2mM PMSF. Glutathione-Agarose beads (Sigma-Aldrich Inc.) suspended in PBS were added to each sample to a final concentration of 5µL beads/100µg total protein and pull-down samples were incubated overnight at 4°C. Beads were subsequently washed three times in WCE buffer supplemented with 0.4% NP40, 0.4% Triton X-100, 1mM DTT and 0.1mM PMSF. Beads were resuspended in SDS loading dye (0.105g/mL SDS, 0.093g/mL DTT, 0.35M Tris HCl pH 6.8 and 30% glycerol), boiled for 5 minutes and centrifuged at 10,000rpm for 10 seconds. The resulting supernatant was collected and analyzed by Western blot.

2.10.2. Using *E. coli* extracts

Extracts were quantified and, for each GST-tagged construct, approximately 400µg total protein was used for each pull-down sample. Samples were brought up to 200µL with lysis buffer and subsequently brought up to 600µL with binding buffer (15mM HEPES pH 7.4, 6mM KCl, 1.2mM EDTA and 12% glycerol) to a final concentration of 0.6% NP40, 0.6% Triton X-100, 10µg/mL aprotinin, 2µg/mL leupeptin, 2.5µg/mL pepstatin, 1mM DTT, 2mM NaF, 2mM NaVO₄, and 0.1mM PMSF. Samples were incubated for 2 hours at 4°C with 30µL Glutathione-Agarose beads. Beads were washed three times with binding buffer supplemented with 0.6% NP40, 0.6% Triton X-100, 1mM DTT and 0.1mM PMSF. Each sample was then incubated with 200µg ΔN c-Raf extract and brought up to 800µL with binding buffer to a final concentration of 0.6% NP40, 0.6% Triton X-100, 10µg/mL aprotinin, 2µg/mL leupeptin, 2.5µg/mL pepstatin, 1mM DTT, 2mM NaF, 2mM NaVO₄, and 0.1mM PMSF. Samples were again incubated for 2 hours at 4°C and beads were washed with binding buffer supplemented with 0.6% NP40, 0.6% Triton X-100, 1mM DTT and 0.1mM PMSF. Beads were resuspended in SDS loading dye, boiled for 5 minutes and centrifuged at 10,000rpm for 10 seconds. The resulting supernatant was collected and analyzed by Western blot.

2.11. Statistical analyses

Using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA), one-way analysis of variance (ANOVA) was performed to compare multiple groups and two-sample t-test assuming unequal variance was performed to compare pairs of groups. Graphed data are presented as mean \pm standard error of the mean (SEM) and are determined to be significant when $p < 0.05$. The number of independent replicates for each experiment is denoted as N.

Chapter 3 – Results

3.1. Endogenous RanBPM and c-Raf are found in a complex

Previous co-immunoprecipitation and pull-down experiments in our laboratory have demonstrated that RanBPM and c-Raf exist together in a complex (24). Since this was shown using ectopically expressed protein constructs, we sought to confirm that the complex occurs with the endogenous proteins in cells. The *in situ* PLA, an assay that allows visualization of protein-protein interactions in cells using fluorescence microscopy (Figure 3.1) (104), was thus used to visualize the interaction between endogenous RanBPM and c-Raf in HeLa cells.

To summarize the PLA, fixed and permeabilized cells are incubated with primary antibodies against two proteins of interest. Two types of specialized secondary antibodies fused to short DNA strands, called PLA probes, are then incubated with the cells to bind their respective primary antibodies. When two different PLA probes come within 40nm of each other, the DNA strands are ligated together and amplified by a polymerase in a process termed rolling-circle amplification. The amplified DNA product is then hybridized with a fluorescently labeled complementary oligonucleotide probe, which is then visualized by fluorescence microscopy. Each fluorescent dot seen represents a protein-protein interaction between the two proteins of interest.

Stable HeLa cell lines expressing either a control or RanBPM shRNA (clone 2-7), which were previously generated in our laboratory (18), were used in this experiment. To ensure the PLA probes did not confer any background signal, a control was performed where HeLa control cells were incubated without primary antibodies. To gauge the specificity of the RanBPM primary antibody, another control was included where HeLa 2-7 cells were incubated with primary antibodies against RanBPM and c-Raf. As expected, fluorescent dots representing interactions were not observed in either negative control (Figures 3.2A and 3.2B).

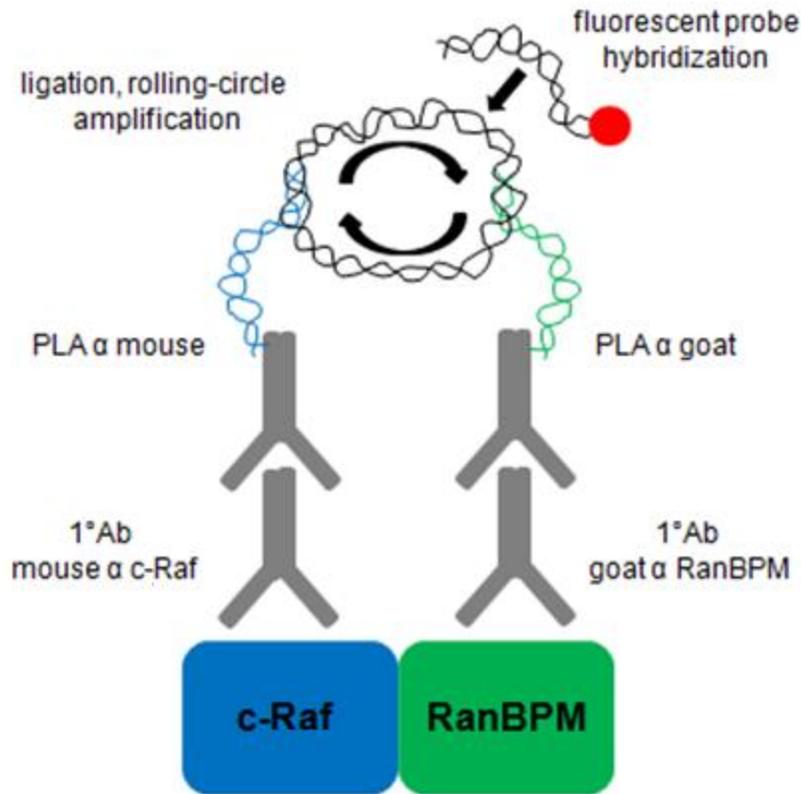


Figure 3.1. Summary of the *in situ* PLA. Primary antibodies against two proteins of interest are incubated with fixed and permeabilized cells. Secondary antibodies attached to short DNA strands, called PLA probes, are incubated with the cells and allowed to bind the primary antibodies. When two PLA probes come within 40nm of each other, ligation with special oligonucleotides anneal the two PLA probe DNA strands and allow them to form a circle. This circle of DNA is amplified using a polymerase, and this amplified product is hybridized with a fluorescently labeled oligonucleotide probe. Each protein-protein interaction is represented as a fluorescent dot which can be visualized by fluorescence microscopy.

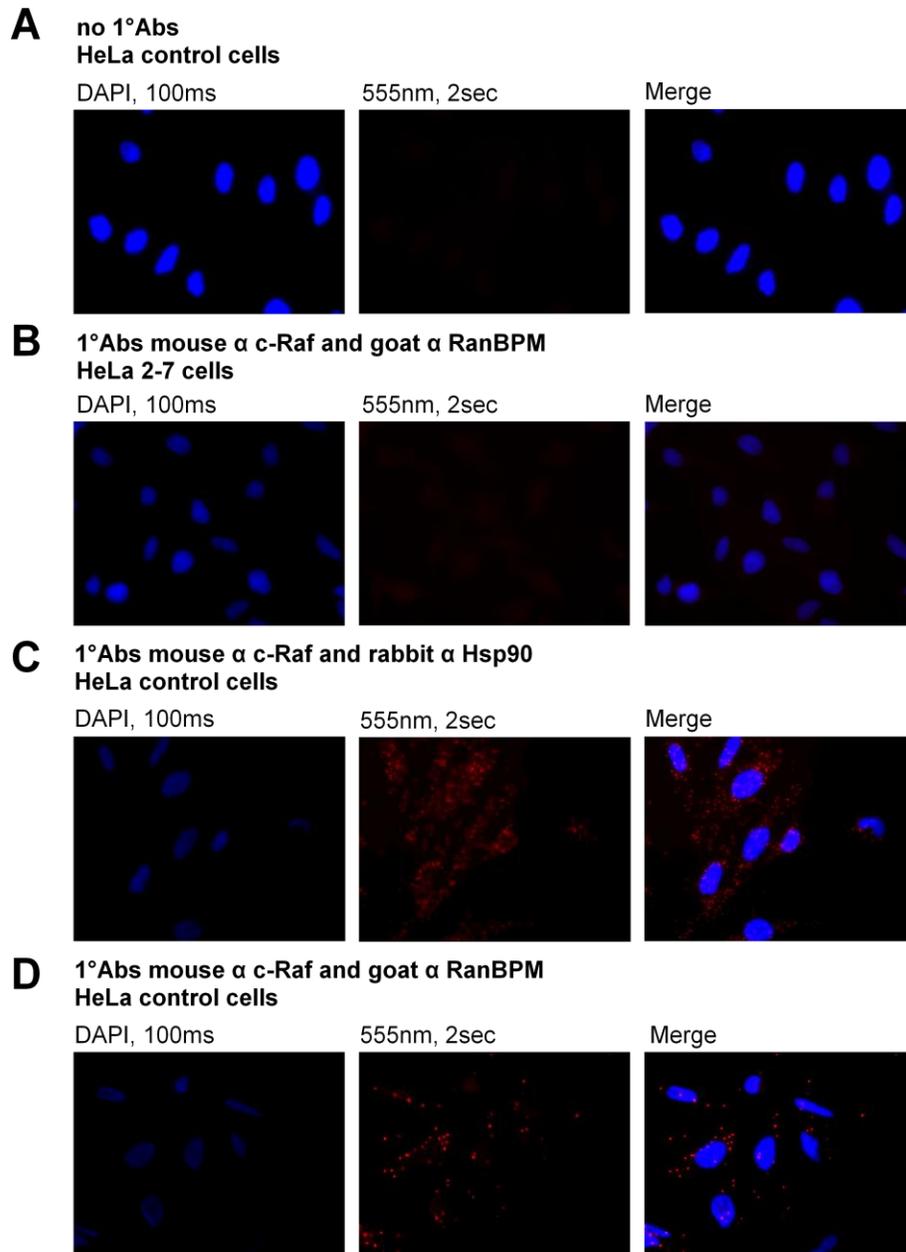


Figure 3.2. Endogenous RanBPM and c-Raf are found in a complex. An *in situ* PLA was performed in **(A)** HeLa control cells, without the addition of primary antibodies (negative control); **(B)** HeLa 2-7 cells, using primary antibodies against c-Raf and RanBPM (negative control); **(C)** HeLa control cells, using primary antibodies against Hsp90 and RanBPM (positive control); **(D)** HeLa control cells, primary antibodies against using c-Raf and RanBPM. DAPI staining was used to visualize the nuclei at 358nm, while the PLA dots representing protein-protein interactions were visualized at 555nm.

Given that c-Raf is known to interact with Hsp90 (96), a positive control was included where HeLa control cells were incubated with antibodies against c-Raf and Hsp90. This did expectedly produce fluorescent dots representing interactions (Figure 3.2C). In HeLa control cells in which antibodies against c-Raf and RanBPM were included for the assay, fluorescent dots were observed, confirming that the two endogenously expressed proteins are found together in a complex (Figure 3.2D).

3.2. The N-terminus, CRA and Lish/CTLH domains of RanBPM are required for c-Raf downregulation

Previous experiments in our laboratory have also demonstrated that RanBPM downregulates c-Raf at the protein level (24). Given that RanBPM contains a number of conserved domains, we strove to determine which regions of RanBPM were necessary for downregulation of c-Raf. In order to achieve this, we used a series of RanBPM deletion mutant constructs (Figure 3.3), which have been cloned into the mammalian expression vector pCMV-HA (18,103), to test their effects on levels of Δ N c-Raf. Δ N c-Raf, a constitutively active construct of c-Raf containing only amino acids 325-648 (82), was used instead of full-length c-Raf because RanBPM has been shown to have a greater effect on activated c-Raf (24). By using this construct, we allowed ourselves to observe the most pronounced effect of each RanBPM deletion mutant on c-Raf levels, enabling us to better discern which mutants have lost their ability to downregulate c-Raf. Furthermore, since RanBPM is able to dimerize (13), we opted to perform our experiments in HeLa 2-7 cells to avoid dimerization between mutant RanBPM and endogenous WT RanBPM monomers. Thus, in our system, HeLa 2-7 cells were transfected with pEBG-GST- Δ N-c-Raf as well as either pCMV-HA vector, pCMV-HA-RanBPM or a pCMV-HA RanBPM deletion mutant. Cell extracts were prepared and the effect of each RanBPM mutant on levels of c-Raf was analyzed by Western blot.

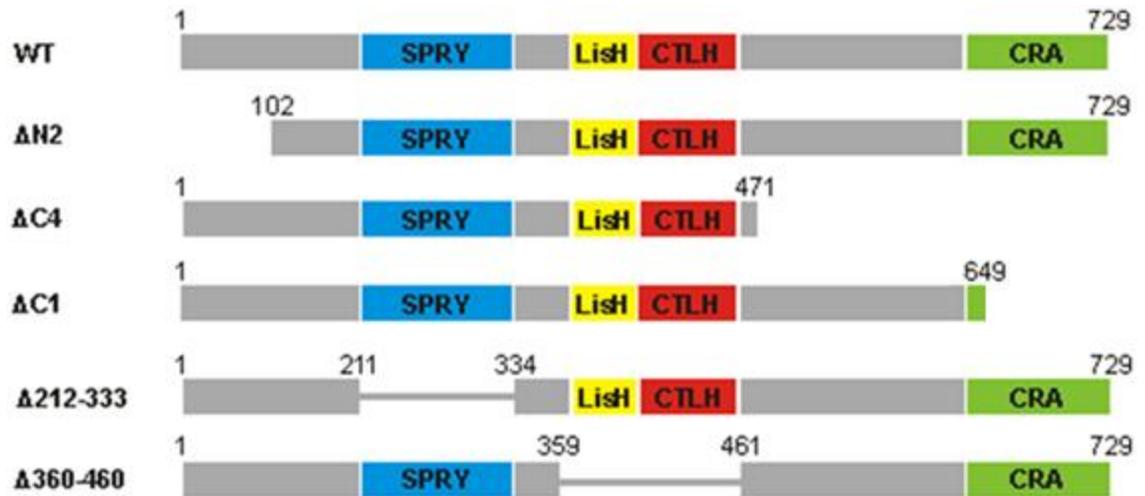


Figure 3.3. Full-length RanBPM and deletion mutants chosen for analysis. WT, Δ N2, Δ C4, Δ C1, Δ 212, and Δ 360 RanBPM constructs were cloned into the mammalian expression vector pCMV-HA.

As previously reported (24), WT RanBPM was able to significantly downregulate Δ N c-Raf compared to the levels of Δ N c-Raf seen in the pCMV-HA control, resulting in a 2.08 ± 0.44 fold decrease of Δ N c-Raf expression (Figures 3.4A-D). Furthermore, Δ 212 RanBPM was also able to downregulate Δ N c-Raf compared to the levels of Δ N c-Raf seen in the pCMV-HA control, demonstrating a significant 1.58 ± 0.45 fold decrease of Δ N c-Raf expression (Figures 3.4A and 3.4C). Although it would appear from the representative image that Δ 212 RanBPM was able to downregulate Δ N c-Raf better than WT RanBPM, Δ 212 RanBPM actually demonstrated better expression in this experiment and therefore more protein was likely available to exert its effect on Δ N c-Raf (Figure 3.4A). Overall, the effects that Δ 212 RanBPM and WT RanBPM demonstrated on Δ N c-Raf were not significantly different from one another (Figure 3.4C), indicating that the SPRY domain is not required for c-Raf destabilization.

Δ N2 RanBPM, however, was unable to effectively downregulate Δ N c-Raf and resulted in a significant 3.14 ± 0.60 fold increase when compared to the levels of Δ N c-Raf seen in response to WT RanBPM (Figures 3.4A and 3.4C). Δ N c-Raf expression in response to this mutant was not significantly different than that observed in the pCMV-HA control (Figure 3.4A and 3.4C). The expression of Δ N2 RanBPM is consistently much lower than that of the other RanBPM deletion mutants, despite identical transfection conditions, and thus our laboratory has hypothesized that the protein is very unstable (16,18). Despite this phenomenon, levels of Δ N2 RanBPM near those of WT RanBPM were achieved in this experiment (Figure 3.4A) and therefore it is reasonable to conclude that Δ N2 RanBPM has lost its ability to downregulate Δ N c-Raf. This implies that the N-terminus of RanBPM is required for its effect on c-Raf.

Δ 360 and Δ C4 RanBPM also did not effectively downregulate Δ N c-Raf, as Δ N c-Raf expression levels were significantly higher than those seen in response to WT RanBPM, demonstrating 4.68 ± 1.15 and 3.34 ± 0.71 fold increases respectively, and were not significantly different than those seen in the pCMV-HA control (Figures 3.4A and 3.4C). This occurred despite the fact that expression of these mutants was remarkably higher than that of WT RanBPM.

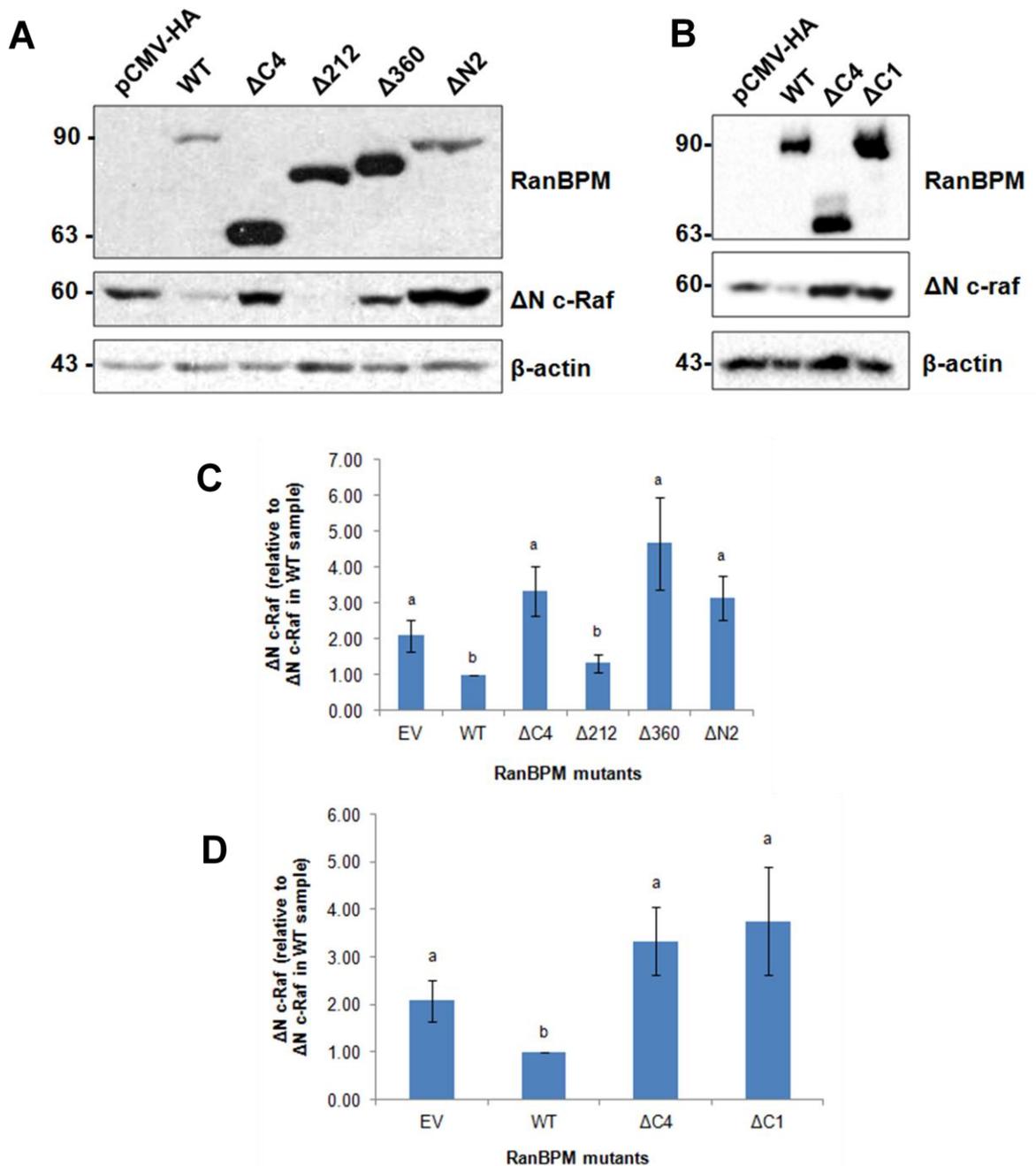


Figure 3.4. $\Delta 360$, $\Delta N2$, $\Delta C4$ and $\Delta C1$ RanBPM deletion mutants do not effectively downregulate ΔN c-Raf compared to WT RanBPM. HeLa 2-7 cells were transfected with pEBG-GST- ΔN -c-Raf and either pCMV-HA, pCMV-HA-RanBPM or pCMV-HA RanBPM deletion mutant constructs. Extracts were analyzed by Western blot. **(A,B)** Representative images are shown. **(C,D)** Multiple experiments were quantified by normalizing ΔN c-Raf levels to the loading control, β -actin, and statistical analyses were performed (N 4–25, SEM shown).

These results suggest that the LisH/CTLH domains and the C-terminus of RanBPM play a role in c-Raf downregulation. However, considering that the $\Delta C4$ deletion removes a very large portion of RanBPM, we decided to repeat the experiment using a construct harboring only a deletion of the CRA domain, namely the $\Delta C1$ RanBPM construct. $\Delta C1$ RanBPM behaved nearly identically to $\Delta C4$ RanBPM in its inability to downregulate ΔN c-Raf and resulted in a 3.76 ± 1.15 fold increase when compared to the levels of ΔN c-Raf seen in response to WT RanBPM, (Figures 3.4B and 3.4D). This implies that within the C-terminus of RanBPM, it is specifically the CRA domain that is needed for c-Raf downregulation.

Altogether, these results demonstrate that the N-terminus, LisH/CTLH and CRA domains are required for c-Raf destabilization, since loss of any of these regions render RanBPM unable to effectively downregulate c-Raf.

3.3. The CRA domain of RanBPM is required for interaction with c-Raf

To continue to characterize the interaction between RanBPM and c-Raf, we aimed to determine the domain or domains of RanBPM required for the interaction. To accomplish this, we used the same system outlined in section 3.2 and used the extracts to perform GST pull-down assays to test which RanBPM deletion mutants have retained their ability to interact with ΔN c-Raf.

Specifically, HeLa 2-7 cells were transfected with either pEBG-GST and pCMV-HA-RanBPM, or pEBG-GST- ΔN -c-Raf and either pCMV-HA-RanBPM or a pCMV-HA RanBPM deletion mutant. Due to its poor stability, $\Delta N2$ RanBPM was not among the mutants tested as we were unable to obtain sufficient levels of the protein to detect it in this type of assay. Cell extracts were prepared and Glutathione-Agarose beads were incubated with the resulting extracts to pull-down GST or GST- ΔN -c-Raf, as well as any RanBPM deletion mutant associated with it. Pull-down samples were analyzed by Western blot.

As anticipated based on previous studies (24), GST- ΔN -c-Raf was able to successfully pull-down WT RanBPM in the positive control, while, as expected for

the negative control, GST alone only pulled-down background levels of WT RanBPM (Figures 3.5A-C). $\Delta 212$ RanBPM and $\Delta 360$ RanBPM both retained their abilities to interact with ΔN c-Raf, as levels of pulled-down RanBPM were not significantly different than those of the positive control but were significantly higher than those of the negative control (Figures 3.5A-C). This indicates that the SPRY and LisH/CTLH domains are not required for the interaction between RanBPM and c-Raf, since deletion of these regions does not nullify the interaction.

However, $\Delta C1$ RanBPM was not able to effectively interact with ΔN c-Raf, as the amount of $\Delta C1$ RanBPM associating with ΔN c-Raf resulted in a significant 2.12 ± 0.19 fold decrease compared to the amount of WT RanBPM associating with ΔN c-Raf, but was not significantly different than the level of interaction seen in the negative control (Figures 3.5A and 3.5C). Altogether, this data suggests that the CRA domain is the only domain tested that appears to be required for the interaction between RanBPM and c-Raf, since deletion of this region abolishes the interaction.

3.4. RanBPM interacts directly with c-Raf through the CRA domain

Further tests were needed to confirm the interaction between the CRA domain of RanBPM and c-Raf. Also, the nature of the interaction, whether it be direct or mediated by another factor, remained to be determined. Therefore, to address this matter, we aimed to repeat pull-down experiments using bacterial extracts. By expressing our mammalian RanBPM and c-Raf constructs in *E. coli*, we ensured that no other mammalian proteins were present to mediate the interaction between our two proteins of interest. Thus, if the interaction was to persist in this system, it was assumed to be direct.

We opted to clone individual domains of RanBPM, as well as WT RanBPM, downstream of GST into the bacterial expression vector pGEX-4T-1 (Figure 3.6). In addition to testing the C1 domain, consisting only of the CRA domain, we took advantage of this relatively simple system to also test the N2

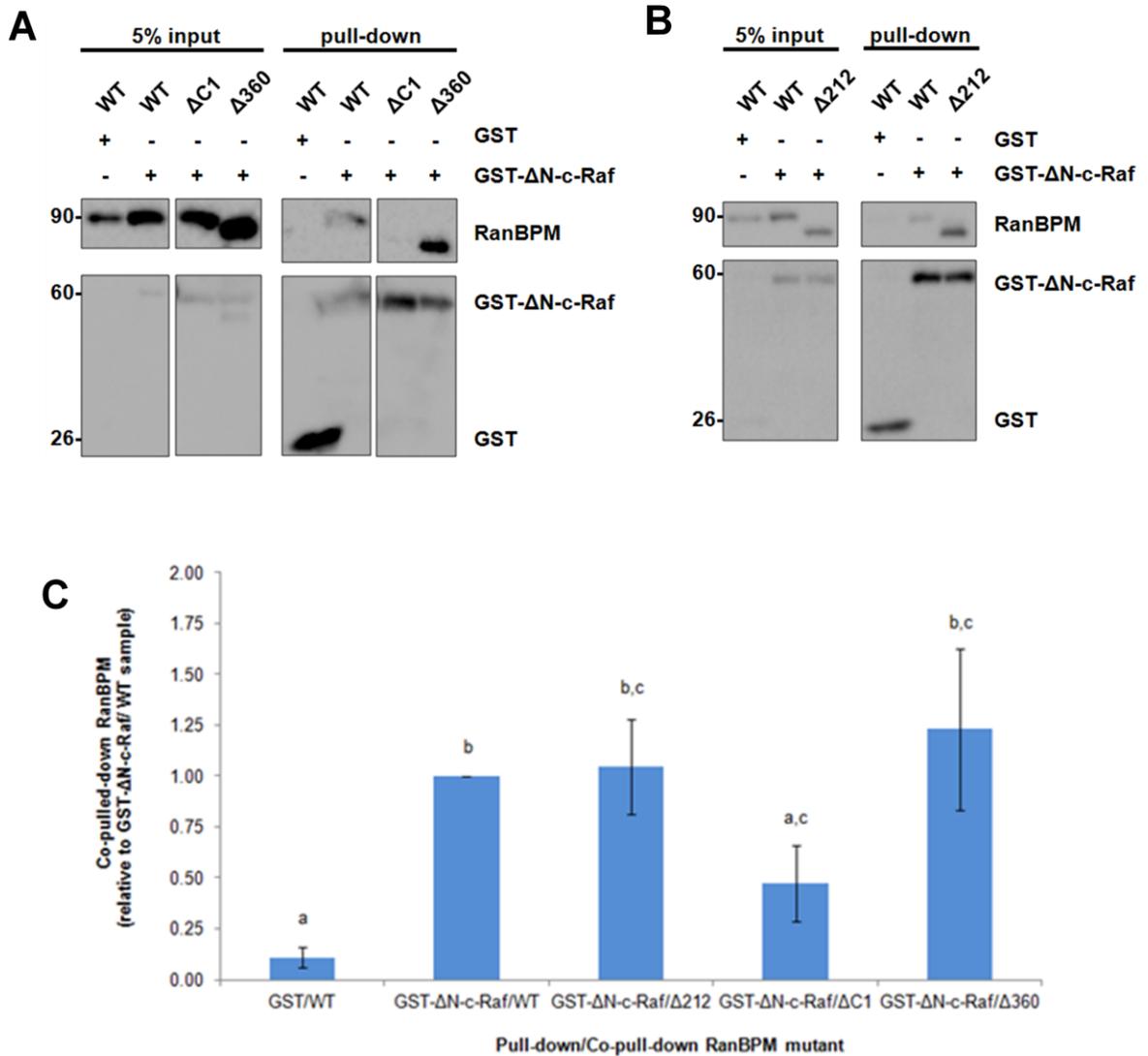


Figure 3.5. $\Delta C1$ RanBPM is unable to interact effectively with ΔN c-Raf. HeLa 2-7 cells were transfected with either pEBG-GST and pCMV-HA-RanBPM or pEBG-GST- ΔN -c-Raf and either pCMV-HA-RanBPM or a pCMV-HA RanBPM deletion mutant. A GST pull-down assay was performed on the resulting extracts and pull-downs were analyzed by Western blot. **(A,B)** Representative images are shown. **(C)** Multiple experiments were quantified by normalizing RanBPM mutant levels to pulled-down GST or GST- ΔN -c-Raf and statistical analyses were performed (N 4–9, SEM shown).



Figure 3.6. Full-length RanBPM and individual domains chosen for analysis. WT RanBPM, N2 domain and C1 domain were cloned into the bacterial expression vector pGEX-4T-1.

domain, which remained unexamined due to the poor level of expression of the Δ N2 RanBPM deletion mutant in previously conducted mammalian cell-based GST pull-down assays (Figure 3.5).

Each GST-tagged construct, as well as GST alone, was expressed separately in *E. coli* and purified using Glutathione-Agarose beads. These constructs were each subsequently incubated with a crude cell lysate from *E. coli* expressing Δ N c-Raf. The GST-tagged constructs were pulled-down and analyzed by Western blot to detect levels of associated Δ N c-Raf.

Both GST-WT-RanBPM and GST-C1 were able to pull-down Δ N c-Raf significantly above background levels pulled down by GST alone, demonstrating 5.02 ± 0.98 and 2.71 ± 0.36 fold increases, respectively (Figures 3.7A and 3.7B). Although it appears that WT RanBPM associates with Δ N c-Raf better than the C1 domain does, the variability within the levels of Δ N c-Raf pulled-down with WT RanBPM was relatively high and in fact the amount of Δ N c-Raf pulled-down with WT RanBPM is not significantly different than that pulled-down with the C1 domain (Figure 3.7B). Therefore, this result confirms that the CRA domain of RanBPM is able to interact with c-Raf and the interaction between RanBPM and c-Raf is direct.

GST-N2, unlike GST-WT-RanBPM and GST-C1, was unable to pull-down Δ N c-Raf significantly better than GST alone (Figures 3.7A and 3.7B). This suggests that the N-terminus of RanBPM is unable to directly interact with c-Raf, although an indirect interaction cannot be ruled out based on these results.

3.5. Endogenous c-Raf and MAEA are found in a complex

Since only the CRA domain of RanBPM is able to interact with Δ N c-Raf but multiple domains play a role in regulating its levels, it is likely that RanBPM is interacting with another protein or complex in order to downregulate Δ N c-Raf. A possible candidate is the CTLH complex, a potential E3 ubiquitin ligase complex which has been shown to include RanBPM and could function to target c-Raf for proteasomal degradation (46,47). For this reason, a PLA was performed to

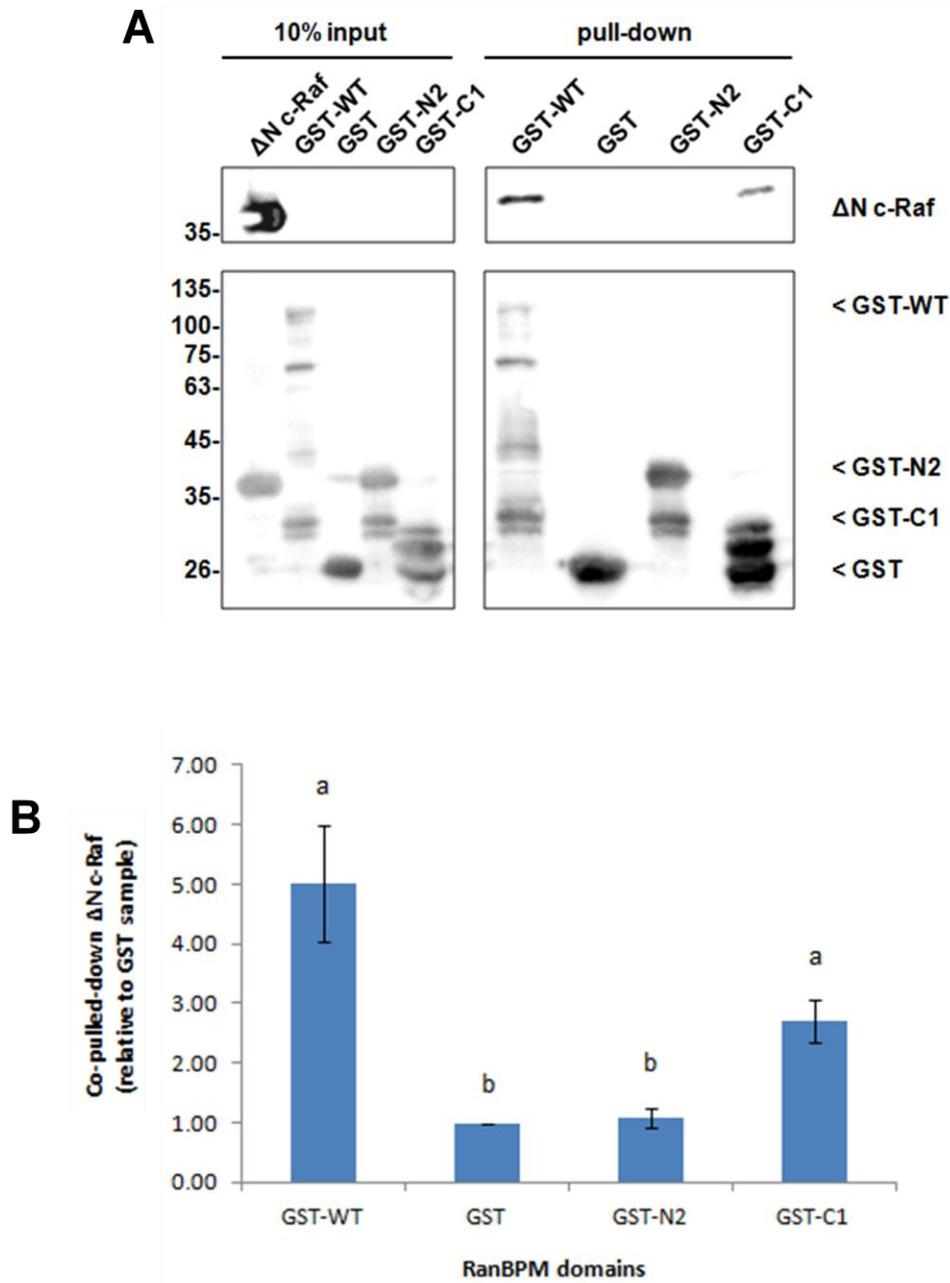


Figure 3.7. WT RanBPM and C1 domain interact directly with ΔN c-Raf. GST pull-down assays were performed using GST, GST-WT-RanBPM, GST-N2-domain and GST-C1-domain *E. coli* extracts as well as ΔN c-Raf *E. coli* extracts. Pull-downs were analyzed by Western blot. **(A)** A representative image is shown. **(B)** Multiple experiments were quantified by normalizing ΔN c-Raf levels to pulled-down GST, GST-WT-RanBPM, GST-N2 or GST-C1 and statistical analyses were performed (N 6, SEM shown).

investigate whether MAEA, a CTLH complex protein, is able to form a complex with c-Raf.

As expected, interactions were not seen in the negative control, namely HeLa cells in which primary antibodies were not added (Figure 3.8A). Since MAEA and RanBPM are known to be found together within the CTLH complex (42,46), a positive control was performed in HeLa cells using primary antibodies against MAEA and RanBPM. Interactions were indeed seen in this positive control (Figure 3.8B). In HeLa cells in which primary antibodies against MAEA and c-Raf were added, interactions were observed, although in noticeably fewer numbers than the positive control (Figure 3.8C). This evidence suggests that endogenous c-Raf does form a complex with MAEA and could associate with the CTLH complex as a whole.

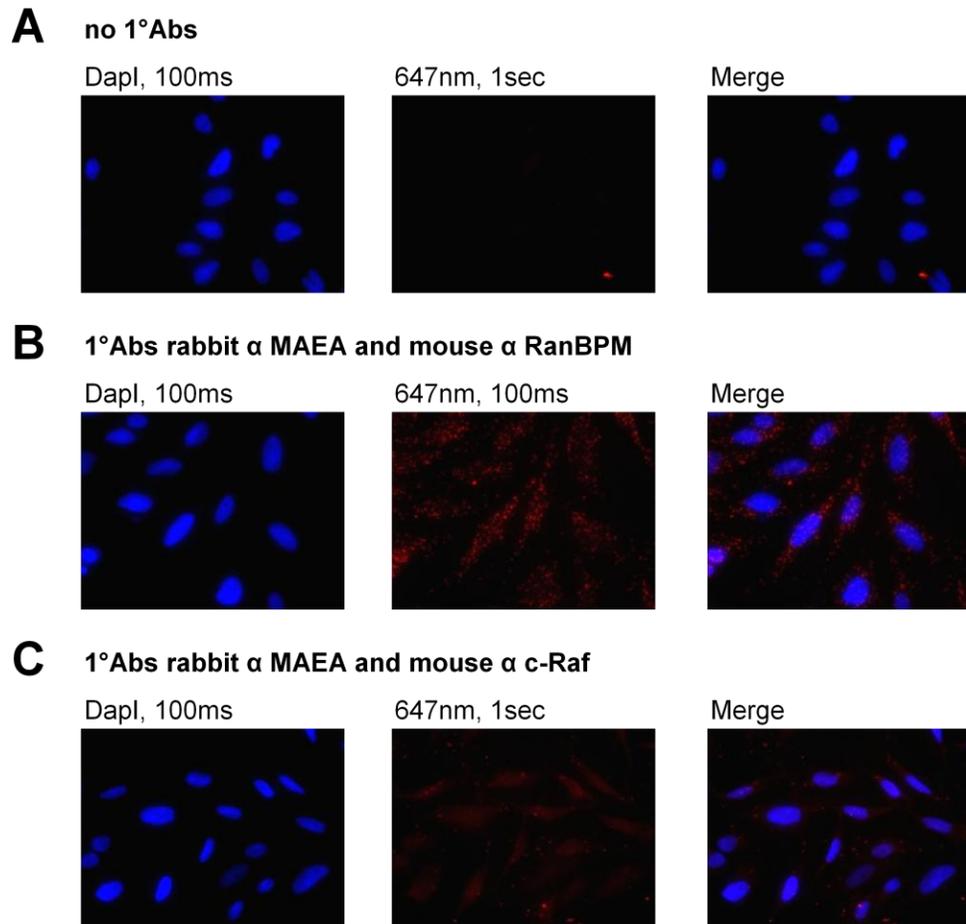


Figure 3.8. Endogenous MAEA and c-Raf are found in a complex. An *in situ* PLA was performed in HeLa cells **(A)** without the addition of primary antibodies (negative control), **(B)** using primary antibodies against MAEA and RanBPM (positive control) and **(C)** using primary antibodies against MAEA and c-Raf. DAPI staining was used to visualize the nuclei at 358nm, while the PLA dots representing protein-protein interactions were visualized at 647nm.

Chapter 4 – Discussion

4.1. Summary of findings

The aim of this study was to characterize the interaction between RanBPM and c-Raf. We hypothesized that specific domains of RanBPM are required for direct interaction with c-Raf and regulation of c-Raf stability by a mechanism involving the CTLH complex. Specifically, we sought to determine which domain(s) of RanBPM are required for regulation of c-Raf stability, identify which domain(s) of RanBPM are required for interaction with c-Raf and investigate the mechanism by which RanBPM downregulates c-Raf. In summary, we found that RanBPM and c-Raf in fact do form a complex in cells. The N-terminus, CRA domain and LisH/CTLH domains of RanBPM are required for downregulation of c-Raf but only the CRA domain is required for complex formation with c-Raf (Table 4.1). RanBPM interacts directly with c-Raf and the CRA domain is sufficient for this direct interaction to occur. Finally, the CTLH complex member MAEA and c-Raf are also found together in a complex, suggesting that c-Raf could associate not only with RanBPM and MAEA, but with the entire CTLH complex.

4.2. Model and rationale

Based on the results obtained from this study, we propose a mechanism by which RanBPM downregulates c-Raf (Figure 4.1). Since deletion of the CRA domain of RanBPM was sufficient to abolish the interaction between RanBPM and c-Raf and since the CRA domain alone was shown to be able to interact directly with c-Raf, it has become evident that RanBPM interacts directly with c-Raf through its CRA domain. Deletion of the CRA domain also prevented c-Raf downregulation, presumably as c-Raf was no longer tethered to the protein regulating its stability. Deletion of the LisH/CTLH domains also inhibited c-Raf downregulation, however RanBPM still retained its ability to interact with c-Raf.

Table 4.1. Summary of results of the effects of RanBPM constructs on downregulation of Δ N c-Raf and interaction with Δ N c-Raf.

RanBPM construct	Downregulates ΔN c-Raf	Interacts with ΔN c-Raf
WT RanBPM	YES	YES
Δ212 RanBPM (ΔSPRY)	YES	YES
ΔC4 RanBPM (ΔC-terminus)	NO	NO
ΔC1 RanBPM (ΔCRA)	NO	NO
Δ360 RanBPM (ΔLisH/CTLH)	NO	YES
ΔN2 RanBPM (ΔN-terminus)	NO	ND
C1 domain (CRA)	ND	YES
N2 domain (N-terminus)	ND	NO

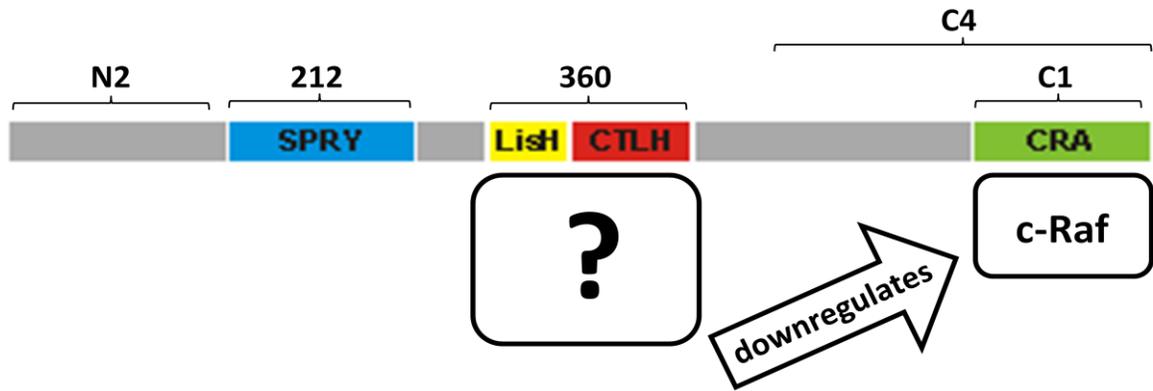


Figure 4.1. Model of the mechanism by which RanBPM downregulates c-Raf. RanBPM directly interacts with c-Raf via its C-terminal CRA domain and presumably interacts with another protein or protein complex, such as the CTLH complex, via its LisH/CLTH domain to downregulate c-Raf.

Therefore, it is hypothesized that some other protein or protein complex is interacting with RanBPM through its LisH/CTLH domains to downregulate c-Raf. Deletion of the N-terminus of RanBPM also resulted in a loss of c-Raf downregulation and this region was also shown to be unable to interact directly with c-Raf. Therefore, the N-terminus might aid the LisH/CTLH domains in mediating the interaction between RanBPM and the unidentified complex potentially responsible for downregulating c-Raf. This is plausible since the N-terminus is a potentially flexible proline-rich region of RanBPM which could fold over to stabilize the protein or, in this case, stabilize interactions with other proteins (16). Given that deletion of the SPRY domain did not perturb the ability of RanBPM to downregulate or interact with c-Raf, we propose that this domain does not participate in regulation of c-Raf.

We have presented a plausible mechanism which suggests the involvement of an additional protein or protein complex in the downregulation of c-Raf. We propose that c-Raf might be targeted for degradation by the CTLH complex in a RanBPM-dependent manner, with c-Raf being tethered to the complex by RanBPM through its CRA domain, and that the CTLH complex interacts with RanBPM primarily through its LisH/CTLH domains. RanBPM is a known member of the CTLH complex and our results show that c-Raf can associate with both RanBPM and MAEA, another member of the CTLH complex. This mammalian complex is comprised of six proteins in total, all of which have known orthologs in *S. cerevisiae* that form the yeast Gid complex (6). Given that the Gid complex is a proven E3 ubiquitin ligase, it has been hypothesized that the CTLH complex may play a similar role in mammalian cells (6). Though this has not experimentally been shown, we propose that the CTLH complex may play a role in the ubiquitination and degradation of target proteins and that it may be targeting c-Raf for degradation in a mechanism that depends on RanBPM as a scaffold for the complex. While the topology of the mammalian CTLH complex has not been elucidated, the idea that RanBPM may act as a scaffold in the complex is consistent with previous studies. It has been shown that Gid1, the yeast ortholog of RanBPM, acts as a scaffold for the Gid complex and interacts

with other members of the complex mainly through its LisH and CTLH domains (47).

Although the major piece of evidence in our study tying the CTLH complex to c-Raf downregulation is the fact that c-Raf and MAEA associate in cells, subtleties in our data are also consistent with the idea that the CTLH complex may be involved in c-Raf downregulation. The number of interactions seen between c-Raf and MAEA were noticeably fewer than the number of interactions seen in the positive control between RanBPM and MAEA. This is compatible with the idea that RanBPM and MAEA are fixed members of the CTLH complex, whereas c-Raf may only associate with the complex temporarily to target it for degradation. Also, previous studies in our laboratory have shown that RanBPM has a greater effect on the active form of c-Raf, which represents only a fraction of the total pool of endogenous c-Raf in the cell (24). This further supports the observation that c-Raf associates with MAEA infrequently, as only activated c-Raf may be targeted for degradation by RanBPM, MAEA and the rest of the CTLH complex.

A large number of studies have described proteins that interact with RanBPM, albeit they seldom provide functional significance for the interactions. A number of these interacting proteins have been described in Chapter 1, but a broader record has been retrieved from the BioGRID (Biological General Repository for Interaction Datasets) version 3.2.114 (Figure 4.2) (105). In addition to the evidence discussed in Chapter 1 of ARMc8, Rmnd5a and MAEA being involved in ubiquitination, the collection of proteins retrieved from the BioGRID that interact with RanBPM involved in ubiquitination, deubiquitination, ubiquitin-like modification or management of ubiquitinated proteins give further weight to the argument that RanBPM and the CTLH complex may play a role in these processes.

For example, RanBPM has been shown to interact with the essential aggresome component HDAC6 (histone deacetylase 6) (Figure 4.2) and our laboratory has shown that RanBPM is essential for aggresome formation (103). Aggresomes are perinuclear structures that accommodate ubiquitinated,



Figure 4.2. RanBPM interactome retrieved from the BioGRID version 3.2.114 showing 71 proteins that interact with human RanBPM. Interactions between RanBPM and other proteins are connected by a red line and interactions between RanBPM interacting partners are connected by a blue line. CTLH complex members are outlined in purple and proteins involved in ubiquitination, deubiquitination, ubiquitin-like modification and management of ubiquitinated proteins are outlined in green.

misfolded or damaged proteins in conditions where the proteasome and the UPS are overwhelmed (106). This crucial role in aggresome formation implies that RanBPM has the ability to handle ubiquitinated proteins targeted for degradation. In addition, RanBPM has been found to interact with the E3 ubiquitin ligase TRAF6 (tumor necrosis factor-receptor-associated factor 6) (Figure 4.2) and to reduce the TGF- β (transforming growth factor β) dependent auto-ubiquitination of TRAF6 (107). Some high-throughput screens studying the ubiquitinome have even found RanBPM among a pool enriched for ubiquitinated proteins (Figure 4.2) (108). This may simply be evidence of RanBPM itself being targeted for ubiquitination, given that RanBPM has been shown to be ubiquitinated as well as deubiquitinated specifically through interaction with USP11 (ubiquitin-specific protease 11) (Figure 4.2) (109). However, there is still substantial reason to suspect that RanBPM, in concert with the CTLH complex, could be playing a role in protein ubiquitination and subsequent degradation by the proteasome.

Although we now propose the CTLH complex as the complex potentially responsible for interacting with RanBPM to downregulate c-Raf, during the course of this study we initially investigated other proteins and complexes which we thought may be playing this role. Initially, we attempted to investigate Hsp90 as a potential c-Raf regulatory mechanism in this context. Hsp90 is a well-characterized molecular chaperone which has been shown to bind and stabilize c-Raf, allowing it to properly participate in ERK pathway signaling (94-96). Since previous results in our laboratory have shown that downregulation of c-Raf by RanBPM inhibits further ERK pathway signaling and that RanBPM expression disrupts complex formation between c-Raf and Hsp90, we hypothesized that RanBPM might be preventing Hsp90 from stabilizing c-Raf (24). However, we were unable to obtain further conclusive evidence of altered acetylation of Hsp90, which reflects Hsp90 activity, in response to RanBPM expression (data not shown). Furthermore, previous preliminary experiments in our laboratory showed no evidence of E3 ubiquitin ligase CHIP complex formation with RanBPM and c-Raf, suggesting CHIP is not involved in the downregulation of c-Raf by RanBPM. Although other studies have shown that CHIP is able to

ubiquitinate c-Raf and send it for proteasomal degradation, it has also been suggested that c-Raf may be ubiquitinated and degraded by CHIP-independent mechanisms (98-100).

4.3. Significance of the CRA domain as a binding-domain for c-Raf

Many studies have identified binding partners for RanBPM and suggested roles for the protein in various cellular processes, but a clear function for RanBPM has yet to be elucidated. Our study further contributes to our growing knowledge of the protein and demonstrates the importance of the CRA domain of RanBPM for its interaction with c-Raf. Some studies have already identified certain regions of RanBPM to be required for interactions with specific proteins. For example, the SPRY domain of RanBPM has been shown to be required for interaction with CDK11^{p46}, L1, MET, TrkA, AR and USP11 (7,8,20,25,35,109). The SPRY domain, along with the LisH domain, has been shown to be sufficient for interaction with BACE1, LRP and APP to increase A β generation (29). The LisH and CTLH domains have both been shown to be required for RanBPM interaction with HDAC6 and only FMRP has previously been shown to interact with the CRA domain of RanBPM (14,103). Our study defines c-Raf as only the second protein to be found to interact with RanBPM through the CRA domain and further confirms this domain to be a protein interaction surface.

The CRA domain has only been reported in a small number of proteins other than RanBPM, namely RanBP10 (Ran-binding protein 10), MAEA, Rmnd5a, Rmnd5b and Twa1 (46,110). It has not been shown, but there is potential for c-Raf to interact with the CRA domain of these proteins as well, although most of these proteins are found within the CTLH complex. RanBP10, the only non-CTLH complex member, shows very high sequence similarity to RanBPM and has also been named for its supposed ability to interact with Ran (110). The CRA domain has been predicted to contain six alpha-helices, and thus has been hypothesized to resemble a DD superfamily domain (14). DD superfamily proteins are generally proteins that propagate apoptotic signals, such

as the death receptors p75NTR and Fas as well as a number of caspases (111). Although c-Raf signaling affects the activities of many downstream proteins involved in apoptosis, an extensive literature search did not reveal any c-Raf interacting partners containing a DD superfamily domain. Thus, this study appears to uncover a novel binding domain for c-Raf, as interacting partners containing a CRA domain or a DD superfamily domain have not yet been reported.

However, it is possible that c-Raf interacts specifically with a sub-section of the CRA domain, such as with the surface of a particular helix, rather than with the entire domain. c-Raf has in fact been shown to interact with the surface of helices in other proteins. For example, residues lysine 49, arginine 56 and arginine 60 along the surface of helix 3 in the protein 14-3-3 have been shown to be important for interaction with c-Raf (112). Although residues on helices often contribute the binding surface for protein-protein interactions, the details of the direct interaction between c-Raf and the CRA domain remain to be examined.

4.4. Consequences of RanBPM-mediated regulation of c-Raf

This study also further contributes to our knowledge of c-Raf regulatory mechanisms within mammalian cells. Although our evidence of RanBPM-dependent CTLH complex downregulation of c-Raf is preliminary, the consequences of this potential novel regulatory mechanism are impactful. As previous work in our laboratory has shown, loss of c-Raf has serious downstream signaling effects, as RanBPM-mediated downregulation of c-Raf and consequent inhibition of the ERK pathway leads to decreased levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL (24). However, ERK1/2 has over 150 other downstream signaling proteins and thus RanBPM-mediated destabilization of c-Raf could affect any number of these signaling pathways and associated cellular processes (54,66). c-Raf is also implicated in non-ERK pathway signaling, such as its negative regulation of the pro-apoptotic proteins ASK1 and MST2 (72,73). Though this has not been shown, RanBPM could partially exert its apoptotic

activity by protecting levels of ASK1 and MST2 through c-Raf downregulation. Altogether, through multiple downstream effectors, c-Raf dictates a number of cellular processes, such as differentiation, proliferation, motility and apoptosis (54,55). Therefore its RanBPM-dependent regulation is a crucial factor in ensuring these functions are properly executed.

c-Raf regulation has long been regarded as a target for cancer therapeutics, since the ERK pathway is known to be upregulated in over one-third of all human cancers (56). Numerous drugs have been developed in an attempt to combat Raf activity and increase tumour cell apoptosis, but drug resistance remains an obstacle and combination drug therapy is often employed to increase the chance of success. Given that RanBPM demonstrates tumour suppressor activity, a RanBPM-derived cancer therapeutic could prove useful and add diversity to the growing number of Raf inhibitors used in the clinic. In addition to the previously mentioned work showing that RanBPM inhibits cell survival and migration, preliminary data from our laboratory provides further evidence of RanBPM as a crucial tumour suppressor (24). Immunocompromised mice injected with RanBPM-deficient HEK cells showed significantly increased tumour formation, primarily localized in the liver, compared to mice injected with HEK cells expressing RanBPM (113). In addition, mouse embryonic fibroblasts generated from RanBPM knockout mice bred in our laboratory showed increased levels of c-Raf compared to those generated from WT mice (113). This provides significant relevance to the concept of c-Raf regulation by RanBPM, as this event has now been shown in a model that closely mimics human physiology.

4.5. Limitations of the study and future studies

Although the work presented in this thesis yielded informative results, some limitations were encountered which could be taken into consideration when planning future studies. Primarily, it was very difficult to obtain even expression of RanBPM deletion mutants upon transfection in HeLa cells in order to fairly assess the ability of each mutant to downregulate c-Raf. Given that RanBPM deletion

mutants exhibit levels of stability different from one another, the amount of DNA used for the transfection of each mutant construct had to be adjusted to obtain similar levels of protein expression among all mutants. However, despite careful optimization, the expression of RanBPM deletion mutants still varied somewhat. Even expression might have been achieved with the generation of HeLa cell lines stably expressing each RanBPM deletion mutant construct. This project remains ongoing in the laboratory.

Furthermore, based on the presence or absence of NLS or NES signals within the sequence of each RanBPM deletion mutant, the subcellular localization of certain mutants could have been altered and hindered their ability to downregulate c-Raf, a predominantly cytoplasmic protein. For example, both $\Delta 212$ and $\Delta 360$ RanBPM demonstrate increased nuclear localization (16). Therefore, 60–70% of the total protein is in fact sequestered within the nucleus and unable to interact with c-Raf, although 30–40% is still nucleocytoplasmic and able to participate in c-Raf regulation (16). Given that $\Delta 212$ RanBPM is mostly nuclear but still demonstrates a strong ability to regulate c-Raf, it is presumed that similar cytoplasmic levels of $\Delta 360$ RanBPM would have also demonstrated this effect if the protein was fully functional in this aspect.

It is also important to note that large protein deletions can have a significant negative impact on the proper folding and stability of a protein. This phenomenon was exemplified perfectly in this study in the case of the $\Delta N2$ RanBPM mutant, where deletion of the N-terminus of the protein resulted in decreased expression, presumably due to protein instability. Although this type of instability was not seen for the other RanBPM deletion mutants, it is difficult to predict whether deletion of the SPRY domain, LisH/CTLH domains or C-terminus resulted in misfolding of RanBPM. If this was the case, it would be impossible to distinguish if the results obtained were in fact due to lack of a required functional domain or simply due to misfolding of the protein. Generating point mutations inhibiting the function of a specific domain is generally a more cautious approach when conducting these types of studies, however given a lack of knowledge on the key residues within each domain of RanBPM, this strategy could not be

employed here. However, future studies could focus on locating specific residues within the CRA domain of RanBPM that abolish its interaction with c-Raf. Subsequent experiments using this mutant could give a more reliable idea of the effect of loss of interaction between RanBPM and c-Raf, since RanBPM folding would be less likely to be affected by a simple point mutation.

Other key future studies include continuing to explore the idea of CTLH complex involvement in RanBPM-mediated c-Raf downregulation. It would be important to provide evidence of RanBPM-dependent c-Raf ubiquitination or proteasomal degradation, although this was attempted during the course of this study and no conclusive evidence was obtained (data not shown). It would also be intriguing to knock-down expression of various CTLH complex members in cells and investigate the effect on c-Raf. Since shRNA knock-down of RanBPM leads to increased levels of c-Raf, knocking-down a CTLH complex member would be expected to yield similar results. Altogether, this would further support the hypothesis that the CTLH complex is involved in c-Raf regulation.

4.6. Conclusion

Overall, this thesis aimed to characterize the interaction between two important regulators of key cellular processes, RanBPM and c-Raf. Although the role and importance of c-Raf has been well-documented in the past, the exact function of RanBPM remains an enigma and ongoing research in the fields of cancer and neurological disease aims to better understand this protein. The work presented in this thesis not only contributes to our knowledge of RanBPM, but also clarifies the relationship between RanBPM and c-Raf by proposing a novel model regarding how RanBPM downregulates c-Raf. Such knowledge is critical in understanding RanBPM as a tumour suppressor and regulator of the ERK pathway, a pathway known to be heavily involved in human oncogenesis.

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Publications

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(2011) Divalent metal ion differentially regulates the sequential nicking
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e23804. doi:10.1371/journal.pone.0023804.

Abstracts

Bérubé-Janzen, W. and Schild-Poulter, C. Characterizing the interaction between RanBPM and c-Raf.
10th Annual Oncology Research and Education Day
London, Ontario
June 21st, 2013

Bérubé-Janzen, W. and Schild-Poulter, C. Characterizing the interaction between RanBPM and c-Raf.
3rd Annual London Health Research Day
London, Ontario
March 18th, 2014

Bérubé-Janzen, W. and Schild-Poulter, C. Characterizing the interaction between RanBPM and c-Raf.
1st Annual Robarts Research Day
London, Ontario
June 9th, 2014

Bérubé-Janzen, W. and Schild-Poulter, C. Characterizing the interaction between RanBPM and c-Raf.
11th Annual Oncology Research and Education Day
London, Ontario
June 20th, 2014

Scholarships and Awards

- CIHR Strategic Training Program in Cancer Research and Technology Transfer Award (\$3100; September 2013 – August 2014)
- Ontario Graduate Scholarship (\$15,000; September 2013 – August 2014)
- NSERC Alexander Graham Bell Canada Graduate Scholarship M (\$17,500; September 2012 – August 2013)
- University of Western Ontario Dean's Honour List (2008 –2009, 2009–2010, 2010–2011, 2011–2012)
- Academic All Canadian Award and Ontario University Athletics Academic Achievement Award, awarded to student-athletes maintaining an average of at least 80% while competing on a varsity team (2008–2009, 2009–2010, 2010–2011, 2011–2012)
- Gordon Risk Bursary, awarded by the University of Western Ontario to a student-athlete with high academic standing and demonstrating financial need (\$500; 2010)
- Laurene Paterson Estate Scholarship, awarded by the Faculty of Science at the University of Western Ontario to a student in the Faculty

of Science with a minimum 80% average and demonstrating financial need (\$2,000; 2010)

- Anne Ferguson Memorial Award in Pharmacology and Toxicology, awarded by the Faculty of Science and the Schulich School of Medicine and Dentistry at the University of Western Ontario to a student studying pharmacology with a minimum 70% average and demonstrating financial need (\$450; 2010)
- Letter of achievement from the Department of Chemistry at the University of Western Ontario (2008)
- Western Scholarship of Excellence, awarded to students entering the University of Western Ontario with a 90-94.9% high school average (2008)

Extracurricular Activities

- Captain of the University of Western Ontario varsity women's golf team (2010–2012) and team member (2008–2012)
- Social committee member in the University of Western Ontario's Varsity Captain's Circle (2010–2011)