11-22-2012 12:00 AM

Characterization of a tumour suppressor function of RanBPM

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

CHARACTERIZATION OF A TUMOUR SUPPRESSOR FUNCTION OF RANBPM

(Spine Title: Characterization of a tumour suppressor function of RanBPM)

(Thesis Format: Integrated Article)

by

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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entitled:

Characterization of a tumour suppressor function of RanBPM

is accepted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Date

Chair of the Thesis Examination Board

Abstract

Ran-binding protein M (RanBPM) is an evolutionarily conserved nucleocytosolic protein that has been proposed to regulate various cellular processes, including protein stability, gene expression, receptor-mediated signalling pathways, cell adhesion, development, and apoptosis. Despite the multitude of functions attributed to RanBPM however, little is known regarding the precise mechanisms by which RanBPM executes these cellular roles. In this work, we seek to address this matter by describing functions for RanBPM in the regulation of apoptotic and pro-survival signalling pathways, and in cellular transformation.

We first identify RanBPM as a pro-apoptotic protein that regulates the activation of the intrinsic apoptotic signalling pathway in response to DNA damage. We show that RanBPM executes its pro-apoptotic functions by modulating the expression and localization of Bcl-2 family proteins. Next, we demonstrate that RanBPM functions as a novel inhibitor of the ERK1/2 signalling cascade, and that RanBPM regulates the expression of Bcl-2 factors through repression of this pathway. We also extend these analyses to show that RanBPM forms a complex with c-Raf, and that it prevents aberrant ERK1/2 signalling by destabilizing the c-Raf-Hsp90 complex, thus maintaining low cellular c-Raf expression. Our studies also implicate an important function for RanBPM in the regulation of gene expression programs. We find that disruption of RanBPM expression affects transcriptional networks involved in the regulation of organism development and tumourigenesis, and that decreased RanBPM levels alter the expression of factors involved in signal transduction through the Notch, Wnt, PI3K, and ERK1/2 pathways. Importantly, our work also reveals that the down-regulation of RanBPM expression is associated with the acquisition of markers of cellular transformation, specifically evasion from apoptosis, sustained proliferative signalling, and increased cellular migration and invasion, suggesting a novel tumour suppressor function for RanBPM.

Taken together, our studies provide insight into the molecular mechanisms by which may RanBPM mediate its diverse biological functions, and reveal that altered RanBPM expression may have important ramifications in the regulation of organism development and disease pathogenesis.

Keywords: RanBPM, Bcl-2, ERK1/2, c-Raf, development, apoptosis, signalling, gene expression, cellular transformation, tumour suppression

Co-authorship Statement

The chapters of this thesis were written by Elnaz Atabakhsh and edited by Dr. Caroline Schild-Poulter.

The data presented in chapter 2 contains portions of the published manuscript: RanBPM has pro-apoptotic activities that regulate cell death pathways in response to DNA damage. Elnaz Atabakhsh, Dawn M. Bryce, Karen J. Lefebvre, and Caroline Schild-Poulter. *Mol Cancer Res* (2009). In this chapter, the experiments in Figures 2.1 and 2.2 were performed by DMB and CSP. EA and CSP performed the experiments in Figure 2.3, and EA and DMB performed the experiments in Figure 2.4. The experiments presented in Figure 2.5 were performed by EA. The manuscript was written and edited by CSP.

The data presented in chapter 3 have been published in the manuscript: RanBPM is a novel inhibitor of ERK signalling. Elnaz Atabakhsh and Caroline Schild-Poulter. *PLoS One* (2012). All experiments in this chapter were performed by EA. The manuscript was written and edited by EA and CSP.

The data presented in chapter 4 have been published in the manuscript: RanBPM expression regulates transcriptional pathways involved in development and tumourigenesis. Elnaz Atabakhsh, Jean H. Wang, Xu Wang, David E. Carter, and Caroline Schild-Poulter. *Am J Cancer Res* (2012). In this chapter the probe preparation and GeneChip hybridization was performed by DEC at the London Regional Genomic Centre, and the experiments presented in Figure 4.2 were performed by JHW and XW. All other experiments, and data and bioinformatics analyses were performed by EA. The original draft of this manuscript was written by EA and edited by CSP. Revisions to the manuscript were made by EA based upon suggestions by CSP.

For
Mamanie, Mahsheed, Dai Bahman
and
My grandparents Sakineh and Yadola

"Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So…explore, dream, discover."

– Mark Twain

Acknowledgements

I want to start by thanking my supervisor, Dr. Caroline Schild-Poulter, for your support and guidance during the course of my degree. Your door has always been open to discuss any idea or problem, whether it was science-related or otherwise. Thank you for your advice, mentorship, and for helping me to become a keen and independent scientist. I am honoured to have trained under your supervision.

To my advisory committee members, Dr. Sean Cregan and Dr. Fred Dick, thank you for your many helpful discussions regarding my research, and for your support and encouragement throughout the last five years. I sincerely appreciate everything that you have done for me.

To past and present members of the Schild-Poulter Lab: Vicki, Louisa, Wesley, Sarah, Xu, Dawn, Jean, and our numerous undergrad students. Thank you for making our lab a great place to work. To Vicki and Louisa, especially: whether we were brainstorming science, laughing at one of my many "isms", or dealing with "ops" and "codes", everyday in the lab with you was great.

I would also like to express my gratitude to Mrs. Marilynne Fuller for your continued and generous support of me and this project. As well, a major thank you to Jeff and Jeremy at Robarts IT for the countless times you helped me out of a computer disaster before I lost my mind, and the immense patience you've shown while helping me deal with my aversion to technology.

On a personal note, I would like to first thank the most important people in my life, my loving family, for supporting me in everything that I do. To my mother Farzaneh, my sister Mahsheed, and my uncle Bahman: Your incredible strength, unwavering love, and constant encouragement have allowed me to always dream big and to become the person I am today. I cannot put into words how much you mean to me- thank you from the bottom of my heart!

And finally, to the group of people who have made my time in grad school truly wonderful: Piya, Brad, Jake, Jess, Lazar, Chris, and Joey. The times spent with you laughing, talking about everything and nothing, celebrating the small victories, or ranting about the trials of science have meant more to me than I could ever express. You are the best friends anyone could ask for.

List of Abbreviations

AD	Alzheimer's disease
AP-1	Activating protein 1
APAF1	Apoptotic protease-activating factor 1
AR	Androgen receptor
ATM	Ataxia telangiectasia mutated
ATR	ATM-related
Aβ	Amyloid β
BAG1	Bcl-2 associated athanogene 1
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
CBF1	C promoter-binding factor 1
CDC37	Cell division cycle 37 (also called p50)
$CDK11p^{46}$	Cyclin-dependent kinase 11 p46 fragment
CHIP	Carboxy terminal of Hsp70-interacting protein
CK1	Casein kinase 1
CR	Conserved region
CRA	CT11-RanBPM
CREB	Cyclic AMP-response element-binding protein
CRD	Cysteine-rich domain
C(t)	Cycle threshold
C-terminal	Carboxy terminal
CTLH	C-terminal to LisH
cyt *c*	cytochrome *c*
Da	Dalton
DDR	DNA damage response
Dll	Delta-like
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA-PK	DNA-dependent protein kinase
DSBs	Double-stranded breaks
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
ErbB2	Epidermal growth factor receptor 2

ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FOX	Forkhead box
Fz	Frizzled
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor bound-protein 2
GSC	Germline stem cell
GSK3	Glycogen synthase kinase
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
Hes	Hairy and enhancer of split
Hey	Hes-related repressor protein
HIPK2	Homeodomain-interacting protein kinase 2
HMG	High mobility group
HOX	Homeobox
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
IR	Ionizing radiation
IPA	Ingenuity pathway analysis
JAG	Jagged
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KDa	Kilodalton
LFA-1	Lymphocyte function-associated antigen-1
LisH	Lissencephaly homology
LRP5/6	Lipoprotein-related receptor proteins 5 and 6
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MET	MET proto-oncogene
MMP	Mitochondrial membrane permeabilization
MMTV	Mouse mammary tumour virus
MTOC	Microtubule organizing centre
NF-κB	Nuclear factor kappa-B
NICD	Notch intracellular domain
N-region	Negative-charge region
N-terminal	Amino terminal

Oct-1	Octamer factor 1
OMM	Outer mitochondrial membrane
p75NTR	p75 neurotrophin receptor
PANTHER	Protein analysis through evolutionary relationships
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide-3 kinase
pRB	Retinoblastoma protein
PUMA	p53-regulated modulator of apoptosis
qRT-PCR	Quantitative reverse-transcriptase PCR
RanBPM	Ran-binding protein M (also called RanBP9)
RanBPM si-mt	RanBPM shRNA point-mutant expression construct
RBD	Ras-binding domain
RON	Recepteur d'origine nantais
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
Ser/S	Serine
Sos	Son of sevenless
SOX	SRY-related HMG box
SPRY	Sp1A and ryanodine receptors
SRC	v-src sarcoma
TAF4	TATA box binding protein-associated factor 4
TCF/LEF	T cell factor/lymphoid enhancer factor
TGFβ	Transforming growth factor β
TM	Transmembrane
TNF	Tumour necrosis factor
TRIM5	Tripartite motif 5
Trk	Tropomyosin-related kinase
Tyr/Y	Tyrosine
UV	Ultraviolet
WLS	Wntless

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Chapter 1

1. Introduction

1.1 General introduction

On average, the human body generates 60 billion cells per day, and must eliminate the same number of cells daily in order to maintain cellular homeostasis [1]. This requires the balance between signals that stimulate cell proliferation and those that mediate cell death, and not surprisingly, deregulation of either of these processes can result in the pathogenesis of diseases such as cancer [1-3]. In humans, cancer development (tumourigenesis) occurs as a result of complex, multi-step genetic alterations that drive the progression from a normal cell to a transformed, malignant cell [4]. These alterations arise from genomic instability that causes gain-of-function mutations that generate proto-oncogenes and loss-of-function mutations in tumour suppressors, which together disrupt control over cellular growth and proliferation [4-6]. Different combinations of mutations within these genes are attributed to the over 100 different types of human cancers that have been identified to date [4]. Over the last thirty years, intensive studies on the mechanisms that govern malignant transformation have led to the proposal that cancer occurs as a result of broad genetic alterations that disrupt a small number of key cellular processes [4, 5]. Based upon this notion, in 2000 Hanahan and Weinberg proposed the six hallmarks of cancer, which were defined as: uncontrolled cellular replication, failure to respond to growth-suppressor signals, sustained proliferative signalling, evasion from apoptosis, increased angiogenesis, and enhanced cellular migration and invasion [5]. These hallmarks represent the changes in cellular physiology that mediate the progression of cells from a normal to a malignant state, via a series of intermediate stages that define the physiology of pre-metastatic disease [5].

At its core, cancer is a disease that occurs as a result of loss of cellular homeostasis. Therefore, in order to understand how cancer develops, it is also necessary to attain a detailed understanding of how normal cells balance the numerous intrinsic and extrinsic signals that stimulate growth or promote death. The current focus of many cancer researchers is to unravel the complex regulatory networks that mediate normal

cellular growth, proliferation, survival, and death, and couple this with elucidating the mechanisms by which cells disrupt control of these networks to become cancerous. The aim of this thesis is to contribute to this field of research by characterizing a novel function for Ran-binding protein M (RanBPM, also called RanBP9) in the regulation of signalling pathways that govern cell survival and cell death, and the implications of this role for RanBPM in the regulation of cellular transformation.

1.2 RanBPM

1.2.1 Structure and proposed functions

RanBPM (also known as RanBP9) is a 90kDa nucleocytoplasmic protein, initially identified as a binding partner of the Ran GTPase that localized to the microtubule organizing centre (MTOC) [7]. These initial observations of RanBPM were later dismissed however [8], and subsequent studies on this protein have been aimed at identifying its cellular function. The RanBPM protein is ubiquitously expressed [8, 9] and highly conserved, with RanBPM orthologues identified in plants [10], lower eukaryotes (known as Vid30/Gid1 in yeast) [11], and vertebrates species [8], and is characterized by four well-conserved domains (Fig. 1.1). The N-terminus of RanBPM contains a SPRY (Sp1A and ryanodine receptors) domain that is proposed to function as a protein-interaction module [12, 13]. SPRY domains have been identified in over 600 eukaryotic proteins with diverse cellular roles such as calcium and cytokine signalling, and retroviral restriction [12, 13]. The presence of SPRY domains is believed to be of critical importance for the overall function of proteins that contain this domain [12, 13]. For example truncation or deletion of the SPRY domain in the TRIM5 (tripartite motif 5) protein, which is involved in retroviral restriction in primates, completely abolishes its anti-viral activity and also inhibits the anti-viral activity of wildtype TRIM5 [14]. In addition, mutations in the SPRY domain of the Pyrin protein alter its function, and result in an auto-inflammatory condition called familial Mediterranean fever [12, 15, 16]. Interestingly, deletion of the SPRY domain in RanBPM has been found to disrupt its interaction with several of its binding partners [12], suggesting that this domain may also

Figure 1.1 RanBPM domain map. The four conserved regions of the RanBPM protein are comprised of the SPRY, LisH, CTLH, and CRA domains. The relative amino acid position in the human RanBPM protein is indicated for each domain. Figure adapted from [17].

be important for RanBPM function. The central region of the RanBPM protein contains a LisH (Lissencephaly homology) domain that is involved in protein dimerization [18, 19], and a CTLH (C-terminal to LisH) domain, for which a function is currently not known [18]. LisH domains have been found in more than 100 eukaryotic proteins, the majority of which are involved in regulation of microtubule dynamics [18]. Mutations in the LisH domain of proteins have been associated with disease development, including conditions that arise as a result of abnormal cell migration [18]. While a function for the CTLH domain has not been elucidated, structurally conserved CTLH complexes consisting of several LisH- and CTLH-domain containing proteins (including RanBPM) have been identified in plant, yeast, and mammalian cells [10, 20, 21]. The yeast CTLH complex was found to regulate proteasomal and vacuolar degradation of fructose-1,6-bisphosphate [20], while in mammals a component of this complex was reported to participate in the lysosomal trafficking of ubiquitinated proteins [22]. The last 100 amino acids of RanBPM comprise an alpha-helical region called the CRA (CT11-RanBPM) domain, that is proposed to mediate protein-protein interactions [23], and has been identified in several proteins that are components of CTLH complexes [10, 20, 21].

RanBPM has been reported to function in a multitude of cellular processes including regulation of protein stability [24, 25], transcriptional regulation of steroid receptors [26-28], functioning as an adaptor/scaffold for receptor signalling pathways [29-32], and in the regulation of development [33-35] and apoptosis [36-39]. Despite these findings however, the precise modality by which RanBPM functions remains largely undetermined.

1.2.2 Regulation of intracellular signalling pathways

Early studies on RanBPM revealed that the N-terminal region of the protein contains a long stretch of proline and glutamine residues [8]. It is well established that proteins which possess glutamine-rich regions form aggregates [40], a characteristic which has been attributed to the development of several pathological conditions including Huntington's Disease (HD) [40]. The presence of a glutamine-rich region in RanBPM

led to the hypothesis that protein-protein interactions may be important to its cellular function, a notion that was supported by the observation that RanBPM was a component of a large, multi-subunit protein complex exceeding 670kDa in size [8]. This also led to the suggestion that RanBPM may function as a scaffolding protein that integrates signals from multiple pathways within the cell [9, 41]. Consistent with such a role, RanBPM was proposed to localize to the plasma membrane and function as a scaffold for several cell-surface receptors, by coupling receptor activation to the induction of downstream signalling events. For example, interaction of RanBPM with the intracellular kinase domain of the MET proto-oncogene (MET) receptor was found to stimulate extracellular signal-regulated kinase (ERK) 1/2 signalling, and to enhance cell migration [30]. Further, its interaction with the intracellular kinase domain of the tropomyosin-related kinase (Trk) B receptor was found to promote ERK1/2 and Akt activation, and lead to neuronal differentiation [31]. In an opposing role, Cheng *et al.* reported that RanBPM interacted with the cytoplasmic domain of the neural cell adhesion molecule L1, and inhibited L1-mediated ERK1/2 activation and neurite outgrowth [29]. In studies with the LFA-1 (lymphocyte function-associated antigen-1) integrin, RanBPM was observed to bind the cytoplasmic domain of the $\beta_2$integrin LFA-1 at the plasma membrane, and couple LFA-1 with intracellular signalling and transcriptional activation [9]. Together, these findings led to a model for RanBPM function, in which RanBPM mediates intracellular responses to extracellular cues that govern cell function and behaviour.

1.2.3 Functions in developmental regulation

Most studies on RanBPM are centred upon the identification and characterization of its interaction partners, but fail to ascribe a biological significance to these interactions. However, several recent reports have pointed to an important function for this protein in neuronal and germline development. In support of a role in neuronal development, Brunkhorst *et al.* reported that RanBPM interacted with the transcriptional co-activator TAF4 (TATA box binding protein-associated factor 4) in neural stem cells and mediated neuritogenesis during differentiation [35]. Additionally, work by Scantlebury and colleagues determined that in the *Drosophila* nervous system, RanBPM

function was required for proper larval feeding behaviour, response to light, and for coordinated locomotion [42]. With regard to a function in germline development, RanBPM was found to regulate the size and morphology of the microenvironment (niche) that determines germline stem cell (GSC) fate in the *Drosophila* ovary, and to regulate the capacity of this niche for GSCs [34]. Moreover, recent studies using knockout mice demonstrated that RanBPM is critical for normal gonad development in mammals, as absence of RanBPM expression resulted in the loss of all germ cells in the gonads of male and female mice, and rendered them sterile [33].

The importance of RanBPM function in development is further substantiated by the observation that it is a vital gene in *Drosophila*, and that larvae expressing mutant forms of RanBPM exhibit feeding defects that cause them to be markedly smaller than control flies [34, 42]. RanBPM appears to be equally important for normal development in mammals, although unlike in *Drosophila*, deletion of RanBPM in mice is not lethal [33]. Intriguingly, in the study by Puverel *et al.*, over half of RanBPM knockout mice died perinatally, and those which did survive were significantly smaller in size than their normal littermate controls [33]. While the causes of perinatal lethality and growth suppression in these mice have not yet been elucidated, they support the notion that perturbation of RanBPM expression has significant consequences for normal organism development.

1.2.4 Functions in apoptotic signalling

RanBPM has also been linked to the regulation of apoptotic pathways. It was reported to bind the intracellular death domain of the p75 neurotrophin receptor (p75NTR), a member of the tumour necrosis factor (TNF) superfamily that promotes apoptosis in neuronal cells [43]. It was also found to bind a caspase-cleaved fragment of cyclin-dependent kinase 11 ($CDK11p^{46}$) that functions as an effector protein in receptor-activated apoptotic signalling pathways [37]. Additionally, RanBPM interacts with factors involved in transcriptional regulation of intrinsic apoptotic signalling. Kramer and colleagues reported that binding of RanBPM to the transcription factor p73 stabilized

the p73 protein, and enhanced its pro-apoptotic activity [25]. An interaction of RanBPM was also observed with homeodomain-interacting protein kinase 2 (HIPK2), which promotes p53-dependent apoptosis [38]. Recent studies have also identified a function for RanBPM in apoptotic regulation in diseased states. Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized by accumulation of the amyloid β (Aβ) protein in extracellular plaques, and by widespread neuronal and synaptic loss [44]. RanBPM was found to enhance Aβ generation [45] and to augment Aβ-mediated apoptosis and neurodegeneration in neuronal cell lines [39].

1.2.5 Summary

Since its initial discovery nearly 15 years ago, extensive efforts have been made to understand the cellular function(s) of RanBPM. This has been complicated, however, by the fact that the majority of reports on RanBPM identify binding partners for this protein without providing insight into the functional consequences of these interactions. Nevertheless these observations have identified several processes in which RanBPM appears to have a critical role, including apoptosis and the regulation of diverse signalling pathways. They also highlight an important role for RanBPM in development, and suggest a function for RanBPM in disease pathogenesis. While it remains to be determined how RanBPM can govern so many distinct processes, it appears that elucidating the cellular functions of RanBPM may have great implications in understanding normal organism function and the development of pathophysiological conditions.

1.3 Apoptosis

1.3.1 Overview

Initially defined in 1972 as a counterbalance for cell proliferation, apoptosis is a process of programmed cell death that is critical to both physiological and pathological development [2]. Activation of apoptosis can be triggered either by extracellular stimuli

(extrinsic pathway) or intracellular stimuli (intrinsic pathway) that converge upon a family of cysteine proteases called caspases, which are responsible for the execution of cell death [46]. In the extrinsic apoptotic pathway, specialized immune cells produce death-inducing ligands that bind pro-apoptotic receptors at the cell membrane and activate intracellular signalling pathways that lead to caspase activation [46]. Conversely exposure of cells to stresses such as heat shock, growth factor withdrawal, or radiation-induced DNA damage, activates the intrinsic apoptotic pathway that mediates caspase activation via disruption of mitochondrial function [46, 47]. Upon activation, caspases cleave specific cellular substrates to induce apoptotic cell death [47]. The end stages of apoptosis are characterized by chromatin condensation, nuclear fragmentation, cell shrinkage and fragmentation, and ultimately the engulfment and elimination of cells by phagocytosis [1].

1.3.2 Intrinsic apoptotic pathway

DNA is under constant assault from environmental and intracellular factors, which pose a threat to the integrity of the genome [48]. Genotoxic agents such as ionizing radiation (IR) or reactive oxygen species (ROS) can generate DNA lesions and strand breaks [48, 49], the most dangerous form of which is DNA double-stranded breaks (DSBs) [50, 51]. If not repaired properly, DNA damage can lead to genomic instability and chromosomal rearrangements, both of which are critical features of cancer development [52]. Thus cells have developed intricate signalling pathways, collectively known as the DNA damage response (DDR), to sense and repair DNA strand breaks [51, 52]. Alternatively when repair is not possible, the DNA damage response activates cell death pathways that eliminate damaged cells before the compromised genetic material can be propagated [51-53]. In the DDR, DNA lesions signal the recruitment and assembly of repair complexes at the sites of damage, and the activation of checkpoint signalling [53, 54]. Checkpoint signalling mediates cell cycle arrest in order to allow cells sufficient time to repair the damaged DNA and to restore genomic integrity [53, 54]. Successful DNA repair causes the inactivation of checkpoint signalling and re-entry into the cell cycle [53, 54]. However, in instances where DNA damage exceeds a certain

threshold and is too extensive to be repaired, the intrinsic apoptotic pathway is activated to eliminate damaged cells (Fig 1.2) [52, 54]. DNA damage can signal apoptotic activation through several cellular factors including caspase-2, and the transcription factors p53 and E2F1 [49, 51, 55]. P53 is one of the key proteins involved in mediating DNA damage-induced apoptosis. It has been extensively demonstrated to function as a critical tumour suppressor in cells that prevents genomic instability by promoting cell death in response to irreparable DNA damage [56]. Upon its activation by upstream DNA damage signalling cascades, p53 transactivates the expression of pro-apoptotic B-cell lymphoma 2 (Bcl-2) family factors. Pro-apoptotic Bcl-2 proteins trigger mitochondrial membrane permeabilization (MMP), which causes mitochondrial dysfunction and marks the commitment of cells to apoptotic cell death [52]. Mitochondrial dysfunction results in the release of integral mitochondrial proteins such as cytochrome *c* (cyt *c*) from the intermembrane space into the cytoplasm [57]. Cytosolic cyt *c* then recruits the apoptotic protease-activating factor-1 (APAF-1), and the initiator caspase pro-caspase-9, and together these form the death signalling complex known as the apoptosome [57]. Within the apoptosome, pro-caspase-9 undergoes autocatalytic activation and cleaves and activates the effector caspases, caspase -3 and -7 [57, 58]. Effector caspases have a wide range of cellular targets, including factors involved in the cell cycle and DNA repair, as well as cytoskeletal and structural proteins. Cleavage of these substrates promotes downstream signalling events that culminate in the execution of apoptotic cell death.

Apoptosis is a highly regulated process, and the ability to undergo apoptosis is pivotal to preventing genomic instability and chromosomal rearrangements, and to maintaining cellular and tissue homeostasis [1]. Consequently, deregulation of apoptotic pathways is associated with the occurrence of pathological conditions such as cancer. For example, somatic mutations in p53 are among the most common in cancer, occurring in nearly half of all human malignancies [59]. These mutations result in the simultaneous loss of the tumour suppressor activity of p53 and in the acquisition of oncogenic activity by p53, and are often associated with more aggressive and metastatic cancers [59]. Mutations in the expression and activity of several Bcl-2 family proteins have also been associated with the occurrence and progression of certain cancers, and are often

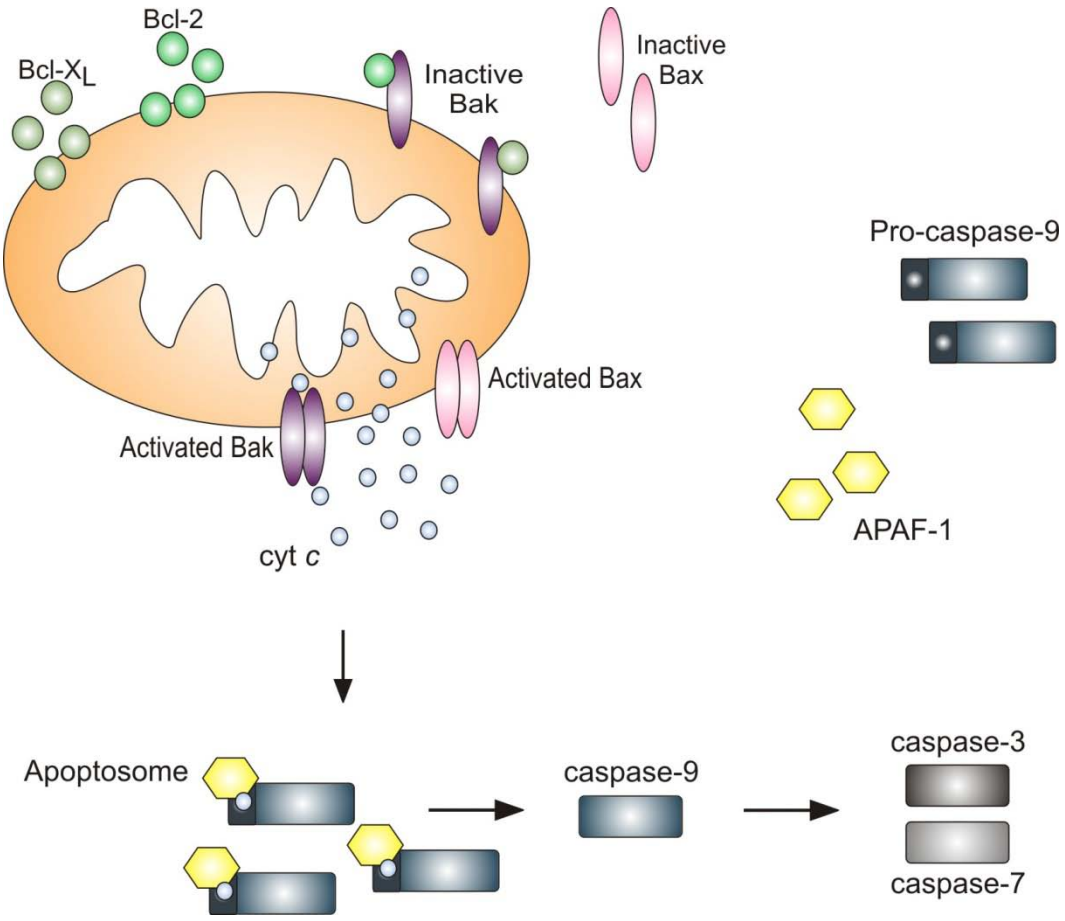

Figure 1.2 Intrinsic apoptotic pathway. In resting cells, Bcl-2 and Bcl-X_L prevent mitochondrial membrane permeabilization by inhibiting Bax and Bak activity. Induction of upstream apoptotic signalling leads to Bax and Bak oligomerization at the mitochondrial membrane, and cytochrome *c* (cyt *c*) release into the cytoplasm. Together with APAF-1 and pro-caspase-9, cyt *c* forms a death signalling complex (apoptosome), in which caspase-9 is activated, and mediates cleavage and activation of the executioner caspases, caspase-3 and caspase-7.

prognostic indicators of poor patient outcome [60-62]. As such, these proteins have become the focus of tremendous research aimed at elucidating the mechanisms that regulate their expression and function under normal conditions, and how these processes are deregulated in cancer.

1.4 Bcl-2 family proteins

1.4.1 Overview

Bcl-2 proteins comprise a large family of pro- and anti-apoptotic factors, with critical functions in apoptotic regulation. In the absence of death stimuli, anti-apoptotic Bcl-2 proteins bind pro-apoptotic Bcl-2 members and retain them in an inactive state, thereby preventing cell death. Upstream apoptotic signalling causes the release and activation of pro-apoptotic Bcl-2 proteins, followed by MMP and cyt *c* release, resulting in cell death. Due to their critical roles in apoptosis, the expression of Bcl-2 proteins is highly regulated, and changes in the relative cellular ratios of the pro- and anti-apoptotic members determines the commitment to cell death. Bcl-2 proteins are divided into three sub-classes: the anti-apoptotic Bcl-2 proteins (Fig 1.3A), the multi-BH (Bcl-2 homology) domain pro-apoptotic proteins (Fig. 1.3B), and the pro-apoptotic BH3-only proteins that as the name indicates contain only the BH3 motif (Fig 1.3C). The BH1-3 motifs in the anti-apoptotic, and multi-domain pro-apoptotic, Bcl-2 proteins form a hydrophobic groove that can be bound by the BH3 motif of BH3-only proteins [63]. In the pro-apoptotic BH3-only proteins, this motif serves as an interaction surface that either binds and represses anti-apoptotic Bcl-2 proteins, or binds and activates pro-apoptotic Bcl-2 proteins [63]. It is believed that for the majority of BH3-only proteins, this motif is required for their death-promoting activity [63, 64]. Finally, the C-termini of Bcl-2 proteins contain hydrophobic regions (known as the transmembrane (TM) domain) that facilitate their interaction with, and their insertion into, intracellular membranes, such as the mitochondria [63].

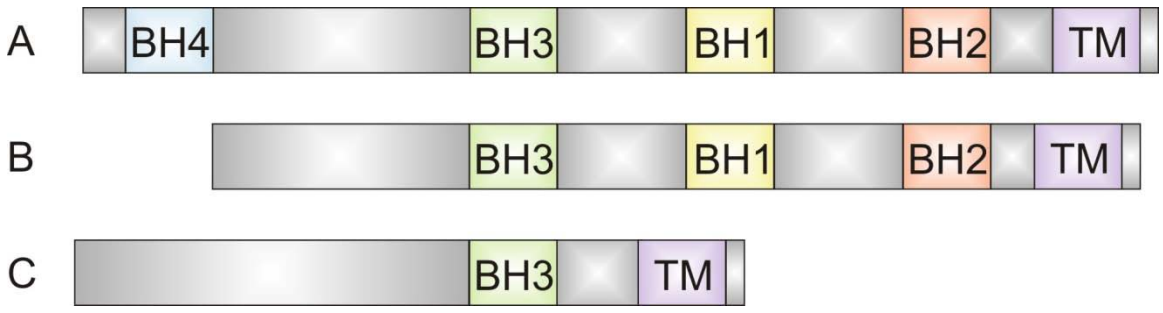

Figure 1.3 Bcl-2 family proteins. The domain structure of the (A) multi-domain anti-apoptotic, (B) multi-domain pro-apoptotic, and (C) BH3-only pro-apoptotic, Bcl-2 proteins is depicted. The respective positions of the Bcl-2 homology (BH), and transmembrane [(TM), purple] domains are indicated for each protein. Figure adapted from [63].

1.4.2 Pro-apoptotic Bcl-2 proteins

The two classes of pro-apoptotic Bcl-2 proteins function to regulate the activation and execution of MMP and cell death. The pro-apoptotic BH3-only proteins act as stress sensors that are activated in response to various stimuli including DNA damage, and function upstream of the mitochondria to activate the pro-apoptotic multi-BH domain proteins [65]. The function of the multi-BH domain pro-apoptotic proteins is to execute mitochondrial permeabilization and cyt *c* release [65].

To date, eight BH3-only Bcl-2 proteins have been identified, including Noxa, p53-upregulated modulator of apoptosis (PUMA), Bad, and Bim, which are each activated by specific death stimuli. Activation of BH3-only proteins is achieved through changes in their expression levels, either via up-regulation of gene expression or changes in post-translational modifications. This increases the cellular ratio of pro-apoptotic Bcl-2 proteins, relative to the anti-apoptotic proteins, and shifts the balance toward cell death [47, 65]. BH3-only proteins can activate the pro-apoptotic multi-BH domain proteins by two distinct mechanisms [64]. They can bind and sequester anti-apoptotic Bcl-2 proteins, thus facilitating the release and indirect activation of the pro-apoptotic multi-BH domain proteins [64, 66]. Alternatively, BH3-only proteins can directly bind the pro-apoptotic multi-BH domain proteins, resulting in their release from inactive heterodimers, and their activation [64, 66]. Based upon their roles as stress sensors and activators of downstream apoptotic signalling, BH3-only proteins are essential to the initiation of apoptosis [64].

The two main multi-BH domain pro-apoptotic Bcl-2 proteins are Bax and Bak, which function by oligomerizing at the outer mitochondrial membrane (OMM), thus causing mitochondrial dysfunction and cell death. Activation of Bax and Bak occurs through changes in their post-translational modifications and sub-cellular localization [64, 66]. In the absence of death stimuli Bax resides in the cytoplasm as an inactive monomer (Fig. 1.2) [57, 65]. Upon activation by BH3-only proteins, it translocates to the mitochondria where it undergoes conformational changes that facilitate its insertion into the OMM [57, 65]. Conversely, Bak resides at the OMM, and is retained in an inactive state through heterodimerization with anti-apoptotic Bcl-2 proteins (Fig. 1.2) [57, 65]. Upon activation Bak is released from these inactive heterodimers, and undergoes

conformational changes that cause its oligomerization [57, 65]. Bax and Bak oligomers form pores or channels in the mitochondrial membrane that disrupt the voltage gradient across the membrane, and lead to loss of mitochondrial integrity. This, coupled with the release of inner mitochondrial proteins such as cyt *c* into the cytosol, commits the cells to apoptotic cell death [47].

1.4.3 Anti-apoptotic Bcl-2 proteins

The central function of the anti-apoptotic members of the Bcl-2 family is to prevent apoptosis by opposing the death-inducing activity of pro-apoptotic Bcl-2 proteins [60, 63]. The five members of this sub-class (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1) are known to protect cells from a variety of cytotoxic insults including IR, UV radiation, and cytokine withdrawal [60, 63]. In resting cells, anti-apoptotic Bcl-2 proteins sequester pro-apoptotic Bcl-2 proteins and neutralize their death-promoting activity. Apoptosis is triggered by "de-repression" of pro-apoptotic Bcl-2 proteins, wherein anti-apoptotic Bcl-2 proteins become inactivated, and mitochondria become sensitized to permeabilization due to enhanced levels of activated Bax and Bak [66]. Further, anti-apoptotic Bcl-2 proteins can localize to the cytoplasmic surfaces of the endoplasmic reticulum (ER), nuclear, and mitochondrial membranes to maintain their membrane integrity, which may also have important implications in regulating cell death [47, 61, 63]. These proteins also have documented roles in regulating cellular and tissue homeostasis, and in development [47, 63, 67].

The execution of these pivotal cellular functions by anti-apoptotic Bcl-2 proteins necessitates intricate regulation of their expression and activity. This is achieved through complex transcriptional and post-translational mechanisms [61]. Extensive studies on Bcl-2 and Bcl-X_L in particular have implicated roles for the cyclic AMP-response element-binding protein (CREB) [68-70] and nuclear factor kappa-B (NF-κB) [68, 71-74] transcription factors in promoting Bcl-2 and Bcl-X_L gene expression, while p53 has been found to repress Bcl-2 gene expression [75, 76]. Bcl-2, Bcl-X_L, and Mcl-1 are also known to be regulated through post-translational modifications that modulate their anti-

apoptotic activity, and protein stability. For example, their phosphorylation can be associated either with enhanced protein stability and anti-apoptotic function [77-79], or with increased ubiquitination and proteasome-dependent degradation [79-84], depending upon the stimulus that triggers the modification. The multi-level regulation of expression and activity of anti-apoptotic Bcl-2 proteins is achieved mainly through the induction of mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) signalling cascades [79, 80, 82, 85-87]. These pathways regulate the expression of anti-apoptotic Bcl-2 factors either through direct phosphorylation of their proteins, or indirectly by modulating the activity of transcription factors that regulate their gene expression. This shifts the balance either toward cell survival or cell death, depending upon the effect of the pathway on cellular levels of anti-apoptotic Bcl-2 proteins.

1.4.4 Bcl-2 proteins and cancer

The ability of cells to undergo apoptosis is critical to tumour suppression. Mutations that result in deregulated expression and activity of Bcl-2 family proteins have important implications in the initiation and progression of several types of cancer [62, 63, 65]. Based upon their roles in promoting cell death, Bax and Bak are proposed to function as tumour suppressors [60, 61, 63]. In support of this notion, Bax was reported to be mutated and/or inactivated in colon tumours [52, 88], and decreased Bax expression was associated with increased tumourigenicity and poor patient outcome [89-91]. Tumour suppressor functions for both Bim [92] and Bid [93] have also been suggested. These observations, as well as the potent role of BH3-only proteins in promoting apoptosis, have made them attractive targets in the development of chemotherapeutic agents that function by reactivating the apoptotic machinery in order to promote tumour cell death [61, 67].

Significant evidence also supports a tumour-promoting function for anti-apoptotic Bcl-2 proteins. Indeed Bcl-2 itself was initially identified based upon a chromosomal translocation event (denoted t(14;18) translocation) occurring in lymphomas. This translocation fuses the Bcl-2 gene to the immunoglobulin heavy chain gene locus,

resulting in aberrant transactivation of Bcl-2, and a marked increase in its expression [62, 94]. Additional studies have determined that the Bcl-2 gene is often amplified and/or overexpressed in many human cancers [62, 95]. Bcl-2 is a proto-oncogene [47, 62], but is unique in that unlike most oncogenes, it does not promote tumourigenesis via enhanced cellular proliferation, but rather through suppression of cell death [65, 67]. Importantly, mutations in Bcl-2 confer resistance to radiation and chemotherapies in patients, and are poor prognostic indicators in many cancers [62, 94, 95]. Likewise, Bcl-X_L expression is associated with increased radio- and chemotherapeutic resistance in many cancers [95, 96], and Mcl-1 expression is reported to be increased in multiple myeloma [97] and chronic lymphocytic leukemia [98]. These collective findings have resulted in substantial efforts aimed at identifying novel therapies that will antagonize the functions of anti-apoptotic Bcl-2 proteins, in order to restore normal apoptotic signalling and response to chemotherapeutic agents in tumours [67, 95]. These strategies include targeting the pathways that modulate the expression and activity of anti-apoptotic Bcl-2 proteins, such as MAPK signalling.

1.5 ERK1/2 signalling pathway

1.5.1 Mitogen-activated protein kinases

Since their initial discovery over 20 years ago, the MAPK superfamily of serine/threonine kinases has become one of the most intensively studied signal transduction modulators in eukaryotes [99, 100]. These evolutionarily and functionally conserved proteins link cell surface receptors to intracellular signal transducers, and have been implicated in the regulation of many important cellular processes including embryogenesis, cellular proliferation, cell differentiation, metabolism, and cell death [99, 101, 102]. Deregulated MAPK signalling has also been attributed to the development of many pathological conditions including inflammatory and degenerative disorders, and cancer [100, 103]. Signalling through the MAPK pathway involves a "phospho-relay" system [101], whereby mitogenic stimuli lead to the sequential activation of three

kinases, each by its upstream kinase, following the general pattern of

MAPKKK → MAPKK → MAPK

that is also regulated at each step by a MAPK phosphatase that serves to attenuate signalling [100-102].

While each MAPK exhibits a certain degree of specificity for its cellular targets (sometimes called MAPK effectors), there is also considerable overlap amongst different MAPKs for these substrates [99, 100]. This overlap allows for the incorporation of signals from numerous stimuli into specific cellular substrates that mediate the response to that stimulus [99]. Thus, regulation of MAPK signalling is achieved through the formation of complexes that integrate various signal inputs and ensure substrate specificity [99]. Within these complexes, scaffolding proteins tether the different components of the signalling pathway to each other, thereby facilitating kinase activation, and determining the specificity of kinases for their targets [99, 100]. These scaffolding complexes can also impact the sub-cellular localization of MAPKs, which has important consequences for MAPK activity, and substrate recognition [99]. Substrates of activated MAPKs include transcription factors, cytoskeletal proteins, other kinases, and upstream components of the MAPK signalling pathway [100, 102, 104]. Upon activation, MAPKs can translocate to the nucleus, where they modulate the activity and DNA binding affinity of transcription factors, thus altering gene expression patterns [100, 102]. In the cytoplasm, active MAPKs can regulate cell motility and morphology through phosphorylation of cytoskeletal proteins [99, 104], and can activate downstream kinases to promote cell cycle progression and cell proliferation [102, 104]. They can also activate negative feedback loops to attenuate their signalling by phosphorylating upstream components of the signalling cascade [102, 105]. This is particularly important as the duration of MAPK signalling determines the functional outcome of signalling, such as whether cells will undergo proliferation or differentiation in response to a given stimulus [100, 102, 105].

To date, four distinct sub-classes of MAPKs have been identified in mammals: ERK1 and 2 (ERK1/2), c-Jun N-terminal kinases/stress activated protein kinases

(JNK/SAPK), p38 kinases, and ERK5 [99, 101, 102], which are each activated by unique MAPKKs to govern distinct cellular responses [99, 102]. Of these, ERK1/2 is perhaps the most well studied MAPK sub-family, which is attributed in part to its pivotal roles in regulating both normal and pathological processes [105].

1.5.2 Overview of ERK1/2 signalling

ERK1 and ERK2 (often referred to as ERK1/2) are ubiquitously expressed, highly related kinases that have important functions in many fundamental cellular and physiological processes, and in the development of various diseases [99, 105, 106]. ERK1/2 signalling can be activated in response to growth factors, cytokines, viral infection, G protein-coupled receptor (GPCR) activation, transforming agents, and cellular stresses [99, 101, 105] to mediate responses such as cell cycle progression and proliferation, cell motility, differentiation, metabolism, cell survival, and apoptosis [105-107]. ERK1/2 signalling is also required for physiological processes such as growth factor response, tissue development and homeostasis, and memory formation, and aberrant signalling through this pathway is known to cause pathologies including cancer, diabetes, and cardiovascular disease [105, 108].

The most well established model of the ERK1/2 pathway is signalling that is activated by the binding of extracellular growth factors to receptor tyrosine kinases (RTKs) at the plasma membrane (Fig 1.4) [99, 105]. Ligand binding initiates the catalytic activity of RTKs, and results in autophosphorylation of tyrosine residues on the cytoplasmic tails of the receptors. Tyrosine phosphorylation leads to the recruitment of the adaptor protein Grb2 (growth factor receptor-bound protein 2) and the guanine exchange factor Sos (Son-of-sevenless) to the membrane, followed by their interaction with the small G-protein Ras. Sos induces Ras activation by mediating GTP loading on Ras. Once activated, Ras recruits the serine/threonine kinase Raf to the membrane and binds it, causing conformational changes that lead to Raf phosphorylation, and activation of its kinase activity. Raf mediates the phosphorylation and activation of MEK1/2, which in turn phosphorylates and activates ERK1/2.

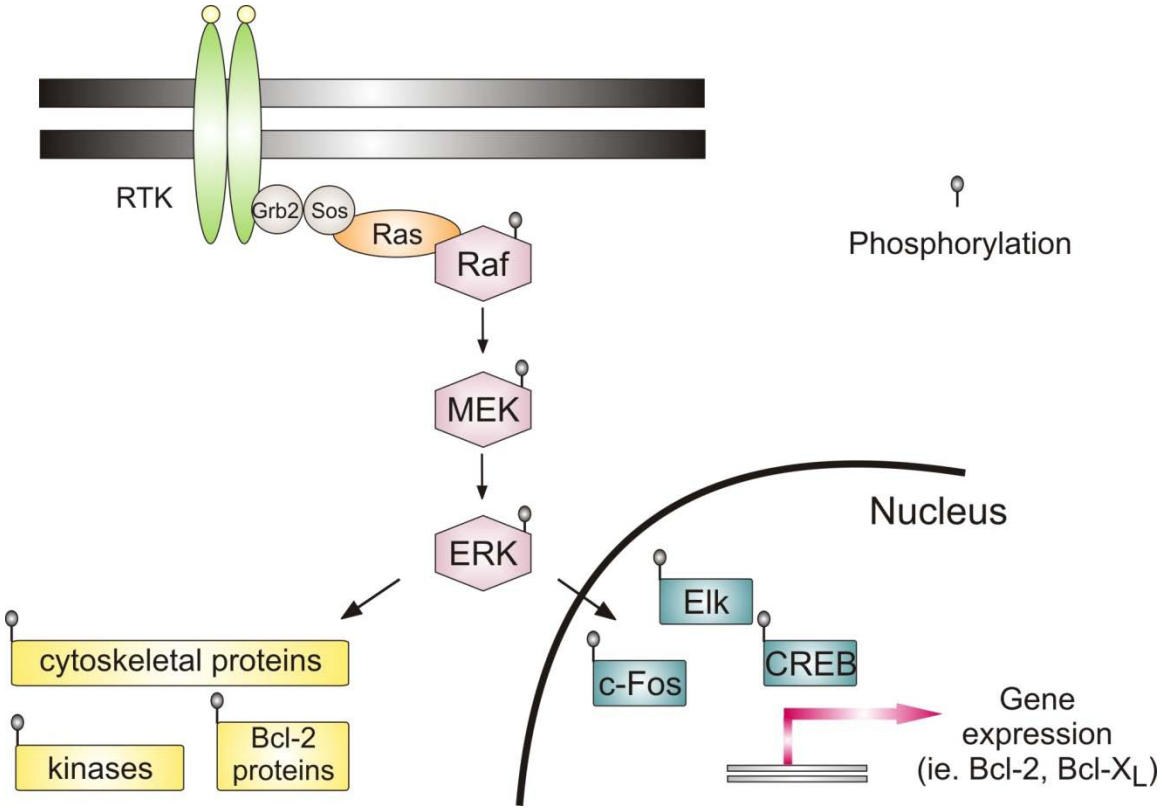

Figure 1.4 ERK1/2 signalling pathway. The binding of growth factors to cell-surface receptor tyrosine kinases (RTKs) results in receptor activation and recruitment of the adaptor protein Grb2, and the guanine exchange factor Sos to the membrane, where they mediate GTP-loading on Ras. Activation of Ras leads to the induction of MAPK signalling and the sequential phosphorylation and activation of Raf, MEK1/2, and ERK1/2. Upon its activation, ERK1/2 phosphorylates numerous nuclear and cytosolic substrates that mediate the specific cellular responses to the stimulus, such as cell proliferation and migration.

To date over 70 cellular substrates of ERK1/2 have been identified [100, 105, 109]. The functional outcome of ERK1/2 activation is dependent, to a great extent, on the substrates that it phosphorylates, which is determined by the stimuli that initiate the signalling pathway [105]. For example, one of the most potent downstream signalling events mediated by ERK1/2 is the activation of transcription factors, including Elk1, c-Fos, Ets, and CREB, achieved through the nuclear translocation of activated ERK1/2 [99, 105]. Activation of transcription factors can govern cellular responses including DNA replication, cell cycle progression, and cell survival [102, 107, 108]. In addition to transcription factors, another important target of activated ERK1/2 is substrates that regulate feedback loops that determine the duration and intensity of ERK1/2 signalling [105]. For example, ERK1/2 can directly mediate inhibitory phosphorylation of MEK [109], Raf [110], and Sos [109] that prevents signal propagation through the cascade. These inhibitory feedback loops ensure appropriate cellular response to the stimulus is achieved (ie. differentiation versus proliferation), and are critical in preventing deregulated ERK1/2 signalling [105].

1.5.3 Raf kinases

1.5.3.1 Overview and structure of Raf kinases

Raf protein kinases link the signals from ligand-mediated receptor activation to downstream signalling pathways, in order to regulate diverse cellular and physiological programs. In mammals, three distinct Raf isoforms encoded by three distinct genes, have been identified: A-Raf, B-Raf, and c-Raf (also known as Raf-1) [111, 112] . Raf proteins are ubiquitously expressed, though A-Raf expression is highest in urogenital organs, and B-Raf expression is most abundant in neuronal tissues [99]. Rafs share a common structure consisting of three conserved regions (CR) with distinct functions, denoted CR1, CR2, and CR3 (Fig 1.5) [111-113]. CR1 and CR2 are located within the N-terminal region of Raf that is required for regulation of Raf activity, whereas CR3 is located within the C-terminal region that contains the Raf catalytic kinase domain

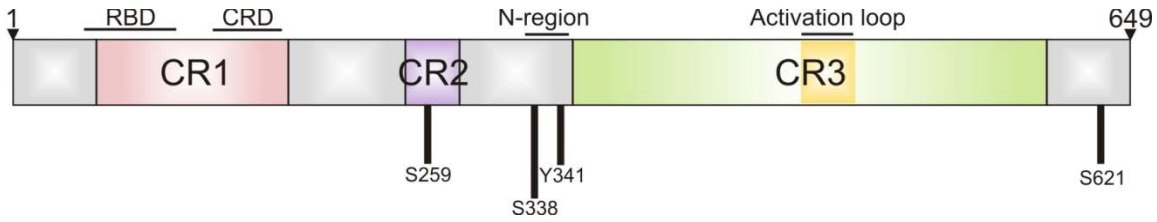

Figure 1.5 Raf protein structure. The mammalian isoforms of Raf (A-Raf, B-Raf, and c-Raf) each consist of three conserved regions: CR1, CR2, and CR3. CR1 (pink) contains the Ras-binding domain (RBD) and cysteine-rich domain (CRD), which are necessary for the recruitment of Raf to the membrane, and for binding to Ras. One of the 14-3-3 binding sites (S259) needed for proper Raf folding is found within CR2 (purple). The catalytic domain of Raf is located within CR3 (green), which also contains the activation loop (yellow) that is pivotal for Raf kinase activity. Immediately upstream of CR3 lies the N-region (negative-charge region) that contains two residues (S338 and Y341) that must be phosphorylated to achieve Raf kinase activation. In the extreme C-terminal region of Raf lies the second 14-3-3 binding site (S621). All amino acid positions indicated correspond to the respective residues in the c-Raf protein. (S, serine; Y, tyrosine). Figure adapted from [112].

[99, 111]. The CR1 of Raf contains the Ras-binding domain (RBD) and cysteine-rich domain (CRD), which facilitate membrane recruitment of Raf, and its interaction with Ras [112, 114]. CR2 is a small region containing several inhibitory phosphorylation sites that are critical to inhibiting Ras binding, and repressing Raf activity [111]. The catalytic domain of Raf is found within CR3, and contains the ATP-binding domain and activation segment [111]. These regions undergo phosphorylation events that are necessary for induction of Raf kinase activity [111]. Since the identification of c-Raf over 20 years ago, Raf proteins have been the subject of intensive study aimed at elucidating the mechanisms that regulate their activation and signalling. While signalling downstream of Raf is well characterized, the mechanisms by which Raf activation occurs remain under study. However, using c-Raf as the model, significant progress has been made in delineating the complex and intricate process of Raf activation [105, 111, 112].

1.5.3.2 Activation of c-Raf

The c-Raf activation/inactivation cycle consists of a series of post-translational modifications, changes in sub-cellular localization, and intra- and inter-molecular protein interactions of c-Raf (Fig 1.6) [110]. In the absence of receptor signalling, c-Raf is held in an inactive state in the cytoplasm. This is achieved through the formation of a "closed" conformation of the protein, where the N-terminal regulatory region folds over the C-terminal catalytic region and inhibits its kinase activity [111, 115]. In this inactive state, a 14-3-3 dimer binds phosphorylated residues in the N-terminal (S259) and C-terminal (S621) regions of c-Raf, and tethers them together, thereby stabilizing the closed conformation [105, 111, 115]. This conformation further represses c-Raf activity by obstructing the interaction surfaces needed for recruitment of c-Raf to the membrane and for its interaction with Ras [112, 115]. Receptor activation mediates the translocation of c-Raf to the membrane, where it is dephosphorylated at S259, releasing 14-3-3 from the N-terminal region of c-Raf, and loosening the closed conformation [105, 111, 115]. These conformational changes allow c-Raf to adopt an "open" conformation that exposes its kinase domain, and enables binding of Ras-GTP to the RBD of c-Raf [111]. In addition to the Ras-binding domain, Ras also binds the CRD of c-Raf, which functions to

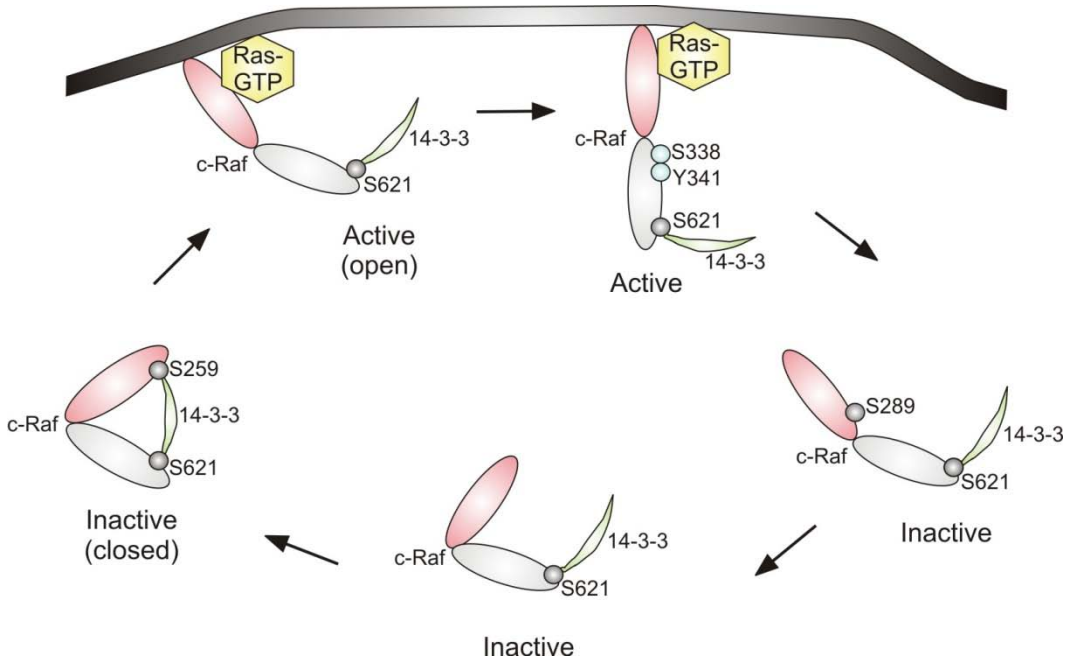

Figure 1.6 C-Raf activation cycle. Phosphorylation of c-Raf at S259 and S621 mediates the binding of 14-3-3 to c-Raf, and the formation of the closed, inactive conformation of the protein. Activated Ras (Ras-GTP) recruits c-Raf to the membrane where S259 is dephosphorylated, allowing c-Raf to adopt an open conformation. Phosphorylation of c-Raf at S338 and Y341 of the N-region facilitates full activation of c-Raf kinase activity, and induction of downstream signalling. Repression of c-Raf activity is achieved by dephosphorylation of S338 and inhibitory phosphorylation at S289, which cause its dissociation from Ras. C-Raf is subsequently re-phosphorylated on S259 to re-form its closed, inactive conformation. (S, serine; Y, tyrosine). Figure adapted from [111].

further stabilize c-Raf membrane localization, and Ras-c-Raf interaction [111]. This also serves as the initiating event in c-Raf catalytic activation [111, 112]. Between the N-terminal regulatory region and C-terminal catalytic region resides the N-region (negatively-charged region) of c-Raf, consisting of Ser^{338}-Ser-Tyr-Tyr^{341} residues [112, 115]. Phosphorylation of S338 and Y341 within this region is essential for the activation of c-Raf kinase activity [111, 113]. While it has been well established that phosphorylation at Y341 is mediated by SRC (v-src sarcoma) and JAK (Janus kinase) family kinases, a great deal of controversy remains over the kinase(s) responsible for phosphorylation of c-Raf at S338, a fact which has been attributed to the diverse stimuli that can signal c-Raf activation [111-113]. In addition to mediating c-Raf activation, phosphorylation of the N-region also facilitates c-Raf-MEK1/2 interaction at the membrane, and the induction of downstream signalling events [111, 113]. Inactivation of c-Raf, and attenuation of its signalling, is achieved through inhibitory phosphorylation at multiple residues including S259 and S289, coupled with dephosphorylation at S338, and binding to 14-3-3 [110, 111]. This restores c-Raf to its closed, inactive conformation in the cytoplasm until the next round of c-Raf signalling is initiated. The complex conformational changes that drive the c-Raf activation cycle underscore the importance of c-Raf protein structure in modulating its activity and downstream signalling. In-depth analyses of the regulation of c-Raf folding and structure have highlighted an important role for molecular chaperones in this process.

1.5.3.3 C-Raf protein folding and stability

1.5.3.3.1 Heat shock proteins

Molecular chaperones are critical to the maintenance of proteostasis, and the prevention of protein misfolding and aggregation within the cell [116]. They function by assisting in the folding of nascent polypeptides, forming of protein complexes, and preventing the aggregation of misfolded or unfolded proteins by mediating either their refolding or degradation [116, 117]. Heat shock protein (HSP) 90 is a highly conserved

molecular chaperone that regulates the folding and stability of over 200 cellular substrates (known as Hsp90 client proteins), with roles in immune response, cell signalling, and protein trafficking [116-118]. Hsp90 can either function alone, or as a component of a multi-chaperone complex with a related molecular chaperone called Hsp70 [119]. Like its counterpart, Hsp70 is a highly conserved chaperone that regulates the folding, trafficking, degradation, and protein-protein interactions of its client substrates [120]. Both Hsp70 and Hsp90 contain an ATPase domain, and utilize the energy from ATP hydrolysis to bind and fold their client proteins [116, 120].

The activity of molecular chaperones is further regulated by a group of cofactors, known as co-chaperones, which either associate with Hsp70 and Hsp90 alone, or together as components of the multi-chaperone complex [116, 119]. In mammalian cells, over 20 co-chaperones have been identified thus far [116], which are grouped into four general categories according to their functions. Co-chaperones can (1) physically link Hsp70 and Hsp90 and mediate the transfer of client proteins between these two chaperone systems, (2) modulate the ATPase activity of chaperones, thus modifying their affinity for substrates, (3) target client proteins to specific chaperones, and (4) recruit chaperones to execute specific cellular tasks such as protein trafficking and degradation [116, 119]. In support of a role in degradation, some co-chaperones have been found to link Hsp70 and Hsp90 to proteasomes. The co-chaperone CHIP (carboxy terminal of Hsp70-interacting protein) is an E3 ubiquitin ligase [121] that binds the Hsp70-Hsp90 complex and mediates the ubiquitination and degradation of unfolded client proteins [116, 119]. BAG1 (Bcl-2 associated athanogene 1) is a co-chaperone that binds the ATPase domain of Hsp70 and opposes its protein folding activity, therefore targeting Hsp70 client proteins to proteasomes for degradation [119]. In addition, certain co-chaperones function on specific classes of protein substrates. For example, the co-chaperone Cdc37 (cell division cycle 37 homologue, also called p50) specifically binds kinases that are Hsp90 client proteins, and assists Hsp90 in their folding and activation [122]. Studies on Raf proteins have uncovered a pivotal role for the molecular chaperone and co-chaperone machinery in regulating the folding, kinase activity, and stability of c-Raf.

1.5.3.3.2 Regulation of c-Raf folding and stability by molecular chaperones

Early studies on c-Raf established that it exists as a component of a high molecular weight protein complex of 300-500kDa, that consisted of Hsp90 and Cdc37 [123, 124]. C-Raf was found to bind Hsp90 via its C-terminal catalytic domain [123, 125] both in its inactive state in the cytoplasm, and when activated and bound to Ras at the plasma membrane [124]. The c-Raf-Hsp90 complex was initially postulated to be necessary for membrane recruitment and activation of c-Raf [126]. However, seminal studies by Schulte and colleagues revealed that in addition to mediating its membrane recruitment, binding of c-Raf to Hsp90 was also critical for stabilization of the c-Raf protein, as disruption of this binding targeted c-Raf for proteasomal degradation [124, 127, 128]. The integration of these findings provided a model wherein the binding of Hsp90 to c-Raf is required to stabilize the tertiary structure of the c-Raf protein and facilitate native c-Raf folding, and to mediate c-Raf activation and signalling [113, 129].

In addition to Hsp90, other components of the molecular chaperone machinery including Hsp70 [113], BAG1 [130], and CHIP [131, 132], have been reported to bind c-Raf and regulate its activity, stability, and degradation. BAG1 binds the catalytic domain of c-Raf and stimulates its kinase activity, thereby promoting the activation of ERK1/2 signalling independently of Ras activation [130]. The same study also reported that Hsp70 competes with c-Raf for binding to BAG1. They found that in resting cells BAG1 tightly binds c-Raf and promotes its activation. However in response to cellular stress such as heat shock, the protein levels of Hsp70 are up-regulated, resulting in the displacement of c-Raf from BAG1, and the suppression of downstream signalling pathways. Therefore, Hsp70 was proposed to function as a negative regulator of c-Raf-BAG1 interaction, and of cell growth signalling [130]. More recently, work by Dogan *et al.* uncovered a role for inhibitor of apoptosis (IAP) proteins in regulating c-Raf stability and degradation through a chaperone-dependent mechanism [132]. They observed that the binding of IAPs to c-Raf interferes with c-Raf folding and disrupts its native conformation, resulting in the recruitment of CHIP to the c-Raf-Hsp90 complex, and the proteasomal degradation of c-Raf [132]. Together, these findings reveal the intricate mechanisms by which c-Raf expression and signalling are modulated within the cell.

1.5.4 Deregulation of Ras-Raf-ERK1/2 signalling in cancer

In the last decade overwhelming evidence has pointed to a central role for deregulated ERK1/2 signalling in promoting cancer development and metastasis, due to the significant growth, migration, and survival advantages afforded to cells harbouring mutations of this pathway [108, 133, 134]. This is reinforced by the finding that components of ERK1/2 signalling are mutated in a significant majority of cancers. Activating mutations in Ras have been observed in 30% of all cancers [135] and occur with particular frequency in pancreatic (90%), colorectal (50%), and thyroid (60%) cancers [134]. Of the Raf kinases, B-Raf is most commonly mutated in cancer [111], with the highest incidence of mutations reported in melanoma (70%) and thyroid cancer (50%) [112, 134]. To date over 100 different mutations in B-Raf have been reported in tumours, though the majority of tumours exhibit a valine to glutamic acid mutation at amino acid 600 (V600E), which disrupts the inactive conformation of the kinase and renders it constitutively active [111, 112]. By comparison, mutations in c-Raf are quite rare [112, 133] but have been found in acute myeloid leukemia [111]. In addition to mutations in Ras and Raf, ERK1/2 is hyperactivated in 30% of human cancers [112], and upstream components of the signalling cascade, including several RTKs, are often mutated in tumours [134]. These staggering statistics have led to tremendous efforts to identify therapeutic strategies capable of inactivating components of the ERK pathway, and thus repress its signalling in cancers harbouring mutations in this pathway.

1.6 Cellular transformation and tumourigenesis

1.6.1 Hallmarks of cancer

Three decades of research into the molecular and physiological processes associated with the development of cancer led to the classification of six essential markers of cell transformation and metastasis [5, 136]. These were termed the "hallmarks of cancer" and were defined as: evasion from apoptosis, uncontrolled cellular replication, increased angiogenesis, enhanced cellular migration and invasion, evasion from growth-suppressor signals, and sustained proliferative signalling [5]. In recent

years, an important role has also emerged for the tumour microenvironment as a key contributor to malignancies [137, 138]. The tumour microenvironment consists of the normal stromal cells that surround the cancer tissue, and is exploited by cancer cells to promote tumour angiogenesis, proliferation, migration, and invasion [137-139]. Current cancer research seeks to provide insight into the mechanisms by which tumour cells manipulate their environment and normal cellular networks to accomplish malignant transformation. This process is believed to be achieved largely through the acquisition of the hallmarks of cancer.

1.6.1.1 Evasion from apoptosis

The process of cellular transformation in itself exposes cancer cells to numerous cellular stresses, such as increased DNA damage arising from hyperproliferation, and accumulation of ROS arising from increased metabolism [137, 140]. While these stresses typically activate apoptotic signalling, cancer cells often develop mechanisms to avoid apoptotic induction. This is accomplished in several ways, such as up-regulation of pro-survival Bcl-2 proteins [61, 67] and the down-regulation of DNA damage sensors such as p53 [59]. Deregulation of apoptosis disrupts cellular and tissue homeostasis, and results in cancer metastasis and resistance to cancer therapies [137].

1.6.1.2 Uncontrolled cellular replication

Normal cells exhibit finite replicative capacities, meaning that they are programmed to undergo a specific number of cell divisions, after which growth stops and cells become senescent [141]. On occasion, cells may acquire the capacity to overcome this senescent state, thus allowing for continued replication [141]. Ultimately however, these cells will enter a second inhibitory state called crisis, which is characterized by wide-spread cell death [5, 141]. However, in highly rare instances, a sub-population of cells are able to also overcome crisis and consequently become immortalized, which is defined by their ability to undergo infinite cycles of cell replication [5]. It is believed that

senescence and crisis function as two important tumour suppressor mechanisms in cells [137]. The number of replications that cells are able to undergo before entering senescence or crisis is suggested to be largely dependent upon the length of telomeres, which protect DNA ends by preventing end-to-end chromosome fusions and genomic instability [136, 141]. Cancer cells are proposed to circumvent senescence and crisis through several mechanisms, such as acquiring mutations in the telomerase gene, thus preventing telomere shortening, or by inactivating factors such as p53 that sense the genomic instability that can be caused by chromosome-end fusions [56, 136, 137]. Uncontrolled cellular replication is associated with tumour growth and progression [137].

1.6.1.3 Sustained proliferative signalling

While in normal cells the expression and release of factors that govern cell growth and division is highly regulated, cancer cells can develop the ability to grow in the absence of growth factor stimulation, and thus exhibit sustained cell proliferation [137]. This is usually achieved through deregulation of signalling pathways that are activated downstream of growth factors [137], such as that of MAPKs and the ERK1/2 signalling pathway [134]. For example, in some cancers hyperproliferation is accomplished through aberrant expression of RTKs that enhance the cellular response to growth factors, or through activating mutations in RTKs that altogether bypasses the need for growth factor activation [134]. In other instances, cancer cells acquire activating mutations in a component of these signalling pathways (such as Ras or Raf) that allows them to circumvent the need for upstream receptor activation [134, 137]. The ability of cancer cells to gain growth factor independence is one of the key processes associated with tumourigenesis.

1.6.1.4 Escape from growth suppression

In cancer, sustained cellular proliferation is frequently coupled with the ability to evade the critical growth suppression mechanisms that exist in normal cells. The

prevention of aberrant cellular growth and proliferation is regulated by tumour suppressors, such as p53 and retinoblastoma protein (pRB), which are accordingly often mutated or inactivated in cancers [136, 137]. P53 is an essential component of the cellular response to intrinsic stresses such as DNA damage, and functions as a tumour suppressor by mediating cell cycle arrest, apoptosis, and DNA repair in damaged cells [59]. The tumour suppressor pRB plays a central role in the regulation of cell cycle progression and apoptosis, and the inactivation of pRB function has been observed in a multitude of cancers [142, 143]. Inactivation of pRB leads to alterations in cell cycle checkpoint activation and cell cycle progression, and in increased genomic instability [142, 144]. Loss of p53 and/or pRB function ultimately impedes the ability to eliminate damaged cells, and thus promotes tumourigenesis.

1.6.1.5 Increased angiogenesis, and enhanced cellular migration and invasion

The hallmarks of cancer outlined thus far are components of the events that initiate cellular transformation, and are involved in the early stages of tumourigenesis and primary tumour development. Cancer progression is characterized by the acquisition of secondary features by tumour cells that mediate metastasis and the formation of secondary lesions in distant tissues. Metastasis marks a key point in cancer progression, as metastatic cancers are predominantly incurable and account for over 90% of all cancer-related deaths [145]. Two key features acquired by metastatic cancers are increased angiogenesis, and enhanced cell migration and invasion.

Angiogenesis represents an important step in the progression of cancer to a metastatic disease [146]. Induction of angiogenesis (known as the angiogenic switch) is characterized by elevated pro-angiogenic signalling and abnormal proliferation of endothelial cells in the tumour microenvironment, that together lead to neovascularisation [146, 147]. Enhanced angiogenesis is required for the exponential growth of tumour cells, and thus mediates disease progression [146, 148].

Metastatic cancer is also the result of an elaborate biological process known as the invasion-metastasis cascade, in which tumour cells invade the local extracellular matrix

(ECM) and surrounding stromal cells, intravasate blood vessels and are transported to secondary sites by the vasculature, extravasate and form micrometastases at these secondary sites, and finally reactivate proliferation at the sites of metastasis to form secondary tumours [145]. A critical step in the invasion-metastasis cascade is epithelial to mesenchymal transition (EMT) [137], where epithelial cells of the primary tumour undergo intricate changes in their architecture and behaviour to become more akin to mesenchymal cells [149]. The acquisition of a mesenchymal phenotype is mediated by changes in gene-expression patterns that alter cell-cell, and cell-ECM contacts, and renders tumour cells highly motile and able to invade distant tissues [149]. EMT also provides protection from apoptosis, which enables cancer cells to survive migration to secondary sites, and to form lesions at these sites [137, 149]. Several cellular networks have been implicated in the regulation of EMT, both in normal and pathological settings, and chief among these is EMT mediated by transforming growth factor β (TGFβ) signalling [150]. Enhanced TGFβ expression and signalling in cancers is associated with increased EMT and migration of tumour cells, and with the induction of angiogenesis in tumours [150, 151].

The processes of angiogenesis and EMT are not novel to pathological conditions, but rather are also pivotal aspects of normal organism development [146, 149]. For example, angiogenesis is required for the *de novo* formation of blood vessels during embryogenesis [146]. This is a highly regulated process, and the factors that signal angiogenesis become quiescent in the adult vasculature [146]. Similarly, EMT is a highly regulated process that is essential for mesoderm formation during early embryogenesis, and for the formation of various tissues, such as bone and muscle, in later stages of embryonic development [149, 150]. Intriguingly, a considerable degree of overlap exists between the pathways that regulate angiogenesis and EMT, and in addition to their critical functions in development, these pathways are also known to contribute to the development and progression of cancer [152].

1.6.2 Deregulated developmental signalling in cancer

Among the distinguishing features of cancer is the acquired ability of cells to manipulate signalling programs that govern normal development, in order to undergo tumourigenesis and metastasis. Therefore it is not surprising that fundamental developmental pathways, such as Notch, TGFβ, and Wnt signalling are often mutated or deregulated in many cancers [153]. Aberrant signalling through these pathways is associated with increased cell proliferation and survival, and enhanced angiogenesis and EMT [152-154]. Thus delineating the mechanisms by which deregulated developmental signalling contributes to cancer development and progression has become highly important to the effective treatment of cancer.

1.6.2.1 Notch signalling

The evolutionarily conserved Notch signalling pathway was first characterized in *Drosophila*, and has since been established as a key component of embryonic development and adult tissue maintenance in metazoans [154, 155]. The four mammalian Notch proteins (denoted Notch 1-4) localize to the plasma membrane as single-pass type I transmembrane receptors, consisting of a large extracellular domain involved in ligand binding and a cytoplasmic domain involved in downstream signalling [154, 155]. Notch receptors are bound by two classes of ligands, known as the Delta-like (Dll 1, 3 and 4) and Jagged (JAG 1 and 2) ligands, which are also expressed as type I transmembrane receptors [154, 155]. Notch signalling is a form of cell-cell communication, and requires the interaction of cells expressing Notch ligands with adjacent cells that express the Notch receptor [154, 156]. Ligand binding triggers the proteolytic cleavage of the receptor, releasing the Notch intracellular domain (NICD) [154, 156]. NICD then translocates to the nucleus and interacts with the DNA binding protein CBF1(C promoter-binding factor 1) to initiate gene expression [154, 156]. To date, the most well established Notch target genes are the Hes (hairy and enhancer of split) and Hey (Hes-related repressor protein) family of basic helix-loop-helix transcription factors [154, 155]. These factors mediate the cellular responses to Notch

signalling, which include embryonic cell-fate determination, differentiation, proliferation, and apoptosis, in a wide range of tissues [152, 154, 156].

A link between aberrant Notch signalling and tumourigenesis was first observed in acute lymphoblastic leukemia, in which a chromosomal translocation event {t(7;9)} resulted in a truncated, constitutively active form of Notch 1 [157]. Subsequent studies in animal models revealed that truncated, constitutively active forms of all four Notch receptors were capable of promoting cellular transformation [155]. The tumourigenic activity of Notch was further corroborated by the finding that Notch receptors, ligands, and effectors are mutated or deregulated in numerous solid tumours including melanoma, non-small cell lung cancer, breast cancer, and ovarian cancer, as well as in hematological malignancies [154, 155]. In addition to tumourigenesis, alterations in Notch signalling have also been associated with cancer metastasis, as activated Notch signalling was found to enhance angiogenesis and EMT [154, 155]. Specifically, ligand-mediated activation of Notch signalling in the tumour microenvironment was reported to promote neovascularisation and angiogenesis in squamous cell carcinomas, and the metastasis of breast cancer to the bone [154]. Intriguingly, Notch signalling can also indirectly mediate metastasis by contributing to EMT programs driven by the TGFβ and Wnt/β-catenin signalling pathways [154, 155]. The cross-talk between these pathways is believed to be an important aspect of developmental regulation, and to contribute to the complexity of treating malignancies bearing mutations in these pathways [152].

1.6.2.2 Wnt/β-catenin signalling

Regulation of developmental programs in the embryo and in adult tissues is also governed by the Wnt signalling pathway. Wnts comprise a large, highly conserved family of secreted growth factors that act as ligands for the Frizzled (Fz) transmembrane, and LRP5/6 (lipoprotein receptor-related proteins 5 and 6), receptors [152, 158]. The founding member of the Wnt family, the proto-oncogene Wnt1, was described in 1982 by Nusse and Varmus as the gene activated by the integration of the mouse mammary tumour virus (MMTV) DNA into virally-induced breast tumours [159]. Since that time

19 Wnt proteins have been identified in mammals, and have been implicated in the regulation of cell fate determination and organogenesis in the embryo, and in the regulation of self-renewal and maintenance in adult tissues [152, 158]. Initiation of Wnt signalling requires the palmitoylation of Wnts on cysteine residues, followed by their secretion from Wnt producing cells by the protein Wntless (Wls) [158, 160]. Secreted Wnts then bind to Fz and LRP5/6 receptors on the surfaces of target cells to trigger intracellular signalling events [158, 160]. The induction of Wnt receptor signalling can activate four distinct pathways within the cell, of which the canonical Wnt/β-catenin pathway is the best understood [158, 161]. In this pathway, β-catenin functions as an effector protein that modulates Wnt target gene expression [162]. Due to its central role in signalling downstream of Wnt activation, the expression of β-catenin is highly regulated [161]. This is accomplished by the cytoplasmic destruction complex, which maintains low cellular levels of β-catenin in the absence of receptor activation by promoting its rapid turnover [158]. In this complex, β-catenin is phosphorylated on a series of serine and threonine residues in its N-terminus by CK1 (casein kinase 1) and GSK3 (glycogen synthase kinase 3), which mark it for ubiquitination and proteasomal degradation [158]. Receptor activation by Wnt ligands inhibits the activity of the cytoplasmic destruction complex, thereby relieving the repression on β-catenin expression [162]. This results in accumulation of stabilized β-catenin in the cytoplasm, followed by its translocation into the nucleus where it binds the TCF/LEF (T cell factor/lymphoid enhancer factor) family of transcription factors to promote gene expression [162]. The transcriptional output of Wnt signalling is often cell-type specific and dependent upon the intended cellular response, such as cell growth and proliferation, cell fate determination, or terminal differentiation [158]. The best characterized gene targets of the TCF/β-catenin complex are positive and negative regulators of the Wnt pathway, cyclin D1, and the transcription factor c-Myc [158].

Since the initial observation of a tumour-promoting function for Wnt1 [159], numerous studies have uncovered a pivotal role for canonical Wnt signalling in cancer development and metastasis. Tumourigenic mutations in the Wnt pathway are frequently observed in tissues which are dependent upon this pathway for renewal or repair, and occur in both hereditary and sporadic cancers [160]. Many of these mutations occur in

genes which are components of the cytoplasmic destruction complex [160]. The result of these mutations is the constitutive stabilization and accumulation of β-catenin, and aberrant Wnt target gene expression [160]. Gain-of-function mutations in Wnt ligands and receptors, and loss-of-function mutations in negative regulators of Wnt signalling, have also been observed in several cancers [153, 160]. Mutations of the Wnt pathway are associated with the development of several solid tumours including melanoma, hepatocellular, gastric, and colon cancers and with hematological malignancies including acute and chronic myeloid leukemias [152, 160]. The tumourigenic activity of Wnt signalling is compounded by the fact that it can also promote cancer metastasis. In this respect Wnt signalling can induce changes in the gene transcription events, and cell adhesion mediated by β-catenin, which cause EMT [163]. In addition cross-talk between the canonical Wnt pathway, TGFβ, and Notch signalling can enhance EMT [160].

1.6.2.3 Summary

How these complex pathways regulate normal organism development and function has only recently begun to be elucidated. Further, how cells gain the ability to reactivate these programs in order to undergo cellular transformation, and exploit them to undergo metastasis, is poorly understood. However, it is clear that acquiring a more detailed understanding of these processes represents an important hurdle that must be surpassed to better understand cancer development and progression.

1.7 Scope of thesis

The observation that perturbations in a few key cellular processes can cause the transition from a normal cell to a cancer cell highlights the delicate balance that must be maintained between signals that promote, or inhibit, cellular growth and survival. Decades of research have significantly advanced our knowledge of how highly intricate cellular networks maintain the balance between life and death. However, the effective detection and treatment of pathological conditions such as cancer requires greater insight

into the mechanisms and factors that "fine-tune" these complex cellular events. Studies on RanBPM have implicated a role for this protein in the regulation of diverse cellular processes such as transcription, cell adhesion, signalling, and apoptosis, which are of importance to both organism development and disease pathogenesis. Despite this knowledge however, the mode of RanBPM function remains elusive. The work presented in this thesis aims to address this matter through characterizing functions of RanBPM in regulation of apoptotic activation, cell signalling, and cellular transformation.

We begin by describing a function for RanBPM in the activation of apoptotic cell death in response to radiation-induced DNA damage (Chapter 2). We show that RanBPM is a pro-apoptotic protein whose ectopic expression promotes apoptotic cell death, whereas down-regulation of RanBPM expression protects cells from apoptosis and enhances cell survival in response to DNA damage. Further, we determine that RanBPM mediates its pro-apoptotic effects by modulating the sub-cellular localization and expression of Bcl-2 family factors. Together, these studies identify RanBPM as a novel regulator of the intrinsic apoptotic signalling pathway.

We then continue our analyses by characterizing the regulation of Bcl-2 family factors by RanBPM, which result in the identification of a role for RanBPM as a novel inhibitor of the ERK1/2 signalling pathway (Chapter 3). The data in this chapter show that RanBPM represses ERK1/2 signalling by regulating c-Raf protein expression and stability, and that down-regulation of RanBPM expression results in hyperactivation of the ERK1/2 pathway. We go on to show that in cultured cells, down-regulation of RanBPM expression results in alterations in cellular behaviour that are associated with cellular transformation and metastasis. These findings identify a central role for RanBPM in the regulation of signalling pathways and processes involved in normal cell function and cancer development.

Finally, the data presented in chapter 4 describe the effects of sustained RanBPM down-regulation on global transcriptional programs in cells. Our gene expression profiling data indicate that RanBPM regulates processes associated with cell signalling, tissue and organ development and maintenance, and cancer. The studies in this chapter

contribute to the characterization of an important role for RanBPM in development, and together with the data obtained in chapters 2 and 3, implicate a function for this protein as a critical tumour suppressor in cells.

1.8 References

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Chapter 2

2. RanBPM has pro-apoptotic activities that regulate cell death pathways in response to DNA damage

2.1 Introduction

The integrity of the genome is under constant threat, not only from environmental toxins and radiation, but also from by-products of normal cellular metabolism. In response to DNA damage, eukaryotic cells trigger signalling pathways to induce cell cycle checkpoints and establish DNA repair complexes [1, 2]. The activation of apoptotic pathways is also an essential component of the DNA damage response, and defects in the activation of apoptosis or in the apoptotic machinery lead to genomic instability [3]. Conversely, chromosomal instability favours the inactivation of apoptotic pathways to select for resistant cells and tumours often harbour inactivating mutations of genes that encode pro-apoptotic factors, such as Bax, or factors that are involved in apoptosis regulation such as p53 [4, 5]. Thus, identifying the factors and mechanisms that link DNA repair and apoptosis is fundamental to our understanding of tumourigenesis and to developing strategies for the prevention of cancer development.

The intrinsic apoptotic pathway is the primary pathway activated in response to DNA damage. The central event in this pathway is mitochondrial membrane depolarization which is controlled by Bcl-2 family factors [6, 7]. Anti-apoptotic family members (such as Bcl-2, Mcl-1 and Bcl-X_L) prevent apoptosis by sequestering and neutralizing the pro-apoptotic members (such as Bax, Bad, Noxa and PUMA) through direct interaction. Pro-apoptotic factors activate membrane permeabilization, releasing mitochondrial intermembrane proteins such as cytochrome *c*, which initiate caspase-dependent apoptosis. Thus, the balance between cell life and death depends on the relative level of expression of pro- and anti-apoptotic members.

RanBPM is a nucleocytoplasmic protein whose function remains largely unknown. Several recent reports have suggested that RanBPM contributes to the regulation of various cell signalling functions, including cell adhesion and migration [8-

11], microtubule regulation [12, 13] as well as the regulation of gene transcription [14, 15]. There is also evidence for RanBPM involvement in signalling pathways elicited by environmental signals. RanBPM is a phosphoprotein whose phosphorylation is modulated by stress stimuli such as osmotic shock and UV radiation [16]. RanBPM is also phosphorylated in response to IR at a consensus site recognized by DNA damage-activated kinases ATM (Ataxia telangiectasia mutated), ATR (ATM-related) and DNA-dependent protein kinase (DNA-PK) [17]. In addition, the participation of RanBPM in apoptotic signalling pathways was suggested based on RanBPM's ability to interact with CDK11p^{46}, a protein implicated in apoptotic signalling cascades [18]. Also, RanBPM was found to interact with the death domain of p75NTR, a member of the TNF receptor family mediating programmed cell death in neurons [19]. Finally, the association of RanBPM with HIPK2 and with p73 has also been suggested to modulate DNA damage-induced apoptotic pathways [20, 21]. However, the functional consequences of these interactions on the regulation of apoptosis remain largely unexplored.

Here, we further the link between RanBPM and apoptosis by establishing a function for RanBPM in promoting apoptosis. We determined that RanBPM expression in Hela cells activates caspase-3 activity and induces cell death. Consistent with these pro-apoptotic capabilities, siRNA-mediated down-regulation of RanBPM compromised the induction of apoptosis and increased cell survival in response to IR. Cells expressing reduced levels of RanBPM also showed altered Bax mitochondrial localization and increased Bcl-2 expression. These results suggest that RanBPM is a DNA damage-activated factor with pro-apoptotic activities capable of regulating the intrinsic apoptotic pathway.

2.2 Materials and methods

2.2.1 Plasmid expression constructs

pCMV-HA-RanBPM (a gift of Dr. Mark Nelson, Department of Surgery, University of Arizona, Tucson, AZ) was described in [18]. pcDNA3-FLAG-RanBPM was generated by sub-cloning of the full-length RanBPM cDNA from pCMV-HA-

RanBPM into pcDNA3-FLAG. pCMV-HA-RanBPM shRNA mutant construct (HA-RanBPM si-mt) was generated by introducing 2 silent point mutations at nt 2152/2153 (TC to AG, numbering with respect to the first ATG of the RanBPM cDNA) in pCMV-HA-RanBPM by site-directed mutagenesis using PfuTurbo (Stratagene, La Jolla, CA, USA). pEGFP-C1 is from Clontech (Mountain View, CA, USA), pCGN-Oct-1 has been described elsewhere [22].

2.2.2 siRNA and shRNA constructs

All siRNAs were purchased from Ambion (Austin, TX, USA): control siRNA (Ambion, #AM4611), RanBPM siRNA#2 (Ambion, #107725, upper strand: 5'-GGAAUUGGAUCCUGCGCAU -3) and RanBPM siRNA#1 (Ambion, #107724, upper strand: 5'-GGCCACACAAUGUCUAGGA-3). To generate shRNA expression constructs, the pSuper.retro.neo expression vector (Oligoengine, Seattle, WA, USA) was digested with BglII and HindIII, and subsequently ligated to double-stranded oligonucleotides corresponding to either control shRNA (Ambion, #AM4611) or RanBPM shRNA #2 (Ambion, #107725).

2.2.3 Cell culture, treatments and irradiation

Hela and HCT116 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Culture medium for Hela and HCT116 control and RanBPM shRNA stable cell lines was also supplemented with 0.35mg/ml G418 (Geneticin, Bioshop Canada, Burlington, ON, Canada). All cell lines were obtained from the American Type Culture Collection. For irradiation experiments, cells were plated the night before irradiation at 50–60% confluency. Irradiations were performed with a Faxitron RX-650 at a dose rate of 1.42 Gy/min.

2.2.4 Transfections assays

Plasmid transfections were carried out with ExGen 500™ (MBI Fermentas, Burlington, ON, Canada). siRNA duplexes were transfected with siPORT™ NeoFX™ (Ambion) following the manufacturer's instructions, with a final siRNA concentration of 20nM. For clonal selection of Hela cells, 0.35mg/ml G418 was added to the media 24h after transfection and carried out for 10-14 days before colony isolation. Two independently derived RanBPM shRNA cell lines [denoted Hela RanBPM shRNA (clone 2-6) and (clone 2-7)] were selected for experimental analyses. Similarly, clonal selection of HCT116 cells was carried out with 0.35mg/ml G418, and experiments were performed using [HCT RanBPM shRNA (clone 2-8) and clone (2-16)] cell lines.

2.2.5 Extracts and western blot analyses

For whole cell extracts, cells were collected in ice-cold PBS and lysed in buffer containing 150mM NaCl, 1mM EDTA, 50mM HEPES (pH 7.4), 10% Glycerol, 0.5% NP40, and supplemented with 1mM PMSF, 1mM DTT, 1µg/ml leupeptin, 10µg/ml aprotinin, 1µg/ml pepstatin, 2mM sodium fluoride, and 2mM sodium orthovanadate. For sub-cellular fractionations, cells were scraped and washed in ice-cold PBS and lysed in mitochondrial lysis buffer (20mM HEPES, 1mM EGTA, 1mM EDTA, 10mM KCl and 1.5mM $MgCl_2$) with 50 strokes of a dounce homogenizer. After centrifugation, the pellet (nuclei) was incubated in nuclear lysis buffer (20mM HEPES, 25% glycerol, 450mM NaCl, 1.5mM $MgCl_2$ and 0.2mM EDTA) and centrifuged to collect the nuclear fraction. The supernatant was centrifuged at 10,000g for 20min to collect the supernatant (cytoplasmic fraction). The pellet (heavy-membrane) was washed in mitochondrial lysis buffer and resuspended in 1% CHAPS buffer (50mM Tris HCl, 110mM NaCl, 50mM HEPES, 10% glycerol and 0.5% NP40), incubated on ice for 15 minutes, and centrifuged to collect the mitochondrial fraction.

For Western blot analysis, extracts were resolved by SDS-PAGE (between 8% and 12%). Gels were transferred on PVDF membrane and hybridized with either of the following antibodies: RanBPM 5M [23] (Bioacademia, Japan), β-actin (I-19, Santa Cruz,

CA, USA), Bax (N-20, Santa Cruz), Bcl-2 (Cell Signalling, Danvers, MA, USA), HA (HA-7, Sigma, Oakville, ON, Canada), γ-tubulin (a kind gift from Dr. Litchfield, University of Western Ontario, London, ON, Canada), Ku70 (AB-4, NeoMarkers), PCNA (clone PC-10, Millipore, Billerica, MA, USA) and Cox IV (Cell Signalling). The blots were developed using the Enhanced Luminol Reagent (Renaissance, NEN Life Sciences).

2.2.6 Caspase assays

Cell extracts were prepared in Lysis buffer (1mM KCl, 10mM HEPES (pH 7.4), 1.5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 5µg/ml leupeptin, 2µg/ml aprotinin, and 10% glycerol). Caspase activity was measured as previously described [24] in caspase assay buffer (25mM HEPES (pH 7.4), 10mM DTT, 10% sucrose, 0.1% CHAPS containing either 10µM caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-(7-amino-4trifluoromethyl-coumarin (DEVD-AFC) or 10µM caspase-2 substrate, N-acetyl-Val-Asp-Val-Ala-Asp-AFC (VDVAD-AFC) (BIOMOL International, L.P., Plymouth Meeting, PA, USA). The fluorescence produced by substrate cleavage was measured on a SpectraMax M5 fluorimeter (excitation 400 nm, emission 505 nm) over a 2h interval. Caspase activity was calculated as the ratio of the fluorescence output in treated samples relative to corresponding untreated controls. For caspase assays of transfected samples, mock-transfected cells were used as controls.

2.2.7 Apoptotic index and survival assays

Apoptosis was assessed by analyzing nuclear morphology in Hoechst 33342 stained cells. Cells were stained live with Hoechst 33342 (1 µg/ml, Sigma-Aldrich, St Louis, MO, USA) and were visualized by fluorescence microscopy (IX70; Olympus, Tokyo, Japan). Images were captured with a CCD camera (Q-imaging, Burnaby, British Columbia, Canada) using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada). A minimum of 500 cells were counted for each sample analyzed, and

the fraction of cells displaying an apoptotic nuclear morphology (chromatin condensation, nuclear blebbing and/or fragmentation) was determined.

For clonogenic assays, cells were plated at single-cell density (200-1000 cells per 6cm dish), irradiated 6-8 h after plating and incubated for 10-14 days to allow for colony growth. Colonies were fixed and stained with crystal violet. Colonies of at least 50 cells were scored as survivors. The number of colonies of irradiated samples was normalized to that of unirradiated controls. For crystal violet staining, the cells were washed with PBS and stained with 0.5% crystal violet in 20% methanol, and rinsed 3 times with PBS.

2.2.8 Statistical analyses

Differences between two groups were compared using an unpaired two-tailed t-test and analysis of variance (ANOVA) was used when comparing multiple groups. Results were considered significant when $P < 0.05$.

2.3 Results

2.3.1 RanBPM overexpression induces cell death

We initially identified RanBPM in a yeast two-hybrid screen as interacting with Octamer factor 1 (Oct-1), a transcription factor previously characterized as a regulator of cell survival in response to DNA damage [22, 25]. Intrigued by preliminary reports implicating RanBPM in signalling pathways elicited by DNA damage [16, 17, 20] we decided to investigate a potential role for RanBPM in the DNA damage response.

In initial transfection experiments, we determined that RanBPM ectopic expression in Hela cells triggered significant cell death 24-48h following transfection, that was not observed upon transfection of similar constructs expressing different cDNAs using identical transfection conditions (data not shown). To confirm a potential effect of RanBPM ectopic expression on cell viability, we expressed RanBPM from a Neomycin selection-containing expression vector and selected transfected cells with G418 for

several days (Fig. 2.1A). While transfection of the vector alone gave rise to numerous G418-resistant colonies, far fewer colonies were observed for cells transfected with RanBPM (20-30 times less), supporting the notion that increased expression of RanBPM reduced cell viability. To determine if RanBPM-induced cell death was due to increased apoptotic activity, we measured caspase activity in RanBPM-transfected Hela cells using a caspase-3 substrate. A strong induction of caspase activity was observed in cells transfected with RanBPM, but not vector alone, suggesting that RanBPM expression activates apoptotic pathways (Fig. 2.1B). Additional caspase analyses performed using extracts from cells transfected with EGFP and Oct-1 expression constructs confirmed that apoptosis was not appreciably induced by overexpression of these proteins, but that the effect was restricted to RanBPM ectopic expression (Fig. 2.1C). Finally to confirm that cell death was triggered specifically by HA-RanBPM expression, we performed indirect immunofluorescence experiments. Cells showing condensed nuclei, typical features of apoptosis, also showed expression of HA-RanBPM detected with an HA antibody (Fig. 2.1D), further linking RanBPM overexpression with cell death. Altogether, these results suggested that increased expression of RanBPM activates apoptotic pathways.

2.3.2 RanBPM down-regulation prevents caspase activation and cell death in response to IR

While our results suggested the possibility of a pro-apoptotic role for RanBPM, we proposed to determine if endogenous RanBPM could fulfill such a function in response to pro-apoptotic stimuli. To investigate a physiological role for RanBPM in the activation of apoptosis, we assessed the effect of RanBPM down-regulation on DNA damage-induced apoptotic induction (Fig. 2.2). The substantial reduction in RanBPM expression obtained through transient siRNA transfection (Fig. 2.2A) resulted in a marked decrease of caspase-3 activation in response to 10Gy of IR (over 2.5-fold decrease at 96h and 120h, Fig. 2.2B). The activation of caspase-2, a stress and DNA damage-induced caspase was also strongly inhibited by RanBPM down-regulation, with over 2-fold reduction of activation at 96h and 120h (Fig. 2.2C). This effect appeared specific to RanBPM as we obtained a similar result with a second RanBPM siRNA

FIGURE 2.1 Ectopic expression of RanBPM in Hela cells induces cell death through apoptotic pathways. A. Cells overexpressing RanBPM display reduced clonogenic potential. Hela cells were transfected with equivalent amounts of pcDNA3-FLAG-RanBPM or empty pcDNA3 vector. Cells were re-plated 24h later at the dilutions indicated and selection was carried out with 0.4mg/ml G418 for 10-14 days at which time cells were stained with crystal violet. **B.** Ectopic expression of RanBPM induces caspase activity. Equivalent amounts of pCMV-HA-RanBPM or pCMV-HA empty vector were transfected in Hela cells. Caspase-3 activity was assayed 24h later. Shown is fold increase of caspase-3 activity in RanBPM-transfected cells over control cells. Results are averaged from seven individual transfections experiments. Error bars indicate the standard deviation of the mean. **C.** Cells were transfected with equivalent amount (250ng) of pCMV-HA-RanBPM, pEGFP-C1 or pCGN-Oct-1. Caspase activity was assayed as in B. Results are from three experiments performed with triplicate samples. Error bars indicate standard deviation. **D.** Cells transfected with pCMV-HA-RanBPM were grown on poly-L-ornithine-treated coverslips, and fixed 30h post-transfection. Immunolabeling for RanBPM was done with an anti-HA antibody and nuclei were stained with DAPI. Arrows indicate cells undergoing apoptosis.

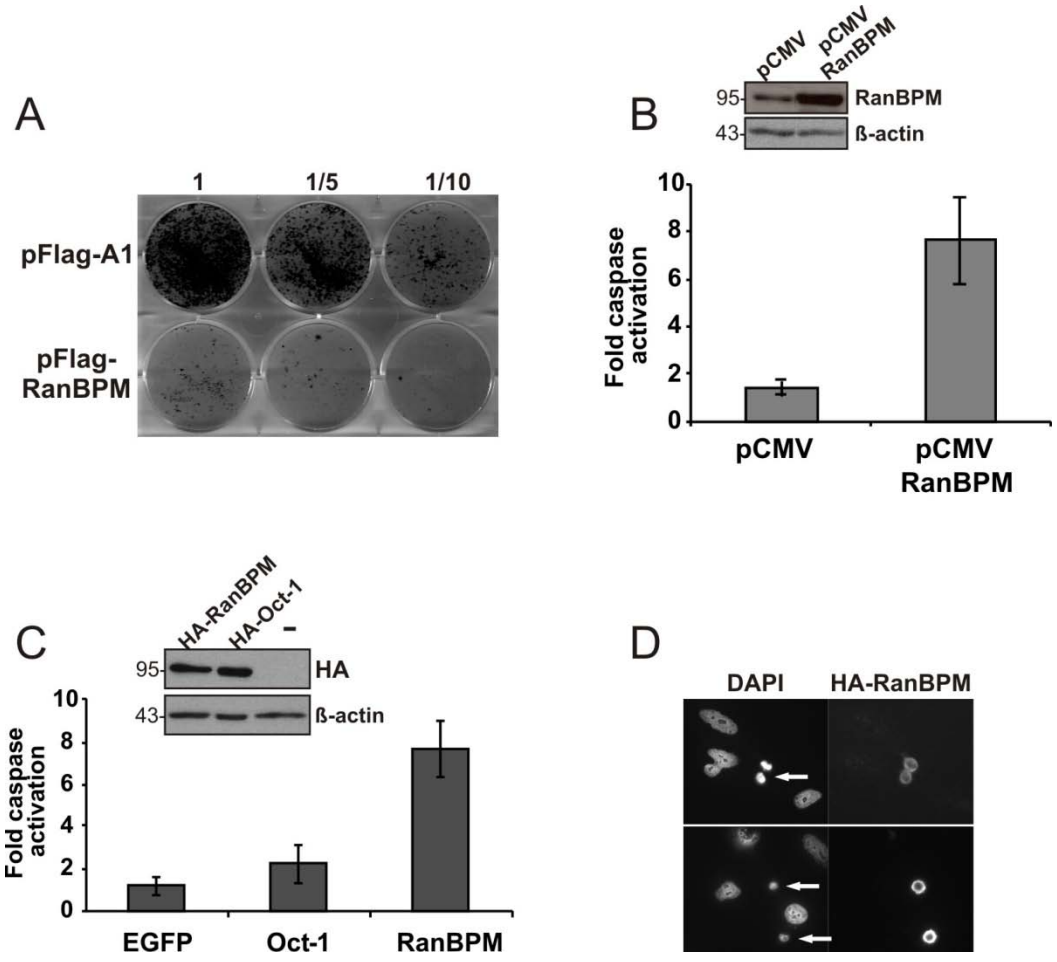
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FIGURE 2.2 Down-regulation of RanBPM expression affects the induction of caspase activity following IR treatment. Hela cells were transfected with control siRNA or RanBPM siRNA#2, split 24h after transfection and plated in 6-well plates for western blot (A) or for caspase assay (B, C). **A.** Unirradiated cells transfected with control or RanBPM siRNA, were harvested at the time indicated after transfection and whole cell extracts were analyzed by western blot with a RanBPM antibody. The membrane was re-hybridized with a β-actin antibody to verify equal loading. **B.** Cells were irradiated with 10Gy of IR or left untreated. Extracts were prepared at the times indicated after IR treatment and assayed for caspase-3 activity using the Ac-DEVD-AFC substrate. Shown is fold increase of caspase-3 activity in irradiated cells over control cells. The results are from three separate experiments with duplicate samples at each time point. Error bars indicate standard deviation and P <0.001 for 96h and 120h. **C.** Extracts prepared in B were assayed for caspase-2 activity using the caspase-2 substrate Ac-VDVAD-AFC. Shown is fold increase of caspase-2 activity in irradiated cells over control cells. Error bars indicate standard deviation and P <0.001 for 96h and 120h. **D.** Hela cells were transfected with control siRNA or RanBPM siRNA#1, split 24h after transfection and plated in 6-well plates for western blot (left panel) or for caspase assay (right panel). *Left* – Unirradiated cells transfected with control or RanBPM siRNA, were harvested at the time indicated after transfection and whole cell extracts were analyzed by western blot with RanBPM and β-actin antibodies. *Right* – Cells were irradiated with 10Gy of IR or left untreated. Extracts were prepared at the times indicated after IR treatment and assayed for caspase-3 activity using the Ac-DEVD-AFC substrate. Shown is fold increase of caspase-3 activity in irradiated cells over control cells. The results are from three experiments with duplicate samples at each time point. Error bars indicate standard deviation.

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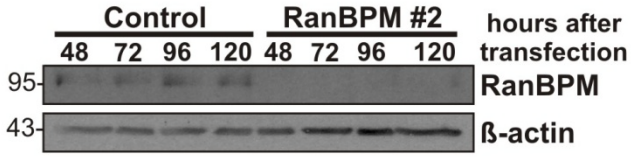
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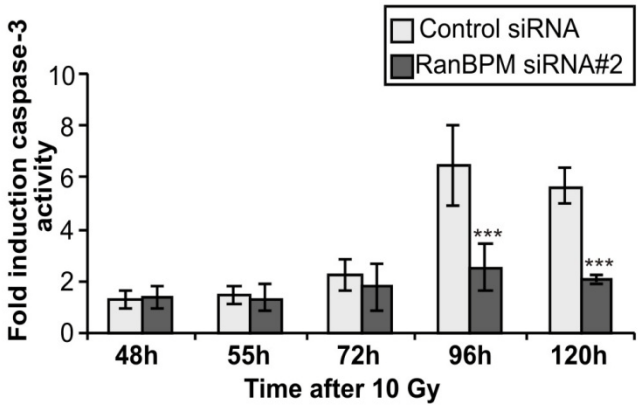
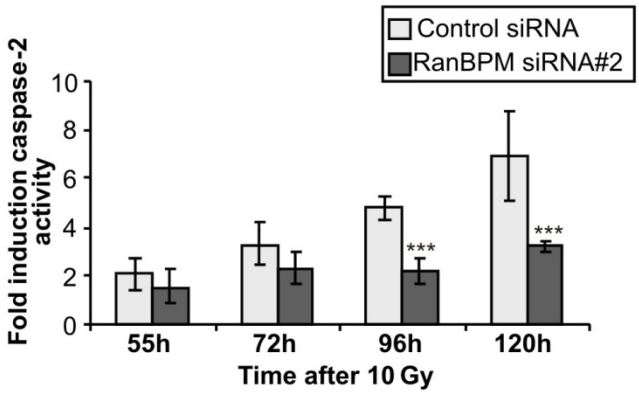
RanBPM
ß-actin

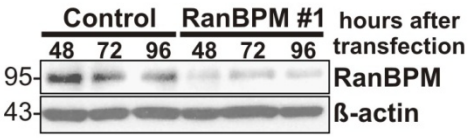
B

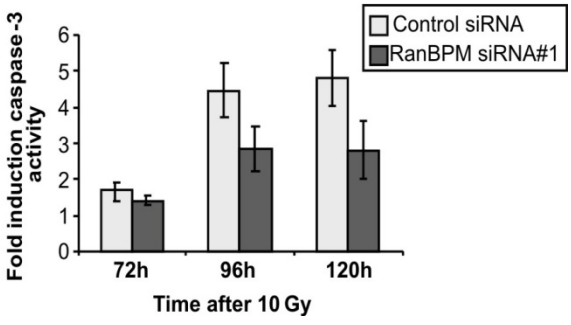

Control siRNA
RanBPM siRNA#2
Fold induction caspase-3 activity
0 2 4 6 8 10
***
***
48h 55h 72h 96h 120h
Time after 10 Gy

C


Control siRNA
RanBPM siRNA#2
Fold induction caspase-2 activity
0 2 4 6 8 10
***
***
55h 72h 96h 120h
Time after 10 Gy

D


Control
RanBPM #1
hours after transfection
48 72 96 48 72 96
95-
43-
RanBPM
ß-actin

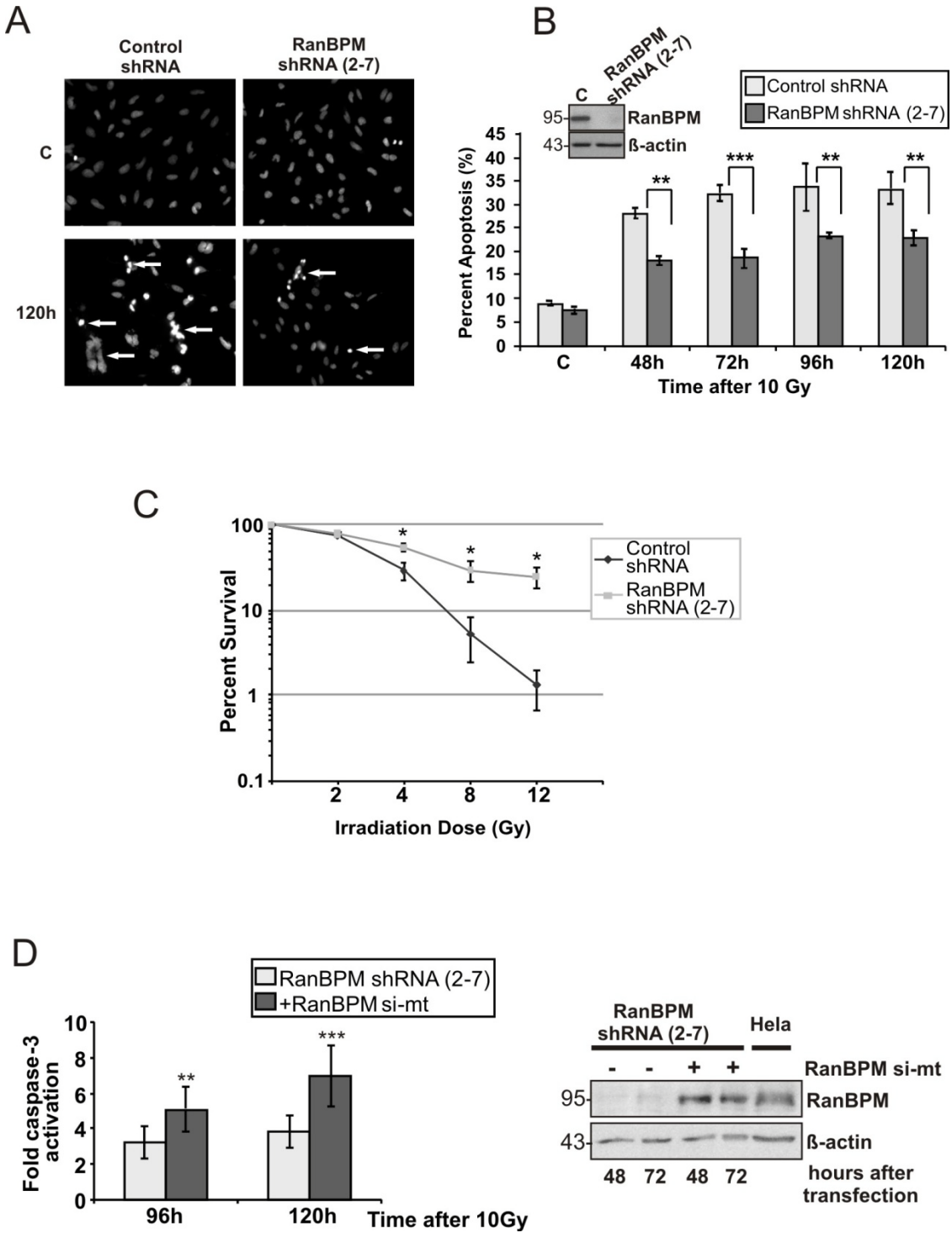

Control siRNA
RanBPM siRNA#1
Fold induction caspase-3 activity
0 1 2 3 4 5 6
72h 96h 120h
Time after 10 Gy

targeting a different region of RanBPM mRNA (Fig. 2.2D). To determine if this decrease in caspase activation correlated with an effect on cell viability, we generated a stable cell line expressing RanBPM siRNA from the pSuper.Neo expression vector in which RanBPM expression was found to be efficiently down-regulated (Fig. 2.3A). We measured the relative number of cells undergoing apoptosis (apoptotic index) after IR treatment by examination of apoptotic cell morphology following staining with Hoechst 33342 in control and RanBPM shRNA cells (Fig. 2.3A, B). Consistent with the reduction of caspase activity observed in our previous assay, we observed a significant decrease in apoptosis induced by DNA damage in RanBPM-deficient cells compared to control shRNA cells. This correlated with an increased overall survival of the RanBPM shRNA expressing cells in colony-forming assay (Fig. 2.3C), further demonstrating the protective effect of RanBPM down-regulation on cell survival following DNA damage. The protective effect conferred by RanBPM down-regulation was particularly apparent at high doses of IR, as over 5-fold increase in survival was observed at 8 Gy (5.3 ± 2.9 for control versus 29.7 ± 8.1 for RanBPM shRNA) and over 10-fold at 12 Gy (1.3 ± 0.6 versus 24.6 ± 6.4). Finally, we verified that the reintroduction of RanBPM in the shRNA down-regulated cell line, through ectopic expression of a RanBPM cDNA bearing a point mutation in the sequence targeted by the shRNA, re-instated caspase activation in response to IR, confirming the specificity of RanBPM function in the activation of apoptotic pathways (Fig. 2.3D). Thus these results suggested that decreased RanBPM expression prevents the activation of apoptosis in response to DNA damage, and promotes cell survival.

2.3.3 RanBPM affects levels and localization of Bcl-2 family factors

IR-induced DNA damage activates apoptosis primarily through the mitochondrial pathway which is controlled by the Bcl-2 protein family [7, 26]. We sought to examine the mechanism of RanBPM pro-apoptotic function by evaluating the effect of RanBPM expression, or lack thereof, on members of the Bcl-2 family. We initiated this analysis by testing the expression and localization of Bax, which is central to the activation of the mitochondrial pathway activated by DNA damage [26]. In most cell types, in response to

FIGURE 2.3 RanBPM down-regulation reduces apoptosis and increases cell survival. **A.** Apoptotic index. Hela cells stably expressing a control shRNA or RanBPM shRNA (clone 2-7) were either irradiated with 10Gy or left untreated (C), and stained with Hoechst 33342. Images were captured with a fluorescent microscope using Northern Eclipse 7.0 software. Images from unirradiated and 120h post-irradiation control shRNA and RanBPM shRNA cells are represented. Arrows indicate fragmented and condensed nuclei considered to exhibit apoptotic morphologies. **B.** To assess apoptotic activation, a minimum of 500 nuclei were counted at each timepoint, and the fraction of cells exhibiting an apoptotic nuclear morphology is indicated. Data represents the mean of four independent experiments with error bars representing standard error, and ** P <0.01, *** P <0.001. The inset shows RanBPM protein levels in whole cell extracts from cells expressing a control shRNA or RanBPM shRNA. Western blot analysis was done with RanBPM and β-actin antibodies as indicated. **C.** Clonogenic assay. Hela cells stably expressing control shRNA or RanBPM shRNA (2-7) were seeded at a single-cell density and either irradiated at the dose indicated or left untreated (C). Cells were incubated for 10-14 days, after which they were fixed and stained with crystal violet and counted. Survival is expressed as the ration of irradiated cells at each dose over control cells. Data represents the mean of three independent experiments with errors bars representing standard error, * P <0.05. **D.** Ectopic expression of RanBPM restores caspase-3 activity in RanBPM-deficient cells. *Left* - Hela cells stably expressing RanBPM shRNA (2-7) were transfected with a pCMV-HA-RanBPM construct bearing a silent point mutation in the RanBPM cDNA sequence targeted by the siRNA (RanBPM si-mt), or with the empty vector. Cells were irradiated (10Gy) 24h after transfection, and incubated for another 96h or 120h, at which time extracts were prepared for caspase-3 assays. Results are averaged from four individual experiments performed in duplicates. Error bars indicate standard deviation, ** P <0.01 and *** P <0.001. *Right* – Representative western blot analysis of RanBPM shRNA (2-7) cells showing the level of expression of transfected RanBPM si-mt (+), in comparison with empty vector transfected cells (-) and control Hela cells (Hela).

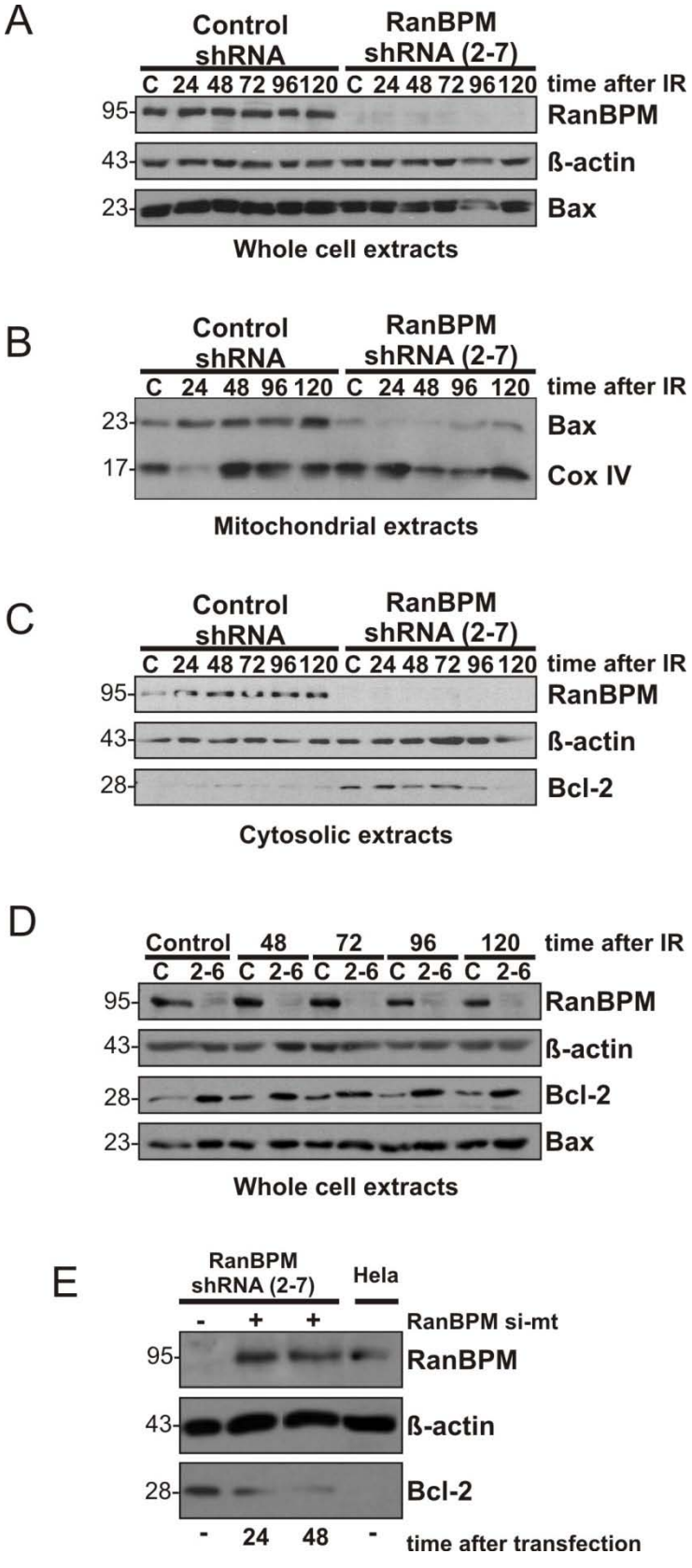

A
Control shRNA
RanBPM shRNA (2-7)
C
120h
B
C
RanBPM shRNA (2-7)
95
43
RanBPM
ß-actin
Control shRNA
RanBPM shRNA (2-7)
Percent Apoptosis (%)
45
40
35
30
25
20
15
10
5
0
**
***
**
**
C
48h
72h
96h
120h
Time after 10 Gy
C
100
10
1
0.1
Percent Survival
*
*
*
Control shRNA
RanBPM shRNA (2-7)
2
4
8
12
Irradiation Dose (Gy)
D
RanBPM shRNA (2-7)
+RanBPM si-mt
Fold caspase-3 activation
10
8
6
4
2
0
**
***
96h
120h
Time after 10Gy
RanBPM shRNA (2-7)
Hela
- - + +
RanBPM si-mt
95
RanBPM
43
ß-actin
48 72 48 72
hours after transfection

apoptotic stimuli, Bax translocates to the mitochondrial membrane, oligomerizes and triggers the release of cytochrome *c* and other proteins present in the intermembrane space [6, 27]. However, in some cell types, including Hela cells, Bax mitochondrial levels do not increase during apoptosis, and Bax activation occurs through its oligomerization and insertion in the mitochondrial membrane [28, 29].

Analysis of whole cell extracts from control shRNA and RanBPM shRNA (clone 2-7) cells showed that Bax protein levels were unaffected by RanBPM down-regulation (Fig. 2.4A, D). However, analysis of heavy membrane fractions revealed that the levels of Bax associated with the mitochondria were strikingly decreased in extracts from RanBPM shRNA cells compared to control shRNA cells (Fig. 2.4B). Since Bax localization and activation are largely dependent on Bcl-2 regulation [7, 30], we analyzed Bcl-2 levels in cells exhibiting reduced expression of RanBPM. Bcl-2 protein levels were found markedly increased in cytoplasmic and whole cell extracts prepared from RanBPM shRNA (2-7) cells (Fig. 2.4C, E). Elevated Bcl-2 expression was also observed in whole cell extracts prepared from a second, independently derived clonal RanBPM shRNA cell line [Hela RanBPM shRNA (2-6)] (Fig. 2.4D). Further, we confirmed that the rise of Bcl-2 protein levels was triggered by the lack of RanBPM expression as re-introduction of RanBPM in the RanBPM shRNA cells (via RanBPM si-mt transfection) reduced Bcl-2 protein levels close to those observed in control shRNA cells (Fig. 2.4E). Thus, RanBPM pro-apoptotic activity is mediated, at least in part, by its regulation of factors of the mitochondrial apoptotic pathway.

Finally, to verify that the effects of RanBPM depletion were not due to a cell line-specific mechanism in Hela cells, we produced HCT116 clonal derivatives expressing either a control or RanBPM shRNA. As was observed for Hela cells, down-regulation of RanBPM in HCT116 cells correlated with a strong increase in Bcl-2 protein levels in two independent clonal-derived cell lines (RanBPM shRNA 2-8 and 2-16) (Fig 2.5A). Further, RanBPM depleted HCT116 cells also displayed decreased caspase activation in response to IR (Fig. 2.5B) and increased survival as measured by clonogenic assays (Fig. 2.5C), confirming that RanBPM also regulates apoptotic pathways in these cells.

FIGURE 2.4 RanBPM down-regulation affects Bax localization and Bcl-2 expression. A. Hela cells stably expressing control shRNA or RanBPM shRNA (2-7) were subjected to 10Gy of IR, or left untreated (C). Whole cell extracts were prepared at the indicated timepoints and 20µg of protein was analyzed by western blot with antibodies directed against RanBPM and Bax, and β-actin was used as a loading control. **B.** Control and RanBPM shRNA (2-7) cell lines were treated as in A, and mitochondrial (heavy membrane) fractions were prepared at timepoints indicated. Western blotting was performed with 10µg of protein extract. Mitochondrial localization of Bax was assessed using a Bax antibody, and Cox IV was used as a loading control **C.** Control shRNA and RanBPM shRNA (2-7) cells were treated as in A, cytosolic fractions were prepared at the timepoints indicated, and extracts were analyzed by western blotting using antibodies against RanBPM, β-actin and Bcl-2. **D.** Control shRNA (C) and RanBPM shRNA (2-6) cells were either irradiated at 10Gy or left untreated (control). Whole cell lysates were prepared at the timepoints indicated, and were analyzed by western blotting using the indicated antibodies. **E.** Down-regulation of Bcl-2 levels through restoration of RanBPM expression. Hela RanBPM shRNA (2-7) cells were either left untransfected (-) or were transfected with pCMV-HA-RanBPM si-mt (+ RanBPM si-mt), and whole cell lysates were prepared 24h and 48h post-transfection. Western blot analysis was performed with 20µg of protein extracts from control shRNA, untransfected RanBPM shRNA (2-7), and RanBPM shRNA (2-7 + RanBPM si-mt) using antibodies directed against RanBPM and Bcl-2. β-actin was used as a loading control.


A
Control shRNA
RanBPM shRNA (2-7)
C 24 48 72 96 120 C 24 48 72 96 120
time after IR
95
RanBPM
43
ß-actin
23
Bax
Whole cell extracts
B
Control shRNA
RanBPM shRNA (2-7)
C 24 48 96 120 C 24 48 96 120
time after IR
23
Bax
17
Cox IV
Mitochondrial extracts
C
Control shRNA
RanBPM shRNA (2-7)
C 24 48 72 96 120 C 24 48 72 96 120
time after IR
95
RanBPM
43
ß-actin
28
Bcl-2
Cytosolic extracts
D
Control
48
72
96
120
time after IR
C 2-6 C 2-6 C 2-6 C 2-6 C 2-6
95
RanBPM
43
ß-actin
28
Bcl-2
23
Bax
Whole cell extracts
E
RanBPM shRNA (2-7)
Hela
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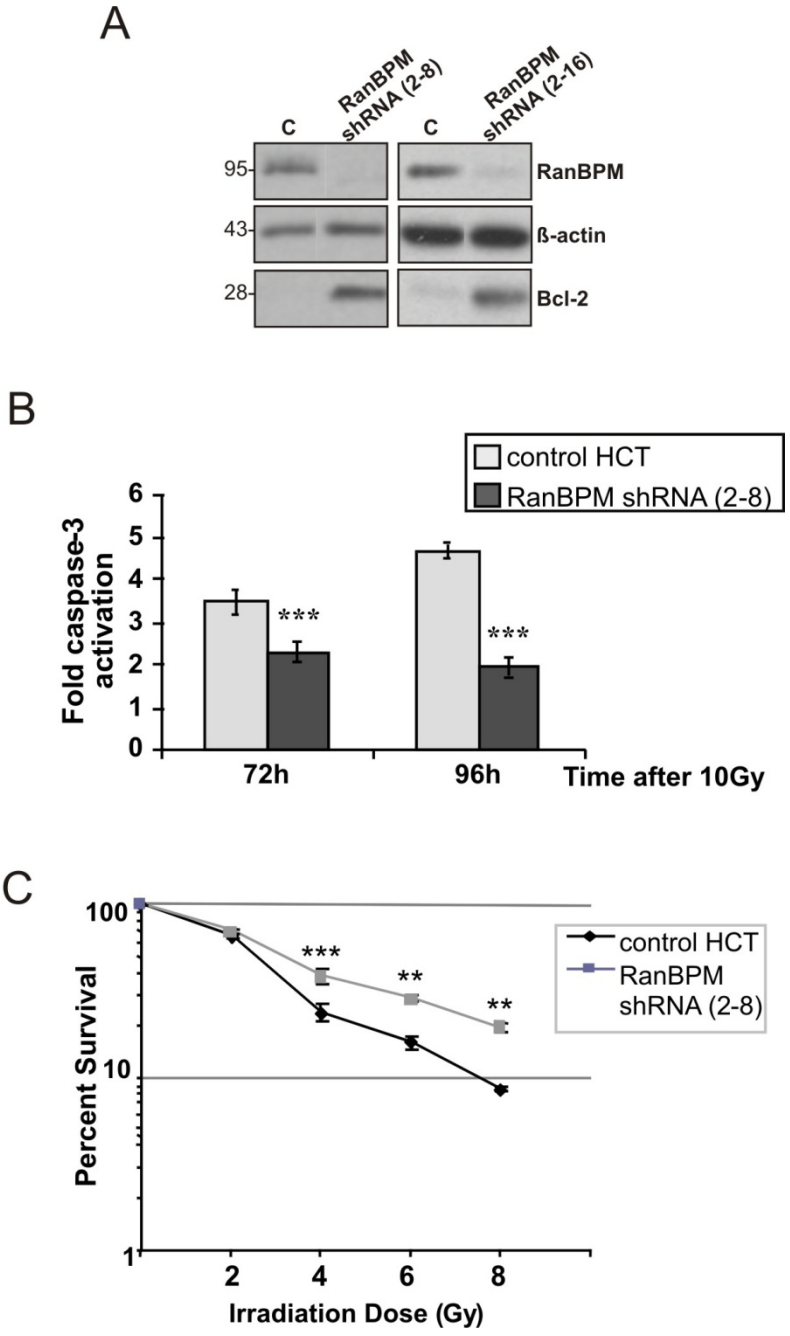
RanBPM si-mt
95
RanBPM
43
ß-actin
28
Bcl-2
- 24 48 -
time after transfection

FIGURE 2.5 RanBPM down-regulation in HCT116 cells. A. Western blot analysis of control and RanBPM shRNA (2-8) and (2-16) HCT116 stable cell lines. Whole cell extracts were analyzed with antibodies against RanBPM, Bcl-2 and β-actin. **B.** Control and RanBPM shRNA (2-8) expressing cells were either irradiated at 10Gy or left untreated. At the timepoints indicated, extracts were prepared and assayed for caspase-3 activity. Results are from four independent experiments with triplicate samples at each time point. Error bars indicate standard deviation and *** P <0.001 for 72h and 96h. **C.** Clonogenic assay. HCT116 control and RanBPM shRNA cells (2-8) were plated at single-cell densities, and either irradiated at the dose indicated or left untreated. Cells were incubated for 6-8 days, after which they were fixed, stained with crystal violet, and counted. Survival is expressed as the ratio of irradiated cells at each dose over control cells. Data represents the mean of four independent experiments with errors bars representing standard error. ** indicates P <0.01 and *** indicates P <0.001.


A
C
RanBPM shRNA (2-8)
C
RanBPM shRNA (2-16)
95-
43-
28-
RanBPM
ß-actin
Bcl-2
B
control HCT
RanBPM shRNA (2-8)
Fold caspase-3 activation
0
1
2
3
4
5
6
***
***
72h
96h
Time after 10Gy
C
100
10
1
Percent Survival
***
**
**
control HCT
RanBPM shRNA (2-8)
2
4
6
8
Irradiation Dose (Gy)

2.4 Discussion

Here, we present evidence that RanBPM functions as an activator of the mitochondrial apoptotic pathway. By the use of two complementary approaches, overexpression and down-regulation, we demonstrated that RanBPM regulates the activation of caspases, modulates the levels and localization of Bcl-2 family members, and regulates cell survival in response to DNA damage.

While overexpression of RanBPM triggered caspase activation, down-regulation of RanBPM prevented the activation of apoptotic pathways in response to DNA damage, thus establishing a function for RanBPM in the activation of apoptosis in response to genotoxic insults. RanBPM depletion had a marked effect on overall cell survival in response to IR. This raises the possibility that RanBPM may also function to regulate other forms of cell death such as necrosis and autophagy that can also be triggered, in addition to apoptosis, in response to DNA damage [31]. Apart from caspase-3, we have found 3 proteins that exhibit changes in their expression, activity, or localization upon RanBPM down-regulation: Bcl-2, Bax and caspase-2. Together, these observations may account for the apoptotic defects observed in RanBPM-deficient cells. Bcl-2 is a primary regulator of the mitochondrial apoptotic pathway, and its overexpression impairs the activation of apoptosis and is linked to cancer development [4, 30]. Bcl-2 functions by interacting with, and neutralizing, members of the pro-apoptotic family. In particular, Bcl-2 has been shown to prevent Bax translocation and oligomerization, either through direct binding or by preventing its activation through BH3-family factors [6, 28, 32, 33]. Thus, the increase in Bcl-2 expression could explain the decrease in mitochondrial Bax observed in RanBPM-down-regulated cells. Consistent with previous observations [34], Bcl-2 up-regulation was not associated with an increase in overall Bax levels.

Bcl-2 levels have been shown to be regulated by several mechanisms and pathways. First, Bcl-2 expression is regulated at the transcriptional level by several transcription factors including CREB, p53, NF-κB, Sp1, and Oct-1 [35-37]. Bcl-2 can also be regulated by post-translational modifications such as phosphorylation and ubiquitination [38]. In turn, several studies have implicated RanBPM in transcriptional regulation [14, 15, 39], while others have linked RanBPM to the regulation of post-

translational modifications such as phosphorylation, ubiquitination, and sumoylation [10, 20, 39]. Thus, RanBPM could control Bcl-2 transcriptionally and/or post-translationally. We noted a steady decrease of Bcl-2 protein levels in response to IR in RanBPM shRNA cells. Such a decrease was previously suggested to be causally involved in apoptotic activation [40-42]. Yet, this IR-dependent (RanBPM-independent) Bcl-2 down-regulation is not sufficient to fully activate apoptotic pathways in RanBPM-deficient cells, suggesting that RanBPM also affects the activation of other pro-apoptotic pathways.

Interestingly, we found that caspase-2 activation in response to IR was abrogated in absence of RanBPM. Several studies indicate that caspase-2 activation is a requirement for mitochondrial membrane permeabilization and the apoptotic response induced by various agents, including DNA damage and H_2O_2 [43-45]. Caspase-2 has been reported to contribute to Bax translocation and oligomerization at the mitochondrial membrane [44, 46]. However, the signals controlling caspase-2 activation are still largely undefined, and caspase-2 activation has been found in turn to occur through both Bcl-2-dependent and Bcl-2-independent mechanisms [47-50]. Further investigation will be needed to understand whether RanBPM controls the activation of both factors or if they are regulated by each other sequentially.

In conclusion, we have characterized a novel pro-apoptotic function for RanBPM and revealed a critical role for this factor in the activation of cell death pathways triggered by DNA damage. Our results predict that in cells exhibiting genomic alterations, RanBPM inactivation would result in decreased cell death and allow for the propagation of potential oncogenic mutations. Interestingly decreased levels, and altered patterns of expression, of RanBPM were previously observed in cancer cells from several tumour samples, suggesting that down-regulation of this protein accompanies cancer development [16]. While further investigations will be needed to explore the molecular details of RanBPM action, our current findings suggest that RanBPM could be an important regulator of pathways that prevent tumourigenesis by promoting the elimination of cells with genomic alterations.

2.5 References

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Chapter 3

3. RanBPM is an inhibitor of ERK signalling

3.1 Introduction

The ERK pathway is activated by a wide range of signals including growth factors, cytokines and external stressors. These signals trigger the activation of transmembrane receptors such as receptor tyrosine kinase (RTK) or G protein-coupled receptors which activate the Ras-Raf-MEK signalling cascade [1, 2]. Activation of Ras is mediated by adaptor proteins, including Sos (son-of-sevenless) and Grb2 (growth-factor-receptor bound 2), which mediate GDP for GTP exchange on Ras, leading to Ras activation [1, 3]. Activation of Ras at the plasma membrane leads to its association with Raf serine/threonine kinases, promoting their activation and in turn phosphorylation and activation of MEK1/2, ultimately resulting in the activation of ERK1 and ERK2 [1, 3]. ERK1 and ERK2 (commonly referred to as ERK1/2 or ERK) are over 80% identical and share many physiological functions. ERK1/2 are promiscuous kinases that have been demonstrated to act on nearly 100 cellular targets, and regulate several diverse cellular functions such as cell cycle progression, proliferation, cell adhesion, transcription, and importantly cell death and apoptosis [3, 4]. The ERK pathway is generally associated with increased cell survival and proliferation and has been shown to be constitutively activated in many tumours [4, 5]. In particular, the ERK pathway is known to inhibit apoptosis by regulating the levels and activity of many apoptotic regulators, including Bcl-2 and Bcl-X_L [4, 6, 7].

RanBPM is a nucleocytoplasmic protein whose function is still elusive, but that has been implicated in a variety of cellular functions, including transcriptional regulation [8, 9], regulation of cell morphology [10, 11] and regulation of receptor-activated intracellular signalling pathways including those activated by MET, TrkA and TrkB [12-15]. Analyses of RanBPM-deficient mice have recently shown a role for RanBPM in gametogenesis in both genders [16]. Several reports have also suggested that RanBPM functions as a regulator of apoptotic pathways through its interaction with several apoptotic factors such as cyclin-dependent kinase CDK11p^{46}, the p75 neurotrophin

receptor (p75NTR), p73, and homeodomain interacting protein kinase-2 (HIPK-2) [17-20]. We have also demonstrated a functional role for RanBPM in DNA-damage induced activation of the intrinsic apoptotic pathway (Chapter 2). We found that down-regulation of RanBPM inhibited the activation of apoptosis in response to ionizing radiation (IR), and consequently led to increased cell survival in both Hela and HCT116 cells. Furthermore, we showed that down-regulation of RanBPM resulted in a substantial up-regulation of Bcl-2 protein levels, suggesting that RanBPM pro-apoptotic function could result at least in part from its ability to regulate the expression of anti-apoptotic factors.

In the present study we provide evidence that the RanBPM-mediated modulation of Bcl-2 expression is linked to its regulation of the ERK pathway. We first demonstrate that similarly to Bcl-2, the protein levels of Bcl-X_L are markedly increased in RanBPM down-regulated cells and that RanBPM controls the expression of these anti-apoptotic factors both at the transcriptional and post-translational levels. Next, we demonstrate that RanBPM down-regulation results in increased ERK1/2 activation that can be reversed upon re-expression of RanBPM. Further, the effect of RanBPM on Bcl-2 expression is dependent on the regulation of the ERK1/2 pathway by RanBPM. We also provide evidence that RanBPM's control of ERK signalling occurs through a regulation of c-Raf levels/stability and that RanBPM associates with c-Raf and affects the interaction of c-Raf and Hsp90. Finally, we show that RanBPM down-regulation promotes cell proliferation and migration, cell transformation properties known to be triggered by deregulated ERK activation. Together, our findings implicate a novel role for RanBPM as an inhibitor of ERK1/2 activation through the regulation of c-Raf stability. They also suggest that loss of RanBPM function, in addition to compromising apoptosis, promotes cellular events leading to cellular transformation, and that these effects could be attributed, at least in part, through a deregulation of the ERK pathway.

3.2 Materials and methods

3.2.1 Plasmid expression constructs

pCMV-HA-RanBPM shRNA mutant construct (RanBPM si-mt) was described in chapter 2. pEGFP-C1 is from Clontech (Mountain View, CA, USA), and pCGN-ΔN-Oct-1 has been reported elsewhere [22]. The pCMV-3xFlag-Bcl-2 construct was a kind gift from Dr. Sean P. Cregan (University of Western Ontario, London, ON, Canada). The kinase-deficient ERK1 construct pCEP4-DN-ERK1 (DN-ERK1) [23] was a kind gift from Dr. Melanie H. Cobb (University of Texas, Southwestern Medical Centre, Dallas, TX, USA). The constitutively active H-Ras construct pSV-3xHA-RasV12 (RasV12) [24] was a kind gift from Dr. Arthur Gutierrez-Hartmann (University of Colorado Denver, Aurora, CO, USA). The constitutively active c-Raf constructs pEBG-GST-ΔN-c-Raf (GST-ΔN-c-Raf) and pCMV-Flag-c-Raf Y340D/Y341D (Flag-Y/Y-c-Raf) [25, 26] were a kind gift from Dr. Zhijun Luo (Boston University, Boston, MA, USA).

3.2.2 siRNA and shRNA constructs

Control siRNA and RanBPM siRNA were purchased from Ambion (Austin, TX, USA) and have been described in chapter 2. Generation of the pSuper-shRanBPM and pSuper-shControl has been outlined in chapter 2.

3.2.3 Cell culture and treatments

Hela and HCT116 control shRNA and RanBPM shRNA stable cell lines were generated previously (Chapter 2). HEK293 control shRNA (clones 1-21 and 1-24) and RanBPM shRNA (clones 1-2 and 1-7) stable cell lines were similarly obtained by clonal selection of cells transfected with pSuper-shRanBPM or pSuper-shControl vectors. Hela, HCT116, and HEK293 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, and 2mM L-glutamine at 37°C in 5% CO_2. Control shRNA and RanBPM

shRNA stable Hela and HCT116 cell lines were maintained in media supplemented with 0.35mg/ml G418 (Geneticin, Bioshop Canada, Burlington, ON, Canada), and HEK293 clonal derivatives were maintained in 0.45mg/ml G418. For serum starvation experiments, HCT116 cells were cultured in media containing 0.1% FBS and HEK293 cells were cultured in serum-free media. For the MEK1/2 inhibitor (U0126) experiments, RanBPM shRNA Hela cells were treated with 10μM U0126 (Cell Signalling, Danvers, MA, USA) or DMSO alone (Sigma, Oakville, ON, Canada) for 24h.

3.2.4 Transfections assays

Plasmid transfections were carried out with ExGen 500™ (MBI Fermentas, Burlington, ON, Canada) according to the manufacturer's protocol, and siRNA duplexes were transfected with siPORT NeoFX (Ambion) as outlined in chapter 2.

3.2.5 Western blot, co-immunoprecipitations and GST-pull down assays

Preparation of whole-cell extracts has been described in chapter 2. In experiments involving c-Raf analysis, the whole cell extract buffer was supplemented with 0.5% Triton-X-100. For Western blot analysis, extracts were resolved by SDS-PAGE (between 8% and 12%). Gels were transferred on PVDF membranes and hybridized with the following antibodies: RanBPM 5M (Bioacademia, Japan), β-actin (I-19, Santa Cruz, Santa Cruz, CA, USA), Bcl-2 (Cell Signalling), Bcl-X_L (Cell Signalling), HA (HA-7, Sigma), Flag (M2, Sigma), phospho-T202/Y204-ERK1/2 (Cell Signalling), ERK1/2 (Cell Signalling), phospho-S217/221-MEK1/2 (Cell Signalling), MEK1/2 (Genscript, Piscataway, NJ, USA), c-Raf (clones C-12 and E-10, Santa Cruz), Hsp90 α/β (clone H-114, Santa Cruz), γ-tubulin (a kind gift from Dr. David Litchfield, University of Western Ontario, London, ON, Canada), GST (GE Health Care Life Sciences, Baie d'Urfe, QC, Canada). The blots were developed using the Western Lightning® Enhanced Chemiluminescence Reagent (Perkin Elmer, Waltham, MA, USA).

In co-immunoprecipitation analyses of RanBPM and c-Raf, 1.5mg of extracts were adjusted to 0.25% Triton X-100, 0.25% NP-40 and 100mM KCl, immunoprecipitations were carried out for 2h at 4ºC with the indicated antibodies, and immunoprecipitates were isolated with Dynabeads® protein G (Invitrogen, Life Technologies, Burlington, ON, Canada). GST pull-down assays were performed overnight in the same conditions with glutathione beads (Sigma). In co-immunoprecipitation analyses of c-Raf and Hsp90, extracts were adjusted to 0.2% NP-40, 0.04% Triton X-100, and 100mM KCl. The amount of protein was adjusted to obtain similar amount of c-Raf immunoprecipitates from control and RanBPM shRNA cells, and immunoprecipitations were carried out overnight at 4ºC with c-Raf (E-10) antibody and were isolated using Dynabeads®.

3.2.6 Quantitative reverse-transcriptase PCR

Total RNA was collected from Hela control shRNA, RanBPM shRNA, and RanBPM shRNA re-expressing RanBPM si-mt, cells using the Qiagen RNeasy RNA Extraction kit (Qiagen, Mississauga, ON, Canada). cDNA was prepared from 2.5µg of total RNA using the SuperScriptII Reverse Transcriptase kit (Invitrogen, Life Technologies). For gene expression analyses of Bcl-2 and Bcl-X_L, 10ng cDNA was incubated with control RNA polymerase II primers (Pol II) (FW: 5' TTGCCTGTGGCTTGATGCG 3' RV: 5' TTTGTTCTTCCCGAGGATCAGC 3'); and 50ng cDNA was incubated with either Bcl-2-specific primers (FW: 5' TTGTTGTTGTTCAAACGGGA 3' RV: 5' ACAAAACCCCACAGCAAAAG 3') or Bcl-X_L-specific primers (FW: 5' GTAAACTGGGGTCGCATTGT 3' RV: 5' CAGGTAAGTGGCCATCCAAG 3'). For c-Raf analysis, 10ng cDNA was incubated with either control GAPDH primers (FW: 5' GTAGCTCAGGCCTCAAGACCTTGG 3' RV: 5' TGCGGGCTCAATTTATAGAAACCG 3') or c-Raf primers (FW: 5' TTAATCGCGGGCGCTTGGGC 3' RV: 5' CCAGCTGACCCTTTTCGGGGC 3'). Quantitative real-time PCR analysis was performed using SYBR green (Bio-Rad, Mississauga, ON, Canada) and the Bio-Rad MyiQ single-colour real-time PCR detection

system. Relative quantification of gene expression was determined by the ΔΔC(t) method, with Bcl-2, Bcl-X_L and c-Raf C(t) values normalized to that of the controls.

3.2.7 Cell growth and cell migration assays

To assess cellular growth rates, control shRNA and RanBPM shRNA HEK293 cells were seeded in triplicate in 6-well dishes, and 24h post-plating cells were placed in serum-free media. At each timepoint cells were trypsinized, counted using a hemocytometer, and the mean number of cells was determined. Percent growth was obtained by dividing the number of cells at each time point by the number of cells at day one.

In cell migration assays, control and RanBPM shRNA HEK293 cells were grown to 100% confluence on 24-well dishes. Cell monolayers were incubated in the presence of 2mM hydroxyurea (Sigma) for 24h to prevent cell proliferation, after which cells were scratched using a sterile 200µl pipette tip, washed, and maintained in DMEM supplemented with 2mM hydroxyurea. Wound closure was assessed at 0h and 24h using a fluorescent microscope (IX70, Olympus), and images were captured using a charge-coupled device camera (Q-imaging). Percent migration was determined by measuring the wound width at each time point using ImageJ software.

3.2.8 Statistical analyses

Statistical differences between groups were analyzed by a student's *t*-test and one-way analysis of variance (ANOVA) using GraphPad (GraphPad Software Inc., La Jolla, CA, USA). Results were considered significant when $P < 0.05$.

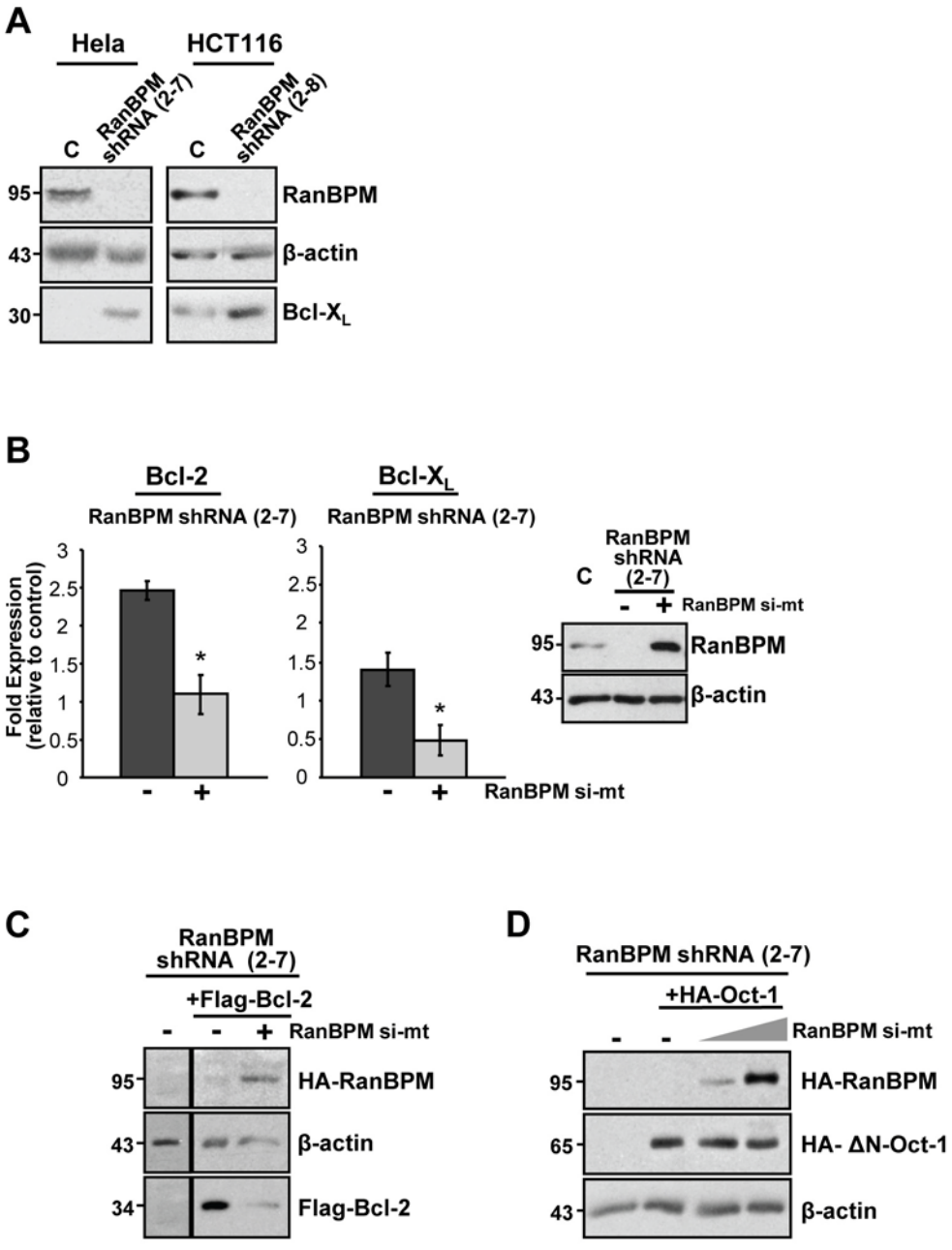
3.3 Results

3.3.1 RanBPM modulates transcriptional and post-transcriptional events that regulate Bcl-2 and Bcl-X_L expression

We showed in chapter 2 that down-regulation of RanBPM expression leads to increased Bcl-2 protein levels in Hela and HCT116 cells. We expanded these analyses to determine whether the expression of other anti-apoptotic Bcl-2 family factors such as Bcl-X_L and Mcl-1 was also altered in the absence of RanBPM. Analysis of whole cell extracts from control shRNA and RanBPM shRNA Hela and HCT116 revealed that Bcl-X_L protein levels were markedly elevated in RanBPM shRNA cells compared to control cells (Fig. 3.1A). However, we found that Mcl-1 protein levels remain unchanged in RanBPM shRNA cells (data not shown).

We carried out quantitative reverse transcriptase-PCR (qRT-PCR) analyses to determine whether RanBPM is involved in the regulation of Bcl-2 and Bcl-X_L gene expression. RanBPM shRNA cells showed increased mRNA levels for both Bcl-2 (2.5-fold increase) and Bcl-X_L (1.4- fold increase) in comparison to control shRNA cells (Fig. 3.1B). To verify that this increase in gene expression was specifically due to RanBPM down-regulation, we re-expressed RanBPM in RanBPM shRNA cells by transfecting a cDNA containing a point mutation in the shRNA target sequence (RanBPM si-mt) (described in chapter 2). Upon RanBPM re-expression, Bcl-2 and Bcl-X_L mRNA expression was reduced to levels near that of control shRNA cells (Fig. 3.1B), thus confirming a role for RanBPM in the transcriptional regulation of Bcl-2 and Bcl-X_L. Previous reports have implicated RanBPM in the regulation of protein stability [17, 27], therefore we sought to determine whether RanBPM may also regulate Bcl-2 and/or Bcl-X_L protein levels. To this end, we expressed a Flag-Bcl-2 construct under the control of the CMV promoter in RanBPM shRNA cells and analyzed the effect of RanBPM re-expression on the Flag-Bcl-2 levels. Expression of RanBPM led to a significant down-regulation of Flag-Bcl-2 protein levels (Fig. 3.1C). To ensure that this was not due to an effect of RanBPM on the CMV promoter, we repeated this experiment using an Octamer transcription factor-1 (Oct-1) expression construct also under the control of a CMV promoter Endogenous Oct-1 protein levels are not affected by RanBPM down- regulation

Figure 3.1 Regulation of Bcl-2 and Bcl-X_L expression by RanBPM. **A.** Down-regulation of RanBPM leads to enhanced Bcl-X_L expression. Whole cell extracts were prepared from Hela and HCT116 control shRNA and RanBPM shRNA cells and were analyzed by western blotting. Blots were hybridized with antibodies against Bcl-X_L, β-actin, and RanBPM. **B**. RanBPM shRNA cells exhibit enhanced Bcl-2 and Bcl-X_L mRNA expression. cDNA from Hela control shRNA, RanBPM shRNA, and RanBPM shRNA cells re-expressing RanBPM via transient transfection of RanBPM si-mt construct was analyzed by qRT-PCR with RNA polymerase II (Pol II), Bcl-2, and Bcl-X_L, specific primers. Relative quantification of Bcl-2 and Bcl-X_L gene expression was determined using the ΔΔC(t) method with Bcl-2 and Bcl-X_L expression normalized to that of the controls. Bars represent values normalized to control shRNA cells. Data represents the mean of three independent experiments with error bars representing standard deviation, and * P <0.05. *Inset*, representative western blot analysis of whole cell extracts to control for the levels of RanBPM using a RanBPM antibody and β-actin as a loading control. **C**. RanBPM expression down-regulates Bcl-2 protein levels. Hela RanBPM shRNA cells were transfected with pCMV-3xFlag-Bcl-2. 24h post-transfection, cells were split and were either transfected with pCMV-HA-RanBPM si-mt (RanBPM si-mt) or empty vector. Whole cell extracts were prepared 48h later and analyzed by western blotting. Expression of ectopic Bcl-2 was determined by hybridization with an anti-Flag antibody. RanBPM expression was assessed with a RanBPM antibody, and β-actin was used as a loading control. **D**. Control experiment to confirm the specificity of RanBPM expression on Bcl-2 protein levels. This experiment was carried out the same as in C, except that RanBPM shRNA cells were transfected with pCGN-HA-ΔN-Oct-1 instead of Flag-Bcl-2. The truncated ΔN-Oct-1 migrates at 65kDa as opposed to full-length Oct-1 (which migrates at 90kDa), allowing for detection of Oct-1 and RanBPM expression in cells transfected with both constructs. Blots were hybridized with anti-HA antibody to verify Oct-1 and RanBPM expression.

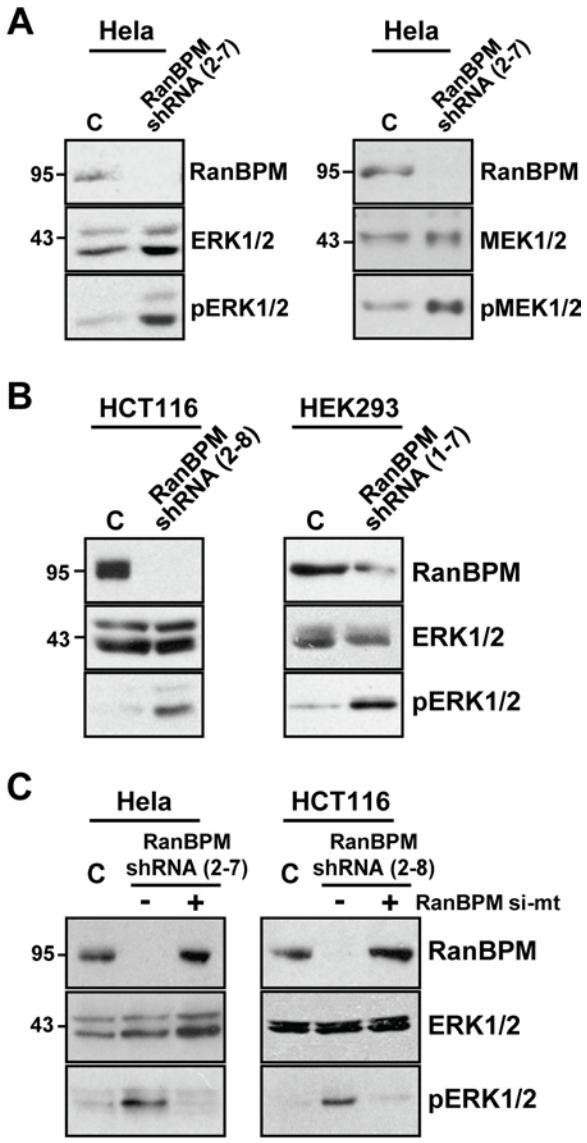

A
Hela
HCT116
C
RanBPM shRNA (2-7)
C
RanBPM shRNA (2-8)
95
43
30
RanBPM
β-actin
Bcl-X$_L$
B
Bcl-2
RanBPM shRNA (2-7)
Bcl-X$_L$
RanBPM shRNA (2-7)
Fold Expression (relative to control)
3
2.5
2
1.5
1
0.5
0
*
*
- +
- +
RanBPM si-mt
C
RanBPM shRNA (2-7)
- + RanBPM si-mt
95
43
RanBPM
β-actin
C
RanBPM shRNA (2-7)
+Flag-Bcl-2
- - + RanBPM si-mt
95
43
34
HA-RanBPM
β-actin
Flag-Bcl-2
D
RanBPM shRNA (2-7)
+HA-Oct-1
- - RanBPM si-mt
95
65
43
HA-RanBPM
HA- ΔN-Oct-1
β-actin

(data not shown). As Oct-1 and RanBPM migrate at the same size on SDS-PAGE (approximately 95kDa), a truncated form of Oct-1 (HA-ΔN-Oct-1 [28]) lacking Oct-1 N-terminus was used so that ectopically expressed HA-Oct-1 and HA-RanBPM would be detected on the same gel. Oct-1 protein levels were found to be unchanged upon RanBPM expression (Fig. 3.1D), indicating that RanBPM does not modulate the activity of the CMV promoter, thus confirming a regulation of Bcl-2 by RanBPM through a post-transcriptional or post-translational mechanism.

3.3.2 RanBPM inhibits ERK1/2 activation

Our findings that RanBPM modulates Bcl-2 and Bcl-X_L expression through mechanisms involving transcriptional and post-translational regulation suggested that RanBPM could regulate signalling pathway(s) that control the expression of both factors. One of the main pathways that regulates Bcl-2 (and Bcl-X_L) both transcriptionally and post-transcriptionally is the ERK pathway [4, 5]. RanBPM was previously shown to participate in ERK1/2 signalling, but the effects of RanBPM on this pathway remain controversial [12, 29, 30]. Thus, we looked at a direct effect of RanBPM down-regulation on ERK activation by comparing ERK1/2 and MEK1/2 phosphorylation in extracts from Hela control shRNA and RanBPM shRNA cells. Both MEK1/2 and ERK1/2 phosphorylation was significantly enhanced in Hela RanBPM shRNA cells compared to control cells (Fig. 3.2A). To verify that this elevated ERK1/2 activation was not specific to Hela cells, we prepared extracts from serum-deprived HCT116 control and RanBPM shRNA cells, and we observed a similar up-regulation in ERK1/2 phosphorylation in RanBPM shRNA HCT116 cells compared to control cells (Fig. 3.2B). In addition, we generated a third stable cell line by expressing either the control shRNA or RanBPM shRNA expression construct in HEK293 cells, which are immortalized but not transformed [31]. We obtained two independently derived control shRNA cell lines [denoted HEK control shRNA (clone 1-21) and (clone 1-24)], and two independently derived RanBPM shRNA cell lines [denoted HEK RanBPM shRNA (clone 1-2) and (clone 1-7)]. Analysis of extracts from these cells revealed that similarly to the effect observed in HCT116 cells, serum starvation led to enhanced ERK1/2 phosphorylation in

Figure 3.2 RanBPM is a negative regulator of ERK1/2 activation. **A**. Enhanced MEK1/2 and ERK1/2 phosphorylation in the absence of RanBPM. Whole cell extracts were prepared from Hela control shRNA and RanBPM shRNA cells (2-7) and analyzed by western blotting. Activation of ERK1/2 was determined by hybridization with phospho-ERK1/2 antibody. Expression of RanBPM in control shRNA cells was verified using a RanBPM antibody, and total ERK1/2 was used as a loading control. **B**. HCT116 control shRNA and RanBPM shRNA (2-8) cells were serum-starved for 18h in 0.1% FBS. HEK293 control shRNA and RanBPM shRNA (1-7) cells were incubated in serum-free media for 24h. Whole cell extract were prepared and analyzed as described in A. **C**. Control and RanBPM shRNA Hela and HCT116 cells were either left untransfected, or were transfected with empty vector or RanBPM si-mt. 24h post-transfection, HCT116 cells were serum-starved in 0.1% FBS, and extracts were prepared 18h later. Western blot analysis was carried out the same as in A.


A
Hela
RanBPM shRNA (2-7)
C
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43
RanBPM
ERK1/2
pERK1/2
Hela
RanBPM shRNA (2-7)
C
95
43
RanBPM
MEK1/2
pMEK1/2
B
HCT116
RanBPM shRNA (2-8)
C
95
43
RanBPM
ERK1/2
pERK1/2
HEK293
RanBPM shRNA (1-7)
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ERK1/2
pERK1/2
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pERK1/2
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RanBPM shRNA (2-8)
- +
RanBPM si-mt
RanBPM
ERK1/2
pERK1/2

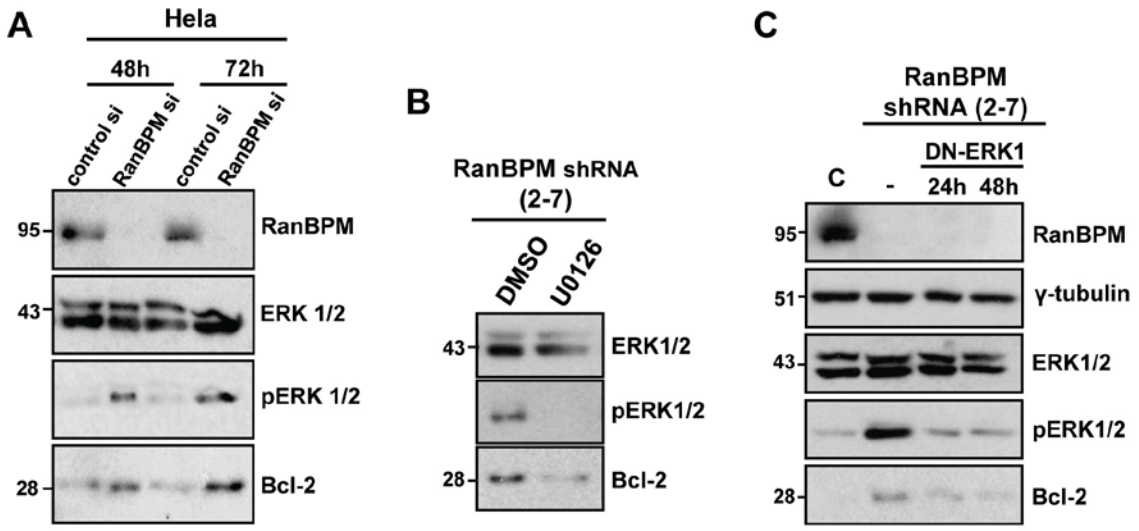
RanBPM-down-regulated HEK293 cells compared to control shRNA cells (Fig. 3.2B). Finally, we verified that the increased ERK1/2 activation was due to a lack of RanBPM expression, as re-expression of RanBPM in both RanBPM shRNA Hela and HCT116 cells led to a marked down-regulation of ERK1/2 phosphorylation to levels near that of control cells (Fig. 3.2C). Together, these analyses suggested that RanBPM inhibits ERK phosphorylation and that down-regulation of RanBPM leads to a constitutive ERK activation.

3.3.3 Inhibition of ERK1/2 signalling down-regulates Bcl-2 protein levels in RanBPM shRNA cells

To further confirm that the enhanced ERK1/2 activation was due to a decrease of RanBPM expression, we performed transient siRNA knockdown experiments in Hela cells (Fig. 3.3A). Transient down-regulation of RanBPM corresponded with a marked increase in ERK1/2 phosphorylation, and this correlated with an increase in Bcl-2 protein levels, suggesting a direct link between RanBPM expression, ERK pathway activation, and Bcl-2 up-regulation.

Since activation of the ERK pathway has been shown to enhance Bcl-2 expression [4, 5, 32], we assessed whether the increased ERK1/2 phosphorylation in RanBPM shRNA cells was responsible for the elevated Bcl-2 protein levels observed in these cells. Treatment of RanBPM shRNA cells with the MEK1/2 inhibitor U0126 completely abolished ERK1/2 phosphorylation, and coincided with a marked down-regulation of Bcl-2 protein expression (Fig. 3.3B). U0126 however is not entirely specific to the ERK1/2 pathway, but can also inhibit MEK5 and thus the whole ERK5 pathway [33]. Therefore we repeated this experiment using a dominant negative ERK1 cDNA construct (DN-ERK1) [34]. Expression of DN-ERK1 led to decreased ERK1/2 phosphorylation and correlated with a significant down-regulation in Bcl-2 protein levels (Fig. 3.3C). These findings support the notion that the enhanced ERK1/2 activation resulting from RanBPM down-regulation promotes the up-regulation of Bcl-2 expression.

Figure 3.3 Regulation of Bcl-2 expression by RanBPM occurs through ERK1/2. A. Transient down-regulation of RanBPM increases ERK1/2 phosphorylation and Bcl-2 protein levels. Hela cells were transfected with control siRNA or RanBPM siRNA. Whole cell extracts were prepared at the indicated timepoints and analyzed by western blotting with antibodies directed against Bcl-2 and phospho-ERK1/2. Down-regulation of RanBPM expression was verified using a RanBPM antibody and total ERK1/2 was used as a loading control. **B.** RanBPM shRNA (2-7) Hela cells were treated with the MEK1/2 inhibitor U0126 or DMSO for 24h. Whole cell extracts were prepared and analyzed by western blot as in A. **C.** Hela control shRNA and RanBPM shRNA (2-7) cells were either left untransfected or were transfected with DN-ERK1, and whole cell extracts were collected 24h and 48h post-transfection. Western blot analysis was performed as in A with γ-tubulin used as a loading control.


A
Hela
48h
72h
control si
RanBPM si
control si
RanBPM si
95
RanBPM
43
ERK 1/2
pERK 1/2
28
Bcl-2
B
RanBPM shRNA
(2-7)
DMSO
U0126
43
ERK1/2
pERK1/2
28
Bcl-2
C
RanBPM
shRNA (2-7)
C
-
DN-ERK1
24h 48h
95
RanBPM
51
γ-tubulin
43
ERK1/2
pERK1/2
28
Bcl-2

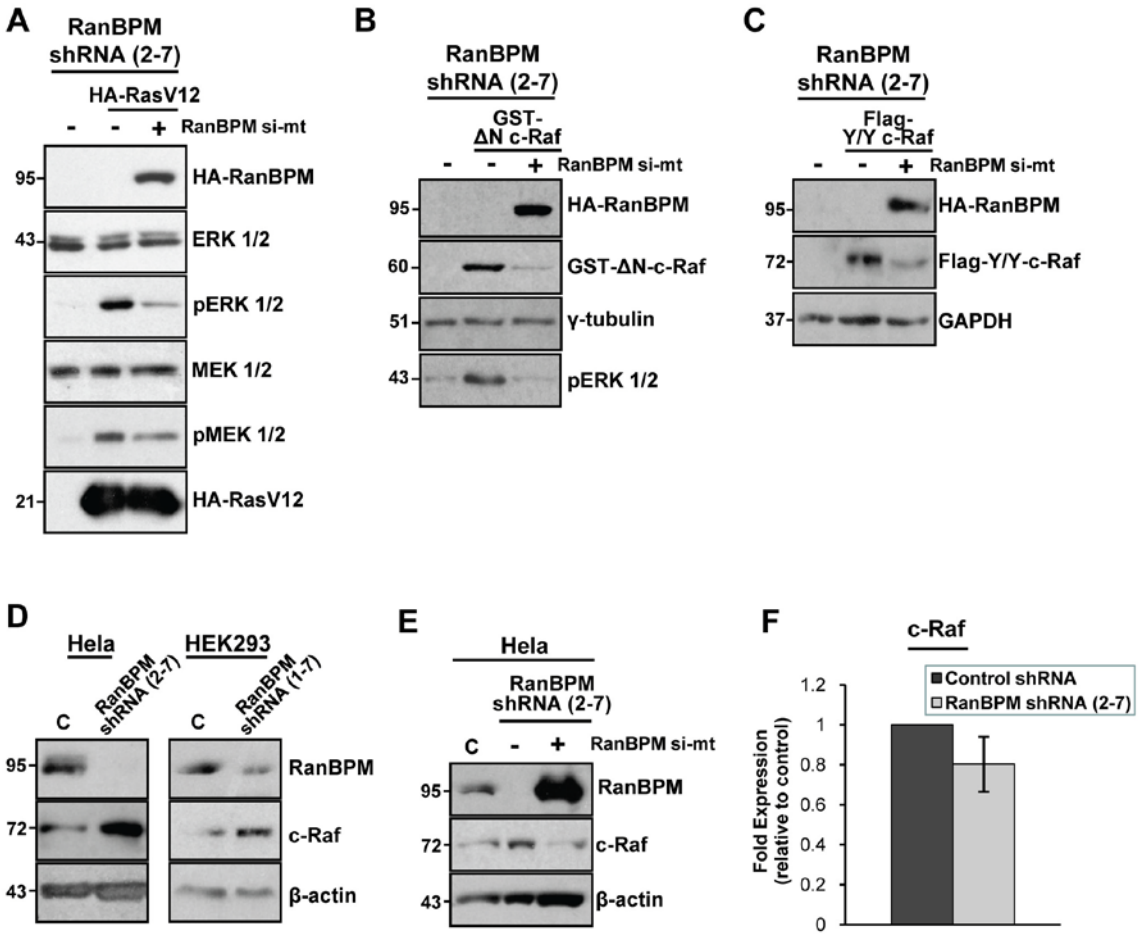
3.3.4 RanBPM targets the ERK1/2 signalling pathway downstream of Ras

We investigated the ability of RanBPM to regulate upstream events in the ERK signalling cascade. Both active forms of Ras and c-Raf have been shown to bypass the upstream components of the pathway for ERK activation [1, 4, 35]. As a first step, we assessed whether RanBPM acts upstream or downstream of Ras to inhibit ERK1/2 activation. We expressed a constitutively active H-Ras construct (RasV12) in Hela RanBPM shRNA cells, either in the presence or absence of ectopically expressed RanBPM, and analyzed the effect of RasV12 and RanBPM expression on the levels of ERK1/2 phosphorylation. As expected, while RasV12 expression resulted in increased ERK phosphorylation, this effect was inhibited by RanBPM expression (Fig. 3.4A). Interestingly, MEK-induced phosphorylation by RasV12 was also reduced upon RanBPM expression. This suggested that RanBPM is able to inhibit MEK and ERK activation by Ras and thus functions to regulate signalling events between Ras and MEK.

3.3.5 RanBPM forms a complex with c-Raf and inhibits c-Raf expression

Using a similar experimental scheme, we next investigated whether RanBPM expression could inhibit MEK and ERK activation by active c-Raf. Co-expression of RanBPM with a constitutively active c-Raf construct (GST-ΔN-c-Raf, containing c-Raf aa 325-648 [25]) in Hela RanBPM shRNA cells had an inhibitory effect on c-Raf-induced ERK activation (Fig. 3.4B). Intriguingly, we found that expression of RanBPM consistently led to a pronounced decrease in GST-ΔN-c-Raf protein levels (Fig. 3.4B, see also Fig. 3.5B). Since the GST-ΔN-c-Raf construct is under an EF-1α promoter that could potentially be affected by RanBPM expression, this experiment was repeated with another constitutively active c-Raf construct (c-Raf Y340D/Y341D). This active c-Raf construct is expressed from a CMV promoter, the transcriptional activity of which is not affected by RanBPM (see Fig. 3.1D). We obtained a similar down-regulation of c-Raf Y340D/Y341D upon RanBPM expression (Fig. 3.4C), suggesting that RanBPM functions to down-regulate c-Raf protein levels.

Figure 3.4 RanBPM inhibits ERK1/2 activation through regulation of c-Raf. A. RanBPM regulates ERK1/2 signalling downstream of Ras. RanBPM shRNA Hela cells were left untransfected, or were transfected with either constitutively active RasV12 and RanBPM si-mt or RasV12 and empty pCMV vector. 24h post-transfection, whole cell extracts were prepared and analyzed by western blotting. MEK1/2 and ERK1/2 activation was assessed using phospho-MEK1/2 and phospho-ERK1/2 antibodies respectively, and their total protein levels were assessed using MEK1/2 and ERK1/2 antibodies. Expression of RasV12 and RanBPM was determined with an HA antibody. **B.** RanBPM expression down-regulates c-Raf protein levels. RanBPM shRNA Hela cells were left untransfected, or were transfected with either the constitutively active c-Raf construct pEBG-GST-ΔN-c-Raf (GST-ΔN-c-Raf) and empty pCMV vector, or GST-ΔN-c-Raf and RanBPM si-mt. 48h post-transfection, whole cell extracts were prepared and analyzed by western blotting. C-Raf expression was determined using a GST antibody, and ERK1/2 activation was assessed using a phospho-ERK1/2 antibody. RanBPM expression was verified using an HA antibody, and γ-tubulin was used as a loading control. **C.** RanBPM shRNA Hela cells were left untransfected, or were either transfected with the constitutively active c-Raf construct pCMV-Flag-Y/Y-c-Raf (Flag-Y/Y-c-Raf) and empty vector, or Flag-Y/Y-c-Raf and RanBPM si-mt. 48h post-transfection, whole cell extracts were prepared and analyzed as in B, with c-Raf levels assessed using a Flag antibody, and GAPDH used as a loading control. **D**. Whole cell extracts were prepared from Hela and HEK293 control shRNA and RanBPM shRNA cells, and endogenous protein levels were analyzed by western blotting with c-Raf and RanBPM antibodies, with β-actin used as a control. **E.** Control shRNA and RanBPM shRNA Hela cells were either left untransfected, or were transfected with empty vector or RanBPM si-mt. 48h post-transfection, whole cell extracts were prepared and analyzed as in D. **F.** RanBPM down-regulation does not affect c-Raf mRNA expression. cDNA from Hela control shRNA and RanBPM shRNA cells was analyzed by qRT-PCR using specific primers for GAPDH and c-Raf. Gene expression was quantified using the ΔΔC(t) method, with c-Raf expression normalized to GAPDH, and expression in RanBPM shRNA cells normalized to control cells (set to an arbitrary value of 1). Data represents the mean of nine independent experiments, with error bars indicating standard error.


A
RanBPM shRNA (2-7)
HA-RasV12
- - + RanBPM si-mt
95
HA-RanBPM
43
ERK 1/2
pERK 1/2
MEK 1/2
pMEK 1/2
21
HA-RasV12
B
RanBPM shRNA (2-7)
GST-ΔN c-Raf
- - + RanBPM si-mt
95
HA-RanBPM
60
GST-ΔN-c-Raf
51
γ-tubulin
43
pERK 1/2
C
RanBPM shRNA (2-7)
Flag-Y/Y c-Raf
- - + RanBPM si-mt
95
HA-RanBPM
72
Flag-Y/Y-c-Raf
37
GAPDH
D
Hela
HEK293
C
RanBPM shRNA (2-7)
C
RanBPM shRNA (1-7)
95
72
43
RanBPM
c-Raf
β-actin
E
Hela
RanBPM shRNA (2-7)
C - + RanBPM si-mt
95
72
43
RanBPM
c-Raf
β-actin
F
c-Raf
Control shRNA
RanBPM shRNA (2-7)
Fold Expression (relative to control)
1.2
1
0.8
0.6
0.4
0.2
0

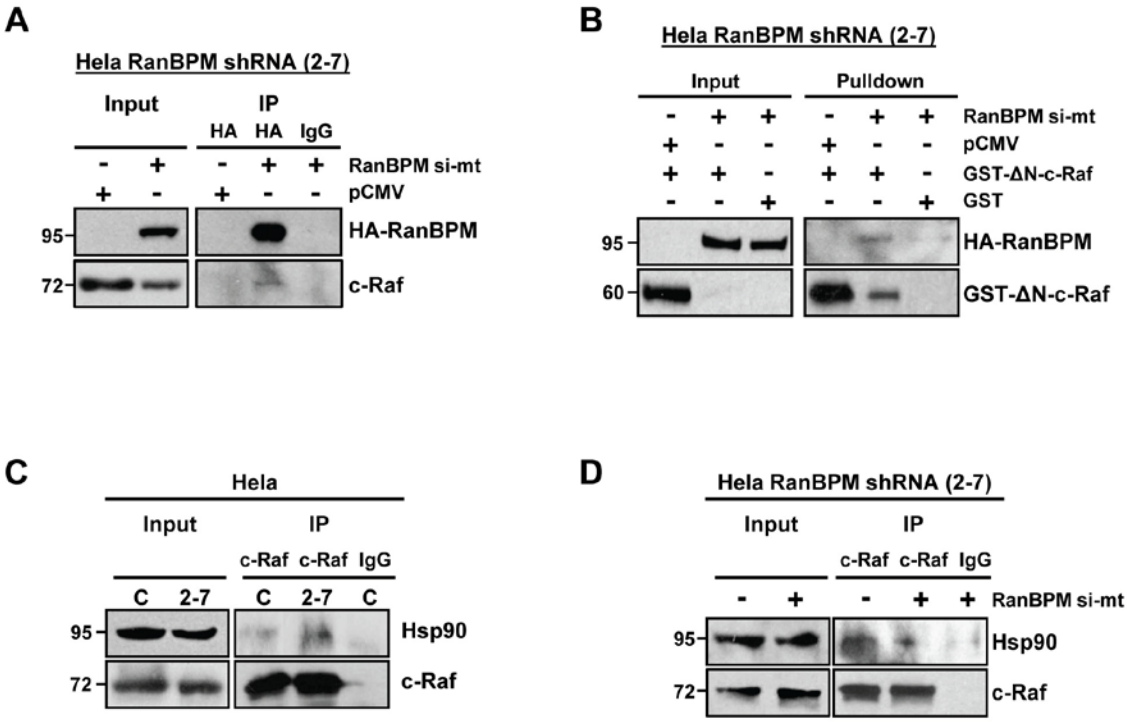
To confirm that RanBPM affects c-Raf expression, we analyzed endogenous c-Raf protein levels in Hela and HEK293 control and RanBPM shRNA cells (Fig. 3.4D). Indeed, RanBPM shRNA cells exhibited elevated c-Raf protein levels, and this effect was specifically due to RanBPM down-regulation, as restoration of RanBPM expression in RanBPM shRNA cells led to a decrease in c-Raf protein levels (Fig. 3.4E). To determine whether up-regulation of endogenous c-Raf protein levels may also be attributed to changes in c-Raf gene expression, we performed qRT-PCR analyses to compare c-Raf gene expression in Hela control and RanBPM shRNA cells. Surprisingly, RanBPM down-regulation resulted in a slight decrease in c-Raf mRNA expression, although this difference was not found to be statistically significant, suggesting that RanBPM does not affect c-Raf expression at the transcriptional level (Fig. 3.4F). Together these findings indicate that RanBPM functions to regulate ERK1/2 signalling by modulating c-Raf protein levels.

To begin investigating how RanBPM promotes c-Raf down-regulation, we first looked into a possible association of RanBPM with the c-Raf complex. A previous study reported the interaction of c-Raf kinase domain with RanBPM in a yeast two-hybrid analysis, but their interaction was not confirmed in mammalian cells [29]. Endogenous c-Raf was found to co-immunoprecipitate with HA-RanBPM re-expressed in RanBPM shRNA cells, suggesting that the two proteins form a complex (Fig. 3.5A). In addition, we determined that GST-ΔN-c-Raf, the levels of which were found to be markedly affected by RanBPM (Fig. 3.4B, 3.5B), was able to interact with RanBPM (Fig. 3.5B). Altogether, these results suggest that RanBPM associates with c-Raf and that this interaction relies on the C-terminal kinase domain of c-Raf.

3.3.6 RanBPM disrupts Hsp90-c-Raf association

Rafs are Hsp90 client proteins, and the binding of Hsp90 to c-Raf is required for proper folding and protein stability of c-Raf [36, 37]. To characterize the mechanism by which RanBPM may regulate c-Raf, we assessed whether RanBPM expression affected the association of Hsp90 with c-Raf. Co-immunoprecipitation of c-Raf from Hela control

Figure 3.5 RanBPM interacts with c-Raf and reduces c-Raf-Hsp90 association. A. Co-immunoprecipitation of RanBPM and c-Raf. RanBPM shRNA (2-7) Hela cells were transfected with empty vector or RanBPM si-mt, and 48 post-transfection whole cell extracts were incubated with either an HA antibody or mouse IgG control. Presence of c-Raf in immunoprecipitates was determined using a c-Raf antibody and RanBPM expression was verified using HA, compared to 5% input extract. **B.** RanBPM shRNA (2-7) Hela cells were transfected with GST-ΔN-c-Raf and either pCMV empty vector or RanBPM si-mt, or with RanBPM si-mt and GST empty vector, and whole cell extracts were prepared 48h post-transfection. Activated c-Raf was pulled down using glutathione beads, presence of RanBPM was verified using an HA antibody, and c-Raf expression was determined using GST, compared to 5% input extract. **C.** Co-immunoprecipitation of Hsp90 with c-Raf. Extracts from Hela control shRNA (C) and RanBPM shRNA (2-7) cells were immunoprecipitated with c-Raf or mouse IgG control antibodies. Equal amounts of immunoprecipitated c-Raf from control and RanBPM shRNA cells were analyzed by western blot with Hsp90 and c-Raf antibodies. Inputs represent 5% of the total protein used for immunoprecipitation. **D.** Hela RanBPM shRNA (2-7) cells were transfected with empty vector (-) or RanBPM si-mt, and whole cell extracts prepared 48 post-transfection were immunoprecipitated and analyzed as in C.

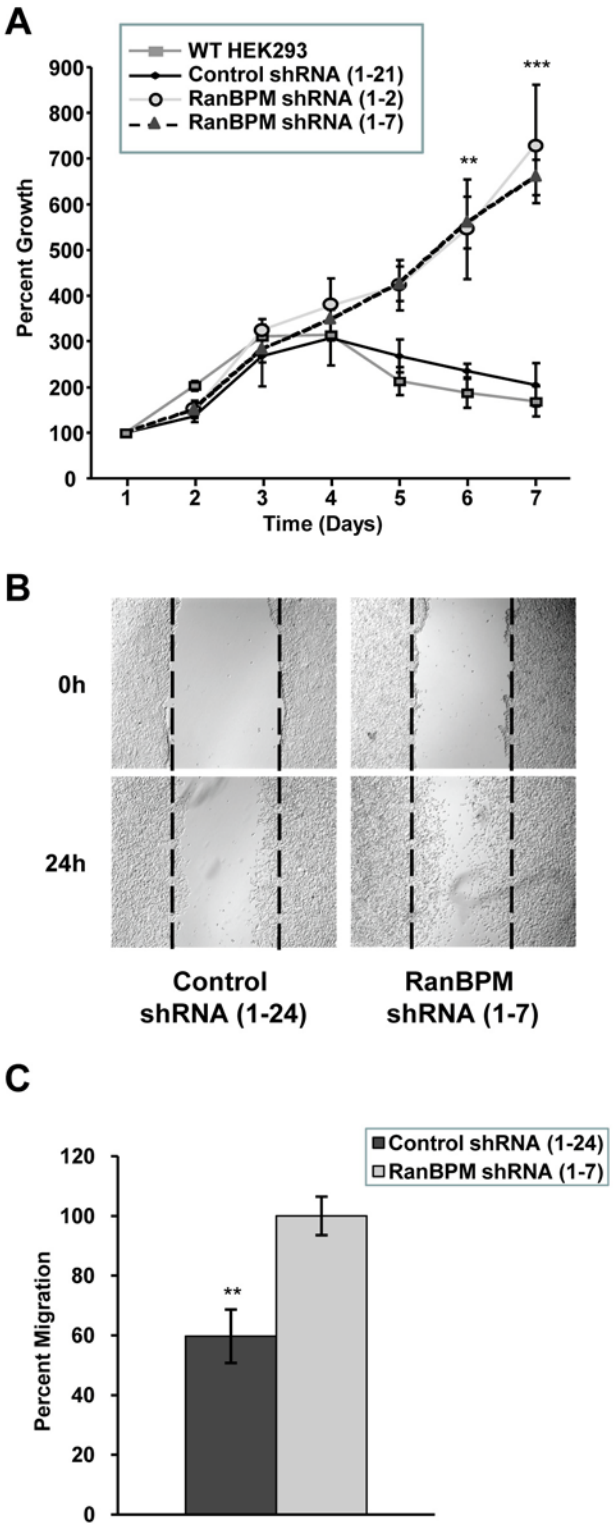

A
Hela RanBPM shRNA (2-7)
Input
IP
HA
HA
IgG
RanBPM si-mt
pCMV
95
72
HA-RanBPM
c-Raf
B
Hela RanBPM shRNA (2-7)
Input
Pulldown
RanBPM si-mt
pCMV
GST-ΔN-c-Raf
GST
95
60
HA-RanBPM
GST-ΔN-c-Raf
C
Hela
Input
IP
c-Raf
c-Raf
IgG
C
2-7
C
2-7
C
95
72
Hsp90
c-Raf
D
Hela RanBPM shRNA (2-7)
Input
IP
c-Raf
c-Raf
IgG
RanBPM si-mt
95
72
Hsp90
c-Raf

and RanBPM shRNA cell extracts revealed an increased amount of Hsp90 co-immunoprecipitating with c-Raf in RanBPM-depleted cells (Fig. 3.5C). In addition, re-expression of RanBPM reversed this effect, substantiating an inhibitory effect of RanBPM on the association of Hsp90 with c-Raf (Fig. 3.5D). These findings indicate that RanBPM may function to destabilize the c-Raf protein by inhibiting the interaction of c-Raf and Hsp90.

3.3.7 Inhibition of RanBPM expression promotes cellular transformation

Activating mutations in K-Ras and N-Ras that result in constitutive activation of the ERK pathway are among the most frequent oncogenic events in human cancers [5, 38]. Our observation that RanBPM down-regulation promotes ERK activation suggested that loss of RanBPM function, in addition to compromising apoptosis, could promote cellular transformation. We first analyzed the effect of RanBPM down-regulation on cell growth in HEK293 cells. We evaluated the growth rate of both RanBPM and control shRNA HEK293 cells upon serum withdrawal. For both HEK293 (WT) and the clonal derivative control shRNA HEK293 (1-21) cells, growth slowed down at approximately four days post serum starvation, and at seven days post serum withdrawal cell growth had almost completely stopped (Fig. 3.6A). However this was not observed with two different clonal derivative RanBPM shRNA cells (1-2 and 1-7). These cells continued to grow in the absence of serum, even at seven days post serum withdrawal. These results indicate that down-regulation of RanBPM expression promotes loss of growth factor dependence. In addition, as previous studies have demonstrated a role for the ERK pathway in promoting cell migration [38, 39], we tested the migratory properties of RanBPM and control shRNA HEK293 cells in a wound-healing assay, which evaluates the ability of cells to move over a cell-free zone created by scraping the middle of the plate with a pipette tip. This assay revealed a significant increase in cell motility of RanBPM shRNA cells, which displayed a 1.7 fold increase in wound closure compared to control cells (Fig. 3.6B, C). Together, these results indicate that loss of RanBPM expression leads to cell signalling alterations that promote aberrant cell proliferation and cell migration.

Figure 3.6 Down-regulation of RanBPM expression enhances cellular transformation. **A.** RanBPM shRNA cells exhibit increased cell growth. Growth rates for HEK293 wild-type (WT), control shRNA (1-21) and RanBPM shRNA (1-2 and 1-7) cells were assessed for seven days. Data represents the mean percent growth for four independent experiments, with error bars indicating standard error, and ** P <0.01 and *** P <0.001. **B.** Confluent monolayers of control shRNA and RanBPM shRNA (1-7) HEK293 cells were cultured in the presence of 2mM hydroxyurea for 24h, scratched using a sterile pipette tip, and wound healing was assessed at the indicated time points using a microscope at 4x magnification. Images from a representative experiment are shown. **C.** Percent wound closure was calculated for control and RanBPM shRNA HEK293 cells. Data represents the mean of four independent experiments with error bars representing standard error, and ** P <0.01.


A
WT HEK293
Control shRNA (1-21)
RanBPM shRNA (1-2)
RanBPM shRNA (1-7)
Percent Growth
Time (Days)
**
***
B
0h
24h
Control shRNA (1-24)
RanBPM shRNA (1-7)
C
Control shRNA (1-24)
RanBPM shRNA (1-7)
Percent Migration
**

3.4 Discussion

This study reveals an important role for RanBPM in repressing ERK activation and signalling. Expanding on our previous findings which showed elevated expression of the anti-apoptotic factor Bcl-2 in RanBPM down-regulated cells, we demonstrate here that Bcl-2 overexpression in these cells is mediated by increased ERK activation that is specifically triggered by the loss of RanBPM expression. We show that the inhibition of ERK signalling by RanBPM is achieved through a regulation of c-Raf protein levels and that RanBPM associates with c-Raf *in vivo*. Finally, we determine that loss of RanBPM expression confers increased cell growth and cell migration, properties known to be induced by increased ERK signalling.

Here, we demonstrate that the regulation of Bcl-2 expression by RanBPM that we documented in chapter 2 is a direct consequence of a regulation of the ERK pathway by RanBPM. Our investigation of a potential effect of RanBPM on the ERK pathway was prompted by the observation that, in addition to Bcl-2, RanBPM also modulated the expression of another anti-apoptotic factor, Bcl-X_L, and that these regulations occurred both at the transcriptional and at the post-translational levels. The ERK pathway has previously been shown to regulate Bcl-2 and Bcl-X_L both transcriptionally and post-transcriptionally [4, 5, 32]. In our studies, we have shown that both transient and stable down-regulation of RanBPM activated ERK phosphorylation, and we have confirmed that this effect was specific to RanBPM, as restoration of RanBPM expression reversed this effect. Further, we have substantiated that RanBPM down-regulation promotes ERK activation in three different cell lines, confirming that the regulation of ERK by RanBPM is not cell-type specific. Moreover, we show that inhibiting ERK in RanBPM down-regulated cells reduces Bcl-2 expression, confirming that ERK signalling is directly responsible for elevated Bcl-2 expression in these cells. Altogether, these experiments demonstrate that RanBPM expression has an inhibitory effect on ERK phosphorylation and signalling.

Previous studies have implicated RanBPM in the regulation of the ERK pathway, however there have been conflicting reports of the outcome of RanBPM expression on ERK activation. In a first study, RanBPM was shown to stimulate this pathway through

an interaction with the RTK MET [12]. While our results are in contradiction with this report, it should be noted that this previous study employed an experimental approach that involved overexpression of a GFP-RanBPM fusion construct. Whether the addition of a bulky fluorescent tag at the N-terminus of RanBPM interferes with RanBPM function is unknown, but since RanBPM has been shown to dimerize (or even multimerize) through LisH domain [40], it is possible that the GFP-RanBPM fusion protein may have had an adverse effect on endogenous RanBPM function. Another study presented evidence that the N-terminal region of RanBPM interacted with the neural cell adhesion molecule L1 and inhibited ERK activation induced by L1 [30]. However, this study relied on overexpression of a truncated form of RanBPM, and the effect of the truncation on RanBPM function was not investigated [30]. In contrast to these reports, a third study reported an inhibitory role of RanBPM on ERK signalling activated by a constitutively active Raf-BXB [29]. This study also described an interaction between RanBPM and the catalytic domain of c-Raf using a yeast two-hybrid assay. Consistent with these findings, we show here that RanBPM associates with the C-terminus of c-Raf using a GST-pull-down assay in mammalian cells. Further, we also show that RanBPM can form a complex with endogenous c-Raf.

Raf proteins are central regulators of the ERK pathway that function by coupling receptor activation to ERK-dependent signalling cascades, and as such their activity is subject to complex regulation [39]. Intensive studies on c-Raf in particular have revealed that c-Raf undergoes an intricate cycle of activation/deactivation that involves multiple interactions with regulators, phosphorylation and dephosphorylation events, and conformational changes [39]. Our data indicate that RanBPM modulates c-Raf expression through a regulation of c-Raf protein levels/stability. First, our qRT-PCR analyses revealed that RanBPM down-regulation did not result in increased c-Raf mRNA levels, indicating that RanBPM does not affect c-Raf gene expression. In fact, RanBPM down-regulation resulted in a slight, albeit not significant, decrease in c-Raf mRNA levels. A possible explanation may be that elevated c-Raf protein expression activates negative feedback loops that repress its transcription. Second, our results show that RanBPM is able to modulate the stability of ectopically expressed c-Raf, as the expression of both active c-Raf point mutant (Y/Y) and ΔN deletion mutant was strongly

affected by RanBPM expression. Interestingly, the down-regulation of c-Raf by RanBPM seemed more pronounced with these transfected active forms of c-Raf than with the pool of endogenous c-Raf proteins which comprises active and inactive forms. This is consistent with the ability of RanBPM to form a complex with the c-Raf kinase domain, and thus suggests that RanBPM preferentially targets active forms of c-Raf.

An important part of c-Raf regulation is its association with chaperone proteins that ensure proper folding and prevent c-Raf degradation [36]. C-Raf protein folding and stability has been shown to be dependent on its association with the chaperone Hsp90, as disruption of Hsp90-c-Raf interaction results in a sharp decrease in c-Raf levels [37, 41, 42]. Phosphorylation of Ser^{621} through c-Raf autophosphorylation has also been implicated in promoting c-Raf stability [43]. We did not find a consistent change in Ser^{621} phosphorylation resulting from RanBPM expression (data not shown). However, we found that RanBPM down-regulation enhanced c-Raf-Hsp90 complex formation. This effect was found to be specifically due to down-regulation of RanBPM, as restoration of RanBPM expression reduced the association of Hsp90 with c-Raf. These findings suggest that the interaction of RanBPM with c-Raf disrupts the c-Raf-Hsp90 complex, leading to its destabilization. For instance, RanBPM may function to destabilize c-Raf by competing with Hsp90 for binding to c-Raf. Such a mechanism was previously proposed to explain the negative regulation of c-Raf by Hsp70. Hsp70 was shown to compete with c-Raf for binding to BAG1, a chaperone that stimulates c-Raf catalytic activity, thus preventing c-Raf activation of proliferation pathways [44, 45]. Alternatively, RanBPM may recruit a protein or protein complex to c-Raf that disrupts the c-Raf-Hsp90 complex. A precedent exists for such a remodelling of Hsp90-chaperone complexes, which is mediated by the co-chaperone CHIP (carboxy terminus of Hsp70-interacting protein) [46]. CHIP was shown to bind Hsp90 substrates and mediate the transfer of client proteins to Hsp70, causing their dissociation from Hsp90 and promoting their proteasome-mediated degradation [41, 46-48]. Whether RanBPM is part of this complex or functions independently in regulating chaperone-dependent Hsp90 client proteins stability/degradation remains to be determined.

It is well established that deregulation of the ERK pathway leading to its constitutive activation is linked with many aspects of tumour development including cell growth, proliferation, differentiation, and migration [4-6]. Our observation that RanBPM down-regulation promotes ERK activation suggests that loss of RanBPM function could promote cellular events leading to cellular transformation. Both cell proliferation and cell migration were found enhanced in HEK293 RanBPM shRNA cells suggesting that RanBPM expression is essential to regulate these two cellular functions. It should be noted that while the increased cell migration observed upon RanBPM down-regulation may be due to increased ERK activation, it could also result from MEK-independent functions of c-Raf, which has been shown to regulate cell motility through a direct regulation of the Rho effector Rokα [38, 39, 49]. Previous reports have suggested a function for RanBPM in repressing oncogenic cellular events by promoting the activity of the tumour suppressor p73 and Mammalian Lethal Giant Larvae-1 (Mgl-1) [17, 50]. Our results not only confirm a tumour suppressor role for RanBPM, but go beyond these observations to show that altering RanBPM expression is in itself sufficient to disrupt regulatory mechanisms that control cell transformation and the establishment of oncogenic pathways. Interestingly, decreased RanBPM expression was previously reported in cancer cells from several tumour samples, suggesting that loss of RanBPM may be linked to tumour development [51]. To confirm a link between these observations, it will be important to determine whether loss of RanBPM expression in tumours correlates with the constitutive activation of the ERK pathway.

3.5 References

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Chapter 4

4. RanBPM expression regulates transcriptional pathways involved in development and tumourigenesis

4.1 Introduction

Cancer development is driven by alterations in cellular pathways leading to the evasion from mechanisms that normally restrict growth, migration and invasion [1]. Tumourigenesis is associated with changes in gene expression and the progression of cell transformation from normal to tumour cells [2, 3]. Altered gene expression can elicit cancer development, and may also occur as the result of downstream signalling from pathways deregulated during cancer progression [4]. These changes in expression have been linked to specific phases of cancer development often reflecting either the type or the stage of cancer progression. To this end gene expression profiling has greatly contributed to our understanding of cancer progression, and to the identification of key genes and pathways which when deregulated promote cancer development [3].

RanBPM was initially identified as a binding partner for the Ran GTPase that localized to the microtubule-organizing center (MTOC) [5], although both of these observations were later dismissed [6]. Several roles for RanBPM have subsequently been proposed in cellular processes including the regulation of cell morphology [7-9], cell adhesion [10-12], cell cycle progression [13] and regulation of neurological functions [13-15]. Most of these functions result from interaction of RanBPM with various proteins, which have been reported to occur both in the cytoplasm and the nucleus. In the cytoplasm, RanBPM has been suggested to function as a scaffold for receptor signalling pathways through interactions with the neuronal cell adhesion molecule L1 [11], the MET receptor (MET proto-oncogene, also called hepatocyte growth factor receptor) [16], and tropomyosin-related kinase (Trk) TrkA [17] and TrkB [14] receptors. The interaction of RanBPM with these receptors is believed to regulate the activation of downstream signalling pathways including the ERK1/2 [11, 16], Akt [14], and NF-κB pathways [18]. In addition, RanBPM was suggested to modulate the stability of proteins, such as the pro-apoptotic transcription factor p73 [19]. In the nucleus, RanBPM has been shown to

interact with the transcriptional regulator TAF4 [15] and the viral early-immediate transcriptional regulator Rta [20]. RanBPM has also been reported to modulate the transcriptional activity of the androgen receptor (AR) [21], the glucocorticoid receptor (GR) [21], and the thyroid hormone receptor (TR) [22]. These data suggest that RanBPM could have wide ranging and important influences on gene expression, either directly through interaction with transcriptional regulators, or indirectly through the modulation of intracellular signalling pathways.

In chapter 2, we established that RanBPM functions to promote apoptosis in response to DNA damage. We showed that down-regulation of RanBPM in Hela and HCT116 cells prevented the activation of the mitochondrial apoptotic pathway and promoted cell survival in response to ionizing radiation treatment. Therefore in the present study, we sought to gain further insight into the pathways and cellular functions that are regulated by RanBPM. Using microarray analyses that compared RanBPM down-regulated cells to those in which RanBPM is expressed at physiological levels, global changes in gene expression elicited by RanBPM down-regulation were investigated. Our analyses reveal that decreased RanBPM expression causes wide spread changes in gene expression that indicate it may be an important mediator in the control of many tumourigenic processes.

4.2 Materials and methods

4.2.1 Cell culture

Hela and HCT116 control shRNA and RanBPM shRNA stable cell lines were previously generated (as outlined in chapter 2). Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Wisent Bioproducts, St. Bruno, QC, Canada), 2mM L-glutamine, and 1mM sodium pyruvate (Life Technologies) at 37°C in 5% CO_2. Control shRNA and RanBPM shRNA stable cell lines were maintained in media supplemented with 0.35mg/ml G418 (Geneticin, Bioshop Canada, Burlington, ON, Canada).

4.2.2 Plasmid expression constructs and transfection assays

The pCMV-RanBPM shRNA mutant expression construct (RanBPM si-mt) has been described in chapter 2. Transfection assays were carried out using ExGen 500^{TM} (Thermo Scientific, Fermentas, Burlington, ON, Canada) as per the manufacturer's instructions.

4.2.3 Western blotting

To prepare whole cell extracts, cells were collected in ice-cold PBS and lysed in buffer containing 150mM NaCl, 1mM EDTA, 50mM HEPES (pH 7.4), 10% Glycerol, 0.5% NP40, and supplemented with 1mM PMSF, 1mM DTT, 1µg/ml leupeptin, 10µg/ml aprotinin, 1µg/ml pepstatin, 2mM sodium fluoride, and 2mM sodium orthovanadate. For western blot analysis, 20µg of protein extracts were resolved on 10% SDS-PAGE, transferred onto PVDF membranes (Bio-Rad, Burlington, ON, Canada), and blots were hybridized with antibodies against RanBPM (5M, BioAcademia, Japan) and β-actin (I-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

4.2.4 RNA extraction, quantitative reverse-transcriptase PCR and statistical analysis

Total RNA was isolated from Hela and HCT116 control shRNA, RanBPM shRNA, and RanBPM shRNA re-expressing RanBPM si-mt, cells using the Qiagen RNeasy RNA isolation kit (Qiagen, Mississauga, ON, Canada). For quantitative reverse-transcriptase PCR (qRT-PCR) analyses, cDNA was prepared from 2.5µg of total RNA using the SuperscriptII Reverse Transcriptase kit (Life Technologies), and gene expression was determined using 10-100ng of cDNA incubated with primers described in Suppl. Table 4.1, using SYBR green (Bio-Rad) and the BioRad MyiQ single-colour real-time PCR detection system. Relative gene expression was quantified via the ΔΔC(t) method with candidate gene values normalized to that of controls. Statistical significance was analyzed using a student's *t*-test, with a $P < 0.05$ indicating significant results.

4.2.5 RNA quality assessment, probe preparation and GeneChip hybridization

All sample labelling and GeneChip processing was performed at the London Regional Genomics Centre (Robarts Research Institute, London, ON, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA, USA). Single stranded complementary DNA (sscDNA) was prepared from 200ng of total RNA as per the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript (WT) Expression Arrays (http://www.ambion.com/techlib/prot/fm_4411973.pdf, Applied Biosystems, Carlsbad, CA, USA) and the Affymetrix GeneChip WT Terminal Labelling kit and Hybridization User Manual (http://media.affymetrix.com/support/downloads/manuals/wt_term_label_ambion_user_manual.pdf, Affymetrix, Santa Clara, CA, USA). Total RNA was first converted to cDNA, followed by in vitro transcription to make cRNA. 5.5 µg of single stranded cDNA was synthesized, end labelled, and hybridized, for 16 hours at 45°C to Human Gene 1.0 sense-target (ST) arrays. All liquid handling steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix) using Command Console v1.1.

4.2.6 Bioinformatics and data analysis

Probe level (.CEL file) data was generated using Affymetrix Command Console v1.1. Probes were summarized to gene level data, background subtraction was performed, and expression values were normalized to log base-2 in Partek Genomics Suite v6.6 (Partek, St. Louis, MO) using the Robust Multiarray Averaging (RMA) algorithm [24]. Partek was used to determine gene level ANalysis Of VAriance (ANOVA) p-values, fold-changes, and Gene Ontology (GO) enrichment, using a Chi-square test. Partek Pathway was also used to find enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, using a Fisher's exact test.

For bioinformatics analyses, a list of genes exhibiting a minimum of 1.2-fold increase or decrease in expression in RanBPM shRNA cell lines compared to control shRNA cell lines was first generated (target gene list). Analysis of genes differentially expressed in RanBPM shRNA cells was performed using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com) and the Protein Analysis Through Evolutionary Relationships (PANTHER) database [25]. For IPA, the target gene list was uploaded alongside the respective HUGO (HUman Genome Organization) gene symbols and fold-change values, and analyzed using Ingenuity Pathway Core Analysis, which generated a list of focus genes. IPA Functional Analysis of this gene list was performed to identify top biological processes affected by decreased RanBPM expression, based upon GO terms and curator-defined ontology terms (Ingenuity® Systems, www.ingenuity.com). IPA was also used to generate cellular networks affected by RanBPM down-regulation. Significance of identified cellular networks was determined by assigning a score to each network. A score is assigned based upon a p-value calculation that determines the likelihood that genes appear within a given network by random chance, and is the negative exponent of the right-tailed Fisher's exact test result (Ingenuity® Systems, www.ingenuity.com). A score of ≥ 3 is considered significant, as it indicates a 1/1000 chance that genes appear within a network by chance.

The PANTHER database uses protein sequences to group proteins into functional families and subfamilies, and uses ontology terms to classify proteins according to molecular functions, biological processes, and protein classes. For PANTHER analyses, the target gene list and HUGO gene symbols were uploaded to the PANTHER website and the top biological processes, molecular functions, and protein classes affected in response to RanBPM down-regulation were identified.

The oPOSSUM analysis system was utilized to identify over-represented Transcription Factor Binding Sites (TFBS) in the list of genes affected by RanBPM down-regulation. Briefly, oPOSSUM compares the occurrence of TFBS within a set of co-expressed genes (target gene list) to a pre-determined background set of genes, in order to identify over-represented sites in the target list [26]. The significance of any identified binding sites is calculated using a Z-score and Fisher score, with a Z-score of

≥10 indicating a significantly over-represented TFBS, in agreement with database publisher's recommendations [27].

4.3 Results

4.3.1 Identification of gene targets regulated by RanBPM expression

We have previously generated clonal stable cell lines in Hela and HCT116 cells expressing either a control or RanBPM shRNA (as described in chapter 2 and shown in Suppl. Fig. 4.1). To identify gene targets that are regulated by RanBPM expression, RNA samples were prepared in triplicate from normally proliferating Hela control shRNA and RanBPM shRNA (clone 2-7) cells, and gene expression profiling was performed using Affymetrix human gene expression arrays. The mean fold-change in gene expression in RanBPM shRNA (2-7) cells compared to control shRNA cells was calculated, and using a 1.2-fold change cut-off, we identified 2621 genes for which expression was altered by RanBPM down-regulation. To minimize the potential that the observed changes in gene expression arose from the derivation of clonal cell lines, RNA samples were prepared in triplicate from a second Hela RanBPM shRNA cell line [denoted RanBPM shRNA (clone 2-6)]. Gene expression was quantified using the parameters outlined above, and we identified 3952 genes differentially expressed in RanBPM shRNA (2-6) cells compared to control shRNA cells. Comparison of the list of differentially expressed genes in the two Hela RanBPM shRNA cell lines identified 1719 genes common to both cell lines (Suppl. Fig. 4.1). Further, to limit possible cell-type specific effects of RanBPM down-regulation on gene expression patterns, RNA samples were also prepared in triplicate from HCT116 cells expressing either a control or RanBPM shRNA [denoted HCT116 RanBPM shRNA (clone 2-8)]. The mean fold-change in expression was calculated, and using a 1.2-fold change cut-off we identified 2226 genes with altered expression in response to RanBPM down-regulation. Combining the list of differentially expressed genes obtained for each cell line we identified a total of 187 genes common to all three cell lines, for which expression was changed by RanBPM down-regulation (Suppl. Fig. 4.1 and Suppl. Table 4.2). Of these, 167 were annotated genes, with 74 genes

down-regulated and 93 genes up-regulated upon RanBPM down-regulation. Our analysis also confirmed a strong decrease in RanBPM expression in all three RanBPM shRNA cell lines, as demonstrated by a 2.46-fold decrease in Hela RanBPM shRNA (2-6), 2.92-fold decrease in Hela RanBPM shRNA (2-7), and 2.76-fold decrease in HCT116 RanBPM shRNA (2-8) cells respectively (Suppl. Table 4.2).

4.3.2 IPA and PANTHER analyses

To gain insight into the functional and biological consequences associated with decreased RanBPM expression, we performed Ingenuity Pathway Analysis on the 167 genes identified above. IPA is a web-based tool that utilizes information from the literature to analyze data obtained from gene expression arrays. This analysis allows for modeling biological interactions and building networks of cellular processes to determine the effects of a given experimental treatment. IPA Functional Analysis of genes that were differentially expressed in RanBPM shRNA cells revealed that down-regulation of RanBPM expression most significantly affects cellular processes associated with cancer, tissue development, and cellular function and maintenance (Table 4.1). Notably, over one-third of the 167 genes analyzed were associated with cancer, suggesting a potentially important role for RanBPM in regulating cellular processes associated with tumourigenesis. To characterize pathways that are affected by down-regulation of RanBPM, we next performed Network Analyses in IPA. This analysis uses a given gene list to build networks of cellular processes, and assigns a score based upon the number of genes from that list that are found within a particular cellular network. Using the gene list from above and a cut-off score of 3 (see methods) we identified seven cellular networks that were significantly affected by the decrease in RanBPM expression. These networks encompass cellular processes such as organ development, tissue morphology, cancer, cell motility, cell signalling, RNA modification, protein synthesis, and molecular transport (Table 4.2). ERK1/2 was a component of the top two cellular networks affected by the down-regulation of RanBPM expression. Additionally, PI3K/Akt, Notch, and NF-κB signalling were components of the most highly affected cellular networks in RanBPM shRNA cells. Together these analyses reveal that down-regulation of RanBPM leads to

Table 4.1. IPA analysis of top biological functions altered in RanBPM shRNA cells

Diseases and Disorders

Name	p-value	# of molecules
Cancer	**2.43E-04 - 3.79E-02**	**57**
Reproductive System and Disease	2.43E-04 - 2.86E-02	19
Gastrointestinal Disease	5.43E-04 - 3.79E-02	16
Organismal Injury and Abnormalities	5.43E-04 - 3.79E-02	10
Inflammatory Response	1.34E-03 - 3.79E-02	19

Molecular and Cellular Functions

Name	p-value	# of molecules
Cellular Function and Maintenance	**2.60E-04 - 3.79E-02**	**22**
Cell Morphology	2.73E-04 - 3.14E-02	12
Cellular Assembly and Organization	2.73E-04 - 3.79E-02	10
Lipid Metabolism	9.00E-04 - 3.79E-02	10
Small Molecule Biochemistry	9.00E-04 - 3.79E-02	20

Physiological System Development and Function

Name	p-value	# of molecules
Tissue Development	**7.03E-04 - 3.79E-02**	**42**
Hematological System Development and Function	2.60E-04 - 3.79E-02	22
Organ Morphology	9.00E-04 - 3.79E-02	10
Reproductive System Development and Function	9.00E-04 - 3.79E-02	9
Connective Tissue Development and Function	1.02E-03 - 3.79E-02	23

Top biological function in each category is bolded

Table 4.2. IPA analysis of cellular networks affected by RanBPM down-regulation

	Genes	Score	Function
1	, , CREB, EDAR, EGLN3, ELF3, **ERK1/2**, , IFNγ, IgG1, IL12, IL18, Interferon α, JNK, L1CAM, MFGE8, p38 MAPK, PDGF BB, , PI3K, , PMAIP1, , SCN9A, SDC4, , , , , SYK/ZAP, TCIRG1, TCR, TLR3, USP18, VEGF	37	Cardiovascular System Development and Function, Organ Development, Organismal Development
2	, AKT, ARL6IP5, ATP1B3, CD93, DHRS3, EMP1, ERAP1, **ERK1/2**, ER, FJX1, FSH, GBP1, GCA, hCG, Histone H3, Histone H4, IFITM3, IgG, , , Lh, , MST1R, NF-κB, , , OAS3, PLCB3, PPAP2B, RNF40, , SACS, ,	28	Protein Synthesis, Cell Signaling, Cancer
3	, APP, Arginase, ATP7A, CCND1, CEBPA, , FBXO4, , GABRE, GBP1, HNF1A, HNF4A, HNMT, HOXB5, KIF5C, , KRT10, LEP, LIPA, , MST1, NCAPH, NPM1, NPR2, PADI4, PERP, ROCK2, , , TIMP3, TLE1, , TP53, ULK2	24	Cell Morphology, Hepatic System Development and Function, Cellular Response to Therapeutics
4	Actin, AHR, ANXA3, C9orf3, , CDC25B, CDK2, CDKN1B, CDKN2C, Cyclin E, EHD1, EMILIN1, FBLN5, FURIN, IGF1R, , MATN2, MEIS2, mir-34, NCOA3, NPDC1, , PRKCDBP, RDM1, RNA Pol II, , , SDC4, SLC20A1, , TGFβ1, TIMP3, TIMP2, TWIST1, ZFP36	22	Cellular Movement, Cancer, Cardiovascular System Development and Function
5	AMY2A, DLL1, ERBB2, EZH2, FAM107B, FOXC2, GALNT3, , , HES1, HOXB6, HSD3B7, IL11, , MARCKS, MFAP5, MIB1, MSLN, NOTCH1, NOTCH2, NOTCH3, NOTCH4, NPC2, PAX7, PPARγ, PRICKLE1, PRSS23, PSEN1, RAB34, RAB27B, RAP1GAP, RETNLA, SLC4A11, TAL1, TFAP2A	20	Tissue Morphology, Cellular Development, Embryonic Development
6	ADD2, ALDH1A3, ANK3, AR, ASAP1, BCAR3, CA2, CAST, DAPK2, DICER1, DOCK1, FOS, FOXH1, GM2A, , GRHPR, IRS1, KIF5B, LAMA3, LHFP, , NTS, PDYN, PLK2, , PRKRA, PTPLAD2, , RAP1GAP, SOS, , TBL1X, TCOF1, , TNNT2	13	RNA Modification, Skeletal and Muscular Development and Function, Tissue Morphology
7	ALDH1A2, CASP1, CASP4, , CCL11, CD274, , CSF3, CTSK, CX3CR1, CXCL1, GZMB, IGE, IgG1, IL1, IL2, IL13, IL25, IL27, IL33, IL18, IL23A, IRF4, ITGAL, , LAMB3, MYD88, , NFATC1, , PDCD1, PSTPIP2, SLPI, TRB, ZBTB32	9	Cell-Cell Signaling and Interaction, Hematological System Development and Function, Immunological Disease

Genes marked in red indicate down-regulated genes, genes marked in green indicate up-regulated genes. ERK1/2 is bolded.

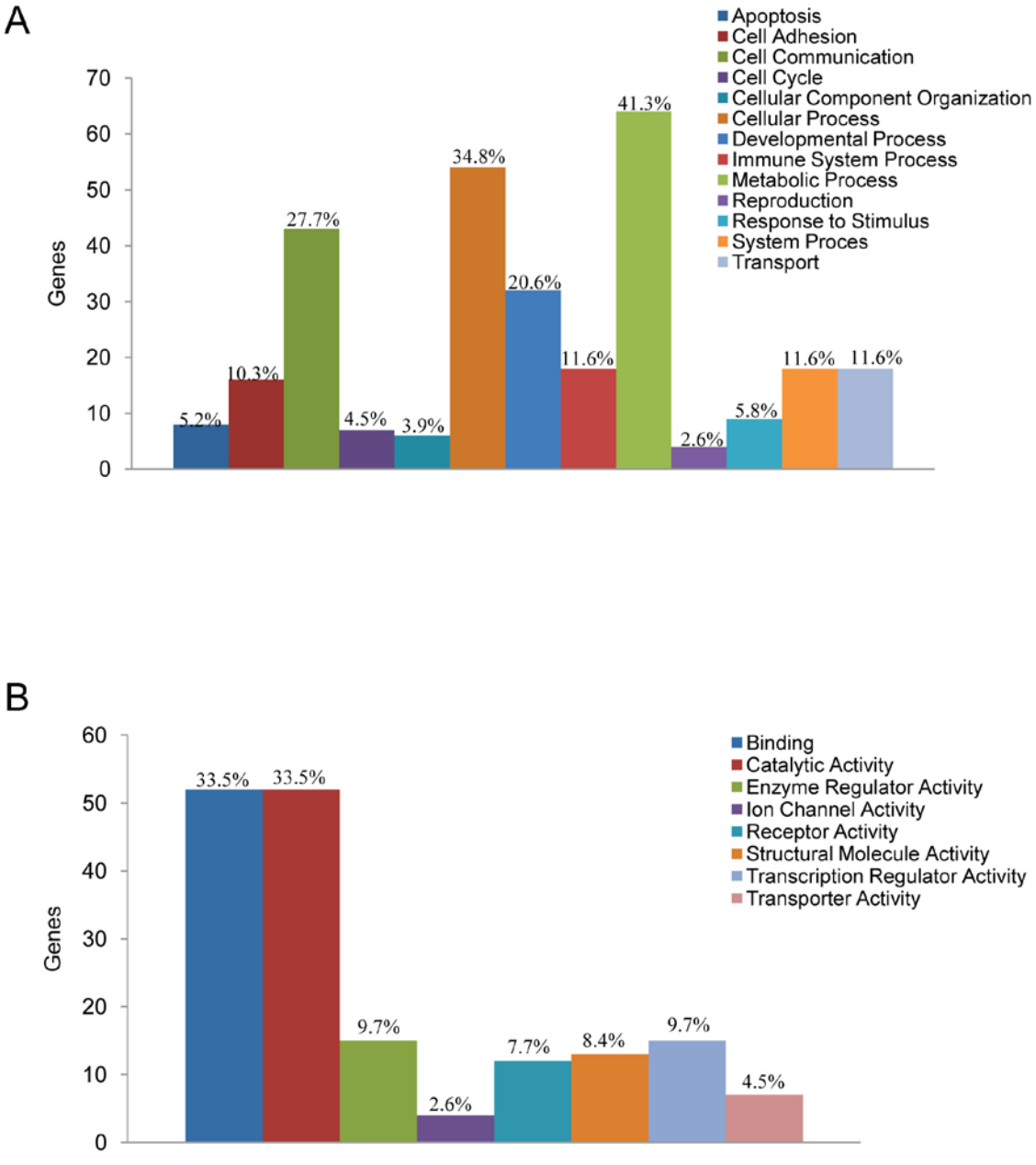
gene expression changes that affect regulation of cell, tissue, and organ development and morphology, as well as biological processes implicated in tumourigenesis.

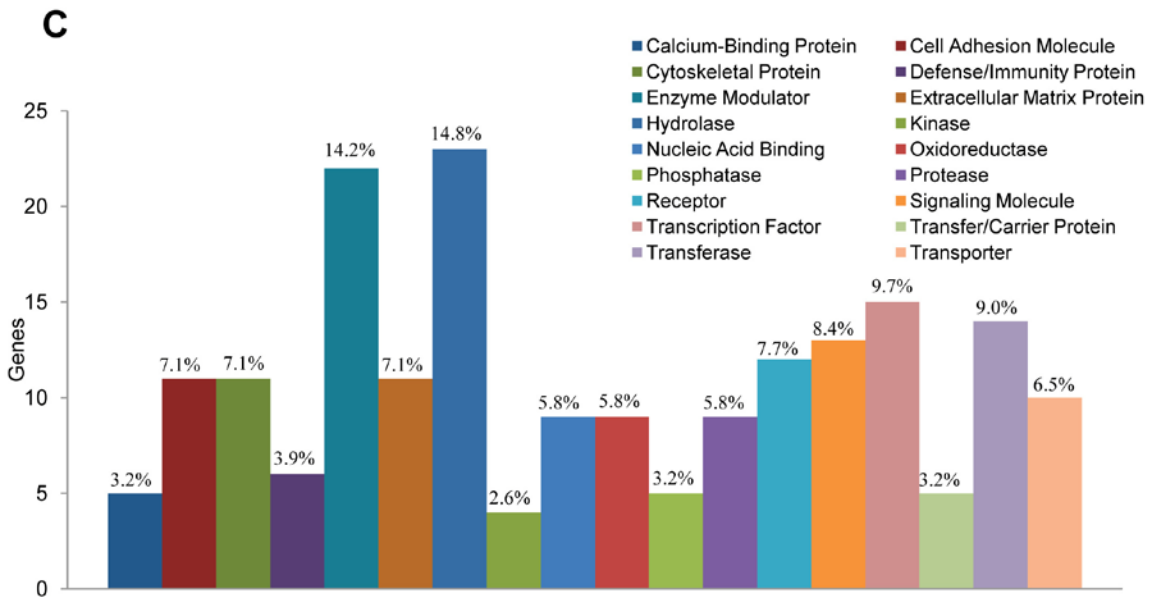
To corroborate the findings from IPA, and further characterize the molecular processes that are altered in cells with decreased RanBPM expression, we analyzed our list of differentially expressed genes using the PANTHER database [25]. Similarly to IPA, PANTHER allows for functional analysis of data gathered from gene expression profiling by using curator-defined groupings of protein sequences to build protein families. These protein families can then be used to identify biological processes, molecular functions, pathways, and protein classes to which groups of genes may be assigned. We first identified the most highly affected biological processes and molecular functions associated with reduced expression of RanBPM (Fig. 4.1A, B). We determined that in RanBPM down-regulated cells, the most highly affected biological processes include cell communication, tissue development, and cellular metabolism; and the most highly affected molecular functions include receptor and protein binding, enzyme catalysis, and transcriptional regulation. PANTHER also allows for the classification of protein classes to which genes from a given gene list may belong. Using the protein class analyses, the expression of transcription factors, receptors, cell adhesion proteins, and cytoskeletal proteins were all found to be affected by the down-regulation of RanBPM expression (Fig. 4.1C). Our analyses using PANTHER verify our findings with IPA and indicate that decreased RanBPM expression leads to changes in gene expression patterns that affect cellular processes involved in both development and cancer.

4.3.3 Validation of selected gene targets

Next, we sought to validate the expression data obtained from our gene arrays by performing qRT-PCR analyses. As our findings with IPA identified cancer as one of the cellular processes most significantly affected by the down-regulation of RanBPM (Table 4.1), we chose to validate gene targets which had previously been linked in the literature to tumourigenesis. Eleven candidate genes were selected, and their expression was analysed in RNA extracts from control and RanBPM shRNA Hela and HCT116 cells

Figure 4.1 PANTHER analysis of cellular processes altered by RanBPM down-regulation. A and B. Gene Ontology (GO) analyses of top biological processes (A) and molecular functions (B) affected by RanBPM down-regulation. The number of genes in target gene list that are annotated to a given function are plotted, with percentages indicating the number of genes that appear in selected gene list divided by the total number of genes assigned to that function. **C.** Protein classes for which expression is most significantly affected in RanBPM shRNA cells. Data is represented as in A and B.

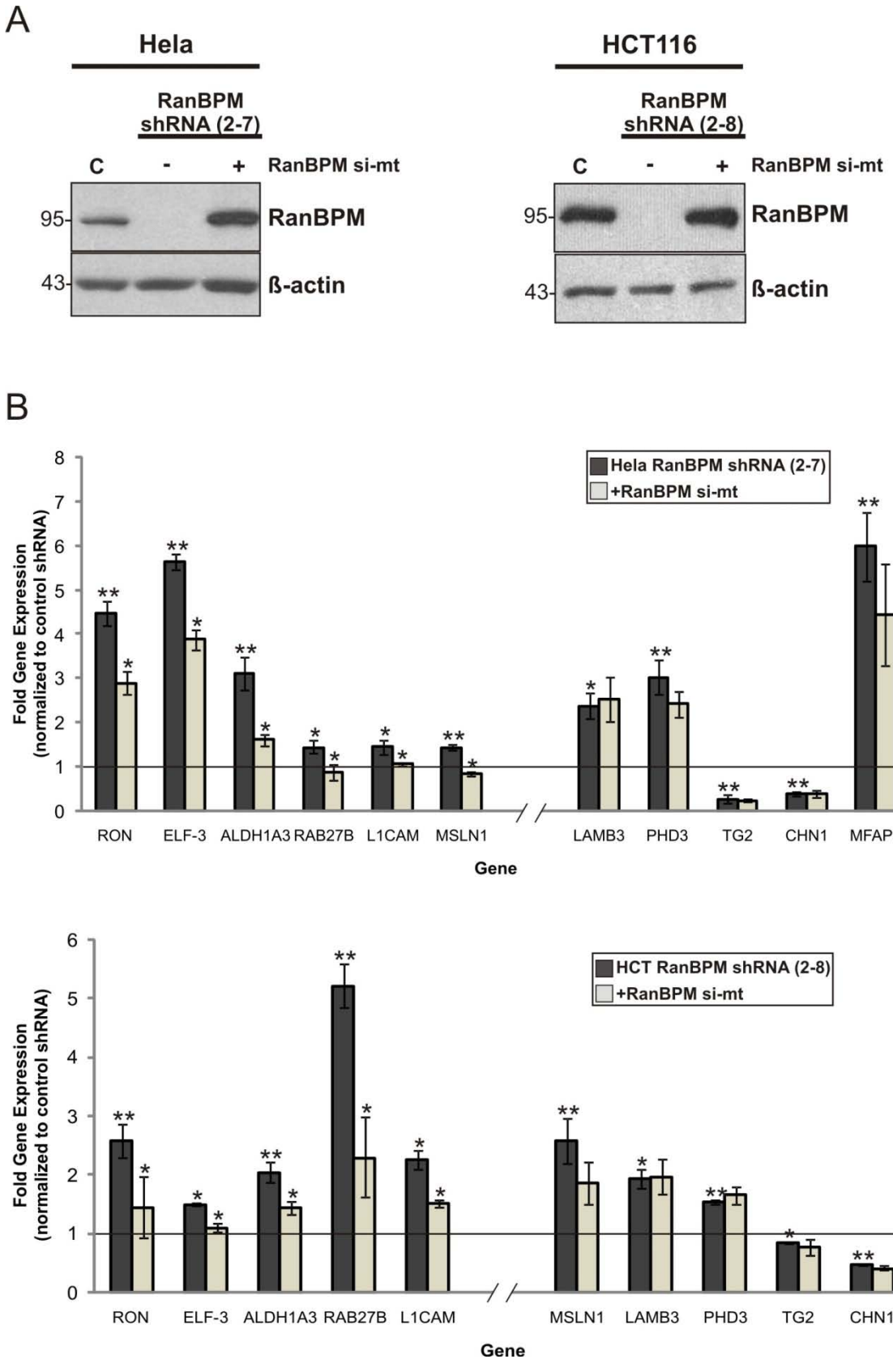

A
Genes
70
60
50
40
30
20
10
0
5.2%
10.3%
27.7%
4.5%
3.9%
34.8%
20.6%
11.6%
41.3%
2.6%
5.8%
11.6%
11.6%
Apoptosis
Cell Adhesion
Cell Communication
Cell Cycle
Cellular Component Organization
Cellular Process
Developmental Process
Immune System Process
Metabolic Process
Reproduction
Response to Stimulus
System Proces
Transport
B
Genes
60
50
40
30
20
10
0
33.5%
33.5%
9.7%
2.6%
7.7%
8.4%
9.7%
4.5%
Binding
Catalytic Activity
Enzyme Regulator Activity
Ion Channel Activity
Receptor Activity
Structural Molecule Activity
Transcription Regulator Activity
Transporter Activity


C
Calcium-Binding Protein
Cell Adhesion Molecule
Cytoskeletal Protein
Defense/Immunity Protein
Enzyme Modulator
Extracellular Matrix Protein
Hydrolase
Kinase
Nucleic Acid Binding
Oxidoreductase
Phosphatase
Protease
Receptor
Signaling Molecule
Transcription Factor
Transfer/Carrier Protein
Transferase
Transporter
Genes
25
20
15
10
5
0
3.2%
7.1%
7.1%
3.9%
14.2%
7.1%
14.8%
2.6%
5.8%
5.8%
3.2%
5.8%
7.7%
8.4%
9.7%
3.2%
9.0%
6.5%

(Fig. 4.2A, B). For 10 of the candidate genes selected, we confirmed the change in expression observed in the gene arrays in both the Hela and HCT116 cell lines (Fig. 4.2B). However MFAP5 (microfibrillar associated protein 5, also known as MAGP2) mRNA expression levels were too low to be reliably measured in HCT116 cells, and therefore the change in expression of MFAP5 was only successfully confirmed in Hela cells. As might be expected we found that for the majority of candidate genes selected, the fold-change in gene expression as determined by qRT-PCR was greater than that observed using the gene arrays [28, 29].

To evaluate the direct contribution of RanBPM down-regulation to the changes in gene expression observed, we ectopically re-expressed RanBPM in Hela and HCT116 RanBPM shRNA cell lines. This was achieved by transiently expressing a RanBPM cDNA containing a point mutation in the siRNA recognition sequence (RanBPM si-mt) in these cells, allowing for restoration of RanBPM expression to near endogenous levels (Fig. 4.2A). RNA extracts were prepared 48h post-transfection from both Hela and HCT116 cells, and gene expression for the 11 candidate genes outlined above was quantified using qRT-PCR. Interestingly, our analyses revealed two categories of genes. The first category comprised genes for which re-expression of RanBPM restored mRNA expression close to levels observed in control cells. These genes, grouped to the left of the axis break in Figure 4.2B, included ELF3 (E74-like factor 3, also known as ESE-1), RON [recepteur d'origine nantais/macrophage stimulating receptor 1 (MST1R)], ALDH1A3 (aldehyde dehydrogenase 1 isoform A3), Rab27B (Rab 27B member Ras oncogene family), and L1CAM (L1 cell adhesion molecule). MSLN1 (mesothelin 1) showed a statistically significant change upon RanBPM re-expression in Hela cells, but not in HCT116 cells. The second category of genes, grouped together to the right of the axis break in Figure 4.2B, comprised genes for which expression was not significantly altered upon restoration of RanBPM expression. Included in this category were TG2 (transglutaminase 2, also called TGM2), PHD3 (prolyl hyrdoxylase 3, also called EGLN3), LAMB3(laminin β3), and CHN1 (chimerin 1). Similarly, MFAP5 expression was not affected by restoration of RanBPM expression in Hela cells.

Figure 4.2 Validation of selected gene targets identified in microarray analyses. A. Hela RanBPM shRNA (2-7) cells (*left)* and HCT116 RanBPM shRNA (2-8) cells (*right*) were either left untransfected (-) or were transfected with pCMV-RanBPM si-mt (+ RanBPM si-mt) expression construct. Forty-eight hours post-transfection, whole cell extracts were prepared and analyzed by western blotting, alongside control shRNA (C) and untransfected RanBPM shRNA extracts. Restoration of RanBPM expression was verified by hybridizing with a RanBPM antibody, and β-actin was used as a loading control. **B.** cDNA from control shRNA, RanBPM shRNA, and RanBPM shRNA +RanBPM si-mt Hela (*top*) and HCT116 (*bottom*) cells was analyzed by qRT-PCR analyses using primers specific to the indicated genes. Fold gene expression was normalized to control shRNA cells. Genes appearing to the left of x-axis break are genes whose expression was responsive to restoration of RanBPM expression, and genes appearing to the right of x-axis break were not responsive to restored RanBPM expression. Data represents the mean of a minimum of three independent experiments with error bars indicating standard error, and * P <0.05 and ** P <0.005.


A
Hela
RanBPM shRNA (2-7)
C
-
+
RanBPM si-mt
95-
RanBPM
43-
ß-actin
HCT116
RanBPM shRNA (2-8)
C
-
+
RanBPM si-mt
95-
RanBPM
43-
ß-actin
B
Hela RanBPM shRNA (2-7)
+RanBPM si-mt
Fold Gene Expression (normalized to control shRNA)
0
1
2
3
4
5
6
7
8
RON
ELF-3
ALDH1A3
RAB27B
L1CAM
MSLN1
LAMB3
PHD3
TG2
CHN1
MFAP5
Gene
HCT RanBPM shRNA (2-8)
+RanBPM si-mt
Fold Gene Expression (normalized to control shRNA)
0
1
2
3
4
5
6
RON
ELF-3
ALDH1A3
RAB27B
L1CAM
MSLN1
LAMB3
PHD3
TG2
CHN1
Gene

4.3.4 Identification of over-represented transcription factor binding sites

The PANTHER analyses outlined above identified transcriptional regulation and transcription factors as one of the most highly affected molecular functions and protein classes altered by the down-regulation of RanBPM. To expand upon this finding we sought to determine whether decreased RanBPM expression affects the expression of subsets of genes regulated by specific classes or families of transcription factors. To test this, we utilized oPOSSUM, a web-based tool that allows for the identification of over-represented TFBS in the promoters of sets of co-expressed genes [27]. Analysis of the 167 genes differentially expressed in RanBPM shRNA cells using oPOSSUM identified 20 transcription factors which contain significantly over-represented TFBS within the promoters of these genes (Table 4.3). We found that the most over-represented TFBS in our gene list was HOXA5 (homeobox A5), a member of the Homeobox family of transcription factors. Of the 167 genes analyzed, 134 contained binding sites for HOXA5 within their promoters. We also identified six members of the Forkheadbox (FOX) family of transcription factors, four additional members of the Homeobox family, and four members of the High Mobility Group (HMG) family of transcription factors, all of which contained over-represented TFBS in the promoters of our differentially expressed genes.

4.4 Discussion

4.4.1 Summary

RanBPM has been implicated in the control of a multitude of cellular processes including regulation of development [7, 8], cell motility [16], transcription [15, 20-22], and apoptosis [19, 23, 30-32]. However, a common modality for the function of this protein remains unclear. The aim of the present study was to utilize gene expression profiling to gain further insight into the cellular functions of RanBPM, by characterizing the impact of RanBPM down-regulation on global cellular signalling events. To identify the functional consequences associated with decreased RanBPM expression we generated a list of genes whose expression was altered in RanBPM shRNA cells, and identified the

Table 4.3. oPOSSUM analysis of over-represented transcription factor binding sites

Transcription Factor	Family	Target Genes	Z-Score
Class: Winged Helix-Turn-Helix			
FOXI1	Forkhead	103	30.93
FOXO3	Forkhead	112	29.9
FOXD1	Forkhead	108	23.8
Foxa2	Forkhead	102	22.82
FOXA1	Forkhead	105	21.82
Foxd3	Forkhead	97	21.76
SPI1	ETS	130	17.32
Class: Helix-Turn-Helix			
Nkx2-5	Homeo	129	31.21
HOXA5	Homeo	134	29.04
Pdx1	Homeo	122	25.42
Prrx2	Homeo	117	24.24
Nobox	Homeo	113	23.05
ARID3A	Arid	130	28.3
Class: Other Alpha-Helix			
SRY	HMG	116	35
SOX5	HMG	111	22.42
Sox17	HMG	110	20.15
SOX9	HMG	110	18.86
Class: Zinc-Coordinating			
Gata1	GATA	117	25.85
Gfi	ββα-zinc finger	118	18.11
Class: Leucine Zipper			
CEBPA	Zipper-type	109	17.2

most highly affected biological processes and cellular networks in these cells. Down-regulation of RanBPM expression was found to significantly affect the expression of factors associated with embryonic, cellular, and tissue development, as well as those involved in cancer development and progression.

4.4.2 Implications in development

Our analyses revealed several components of the Notch and Wnt signalling pathways whose expression was altered upon RanBPM down-regulation. These include JAG1 (Jagged 1), which is a ligand for Notch receptors [33], RUNX2 (runt-related transcription factor 2), which integrates signals from Notch, Wnt, and TGFβ (transforming growth factor β) to regulate bone development and differentiation [34], and RON/MST1R, a receptor tyrosine kinase known to promote phosphorylation and nuclear accumulation of β-catenin in breast and colon tumours [35]. Moreover, Notch signalling was found to be a major component of one of the top cellular networks affected by RanBPM down-regulation. It is well established that signalling pathways such as the Notch/Wnt/Hedgehog pathway, which normally regulate embryonic development, can become deregulated in cancer. For example, Notch signalling normally mediates cell-cell communication in embryogenesis, as well as cell proliferation, differentiation, and apoptosis [33, 36]. Deregulated Notch signalling has been linked to tumour development in the lung, ovaries, breast, and colon, and to enhanced epithelial-to-mesenchymal transition (EMT) of cancer cells [33, 37, 38]. Induction of Wnt signalling occurs upon the binding of Wnt proteins to cell surface receptors, leading to the stabilization and nuclear accumulation of β-catenin [39]. Within the nucleus, β-catenin mediates the expression of Wnt target genes that regulate embryonic signalling events such as proliferation, morphogenesis, and differentiation [34, 39]. Similarly to Notch, Wnt signalling is deregulated in many cancers and in certain cases such as colorectal tumours deregulated Wnt signalling can initiate tumour development [33, 39]. Several studies have also identified an important role for RanBPM in development. RanBPM was found to be required in the *Drosophila* nervous system for larval behaviour associated with feeding, growth, and locomotion [7]. Recent studies in RanBPM knockout mice revealed a critical

role for this protein in normal gonad development and gametogenesis in both males and females [40]. Additionally, RanBPM has been linked to developmental processes occurring through Notch-dependent signalling. For example it was shown to regulate the size, shape, and organization of the germline stem cell (GSC) niche in female *Drosophila* [8]. The development, and capacity of this niche for stem cells, is known to be regulated through Notch expression and signalling [8, 15]. RanBPM was found to regulate neuronal differentiation in *Drosophila* by interacting with TAF4, a transcriptional co-activator that binds transcription factors downstream of Notch signalling to regulate neural stem cell fate and differentiation [15]. Our gene expression data indicate that RanBPM regulates the expression of several factors involved in Notch signalling, further suggesting a possible role for RanBPM in the regulation of Notch-mediated signalling during development.

In addition to factors involved in Notch/Wnt signalling, we also identified several other differentially expressed genes for which a function in both development and cancer has been demonstrated. These include GBP1 (guanylate binding protein 1), a cytokine-activated small GTPase normally involved in cellular proliferation and angiogenesis [41], which is overexpressed in ovarian and oral tumours [42, 43]; NR0B1 (nuclear receptor subfamily 0 Group B member 1) that acts a transcriptional co-repressor in embryonic stem cell development, pluoripotency, and differentiation [44], and is overexpressed in several tumours [44, 45]; and L1CAM, which is involved in neurite outgrowth and axon guidance in normal cells [46], and is overexpressed in numerous cancers, including melanoma, lung, and thyroid cancer [46, 47]. Thus, these findings suggest a complex role for RanBPM in both the regulation of normal cellular processes associated with development, as well as in the progression of diseased states such as cancer.

4.4.3 Implications in signalling

RanBPM has previously been demonstrated to regulate several receptor-mediated signalling pathways, including the ERK1/2 and NF-κB pathways. As such, it was hypothesized to have potential functions in tumourigenesis, although the outcome of

RanBPM function in this process remains controversial due to differing findings regarding its role in activation of signalling cascades such as ERK1/2. While some reports indicate that RanBPM expression promotes activation of ERK1/2 signalling and would therefore enhance cellular transformation [14, 16, 17], other groups including ours (as outlined in chapter 3) have characterized RanBPM as a repressor of ERK1/2 activation and suggest a tumour-suppressor role for this protein [11]. Our gene expression data indicated that several signalling pathways are affected by decreased RanBPM expression, including the ERK1/2 and the PI3K/Akt pathways, both of which were components of the top two cellular networks affected by down-regulation of RanBPM. These pathways are known to play critical functions in cancer development. ERK1/2 signalling regulates many cellular processes including cell cycle progression, cell proliferation, differentiation, migration, and adhesion [48], and aberrant ERK1/2 signalling has been observed in many diseased states including cardiovascular disease and cancer [48, 49]. PI3K signalling is activated by cell-surface receptors and converges upon Akt, which phosphorylates various cellular targets involved in cell growth, survival, metabolism, and autophagy [50]. Similarly to ERK1/2, both PI3K and Akt are often found to be mutated and/or deregulated in cancer [51]. Our data indicate that while gene expression of ERK1/2, PI3K, and Akt is not affected by RanBPM down-regulation, the expression of several factors that regulate these signalling pathways is altered by decreased RanBPM expression, suggesting a tumour-suppressor function for RanBPM. For example, L1CAM and IL-18 have been found to promote ERK1/2 activation and enhance ERK-target gene expression, and are often overexpressed in tumour samples [46, 52, 53]. Our gene expression data reveal that down-regulation of RanBPM leads to increased L1CAM and IL-18 expression, indicating a potential link between the expression of these genes and deregulated ERK1/2 signalling in RanBPM shRNA cells. Similarly, gene expression of the tyrosine kinase RON was up-regulated in cells with decreased RanBPM expression. Overexpression of RON has been observed in multiple tumours, and is associated with enhanced ERK1/2 and Akt activation and signalling [54-56]. Collectively these findings suggest a role for RanBPM in the regulation of signalling pathways that are associated with both normal cellular function and diseased states, and further implicate a potential role for RanBPM as a tumour suppressor.

4.4.4 Implications in transcriptional regulation

In addition to its roles in development and receptor signalling, RanBPM has been proposed to be directly involved in regulation of gene transcription. RanBPM was reported to function as a transcriptional co-activator for AR, GR, and TR, and to mediate their ligand-dependent nuclear translocation [21, 22]. RanBPM was also shown to enhance the sumoylation and transactivation of the early-immediate Epstein-Barr Virus (EBV) protein Rta [20], and to interact with the TAF4 subunit of TFIID (transcription factor IID, also known as TBP) [15, 57]. As our data revealed a wide range of gene targets affected by RanBPM expression, we sought to identify potential transcription factors through which RanBPM may mediate its effects on gene expression. Analysis of the over-represented TFBS in our list of differentially expressed genes revealed that the FOX, Homeobox, and HMG families of transcription factors contain the greatest number of binding sites within the promoters of genes affected by RanBPM down-regulation.

FOX proteins comprise a large family of transcriptional regulators that are divided into subclasses according to their function in modifying chromatin structure. The FOXA subclass (FOXA 1, 2, and 3) plays an important role in development, organogenesis, metabolism, and stem cell differentiation [57]. FOXA proteins have been reported to be overexpressed or amplified in human tumours, especially in breast, prostate, thyroid, lung, and esophageal cancers [57, 58]. The FOXO subclass (FOXO1, 3a, 4, and 6) is involved in insulin and growth factor mediated signalling through PI3K/Akt, and is a downstream target of activated Akt [57, 59]. FOXOs regulate differentiation, metabolism, cell cycle arrest, cell death, and tumour suppression [57]. Overall, we identified six FOX family transcription factors with over-represented binding sites in our list of differentially expressed genes, including FOXA1, FOXA2, and FOXO3. Interestingly, deregulation of FOXA proteins has been linked to hormone-sensitive malignancies, and is suggested to mediate tumourigenesis through regulation of steroid hormone receptors [58]. As RanBPM has been reported to function as a co-activator of AR, it is tempting to hypothesize that it may function to regulate target gene expression through an AR/FOXA1-dependent process. Additionally, FOXA1 has been reported to mediate chromatin opening and enhance the DNA binding of the GR at the

mouse mammary tumour virus (MMTV) promoter [60]. RanBPM was also reported to enhance the transcriptional activity of GR [21], further suggesting a potential link between RanBPM and FOXA1 in the regulation of steroid receptor-mediated gene expression.

Our oPOSSUM analyses identified five members of the Homeobox family of transcription factors as being over-represented in the promoters of our differentially expressed genes. Homeobox transcription factors play a pivotal role in the regulation of embryonic development [61], regulate homeostasis, cell differentiation, and organ function in adult tissues [62-64] and their expression is often deregulated in cancer [65-68]. One such example is HOXA5, which during development regulates organogenesis in lung, mammary, and tracheal tissues, and in adult tissues regulates mammary gland development and function [64, 69]. HOXA5 is also believed to function as a tumour suppressor by transactivating p53 to promote p53-dependent and p53-independent apoptotic signalling [65]. Consequently, HOXA5 expression is decreased in tumours of the breast, colon, and lung, and this expression is believed to be regulated at least in part through epigenetic modifications of the HOXA5 gene in these tumours [65, 68]. HOXA5 binding sites are the most highly over-represented in our list of genes, as they are found in the promoters of 134 of 167 genes analyzed. The HMG protein family consists of a unique group of transcription factors that bind to the minor groove of DNA and regulate gene expression through modifications of the DNA structure and through interaction with other factors [70]. Analyses in oPOSSUM identified four members of the HMG family with over-represented TFBS in our gene list including SRY (sex-determining region on Y-chromosome), and the SOX (SRY-related HMG Box) proteins SOX5, SOX9, and SOX17. HMG proteins are critical in cell lineage specification and cell maturation during development, and the SOX proteins in particular have been proposed to function in determining stem cell identity, fate, and maintenance in multiple tissues [70, 71]. SOX proteins were reported to enhance the DNA-binding affinity of steroid hormone receptors such as AR, and this has implications in both development and cancer. Deregulated expression of SOX9 has been observed in prostate cancer, and is linked to prostate cancer progression [72, 73]. The binding sites of SOX5, SOX9, and SOX17 are significantly over-represented in our list of genes affected by decreased RanBPM expression.

Collectively, our analyses of the over-represented TFBS in our list of differentially expressed genes identified several transcription factors which regulate key processes in development, and whose function is often deregulated in cancer. These findings further implicate a role for RanBPM in the regulation of pathways that govern the critical balance between development and tumourigenesis.

All of the candidate genes selected for validation by quantitative RT-PCR confirmed the initial results obtained in the microarray analyses. All nine genes up-regulated in RanBPM shRNA cells have previously been reported to be overexpressed in various cancers and/or tumours. For instance, overexpression of RON has been linked to human cancers such as breast, prostate, colorectal, and ovarian carcinomas [35, 55, 74, 75]. RON hyperactivity has been shown to lead to increased cell proliferation, motility, and transformation, and to the inhibition of apoptosis and anoikis [76]. Similarly, ELF3 overexpression has been detected in breast, prostate, colon, and cervical tumours, and is associated with cell transformation [77, 78]. ELF3 is believed to promote tumourigenesis through transcriptional regulation of several known oncogenes, including TGFβ [78]. Abnormal expression of L1CAM has also been observed in various cancer types and linked to cell proliferation, migration, invasion, and metastasis of cancer cells [47]; and Rab27B [79], ALDH1A3 [80], MSLN1 [38], LAMB3 [81], PHD3 [82], and MFAP5 [83] levels have all been reported to be increased in various cancer types. Further, both TG2 and CHN1, the expression of which is strongly down-regulated in RanBPM shRNA cells, have been linked to tumourigenesis, and CHN1 has been proposed to function as a tumour suppressor [84, 85]. Overall, these findings suggest that RanBPM functions to prevent aberrant gene expression that may lead to oncogenesis. This reinforces the notion that has previously been inferred in several studies, that RanBPM may function as a tumour suppressor [10, 19, 23].

4.4.5 Potential implications in epigenetic regulation

The quantitative RT-PCR analysis of target gene expression following re-expression of RanBPM in Hela and HCT116 RanBPM shRNA cells revealed two

categories of genes. The first group comprises genes which responded to RanBPM re-expression, and consists of RON, ELF3, Rab27B, L1CAM, and ALDH1A3. For these genes, re-expression of RanBPM reversed the effect observed upon RanBPM down-regulation, at least partially. Analysis of the promoters of these genes using oPOSSUM did not reveal any common TFBS. While we cannot rule out a direct effect of RanBPM at these gene promoters, an alternate possibility is that RanBPM modulates signalling pathways that regulate the expression of these genes. The second group comprises genes which did not show a transcriptional response to restoration of RanBPM expression. This group consists of LAMB3, PHD3, TG2, CHN1, and MFAP5. Analysis of samples prepared 72h post-transfection with RanBPM si-mt showed identical results (data not shown). This suggests the possibility that RanBPM down-regulation establishes long-term changes in gene expression programs, such as epigenetic modifications, that cannot be reversed by transient re-expression of RanBPM. Interestingly several candidate genes, and transcription factors identified by oPOSSUM analysis, that are affected in response to down-regulation of RanBPM are known to be regulated through epigenetic modifications. For example, LAMB3 expression, which is up-regulated in gastric cancer cells, was shown to be regulated by demethylation of its promoter [86]. Down-regulation of TG2 expression is linked to several types of cancer [84], and has been shown to result from aberrant hypermethylation of the TG2 promoter in brain and breast tumours [87, 88]. Additionally, expression of the HOXA5, SOX9, and SOX17 transcription factors is regulated through epigenetic mechanisms. As discussed above, HOXA5 promoters are hypermethylated in breast and lung cancers which results in silencing of HOXA5 expression, and may correlate with decreased p53 activation and decreased apoptosis in breast tumours [68]. SOX9 has been reported to be hypermethylated in mantle cell lymphoma (MCL), and this hypermethylation is associated with decreased SOX9 expression in these tumours [89]. Hypermethylation of SOX9 in MCL tumours also correlated with higher proliferation, increased chromosomal abnormalities, and reduced overall patient survival [89]. The promoter region of SOX17 is hypermethylated in mammary, gastric, and hepatocellular carcinomas, thereby silencing SOX17 and leading to aberrant activation of Wnt signalling [90, 91].These findings suggest that RanBPM may have broad effects on gene transcription, and may function both directly on gene

promoters, and indirectly through modification of epigenetic programs, to regulate gene expression.

4.4.6 Conclusion

Overall, the results of this study indicate that alterations in the expression of RanBPM has profound and wide ranging effects on genes and pathways that play important roles in the regulation of developmental programs, and are linked to tumourigenesis when disrupted. RanBPM may therefore have a central role in controlling the activity of several signalling pathways that function to coordinate cell proliferation and differentiation during mammalian development and that are tightly regulated in adult tissues to maintain homeostatic regulations and prevent tumourigenesis.

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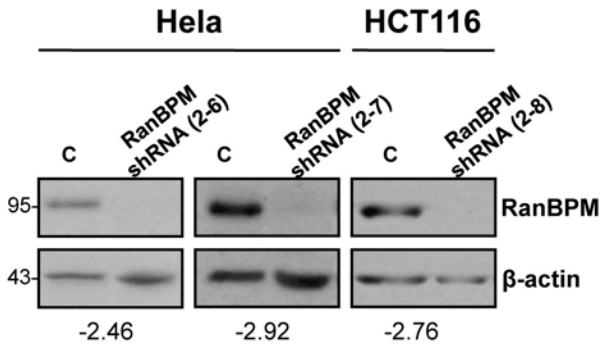
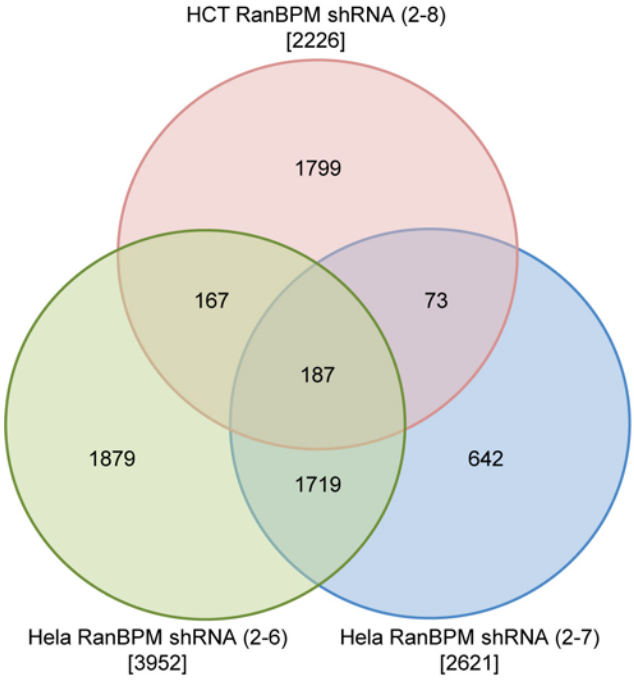
4.6 Supplementary materials

Supplementary Figure 4.1 Analysis of Hela and HCT116 stable cell lines. *Top* – Whole cell extracts from Hela control shRNA, RanBPM shRNA (2-6), RanBPM shRNA (2-7); and HCT116 control shRNA and RanBPM shRNA (2-8) were analyzed by western blotting. Down-regulated RanBPM expression was verified using a RanBPM antibody, and β-actin was used as a loading control. Fold-decrease in mRNA expression for each cell line is also indicated. *Bottom* – Venn Diagram of differentially expressed genes in RanBPM shRNA cell lines. For each cell line, the total number of differentially expressed genes is indicated in square brackets, and differentially expressed genes common between cell lines are indicated in overlapping regions of circles.


Hela
HCT116
C
RanBPM shRNA (2-6)
C
RanBPM shRNA (2-7)
C
RanBPM shRNA (2-8)
95-
43-
RanBPM
β-actin
-2.46
-2.92
-2.76


HCT RanBPM shRNA (2-8)
[2226]
1799
167
73
187
1879
642
1719
Hela RanBPM shRNA (2-6)
[3952]
Hela RanBPM shRNA (2-7)
[2621]

Supplementary Table 4.1. Primers for candidate gene validation by qRT-PCR

Gene	Forward Primer	Reverse Primer
RON (MST1R)	5’ AGGGTGTGGAGCGCTGTTGTG 3’	5’ CTTCCAGGCCAGGCGGGTTG 3’
ELF3 (ESE-1)	5’ AGAAGAGCAAGCACGCGCCC 3’	5’ AGCCTCGGAGCGCAGGAAC 3’
MSLN1	5’ AGGCTCAGCGCCACGCACTC 3’	5’ CCAGGGAGGGAGGCACCGTG 3’
ALDH1A3	5’ AGGCGGAGCGTGGAGTATGC 3’	5’ ACTGCTTTTGATCAATCTGAGGCCC 3’
CHN1	5’ TTCAAGGTGCATACATTCAGAGGGC 3’	5’ ACCACAATCTGCACATTTCACTCCC 3’
LAMB3	5’ CCATTGCAGCCAGGCTCCCC 3’	5’ GCTCGGCTCCTGGCTTCCTC 3’
TG2 (TGM2)	5’ ACCTCATCAAGGTGCGGGCC 3’	5’ TGGGCTCCCCAAGGATCCGG 3’
L1CAM	5’ CGCAGCAAGGGCGGCAAATAC 3’	5’ TCTCCAGGGACCTGTACTCGC 3’
PHD3 (EGLN3)	5’ GCCACGTGGACAACCCCAACG 3’	5’ CAGGATCCCACCATGTAGCTTGGC 3’
MFAP5 (MAGP2)	5’ TCAGCAGCCAAAGGACTCGGTG 3’	5’ CCCCAGGGGTATCCAGTCAGAGG 3’
RAB27B	5’ CGGGACAAGAGCGGTTCCGG 3’	5’ GCTTGCAGTTGGCTCATCCAGT 3’

RON, recepteur d'origine nantais/macrophage stimulating receptor 1 (MST1R); ELF3, E74-like factor 3 (ESE-1); MSLN1, mesothelin 1; ALDH1A3, aldehyde dehydrogenase 1 isoform A3; CHN1, chimerin 1; LAMB3, lamininβ 3; TG2, transglutaminase 2 (TGM2); L1CAM, L1 cell adhesion molecule; PHD3, prolyl hydroxylase 3 (EGLN3); MFAP5, microfibrillar associated protein 5 (MAGP2); RAB27B, RAB27B member Ras oncogene family.

Supplementary Table 4.2. List of genes affected by RanBPM down-regulation

		Hela RanBPM sh (2-6)		Hela RanBPM sh (2-7)		HCT RanBPM sh (2-8)	
Gene Symbol	Accession No.	p-value	Fold Change	p-value	Fold Change	p-value	Fold Change
Down-regulated Genes							
MLLT11	NM_006818	1.21E-05	-1.94805	1.78E-04	-1.65798	7.68E-03	-1.42647
PLA2G4A	NM_024420	9.94E-04	-1.98148	2.50E-02	-1.50694	1.04E-01	-1.38663
CAMSAP1L1	NM_203459	1.86E-04	-1.28092	7.56E-04	-1.23381	4.25E-04	-1.2971
C1orf71	NM_152609	3.65E-04	-1.26617	5.95E-06	-1.43517	5.50E-03	-1.20895
PTGS2	NM_000963	1.55E-03	-2.23247	7.75E-05	-3.13005	4.15E-01	-1.21651
KDM5B	NM_006618	2.38E-01	-1.20273	2.37E-01	-1.20358	9.98E-02	-1.35748
ADD3	NM_016824	1.44E-01	-1.20664	3.16E-04	-1.7965	1.73E-01	-1.22262
KLC2	NM_022822	7.57E-06	-1.45389	2.19E-04	-1.30358	8.60E-03	-1.20564
PANX1	NM_015368	1.69E-02	-1.28365	6.50E-02	-1.20168	1.01E-01	-1.20387
TARBP2	NM_134323	1.36E-07	-1.49385	1.64E-07	-1.48434	1.02E-03	-1.20971
NAV3	NM_014903	3.51E-02	-1.25264	4.96E-03	-1.38175	5.98E-07	-2.70703
ISCU	NM_014301	1.11E-07	-1.56273	6.43E-08	-1.59608	1.68E-05	-1.38687
RERG	NM_032918	7.97E-02	-1.38315	1.20E-02	-1.64535	2.20E-02	-1.66901
SSH1	NM_001161330	1.66E-02	-1.2583	1.96E-02	-1.24929	6.79E-05	-1.74098
GNG2	NM_053064	1.17E-04	-1.56726	1.07E-02	-1.28025	7.66E-02	-1.20227
ADAM21	NM_003813	2.33E-01	-1.23904	2.48E-01	-1.23016	3.64E-01	-1.20459
ADAM21	NM_003813	6.46E-03	-1.49927	7.95E-03	-1.47916	5.32E-02	-1.35929
ATPBD4	NM_080650	4.97E-03	-1.38728	2.73E-02	-1.27256	1.25E-01	-1.20153
MNS1	NM_018365	2.12E-03	-1.24488	4.02E-03	-1.22116	1.03E-02	-1.21957
MFGE8	NM_005928	2.91E-04	-2.09135	3.83E-03	-1.69699	3.03E-01	-1.20496
FGF11	NM_004112	1.46E-01	-1.30058	2.13E-01	-1.24941	3.02E-01	-1.23479
STARD3	NM_006804	1.64E-06	-1.32588	2.20E-06	-1.31601	1.74E-04	-1.22799
PITPNC1	NM_181671	7.03E-05	-2.5873	2.73E-06	-3.68728	3.87E-02	-1.55431
FXR2	NM_004860	1.81E-05	-1.24529	3.34E-05	-1.22921	2.13E-04	-1.21627
ALDOC	NM_005165	4.77E-02	-1.53452	8.64E-02	-1.43808	2.97E-01	-1.27832
GPR125	NM_145290	2.30E-03	-1.27392	1.75E-03	-1.28586	2.09E-02	-1.21458
MRPL34	NM_023937	1.87E-07	-1.58934	4.04E-08	-1.69516	2.88E-05	-1.39995
OLFM2	NM_058164	5.18E-03	-1.79105	1.79E-02	-1.60066	1.52E-01	-1.35655
ZNF585B	NM_152279	6.12E-02	-1.31428	7.42E-03	-1.52573	1.61E-05	-2.78181
TANK	NM_004180	3.16E-02	-1.23583	4.09E-02	-1.22079	5.57E-05	-1.81299
CHN1	NM_001822	3.27E-02	-1.23527	5.21E-03	-1.34482	3.19E-04	-1.6401
SATB2	NM_015265	7.03E-05	-1.53953	2.17E-03	-1.33286	4.69E-03	-1.34527
SCG2	NM_003469	1.58E-01	-1.3339	4.69E-03	-1.92649	1.23E-01	-1.44325
SERPINE2	NM_001136529	4.12E-03	-1.94032	3.54E-01	-1.20136	4.16E-01	-1.20337
SPTLC3	NM_018327	3.38E-07	-2.4565	2.13E-06	-2.14408	7.89E-02	-1.23241
JAG1	NM_000214	2.96E-03	-1.98526	2.63E-01	-1.24633	4.81E-02	-1.60611
GGT7	NM_178026	2.31E-02	-1.2819	2.35E-02	-1.28085	6.66E-02	-1.24981
TGM2 (TG2)	NM_004613	2.65E-04	-1.8711	1.45E-04	-1.95422	1.35E-02	-1.51768
ADRBK2	NM_005160	2.26E-07	-1.89169	6.79E-06	-1.59868	9.37E-03	-1.25698
MRAS	NM_012219	7.07E-06	-2.08349	6.24E-05	-1.80706	1.04E-01	-1.22871

ST6GAL1	NM_173216	3.29E-02	-1.25882	7.53E-02	-1.20493	2.22E-03	-1.52625
TBC1D5	NM_014744	1.17E-08	-2.1308	2.04E-07	-1.81176	2.12E-03	-1.30354
TNNC1	NM_003280	1.45E-03	-1.47697	2.47E-05	-1.85436	1.22E-02	-1.38503
CBLB	NM_170662	7.24E-03	-1.42617	2.10E-03	-1.5327	8.45E-02	-1.27219
METT5D1	NM_001113528	1.28E-03	-1.24527	3.23E-03	-1.2131	1.11E-02	-1.20096
B3GALNT1	NM_001038628	8.34E-04	-1.51373	1.34E-02	-1.31586	5.77E-02	-1.2595
DCUN1D1	NM_020640	9.28E-04	-1.42207	3.69E-02	-1.21187	7.44E-02	-1.20334
TBC1D19	NM_018317	3.21E-02	-1.20661	9.70E-03	-1.26742	1.95E-06	-2.0792
SLC7A11	NM_014331	8.25E-02	-1.39848	1.98E-01	-1.27329	3.52E-01	-1.21972
MAML3	NM_018717	3.45E-04	-4.52242	1.55E-01	-1.60779	4.80E-01	-1.30168
PDGFC	NM_016205	4.80E-03	-1.70882	3.39E-02	-1.45408	1.09E-01	-1.36899
C5orf23	BC022250	2.47E-04	-1.92466	1.39E-01	-1.22998	2.23E-01	-1.21372
PCDHB16	NM_020957	1.73E-06	-2.43184	7.53E-04	-1.60758	1.63E-01	-1.20361
RUNX2	NM_001024630	1.74E-02	-1.36279	2.72E-03	-1.52094	8.85E-04	-1.75488
DCBLD1	NM_173674	1.58E-04	-1.97271	1.68E-02	-1.42643	3.21E-02	-1.43175
C6orf211	BC011348	1.05E-03	-1.53886	4.73E-04	-1.60851	2.72E-02	-1.34316
SERPINB9	NM_004155	2.98E-02	-1.41724	4.75E-02	-1.36721	2.45E-01	-1.22258
RANBP9 (RanBPM)	NM_005493	1.21E-09	-2.46026	1.37E-10	-2.92317	1.62E-09	-2.76113
POPDC3	NM_022361	1.20E-07	-3.88063	2.63E-06	-2.80793	9.01E-02	-1.3191
MAN1A1	NM_005907	2.89E-01	-1.44414	2.47E-04	-5.24997	2.23E-01	-1.63354
CHN2	NM_004067	1.17E-03	-1.33711	6.35E-03	-1.25631	2.55E-06	-1.8985
GNAI1	NM_002069	1.22E-03	-1.30292	6.05E-04	-1.33507	1.02E-03	-1.36712
ANKIB1	NM_019004	2.15E-09	-1.99628	3.42E-09	-1.94551	1.09E-05	-1.46284
FLJ30064	AK054626	1.19E-02	-1.23465	1.20E-02	-1.23407	4.30E-02	-1.20536
PRKAR2B	NM_002736	3.45E-04	-1.51304	9.27E-03	-1.30104	8.69E-03	-1.35962
ELMO1	NM_014800	3.04E-10	-5.47252	2.36E-09	-4.22104	6.24E-02	-1.26619
JHDM1D	NM_030647	3.03E-01	-1.22543	2.24E-01	-1.2735	7.07E-03	-2.01056
SQLE	NM_003129	2.04E-02	-1.36587	5.01E-02	-1.29043	2.42E-02	-1.41606
IMPAD1	NM_017813	6.16E-04	-1.37208	4.27E-03	-1.27602	1.43E-03	-1.38843
FBXO32	NM_058229	1.68E-01	-1.43967	1.86E-01	-1.41688	4.74E-01	-1.23644
NR0B1	NM_000475	9.24E-05	-1.35907	2.86E-03	-1.22408	9.06E-03	-1.21559
TMSB15A	NM_021992	2.40E-03	-1.50078	5.97E-03	-1.42557	2.45E-02	-1.37353
CAPN6	NM_014289	1.61E-02	-1.44132	1.39E-03	-1.70707	8.87E-07	-3.77299
CLIC2	NM_001289	5.70E-04	-2.21894	3.48E-02	-1.5143	2.33E-01	-1.28961
Up-regulated Genes							
TMEM56	NM_152487	1.54E-05	1.58243	1.20E-04	1.45086	8.57E-03	1.27857
AMY2A	NM_000699	1.97E-04	1.89055	2.42E-03	1.59469	8.81E-02	1.30332
DNM3	NM_015569	3.70E-05	2.27619	5.53E-02	1.32742	2.18E-01	1.22254
ELF3 (ESE-1)	NM_001114309	8.38E-10	5.40777	1.61E-08	3.76364	6.03E-01	1.20112
DUSP5P	NR_002834	1.30E-04	1.85352	5.92E-04	1.68014	1.16E-01	1.25092
DUSP5P	AK055963	3.46E-04	1.71938	2.55E-02	1.32929	1.28E-01	1.23605
DHRS3	NM_004753	1.22E-03	4.07971	2.40E-01	1.52307	3.94E-01	1.41615
PPAP2B	NM_003713	1.56E-05	1.6272	8.49E-04	1.37031	3.52E-02	1.21952
GBP1	NM_002053	3.57E-06	1.89229	4.01E-05	1.65635	2.62E-02	1.2726

LAMB3	NM_001017402	9.58E-03	1.46725	1.51E-04	1.94691	1.52E-01	1.24829
TSPAN15	NM_012339	8.49E-08	2.99291	2.66E-06	2.24679	6.67E-02	1.26809
C10orf116	NM_006829	5.53E-02	1.31188	1.29E-01	1.23276	2.10E-01	1.2172
FAM107B	BC072452	4.50E-04	1.36372	7.20E-03	1.23625	2.46E-03	1.33376
FAM111B	NM_198947	1.29E-03	1.47392	3.45E-03	1.403	5.37E-03	1.44156
PLCB3	NM_000932	1.73E-06	1.23013	3.31E-06	1.21553	9.54E-06	1.22635
TCIRG1	NM_006019	3.70E-06	1.42594	6.37E-05	1.30811	6.60E-04	1.26936
PRSS23	NM_007173	8.55E-02	1.21509	7.26E-02	1.22687	1.59E-02	1.39761
GLB1L2	NM_138342	1.99E-04	1.29167	3.31E-04	1.27335	4.54E-03	1.21882
IFITM3	NM_021034	1.05E-03	1.52729	3.90E-02	1.2609	1.39E-01	1.20209
PRKCDBP	NM_145040	1.45E-02	1.2689	1.27E-02	1.27643	7.47E-03	1.36171
IL18	NM_001562	1.46E-02	1.47751	9.54E-02	1.28318	1.79E-01	1.2554
EMP1	NM_001423	3.10E-04	2.03037	2.86E-02	1.43137	1.13E-01	1.33105
SRGAP1	NM_020762	1.07E-02	1.2594	2.89E-02	1.20969	7.36E-06	1.89828
MFAP5 (MAGP2)	NM_003480	5.91E-08	3.81188	2.38E-06	2.63256	1.87E-01	1.21632
PRICKLE1	NM_153026	8.65E-04	2.53978	2.63E-01	1.28881	9.80E-02	1.56251
PCOTH	NM_001014442	1.26E-01	1.22419	9.32E-03	1.45778	1.41E-01	1.25062
LHFP	NM_005780	7.48E-06	2.2658	5.44E-04	1.68312	8.80E-04	1.76235
THTPA	NM_024328	1.22E-06	1.24314	7.77E-06	1.20133	1.37E-05	1.22202
PLEKHG3	NM_015549	1.15E-06	1.64696	1.93E-06	1.6097	5.16E-03	1.25555
LOC388022	AK131040	6.07E-03	1.35422	1.88E-04	1.61103	3.06E-04	1.68425
AP1G2	NM_003917	5.58E-05	1.33624	4.50E-04	1.25886	3.59E-03	1.22432
DHRS1	NM_001136050	3.93E-05	1.78477	1.62E-03	1.45926	7.44E-02	1.23808
EGLN3 (PHD3)	NM_022073	2.31E-04	1.62416	1.85E-04	1.64412	7.00E-02	1.24591
FBLN5	NM_006329	4.78E-04	2.09136	2.18E-02	1.51535	3.30E-01	1.20486
ALDH1A3	NM_000693	6.93E-06	2.48605	3.50E-04	1.83368	5.74E-02	1.35584
MEIS2	NM_172316	9.81E-04	1.25657	6.74E-04	1.2704	5.62E-04	1.32651
MSLN	NM_005823	5.96E-04	1.90214	3.40E-02	1.40282	8.56E-02	1.35907
TMC5	NM_001105248	4.30E-04	1.96946	9.77E-02	1.29488	1.02E-01	1.34208
HSD3B7	NM_025193	1.63E-06	1.53082	8.75E-05	1.33532	1.59E-04	1.36754
C16orf93	NM_001014979	2.82E-03	1.25639	9.19E-03	1.20921	8.97E-03	1.24636
CDRT1	NM_006382	1.98E-04	1.73327	4.81E-02	1.26431	5.32E-03	1.51366
CDRT1	NM_006382	4.79E-04	1.62109	5.34E-03	1.41757	2.28E-02	1.36553
ULK2	NM_014683	9.18E-05	1.42662	8.44E-05	1.43116	5.74E-04	1.39582
RDM1	NM_145654	3.30E-02	1.24351	5.96E-02	1.20757	7.75E-02	1.22423
HOXB5	NM_002147	8.07E-04	1.57366	2.60E-02	1.30036	1.91E-02	1.38102
HOXB6	NM_018952	2.11E-04	1.85847	1.02E-02	1.44224	4.62E-02	1.36399
SCARNA17	NR_003003	6.81E-04	1.33125	2.27E-03	1.27697	2.25E-02	1.21306
RAB27B	NM_004163	3.19E-04	2.14986	4.13E-02	1.42978	9.87E-02	1.38312
PMAIP1	NM_021127	3.22E-04	1.33759	1.17E-03	1.28228	1.17E-05	1.60819
PSTPIP2	NM_024430	1.83E-06	1.63595	5.71E-05	1.42369	1.87E-04	1.43202
ALPK2	NM_052947	8.31E-06	3.5668	1.72E-03	2.02592	3.66E-01	1.21451
ZNF91	NM_003430	2.98E-04	1.72095	3.93E-02	1.28992	3.16E-04	1.86383
HNMT	NM_006895	9.08E-03	1.33786	6.32E-02	1.21293	2.45E-02	1.32208

DKFZp434H1419	AK125369	7.62E-03	1.3181	7.73E-03	1.31719	2.10E-02	1.30412
EDAR	NM_022336	1.15E-02	1.28525	4.95E-02	1.20299	2.87E-02	1.27402
GALNT3	NM_004482	5.55E-07	2.28147	8.67E-07	2.20981	3.34E-03	1.45717
SCN9A	NM_002977	6.71E-05	2.68432	2.36E-04	2.36665	1.29E-01	1.37881
SLC4A11	NM_032034	4.11E-05	1.3121	1.18E-04	1.2752	2.28E-05	1.39485
CTD-2514C3.1	NR_004846	1.10E-03	1.61351	2.96E-02	1.32356	3.71E-02	1.36039
SDC4	NM_002999	2.26E-02	1.27084	1.62E-02	1.29191	8.18E-02	1.22376
USP18	NM_017414	4.57E-04	1.70765	6.16E-03	1.45632	1.14E-01	1.25282
MST1R (RON)	NM_002447	1.75E-04	1.59982	1.30E-05	1.84799	3.40E-03	1.45303
FRAS1	NM_025074	1.63E-04	1.88825	1.89E-02	1.38504	2.10E-01	1.20351
ANXA3	NM_005139	2.40E-03	1.30439	4.91E-03	1.27043	2.93E-02	1.22156
CCDC109B	NM_017918	8.21E-03	1.40159	4.98E-03	1.44161	6.45E-03	1.50005
TLR3	NM_003265	2.85E-07	2.40756	1.99E-05	1.81232	6.08E-02	1.24193
FBXO4	NM_012176	2.10E-02	1.422	2.49E-02	1.40495	2.29E-01	1.21585
SH3RF2	NM_152550	1.24E-02	1.53236	2.80E-02	1.43895	5.00E-02	1.44378
ERAP1	NM_001040458	1.38E-06	1.63246	1.48E-05	1.48083	4.36E-04	1.37218
MRAP2	NM_138409	2.00E-05	2.13295	7.38E-06	2.30291	3.67E-02	1.36787
PLEKHG1	NM_001029884	7.22E-03	1.67107	4.12E-02	1.4414	1.69E-01	1.31205
MBOAT1	NM_001080480	7.64E-07	1.71621	1.40E-05	1.51059	6.26E-03	1.2591
C6orf132	ENST0000034186	2.99E-07	2.60787	1.76E-06	2.26832	1.20E-02	1.4013
COBL	NM_015198	4.52E-02	1.33178	7.54E-02	1.28395	1.01E-01	1.30195
MATN2	NM_002380	9.63E-04	1.50342	3.61E-03	1.4057	3.65E-02	1.29524
RHPN1	NM_052924	1.23E-04	1.29147	3.74E-04	1.25316	4.87E-03	1.20379
C8orf51	NR_026785	8.67E-07	1.27628	5.59E-06	1.22846	8.06E-05	1.20001
C8orf73	NM_001100878	1.04E-02	1.37454	6.80E-02	1.23581	1.03E-02	1.44525
NPR2	NM_003995	1.58E-05	1.46282	6.67E-05	1.38947	8.63E-03	1.22615
GRHPR	NM_012203	2.38E-03	1.2851	1.60E-02	1.20268	2.80E-02	1.20965
FAM189A2	NM_004816	2.68E-02	1.46127	2.05E-03	1.79215	1.39E-01	1.31887
C9orf3	AF043897	1.69E-05	1.48498	2.18E-05	1.47055	2.43E-06	1.73043
NIPSNAP3A	NM_015469	1.28E-05	1.44175	2.96E-03	1.21701	9.71E-03	1.20717
ARRDC1	NM_152285	4.09E-07	1.74846	5.70E-07	1.72052	4.51E-03	1.26792
PTPLAD2	NM_001010915	2.21E-07	2.42495	3.35E-06	2.00393	3.25E-02	1.28292
TLE1	NM_005077	7.80E-05	1.98957	6.76E-04	1.71392	1.49E-01	1.24041
HDHD3	NM_031219	2.73E-02	1.21184	1.79E-03	1.35297	2.20E-02	1.26123
PTRH1	NM_001002913	8.47E-03	1.25899	2.30E-02	1.211	5.87E-03	1.32616
NPDC1	NM_015392	5.95E-04	1.43109	1.55E-03	1.37363	5.30E-02	1.21613
SCML1	NM_001037540	3.55E-05	1.47592	3.27E-06	1.63206	4.95E-04	1.40074
FAM156A	NM_014138	9.97E-03	1.21367	5.06E-04	1.34309	8.82E-03	1.25655
GABRE	NM_004961	6.66E-05	1.81362	2.29E-03	1.47851	4.39E-03	1.5082
GABRE	U92285	2.59E-04	2.57423	4.17E-02	1.53774	3.39E-01	1.24385
L1CAM	NM_000425	8.88E-03	1.31114	2.74E-03	1.38376	3.21E-02	1.27652

Chapter 5

5. Discussion and future directions

5.1 Summary of findings

RanBPM was first described as a factor involved in Ran-mediated microtubule nucleation. However, this notion was quickly dismissed and many studies have since attempted to elucidate the cellular functions of RanBPM. While some progress has been made in understanding its role, the diverse processes in which RanBPM has been proposed to participate have made defining a unified function for this protein difficult. In this thesis, we aim to address this matter by describing a function for RanBPM in the regulation of signalling pathways that mediate cell survival and apoptosis. Further, this work suggests a previously unknown function for RanBPM as a tumour suppressor, and also expands upon a role for RanBPM in development.

Although RanBPM has been implicated in apoptotic regulation, a specific mechanism for such a role has not been demonstrated thus far. In chapter 2, we identify RanBPM as a pro-apoptotic protein that regulates the intrinsic apoptotic signalling pathway. In this chapter we also provide evidence that RanBPM modulates the localization and expression of Bcl-2 family proteins, thus identifying a novel mechanism by which RanBPM executes its pro-apoptotic activity. We go on to show in chapter 3 that the effect of RanBPM on the expression of Bcl-2 family proteins is mediated at least in part, through repression of the ERK1/2 pathway. RanBPM has previously been proposed to regulate ERK1/2 signalling, however, its role in this pathway remains controversial. The studies in chapter 3 reveal that RanBPM inhibits ERK1/2 signalling through destabilization of the c-Raf protein, thus uncovering a novel function for RanBPM in the regulation of the ERK1/2 cascade. Finally, the findings in chapter 4 reveal that altered RanBPM expression affects transcriptional programs involved in cellular signalling, regulation of organism development, and tumourigenesis. This is the first example of a characterization of global gene expression patterns regulated by RanBPM. The data presented in this thesis also suggest a potential role for RanBPM as a novel tumour suppressor in cells. We ascribe this function to RanBPM based upon the

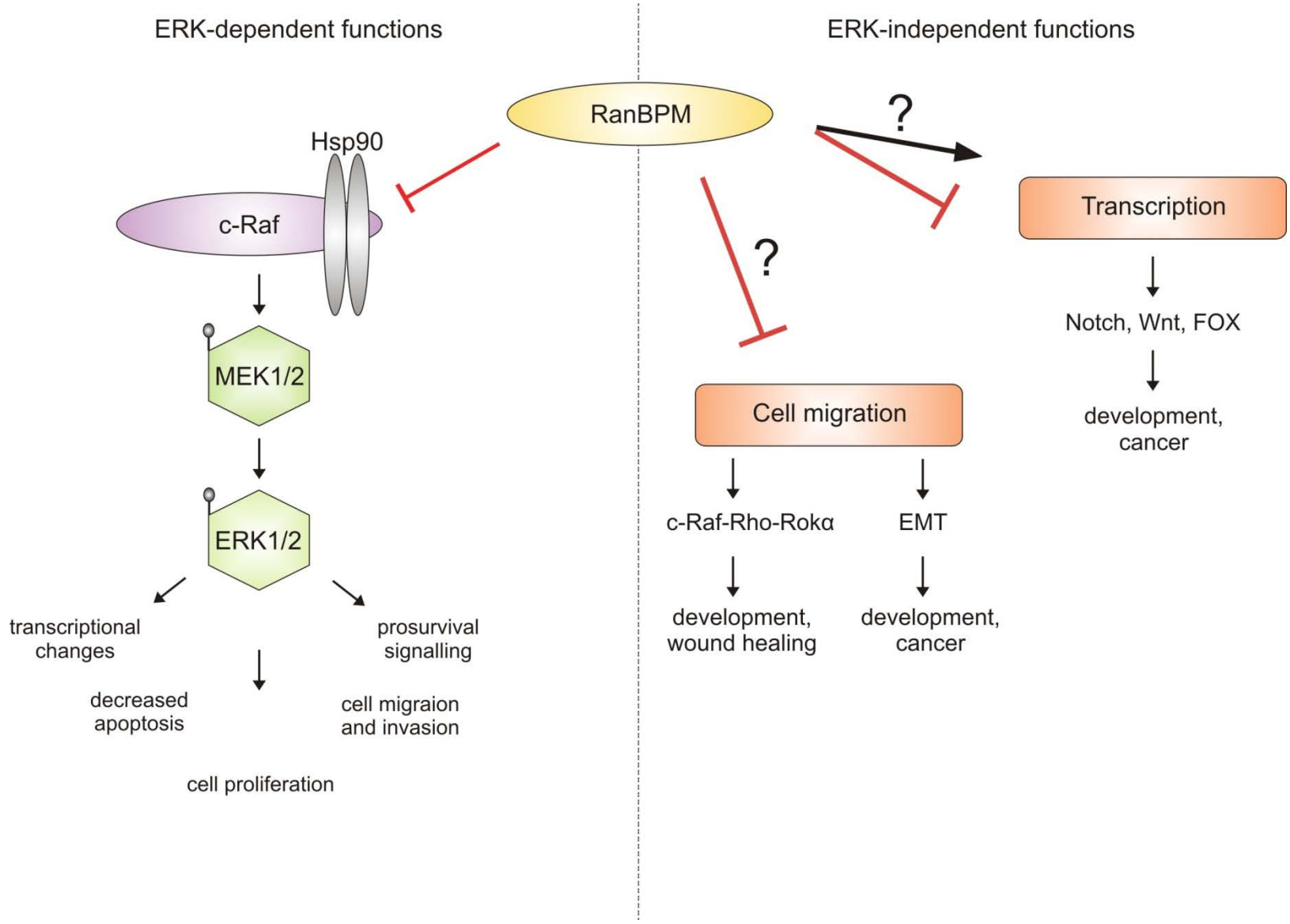
observation that down-regulation of RanBPM expression is associated with the acquisition of multiple hallmarks of cellular transformation. Together, our findings assign a biological significance to the molecular functions of RanBPM by describing how perturbations in RanBPM expression affect normal and pathological development.

5.2 Functions for RanBPM in tumour suppression

A critical aspect of normal cellular function is the ability to balance the extracellular cues that signal either proliferation, or growth suppression [1, 2]. For example, cells in a given tissue are able to stimulate the growth of adjacent cells by secreting mitogenic stimuli, such as growth factors. This triggers growth-inducing signalling cascades, and leads to cell cycle progression and/or differentiation [1, 2]. Conversely, cells can also secrete factors that convey growth-restrictive signals in neighbouring cells [2]. These stimuli activate signalling pathways that prohibit cell proliferation by halting cell cycle progression, inducing differentiation, or activating apoptosis [2]. Importantly, the inability to properly respond to these extracellular cues is associated with the occurrence of pathological conditions such as developmental abnormalities and cancer [1].

A common feature of cancer is chromosomal rearrangements that cause the amplification of oncogenes, and the deletion of tumour suppressors [3]. In its most simple form, an oncogene is defined as a gene that conveys growth-inducing signals in cells, while tumour suppressors are genes whose products potentiate growth-restrictive signals within cells [2]. Thus, mutations that promote the growth-inducing activity of oncogenes, and abolish the restrictive activity of tumour suppressors, together cause the deregulated cellular growth that is observed in cancer [2, 4]. Our analyses of the cellular functions of RanBPM suggest a role for this protein as a tumour suppressor, as absence of RanBPM expression in cells disrupts apoptotic activation, leads to growth factor independence, and increases rates of migration and invasion (Fig. 5.1).

Figure 5.1 ERK-dependent and ERK-independent functions of RanBPM. *Left* – Novel function for RanBPM in the repression of c-Raf-mediated ERK1/2 signalling. Destabilization of the c-Raf-Hsp90 complex by RanBPM inhibits signalling downstream of c-Raf. This has implications in the regulation of cell survival, cell proliferation, cell migration, apoptosis, and transcriptional programs mediated by activated ERK1/2 signalling. *Right* – Regulation of gene expression by RanBPM may occur independently of the ERK1/2 cascade, through Notch and Wnt signalling pathways, and FOX family transcription factors. RanBPM may also inhibit the migratory capacity of cells independently of ERK1/2, by regulating EMT programs or the c-Raf-Rho-Rokα signalling pathway.


ERK-dependent functions
ERK-independent functions
RanBPM
?
Hsp90
c-Raf
?
Transcription
Notch, Wnt, FOX
development,
cancer
MEK1/2
Cell migration
ERK1/2
c-Raf-Rho-Rokα
EMT
transcriptional
changes
prosurvival
signalling
development,
wound healing
development,
cancer
decreased
apoptosis
cell migraion
and invasion
cell proliferation

5.2.1 Down-regulation of RanBPM expression confers resistance to apoptosis

Pro-apoptotic factors comprise an important class of tumour suppressor genes, and are often mutated or inactivated in cancer [5]. The first piece of evidence for a potential tumour suppressor role for RanBPM arises from the observation that RanBPM is a critical pro-apoptotic factor (Chapter 2). In this chapter, we show that ectopic expression of RanBPM leads to caspase-mediated apoptotic cell death. Conversely, the down-regulation of RanBPM expression was able to protect cells from apoptotic activation induced by DNA damage, and corresponded with an order-of-magnitude increase in cell survival upon exposure to IR. In the absence of RanBPM expression, we also observed a marked up-regulation in the cellular levels of anti-apoptotic Bcl-2 proteins, and a concurrent decrease in mitochondria-associated Bax. This suggests that down-regulation of RanBPM causes a shift in the cellular ratios of anti- and pro-apoptotic Bcl-2 proteins within the cell. We hypothesize that this prevents genotoxic stimuli from properly signalling mitochondrial permeabilization, thus blocking the activation of cell death. Based upon the data presented in chapter 2, we propose that RanBPM is required for the proper execution of apoptosis, and that disruption of its pro-apoptotic activity contributes to the acquisition of a transformed phenotype in cells.

In the future, it will be important to determine whether, in addition to IR-induced DNA damage, RanBPM can also participate in apoptotic activation in response to other forms of cellular stress. For example, exposure to cytotoxic factors can cause ER dysfunction, consequently disrupting calcium homeostasis and protein folding, and leading to apoptotic cell death [6]. Assessing such a function for RanBPM will help to determine whether RanBPM is a general apoptotic factor that can be activated downstream of various stress stimuli, or whether it may be involved in "sensing" specific types of cell stress and mediating the apoptotic response to these stresses. A potential role for RanBPM as a stress sensor is supported by our observation that the induction of caspase-2 activity is abrogated in RanBPM-deficient cells upon exposure to IR. Caspase-2 can be activated by various forms of cellular stress, including DNA damage and ER dysfunction, to trigger pro-apoptotic signalling [7]. While the precise mechanisms of caspase-2-mediated apoptosis remain enigmatic, it has been suggested to promote

apoptosis through both p53-dependent and p53-independent mechanisms [7]. In addition, caspase-2 has been shown to localize to the nucleus and to participate in DNA damage-dependent signal transduction [7, 8]. These observations have also led to the notion that caspase-2 is an important tumour suppressor that safeguards cells from undergoing transformation by responding to specific forms of cellular stress and promoting apoptotic induction, even when p53-dependent apoptotic pathways have become inactivated [7, 8]. As RanBPM is also known to localize to the nucleus [9-11], it will be intriguing to investigate whether RanBPM may be involved in promoting the induction of caspase-2 activity to further enhance the apoptotic response of cells exposed to certain types of genotoxic or cytotoxic stress.

5.2.2 Decreased RanBPM expression is associated with loss of growth factor dependence in cells

Factors that function as negative regulators of growth factor-mediated signalling often exhibit tumour suppressor activities in cells [12-14]. Deletion or inactivation of these negative regulators can contribute to growth factor independence, which is often characterized by the constitutive activation of a component of a signalling pathway that thereby vacates the need for upstream ligand-mediated receptor activation [14]. Here we propose that RanBPM functions as a negative regulator of the ERK1/2 signalling pathway (Chapter 3). We found that down-regulation of RanBPM expression in cultured cells results in the hyperactivation of MEK1/2 and ERK1/2. We also show that the elevated Bcl-2 protein levels observed in the absence of RanBPM is specifically due to the hyperactivation of ERK1/2 signalling. Further, we demonstrate that RanBPM functions to regulate this pathway through the destabilization of the c-Raf protein. RanBPM was found to form a complex with c-Raf, and its expression corresponded with a marked down-regulation in c-Raf protein levels. Intriguingly, this latter effect of RanBPM was especially pronounced on activated forms of the c-Raf kinase. Finally, decreased c-Raf protein levels were found to be mediated through the disruption of the c-Raf-Hsp90 complex by RanBPM. Based upon these findings we hypothesize that in RanBPM-expressing cells, RanBPM prevents aberrant ERK1/2 activation by maintaining

low cellular levels of c-Raf. In the absence of RanBPM expression however, this repression on c-Raf is removed, leading to the deregulation of ERK1/2 signalling. The data presented in chapter 3 indicate that loss of RanBPM expression confers growth factor independence upon cells, thus further contributing to the acquisition of a transformed phenotype in these cells.

Our findings that RanBPM disrupts the c-Raf-Hsp90 protein complex suggest a mechanism by which RanBPM may mediate low c-Raf protein expression. Molecular chaperones are known to be essential for the folding and stability of c-Raf, and blocking c-Raf-Hsp90 complex formation was found to cause its rapid ubiquitination and degradation by proteasomes [15, 16]. Therefore, it will be necessary to assess whether RanBPM expression is associated with increased c-Raf protein turnover. Rapid protein turnover is one of the mechanisms by which cells "fine-tune" the duration of, and response to, signalling cascades. For example, activation of Notch signalling causes the rapid degradation of transcription factors that mediate neuronal differentiation, thereby promoting stem cell maintenance in the nervous system [17]. Likewise, the absence of upstream Wnt activation stimulates the rapid turnover of β-catenin to prevent aberrant Wnt target-gene expression [18]. Therefore, it is plausible that RanBPM may function to "fine-tune" ERK1/2 signalling by maintaining low cellular levels of c-Raf. This role for RanBPM is supported by the observation that it is a component of the evolutionarily conserved CTLH complex that is proposed to mediate the ubiquitination and degradation of various cellular proteins [19-21]. Whether RanBPM regulates c-Raf by promoting its ubiquitination and proteasomal degradation is the subject of on-going investigation in our lab.

The mechanism by which RanBPM destabilizes the c-Raf-Hsp90 complex will also need to be elucidated. One proposed mechanism would be that RanBPM directly competes with Hsp90 for binding to c-Raf. A similar role was previously described for Hsp70, which competes with c-Raf for binding to the co-chaperone BAG1 [22]. In this study, binding of c-Raf to BAG1 was found to be necessary for c-Raf kinase activity, and induction of signalling downstream of c-Raf [22]. Disruption of the c-Raf-BAG1 interaction by Hsp70 was thus suggested to facilitate the repression of mitogenic

signalling by c-Raf in response to heat shock [22]. RanBPM may function in a similar manner in order to attenuate c-Raf-mediated ERK signalling. The binding of Hsp90 to c-Raf is not only required for stabilizing c-Raf protein structure, but also for c-Raf catalytic activity and signalling [16]. Therefore destabilization of this complex by RanBPM may provide a means of preventing aberrant ERK1/2 signalling, or attenuating activated ERK1/2 signalling in cells. This notion is supported by our observation that, in addition to modulating c-Raf protein levels in resting cells, RanBPM also robustly down-regulates activated forms of c-Raf. Therefore in this model of RanBPM function, we propose that RanBPM would compete with Hsp90 for binding to c-Raf in resting cells, in order to maintain low c-Raf expression. Upon c-Raf activation, the affinity of RanBPM for c-Raf, or its competition with Hsp90 for binding to c-Raf, would be enhanced and ultimately lead to the down-regulation of c-Raf and repression of its downstream signalling. Alternatively, the interaction of RanBPM with c-Raf may mediate the recruitment of a protein complex that causes the dissociation of c-Raf from Hsp90, thus leading to its destabilization. One of the cellular functions of the molecular chaperone complex is to shuttle client proteins that cannot be properly folded to the proteasomes for degradation [23, 24]. This often requires client-protein transfer, wherein co-chaperones facilitate the physical transfer of protein substrates from one chaperone to another, in order to ubiquitinate client proteins and target them to proteasomes [25]. For example, such a mechanism has previously been described for the ubiquitination and degradation of the Hsp90 client protein ErbB2 (epidermal growth factor receptor 2), by the co-chaperone CHIP. CHIP was reported to bind the Hsp90-ErbB2 complex, and mediate the stepwise dissociation of ErbB2 from Hsp90, followed by its binding to Hsp70, and its ubiquitination and shuttling to proteasomes for degradation [26]. As c-Raf is a known CHIP client protein [27, 28], RanBPM may function to promote CHIP-dependent transfer of c-Raf from Hsp90 to Hsp70, thereby destabilizing the c-Raf-Hsp90 complex. If RanBPM is found to function in this manner, it will also be important to determine whether its binding to c-Raf corresponds with enhanced proteasomal degradation of c-Raf.

A critical physiological outcome of growth factor independence is the ability to undergo sustained proliferative signalling, even in the absence of receptor or growth-

factor stimulation [14]. In chapter 3, we demonstrate that RanBPM-deficient cells exhibit a sustained proliferative phenotype, as they are able to grow under serum-free conditions. We propose that this occurs as a result of hyperactivated ERK1/2 signalling in these cells. Collectively, these findings characterize the molecular and physiological consequences associated with RanBPM down-regulation (Fig. 5.1), and serve to further implicate a role for this protein in tumour suppression.

5.2.3 Absence of RanBPM expression corresponds with enhanced cellular migration and invasion

Cytoskeletal proteins are an important class of ERK1/2 effector proteins, and one of the physiological outcomes of ERK1/2 activation is cell migration [29-31]. Thus, it is not surprising that deregulated ERK1/2 signalling can increase cell migration and invasion, and lead to tumour metastasis [29]. In chapter 3 we show that in addition to apoptotic resistance and loss of growth factor dependence, down-regulation of RanBPM expression in cultured cells is also associated with increased rates of cellular migration and invasion. As tumour suppressors are known to regulate processes associated with cell motility [32], these observations further support the potential of such a role for RanBPM. In the future, it will be necessary to determine how RanBPM-deficient cells acquire their enhanced invasive and migratory capacities (Fig. 5.1). This phenotype may be a result of sustained ERK1/2 activation, or may be attributed to the elevated c-Raf levels observed in these cells. C-Raf is known to mediate cell migration and invasion in certain cell types by signalling through the Rho family of small GTPases, in a process independent of its kinase activity [33]. Thus, it is plausible that the increased cell motility observed in RanBPM-deficient cells occurs in an ERK-independent manner, highlighting yet another signalling pathway in which RanBPM may function. Alternatively, RanBPM may affect rates of cellular migration and invasion through a process that is completely independent of its functions on c-Raf and ERK1/2. Specifically, the data outlined in chapter 4 reveal that RanBPM regulates components of the Notch, Wnt, and TGFβ signalling pathways, all of which are well-established modulators of the EMT program [34, 35]. Whether the down-regulation of RanBPM

expression is associated with the acquisition of a mesenchymal phenotype, and whether this is the cause of the enhanced migratory and invasive capabilities observed in RanBPM-deficient cells, will need to be investigated.

5.3 Regulation of diverse transcriptional programs by RanBPM

The responses to the broad extrinsic cues that cells receive are mediated by changes in gene expression patterns that drive processes such as proliferation, differentiation, and cell death [1, 36]. During embryogenesis, for example, transcriptional changes arising from signalling through the Notch, Wnt, and TGFβ pathways facilitate cell fate determination and organogenesis across a wide range of tissues [36, 37]. Similarly, these pathways regulate maintenance, renewal, and repair in adult tissues [36]. Alterations in these transcriptional programs can result in developmental abnormalities, and in diseases such as cancer. Thus, delineating the complex gene regulatory networks of the cell has important implications in understanding both normal and pathological development [38]. The gene expression profiling data presented in chapter 4 implicate a role for RanBPM in regulating the expression of genes involved in organism development and tumourigenesis.

With regard to a role in development, we found that one-quarter of the genes whose expression is altered in response to RanBPM down-regulation are involved in tissue development, and include components of the Notch, Wnt/β-catenin, and TGFβ pathways. Included in this group of genes are factors implicated in the regulation of cellular proliferation, stem cell differentiation, neurite outgrowth, and organ morphogenesis. Additionally, we found that the promoters of many RanBPM target genes contain binding sites for transcription factors that have key functions in development, including the FOX, HMG, and Homeobox family factors. Together, these observations suggest that RanBPM may function to regulate developmental processes both directly and indirectly. With respect to a direct function in development, RanBPM may modulate the expression of components of signalling pathways in order to execute specific developmental responses. For example, we found that RanBPM down-regulation

corresponded with decreased expression of the Notch ligand JAG1. During embryogenesis, ligand-mediated Notch signalling is essential for cellular differentiation, proliferation, apoptosis, and migration [39]. Defects in this pathway, arising from mutations in either Notch ligands or receptors, are associated with severe developmental defects [36]. Specifically, mutations of JAG1 lead to a developmental disorder known as Alagille syndrome, which is characterized by growth retardation and abnormalities in the development of cardiac, renal, ocular, vascular, and hepatic tissues [36, 40]. Intriguingly, in their recent studies of RanBPM function in development, Puverel and colleagues reported that over half of RanBPM-null mice died perinatally, and those which did survive exhibited severe growth retardation [41]. While the causes of the growth and survival abnormalities observed in these mice have not yet been determined [41], it is tempting to propose that they may be a result of defects in Notch signalling. To assess this, it will be important to determine whether JAG1 expression is down-regulated in RanBPM-null mice compared to wildtype controls, and if so, whether this indeed disrupts JAG1/Notch-dependent pathways in RanBPM-null cells obtained from the tissues of these mice. RanBPM may also indirectly function in development by modulating the activity or function of transcription factors that regulate the expression of groups of genes involved in various aspects of organism development. This function for RanBPM is supported by the fact that it is a known transcriptional co-activator of steroid hormone receptors, including the androgen receptor (AR) [42]. AR is involved in the development, function, and maintenance of male reproductive organs and more recently has been suggested to function in a similar role in female reproductive organs [43, 44]. AR has been shown to interact with the transcription factor FOXA1 to regulate the expression of AR target genes during prostate development [45]. In addition, the interaction of AR with the transcription factor FOXO3a has been found to regulate gene expression patterns associated with development, growth, and apoptosis in the prostate and ovary [46, 47]. As our data in chapter 4 indicate that RanBPM down-regulation alters the expression of FOXA1- and FOXO3a-target genes, we postulate that a potential function for RanBPM may be to regulate developmental programs driven by FOXA1/AR- and FOXO3a/AR-dependent transcription. This hypothesis is corroborated by recent findings that highlight a critical function for RanBPM in the development and

function of mammalian reproductive organs [41]. This study reported that RanBPM knockout led to severe gonadal defects that prevented spermatogenesis and resulted in sterility in male mice, and to sterility and premature ovarian failure in female mice [41], the latter being a characteristic that has previously been attributed to the deletion or mutation of FOXO3a [48]. Therefore, future experiments will need to address whether the gonadal abnormalities observed in RanBPM-null mice arise due to defects in the FOX/AR transcriptional program. This may be assessed using gene expression profiling comparing the expression of FOX/AR gene targets involved in development in control and RanBPM-null mice. It will also be important to verify that any observed changes in transcriptional output occur due to the absence of RanBPM, by assessing whether re-expression of RanBPM can restore normal gene expression patterns in RanBPM-deficient cells.

The data in chapter 4 also strongly implicate a role for RanBPM in tumourigenesis, as more than one-third of all RanBPM target genes identified in our analyses have been associated with cancer. We found that down-regulation of RanBPM expression affected signalling pathways that, when perturbed, can promote cellular transformation and tumour metastasis. For example, decreased RanBPM expression corresponded with a marked up-regulation in the expression of the RTK RON, which drives tumourigenesis by enhancing signalling through the ERK1/2 and Akt signalling pathways [49, 50]. Interestingly, we also noted that many of the transcription factors identified in our analyses that normally function in development can become deregulated to promote oncogenesis. For example, in addition to their aforementioned roles in normal biological processes, most FOX transcription factors have also been found to contribute to oncogenic transformation by functioning as either tumour suppressors or oncogenes [48]. FOXA1, for example, exhibits oncogenic activity in certain tissue subtypes, as its overexpression positively correlates with metastatic lesions in the thyroid and prostate [45, 51]. Conversely FOXO3a is an established tumour suppressor, and its deletion corresponds to enhanced cell cycle progression and inhibition of apoptosis [51]. Inactivation of FOXO3a is associated with tumourigenesis in a broad range of tissues, particularly in haematological cancers [48, 51]. In addition to FOX transcription factors, the deregulated activities of Homeobox (ie. HOXA5), [52, 53] and HMG (ie. SOX9 and

SOX17) [54, 55] families of transcription factors are associated with cancer development and metastasis in several tissues. An important question that arises based upon these observations is whether the expression of RanBPM may correlate with the oncogenic activity of specific groups of transcription factors. While it is known that RanBPM is ubiquitously expressed in normal tissues [10], little is known regarding its expression patterns in cancerous tissues. However, one study did report that the expression of RanBPM was either significantly down-regulated, or completely absent, in patient tumour samples from the lung, kidney, and breast, when compared to RanBPM expression in the normal adjacent tissue [9]. As FOXA1 is suggested to strongly contribute to breast cancer development, but not metastasis [45, 51], it will be highly informative to determine whether RanBPM expression may negatively correlate with FOXA1 expression in breast cancer. A similar analysis could be applied to tumour samples from the lung, as it has been reported that the FOXA1 gene locus is amplified in lung cancers [45]. In addition, the expression of HOXA5 [53] and SOX17 [56] is known to be decreased in breast cancers, therefore whether RanBPM expression positively correlates with the expression of these factors in breast cancer will also be of interest to investigate. The proposition that RanBPM may regulate the activity of these transcription factors is supported by previous findings that RanBPM promotes the nuclear localization of steroid hormone receptors and enhances their DNA-binding affinities [42, 57], and that RanBPM itself exhibits a predominantly nuclear localization in resting cells [9-11]. Therefore, it is tempting to hypothesize that one of the roles of RanBPM within the nucleus may be to modulate the function or activity of specific groups of transcription factors, and that disruption of RanBPM expression would impede this regulation, and thus lead to aberrant gene transcription patterns that promote tumourigenesis. Collectively, the findings in chapter 4 suggest that RanBPM can have wide-reaching effects on cellular transcription programs, which when disrupted, may lead to malignant transformation.

5.4 Oncogenic and metastatic potential of RanBPM *in vivo*

The data presented in this thesis underscore a potentially critical function for RanBPM in regulating tumour suppressor programs in cultured cells. However, an important question that remains to be answered is whether RanBPM can exert such a function in animal models that more closely reflect normal mammalian physiology. This may be addressed through the use of mouse models of tumour formation, and RanBPM-null mice.

The ability of RanBPM to promote tumourigenesis *in vivo* can be addressed by xenografting either control, or RanBPM-deficient cells into athymic nude mice, and measuring the tumour volume in these animals post-injection. We hypothesize that if the absence of RanBPM expression is oncogenic, then mice injected with RanBPM-deficient cells should exhibit increased tumour volume, compared to those injected with control cells expressing physiological levels of RanBPM. To ensure that any observed increase in tumourigenicity occurs specifically due to decreased RanBPM expression, the ability of cells re-expressing RanBPM to rescue the oncogenic phenotype of RanBPM down-regulation will also need to be assessed in these mice. In addition, a role for RanBPM in tumourigenesis can be studied in genetically engineered RanBPM knockout mice, by determining whether these mice may be "pre-disposed" to cancer and exhibit increased rates of spontaneous tumour formation, compared to normal littermate controls. An intriguing aspect of utilizing this model to study the oncogenic potential of RanBPM will be determining which tissues may be pre-disposed to tumourigenesis as a result of RanBPM deletion. Based upon previous reports of RanBPM expression in human cancers [9], we hypothesize that tumours may be particularly likely to develop in the breast and lung tissues of RanBPM-null mice. This notion is also corroborated by our findings in chapter 4 that RanBPM-deficient cells exhibit enhanced expression of genes such as ELF-3, L1CAM, and RON, which are all known drive the development of cancerous lesions in the breast or lung [58-60]. In addition, the expression and activity of transcription factors with over-represented binding sites in the promoters of RanBPM-target genes, such as HOXA5 and FOXA1, are also known to be deregulated in these cancer subtypes [45, 53], further suggesting that ablation of RanBPM expression may

indeed pre-dispose these tissues to tumourigenesis. These mouse models will also be beneficial to assessing whether RanBPM down-regulation is associated with metastatic disease. Our data suggest that RanBPM modulates the invasive and migratory capacity of cells, and may also be involved in EMT of cells (Fig. 5.1). Therefore, it will be interesting to determine whether the number of secondary-site lesions is increased in RanBPM-null mice, compared to controls. For example, advanced stages of breast and lung cancer may be associated with bone metastasis, thus it will be necessary to assess RanBPM-null mice for cancerous lesions of the bone. Further, if RanBPM is found to promote metastasis, the cells from these secondary lesions may be isolated and analyzed for markers of EMT, such as decreased E-cadherin expression or increased vimentin expression [34], and for hyperactivated ERK1/2 signalling. These analyses would provide insight into potential functions for RanBPM in driving EMT and metastatic programs in cancer.

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  University of Western Ontario, London, Ontario, Canada

- **Honours B.Sc., Specialization in Biochemistry** **2007**
  University of Ottawa, Ottawa, Ontario, Canada

RESEARCH EXPERIENCE

Ph.D. Candidate, Department of Biochemistry **2007-12**
University of Western Ontario
Supervised by Dr. Caroline Schild-Poulter, Robarts Research Institute

Honours Thesis Project, Department of Biochemistry **2006-07**
University of Ottawa
Supervised by Dr. Jonathan Lee, University of Ottawa

SELECTED PROFESSIONAL EXPERIENCES

Teaching
- Teaching assistant-Biochemistry 2280 **2011**
  University of Western Ontario

Teaching
- Teaching assistant-Biochemistry 3382 **2012**
  University of Western Ontario

Laboratory Technician, University of Ottawa **2006**
- Lab of Dr. Jonathan Lee, University of Ottawa

PUBLICATIONS

1. **E. Atabakhsh**, D.M. Bryce, K.J. Lefebvre, and C. Schild-Poulter. RanBPM has pro-apoptotic activities that regulate cell death pathways in response to DNA damage. *Mol. Cancer Res.*, December 2009.

2. **E. Atabakhsh** and C. Schild-Poulter. RanBPM is an inhibitor of ERK signaling. *PLoS One,* October 2012.

3. **E. Atabakhsh**, J. H. Wang, X. Wang, D. E. Carter, and C. Schild-Poulter. RanBPM expression regulates transcriptional pathways involved in development and tumorigenesis. *Am. J. Cancer Res.,* August 2012.

SELECTED NON-REFEREED CONTRIBUTIONS

- **E. Atabakhsh*** and C. Schild-Poulter. Inhibition of the ERK1/2 pathway by RanBPM. American Association for Cancer Research Annual Meeting. Chicago, IL (2012)

- **E. Atabakhsh*** and C. Schild-Poulter. Inhibition of the ERK1/2 pathway by RanBPM. London Health Research Day. London, ON (2012)

- **E. Atabakhsh*** and C. Schild-Poulter. Pathways of RanBPM-mediated apoptosis. Oncology Research and Education Day. London, ON (2011)

- **E. Atabakhsh*** and C. Schild-Poulter. Pathways of RanBPM-mediated apoptosis. American Association for Cancer Research Annual Meeting. Orlando, FL (2011)

- **E. Atabakhsh***, D. M. Bryce, and C. Schild-Poulter. Pathways of RanBPM-mediated apoptosis. AACR Special Conference: Cell Death Mechanisms and Cancer Therapy. San Diego, CA (2010)

- **E. Atabakhsh**, D.M. Bryce, K.J. Lefebvre, and C. Schild-Poulter*****. RanBPM regulates the activation of apoptotic pathways in response to DNA damage. Keystone Symposia: Cell Death Pathways. Whistler, BC (2009)

AWARDS AND FELLOWSHIPS

- Ontario Graduate Scholarships in Science and Technology ($15,000/yr) 2010-12
- Graduate Thesis Research Award ($510) 2011
- Department of Biochemistry Publication Incentive ($500) 2010
- Western Graduate Research Scholarship ($7,000/yr) 2007-12
- Dean's Honour List 2006-07
- University of Ottawa entrance scholarship ($2000) 2003