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Characterization of RanBPM Subcellular Localization and Function in HDAC6 Regulation

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Graduate Program in Biochemistry

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Abstract

RanBPM has been shown to interact with numerous proteins implicating it in a variety of cellular processes including apoptosis, transcription regulation, cell migration, adhesion and morphology and has been shown to have tumour suppressive functions. RanBPM has been hypothesized to be a scaffolding protein and is part of a large protein complex termed the CTLH complex. Although the homologous complex in yeast has been demonstrated to have E3 ubiquitin ligase activity, whether the evolutionarily conserved human complex retains this activity remains to be determined. In this work we aim to characterize the regions of RanBPM that regulate its subcellular localization and identify structures that retain RanBPM in the nuclear and cytoplasmic compartments. In addition, we aim to characterize the role of RanBPM in aggresome formation and characterize its association with HDAC6.

In this study we have identified several domains and motifs regulate RanBPM nuclear and cytoplasmic localization. In particular, RanBPM comprises two motifs that can confer nuclear localization, one proline/glutamine-rich motif in the extreme N-terminus and a second motif in the C-terminus. We also identify a nuclear export signal and demonstrate that the SPRY, LisH and CTLH domains function to retain RanBPM in the cytoplasm. We demonstrate that RanBPM associates with chromatin in the nucleus and microtubules in the cytoplasm. We reveal that RanBPM is an essential component of aggresomes induced by both ionizing radiation and proteasomal inhibition. Aggresomes are aggregates of misfolded ubiquitinated proteins that form in response to proteasomal impairment. RanBPM was found to interact with HDAC6, a central regulator of aggresome formation, and inhibits deacetylase activity. We found that the RanBPM-mediated inhibition of HDAC6 α-tubulin deacetylase activity is dependent on its association with HDAC6. We show that HDAC6 does not require RanBPM to associate with microtubules, but that RanBPM requires HDAC6 to associate with microtubules. Furthermore, we demonstrate that components of the CTLH complex associate with both microtubules and HDAC6. Lastly, RanBPM was found to inhibit HDAC6-mediated cell migration.

This suggests that the tumour suppressor functions of RanBPM stem, in part, from an inhibition of the oncogenic activities of HDAC6.
Keywords

RanBPM, CTLH complex, subcellular localization, microtubules, α-tubulin, aggresome, HDAC6, cell migration, tumour suppressor
Questa tesi è dedicata al mio nonno Luigi.

Il mio nonno diceva sempre: "Chi lavora con la testa non ha bisogno di lavorare con le mani". È vero nonno! Tu hai sempre lavorato con le mani facendo innumerevoli sacrifici per la nostra famiglia. E sono stati questi tuoi sacrifici che mi hanno permesso di poter "lavorare con la testa". Tu mi dicevi sempre che dovevo pensare solo a studiare e tu avresti pensato al resto. E l'hai fatto nel vero senso della parola! Mi hai dato tutto quello che mi serviva e più. Io non so come ringraziarti Nonno, per tutto quello che hai fatto per me, e per i buoni esempi di vita che mi hai insegnato. Ti sarò grata e in debito per sempre. E ora che non ci sei più, mi manchi sempre tanto. Ti voglio bene Nonno e mi manchi tanto.
Co-Authorship Statement

Chapters 1, 4 and 5 were written by Louisa Salemi and edited by Dr. Caroline Schild-Poulter. Matthew Maitland generated Figure 1-4.

The data in Chapter 2 of this thesis is published in: Characterization of RanBPM Molecular Determinants that Control Its Subcellular Localization. Louisa M. Salemi, Sandra O. Loureiro and Caroline Schild-Poulter. PLoS One (2015). All experiments and data analysis in this chapter were performed by LMS with some exceptions. Sandra Loureiro conducted the experiments for Figures 2-5A, B, C, D, F, G and 2-6A. Caroline Schild-Poulter, Patricia Christian, Tung Bui, Matthew Maitland, Matthew He, Sarah Meulendyks and Louisa Salemi generated pCMV-HA-RanBPM mutant constructs and pHM830 and pHM840 constructs. The manuscript was written and edited by LMS and CSP.

The data in Chapter 3 of this thesis is published in: Aggresome formation is regulated by RanBPM through an interaction with HDAC6. Louisa M. Salemi, Ahmad W. Almawi, Karen J. Lefebvre and Caroline Schild-Poulter. Biology Open (2014). All experiments and data analysis in this chapter were performed by LMS with some exceptions. Ahmad Almawi conducted the experiments for Figures 3-1C, 3-2A, B, C and 3-4A. Karen J. Lefebvre and Caroline Schild-Poulter performed the experiments for Figures 3-1A and B. The manuscript was written by CSP and edited by LMS.

All experiments and data analysis in Chapter 4 of this thesis were performed by LMS, except for Figure 4-5, which was performed by Eyal Yefet. Juliana Engel, Nadun Chanaka Karunatilleke, Ryan Scanlan and Louisa Salemi generated pcDNA-HA-HDAC6-FLAG mutant constructs and pET28a-HDAC6-CAT2. Wesley Berube-Janzen generated pGEX4T1-GST-WT-RanBPM.
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To the CSP lab, thank you for making every day fun. To Elnaz, thanks for being my big sister in the lab and taking so much time to answer my questions, even after you moved on. To Vicki, thank you for always letting me talk at you whether about science or just a weird dream - you still continue to be my sounding board. I am forever grateful for those ‘watch’ nights where you stayed late or ran errands with me just because. To Wesley, Sarah, Elizabeth, Christina and Matty, thank you for making work fun. You guys were always a source of encouragement, great friendship and humour (mostly at my expense). To Chris and Sanna, best of luck starting your degrees. To Xu, thank you for your constant willingness to help and for always taking care of everything to make day-to-day life in the lab easier. You are the most fun ‘lab mom’ ever.

Thank you to all of the undergrads I had the pleasure of teaching in the lab. Your bright faces and enthusiastic questions always reminded me of why I love science.

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understanding of my schedule and for editing - I can’t thank you all enough. To my family, I can certainly say I wouldn’t have been able to do this without you guys. To Nonno, Nonna, Mom and Dad, for all of your sacrifices over the years so that I could make it this far and never have to worry about anything except my own work. For all of the food and financial support, and most importantly all of your encouragement during the hard times that motivated me not to give up. Thank you Mom for all those surprises throughout the years that always made me smile. To Alfonso for pretending to understand what I do and for being a person I can always talk to about anything.

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<td>BUZ</td>
<td>Binder of ubiquitin zinc finger</td>
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<tr>
<td>C&gt;C&gt;N</td>
<td>Completely cytoplasmic</td>
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<td>C&gt;N</td>
<td>Cytoplasmic greater than nuclear</td>
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<td>E1</td>
<td>Ubiquitin activating enzyme</td>
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<td>E2</td>
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<td>Enhanced chemiluminescence</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>Estrogen receptor</td>
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<td>ErbB2</td>
<td>Epidermal growth factor receptor 2</td>
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<td>ERK</td>
<td>Extra-cellular signal regulated kinase</td>
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<td>FBPase</td>
<td>Fructose-1,6-bisphosphatase</td>
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<td>FBS</td>
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<td>FL</td>
<td>pcDNA-HDAC6-Full Length-FLAG</td>
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<td>FMRP</td>
<td>Fragile X mental retardation protein</td>
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<td>FOX</td>
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<td>GFP-β-gal</td>
<td>Green Fluorescent Protein-β-galactosidase</td>
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<td>Gid</td>
<td>Glucose-induced degradation</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>G protein-coupled receptor kinase 2</td>
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<td>Glycogen synthase kinase 3 β</td>
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<td>Gray</td>
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<td>HA</td>
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<td>HA-RanBPM si-mt</td>
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<td>HAT</td>
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<td>Herpes virus associated ubiquitin-specific protease</td>
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<td>HCC</td>
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<td>Histone deacetylase</td>
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<td>HIPK2</td>
<td>Homeodomain-interacting protein kinase 2</td>
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<td>Human immunodeficiency virus</td>
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<td>HMG</td>
<td>High Mobility Group</td>
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<td>HNRNPM</td>
<td>Heterogeneous nuclear ribonucleoprotein M</td>
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<td>ICGC</td>
<td>International Cancer Genome Consortium</td>
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<td>IR</td>
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<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
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<td>MAEA</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>MET</td>
<td>Proto-oncogene, receptor tyrosine kinase</td>
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<td>Mgl1</td>
<td>mammalian homolog of Drosophila tumour suppressor protein lethal</td>
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giant larvae
mGlu Metabotropic glutamate receptors
Mirk Minibrain-related kinase
MM Multiple myeloma
MOP Mu opioid receptors
MRCT LH Mulkelin/RanBPM/CTLH
MT Microtubule
MTOC Microtubule organizing centre
MVH Mouse vasa homolog
MYH9 Myosin heavy chain 9
N=C Nuclear equal to cytoplasmic
N>>C Completely nuclear
N>C Nuclear greater than cytoplasmic
NES Nuclear export signal
NES Mut pCMV-HA-RanBPM-NES mutant
NFAT Nuclear factor of activated T cells
NF-κB Nuclear factor kappa B
NGF Nerve growth factor
NHL Non-Hodgkin lymphoma
NLS Nuclear localization signal
NLS1 Mut pCMV-HA-RanBPM-NLS1 mutant
NLS2 Mut pCMV-HA-RanBPM-NLS2 mutant
NPC Nuclear pore complex
NSC Neural stem cells
NSG NOD scid gamma
Nur77/TR3 Nuclear hormone receptor/orphan nuclear receptor
OSCC Oral squamous cell carcinoma
p75NTR p75 neurotropin receptor
PABPC Polyadenylate-binding protein 1
PBGD Porphobilinogen deaminase
PBMC Peripheral blood mononuclear cells
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
PD Parkinson's Disease
PI3K Phosphoinositide-3 kinase
Pin1 Peptidyl-prolyl isomerase
PKC Protein kinase C
PLK1 Polo like kinase 1
PMSF Phenylmethylsulfonyl fluoride
PVDF Polyvinylidene difluoride
RanBPM Ran-binding protein microtubule organizing center, RanBP9
RING Really interesting new gene
RMND5A Required for meiotic nuclear division homolog A
ROS Reactive oxygen species
<table>
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<th>Full Form</th>
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<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SE</td>
<td>Standard error</td>
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<td>Serine glutamine containing tetradecapeptide</td>
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Chapter 1

1 Introduction

1.1 Cancer

In 2015 it was estimated that 196,900 people in Canada were diagnosed with cancer and that 78,000 Canadians died from this disease [1]. Cancer is an incredibly complex set of diseases defined as the uncontrolled or abnormal division of cells in any part of the body [2], and in fact there are over 100 different types of cancer identified [3]. As diverse as these cancers may be, most if not all, have acquired a common set of molecular, biochemical and cellular traits, termed the hallmarks of cancer. These hallmarks that include, self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis allow normal cells to overcome the checks and balances which regulate normal cell growth and proliferation and become cancerous [4]. Cancer development (tumourigenesis) is a multi-step process and through genomic instability cells acquire the mutant alleles of either proto-oncogenes or tumour suppressor genes, acquiring the hallmarks of cancer [3]. Self-sufficiency in growth signals, which allows cells to continue to divide without stimulation from the extracellular environment and insensitivity to anti-growth signals result in disruption of the important homeostatic mechanism. The delicate balance of homeostasis is achieved through the regulation of cell proliferation and apoptosis and disruption of this balance gives rise to cancer. Apoptosis can be activated to eliminate cells that have extensive DNA damage. Therefore evasion of apoptosis can contribute to genomic instability. Together, those hallmarks can contribute to limitless replicative potential. Non-cancer cells have a defined number of cell divisions, regulated by the length of their telomeres, however cancer cells are able to circumvent this regulatory pathway maintaining their telomeres at above critical length and therefore continue to divide. Angiogenesis is a tightly regulated process in development and is essential to provide cells with oxygen and nutrients from the blood stream. However once tumours become too large and therefore too far away from existing vasculature, lack of oxygen can threaten further expansion. Initially, tumour cells lack the ability to elicit new
blood vessel growth however; tumour cells can circumvent the regulations of angiogenesis and activate the angiogenic switch, thereby promoting the generation of new vessels. Metastasis is responsible for 90% of cancer cell deaths. Metastasis occurs when cells leave the primary tumour after having acquired all of the other cancer hallmarks and travel through the blood stream of the lymphatic system and take up residence in a new area of the body.

These hallmarks highlight the importance of regulation to maintain cellular homeostasis and how perturbation of this balance can result in tumourigenesis. Understanding the cellular mechanisms that underlie these hallmarks will provide insight into the transition of a normal to a malignant cell and provide opportunities to generate novel therapies to stop this progression.

1.1.1 Apoptosis

1.1.1.1 Overview

Apoptosis is a form of programmed cell death carried out by a highly regulated signal cascade. Apoptosis can be activated by either the extrinsic pathway, mediated by death receptors at the cell membrane or the intrinsic pathway, mediated by the mitochondria [5]. Both pathways converge upon the activation of executioner caspases. Caspases are cysteine proteases that are responsible for the cleavage of cellular substrates leading to chromatin condensation, membrane blebbing, and DNA fragmentation, ultimately causing cell death [5]. Multiple stimuli including extracellular stresses such as the limitation of growth factors, immune responses and intracellular stresses such as DNA damage, oncogene activation and cell cycle arrest due to telomere shortening can activate apoptosis [6,7]. Evasion of apoptosis is a characteristic frequently acquired by cancerous cells, giving them a selective advantage and contributing to tumourigenesis [5].

1.1.1.2 Intrinsic pathway

The intrinsic apoptotic pathway is regulated by pro- and anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members [8]. The balance of expression of these proteins is the critical determining factor responsible for the activation of cell death. Bax is a pro-apoptotic
factor that oligomerizes and forms a channel in the outer mitochondrial membrane causing membrane permeabilization [8]. This allows the release of pro-apoptotic proteins, such as cytochrome c, leading to the activation of caspases. Bcl-2 is a pro-survival factor preventing apoptosis. It directly interacts with Bax preventing its oligomerization and hindering the release of pro-apoptotic factors, promoting cell survival [8].

1.2 RanBPM

1.2.1 Overview

RanBPM (Ran-binding protein microtubule organizing center, also known as RanBP9) was first described in 1998 and was identified as a 55 kilodalton (kDa) protein found to interact with Ran, a GTPase (guanine triphosphatase) involved in nucleocytoplasmic transport, by a yeast two-hybrid screen [9]. RanBPM was found to be localized to the centrosome, hence named Ran-binding protein microtubule organizing center [9]. However, these findings were later dismissed as it was found that the 55 kDa protein product initially identified was a truncation of a longer 90 kDa protein [10]. The full sized RanBPM protein was able to only weakly associate with Ran and had no role in nucleocytoplasmic transport [10]. New antibodies generated against the full sized RanBPM did not show centrosomal localization and instead displayed staining throughout the whole cell while being concentrated in the nucleus [10]. Over the next 18 years, many studies identified RanBPM as interacting with various proteins, suggesting its involvement in many cellular processes. While no specific function could be attributed to RanBPM based on its primary structure, some studies demonstrated a biological effect of RanBPM attributed to these interactions, while in other studies, a biological function was implied only based on the specific interaction identified. RanBPM’s function only started to emerge from studies in yeast, which showed that the RanBPM yeast homolog (Gid1, Vid30) is part of a large E3 ligase complex [11]. Here, we review RanBPM’s role in apoptosis, transcription regulation, cell migration, adhesion and morphology and how these pathways contribute to RanBPM’s function in cancer, development as well as Alzheimer’s Disease.
1.2.2 Domains, conservation and expression

Unlike other Ran binding proteins, with the exception of RanBP10, RanBPM does not have a Ran binding domain [9], instead has Sp1A kinase and ryanodine receptor (SPRY), lissencephaly type-1 like homology (LisH), C-terminal to LisH (CTLH) and CT-11-RanBPM (CRA) domains and an unstructured proline and glutamine rich N-terminal domain (Figure 1-1). The SPRY domain is known to mediate protein-protein interactions. The LisH domain is known to mediate protein dimerization and tetramer formation. The CTLH domain is a domain, whose function remains unknown, that is usually found adjacent to the LisH domain in a subset of LisH-containing proteins. [12-14]. The C terminal CRA domain is made up of six α-helices that resemble the death domain superfamily [15]. RanBPM shares similar domain architecture with its paralogue RanBP10 and they have been shown to associate [16], however this review will only focus on RanBPM.

RanBPM is ubiquitously expressed in different tissue types with higher expression observed in the brain, heart, skeletal muscles and testes [17,18]. RanBPM is well conserved in mammals, in fact the mouse and human proteins are over 90% identical and their differences fall within the N-terminus [10]. RanBPM orthologues have also been identified in plants [19]. A RanBPM homologue, vacuolar import and degradation (Vid) 30 or glucose induced degradation (Gid) 1 has been identified in yeast [11,20]. Gid 1 also contains SPRY, LisH, CTLH and CRA domains [11]. RanBPM has been shown to be part of a large 670 kDa protein complex termed the CTLH complex [10,21,22]. Similarly, Gid1 has been found to be a component of a large protein complex made up of several other Gid proteins, called the Gid complex [11,20,22]. The human homologs of the majority of these proteins have been found to be part of the human CTLH complex [23]. Therefore, RanBPM and most of the CTLH complex members are well conserved in eukaryotic lineages.

RanBPM is localized both in the nucleus and the cytoplasm [10] and has also been found to be present at the plasma membrane as well as within the chromatin fraction [18,24,25]. RanBPM is deubiquitinated by ubiquitin specific protease 11 (USP11) and degraded by the ubiquitin-proteasome pathway [31] and RanBPM mRNA is regulated by microRNA-
Figure 1-1 RanBPM schematic.

Schematic diagram of full length wildtype RanBPM. The conserved domains are SPRY (Sp1a kinase and ryanodine receptor), LisH (lissencephaly type-1 like homology), CTLH (C-terminal to LisH) and the C terminal CRA (CT-11-RanBPM). Figure adapted from [13,26].
RanBPM has been demonstrated to be phosphorylated after genotoxic stress, for example, ultraviolet (UV) treatment and osmotic shock [24]. Cyclin-dependent kinase 11 p46 fragment (CDK11\textsuperscript{p46}) and polo like kinase 1 (PLK1) phosphorylate RanBPM \textit{in vitro} [29,30]. Although these reports suggest that RanBPM is subjected to a number of post-translational modifications, for the most part, the outcome of these modifications remains unknown. In addition, Ataxia telangiectasia mutated (ATM) phosphorylates RanBPM at an ATM consensus sequence following ionizing radiation (IR) induced DNA damage and RanBPM subcellular localization has been reported to change upon IR treatment in an ATM-dependent manner [27,28]. Following IR induced ATM phosphorylation, RanBPM immediately relocalized to the nucleus and this is dependent on active ATM as inhibition of ATM prevents this nuclear accumulation [28]. At later timepoints, RanBPM was found to relocalize to the cytoplasm [28]. Our lab initially demonstrated this cytoplasmic relocalization at 24 hrs after IR persisting to 72 hrs [13]. Palmieri \textit{et al.}, showed that downregulation of RanBPM results in reduced acetylation and in turn reduced activation of ATM [28]. Therefore ATM phosphorylation of RanBPM regulates its subcellular localization, suggesting that post-translational modifications of RanBPM have the ability to regulate its function.

\subsection*{1.2.3 Functions}

RanBPM consists of protein-protein interaction domains and is part of a large complex, therefore, it is not surprising that RanBPM has been hypothesized to be a scaffolding protein and has been demonstrated to have many functions throughout the cell. It has been shown to be associated with many protein partners and has been suggested to integrate different signaling pathways implicating it in a variety of cellular functions both in the cytoplasm and nucleus [24,33-35] (Figure 1-2, Table 1-1). Those functions include apoptosis, transcription regulation and regulation of adhesion, migration and morphology.

\subsection*{1.2.3.1 Role in apoptosis}

Work in our laboratory identified a pro-apoptotic role for RanBPM through modulation of some members of the Bcl-2 family of proteins, which are known to mediate the intrinsic apoptotic pathway. Firstly, our lab showed ectopic expression of RanBPM
Figure 1-2 Pathways and functions attributed to RanBPM.

Summary of the main processes and functions that have been ascribed to RanBPM. Proteins listed in the arrows are those demonstrated to interact with RanBPM, implicating RanBPM in the circled function.
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<td>CRA Domain</td>
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<td>[58]</td>
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<tr>
<td>mGlu Receptors</td>
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<td>Obscurin</td>
<td>aa 108-729</td>
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resulted in increased caspase activation [13]. We demonstrated that the SPRY domain actually inhibits apoptotic activation as deletion of this domain resulted in increased caspase activation. Conversely, the CRA domain induces apoptotic activation as deletion of this domain severely reduces caspase activation. In addition, downregulation of endogenous RanBPM resulted in decreased apoptotic activation and increased cell survival following IR [13]. Similarly, in the context of gastric cancer, knockdown of RanBPM resulted in increased cell survival after exposure to two different chemotherapeutic agents [78]. Our lab showed that the apoptotic activation of RanBPM is mediated through the Bcl-2 family of proteins [13]. Stable RanBPM downregulated cells showed increased Bcl-2 and Bcl-XL protein and mRNA levels, both are anti-apoptotic Bcl-2 family members [13,79]. Following IR, RanBPM shRNA cells had decreased Bax mitochondrial localization, a pro-apoptotic Bcl-2 family member responsible for mitochondrial permeabilization releasing cytochrome-c activating the caspase cascade ultimately resulting in apoptosis [13]. Similarly, another group confirmed that overexpression of RanBPM resulted in decreased Bcl-2 protein levels and increased Bax oligomerization. They also demonstrated that cells with overexpressed RanBPM had increased mitochondrial membrane permeability and more abundant cytoplasmic cytochrome c staining when compared to control cells, which showed mitochondrial cytochrome c staining, demonstrating that RanBPM overexpression induces mitochondrial dysfunction resulting in the activation of apoptosis [80]. Interestingly, neurons generated from RanBPM transgenic mice with increased RanBPM expression exhibited compromised mitochondrial and these neurons had increased amyloid-β (Aβ)-induced apoptosis [81,82]. Altogether, these studies suggest that RanBPM has a role in regulating the intrinsic apoptotic pathway promoting its activation.

Interestingly, RanBPM has been suggested to have additional roles in other aspects of apoptotic activation through its interaction with various proteins functioning in regulating the apoptotic process. For example, RanBPM is able to bind to Homeodomain-interacting protein kinase 2 (HIPK2), which may promote apoptosis through its interaction with the death receptor tumour necrosis factor receptor type 1-associated death domain protein (TRADD) [36]. RanBPM has been shown to interact with the intracellular death domain of p75 neurotrophin receptor (p75NTR), which is part of the tumour necrosis factor
(TNF) receptor superfamily. Numerous adapter proteins have been shown to bind to the cytoplasmic domain of p75NTR and promote apoptosis [37]. RanBPM also interacts with a caspase-processed fragment of CDK11\(^{p10}\), CDK11\(^{p46}\), which further stimulates downstream apoptotic signaling and its overexpression can induce apoptosis [29]. These studies, however, did not investigate the functional outcome of these interactions, therefore, how RanBPM influences the function of these factors in the apoptotic process is unclear. Finally, RanBPM was shown to stabilize pro-apoptotic transcription factor p73 through inhibition of its ubiquitination and subsequent degradation [38]. RanBPM overexpression was also found to upregulate p73 mRNA [80], indicating that it stabilizes p73 at both the transcription and protein level. Again, how these regulations are achieved remains unknown, but collectively, these reports indicate that RanBPM may regulate apoptosis at various steps in the apoptotic cascade activation.

1.2.3.2 Transcription regulation

As RanBPM is nuclear and has been shown to associate with chromatin, it is possible that RanBPM can function in transcription regulation [25]. Microarray analysis of stable control or RanBPM shRNA cells performed in our laboratory revealed global gene expression changes upon RanBPM downregulation. Analysis of the transcription factor binding sites within the promoters of these genes identified homeobox A5 (HOXA5), Forkhead box (FOX) and High Mobility Group (HMG) proteins as being over represented suggesting a role for RanBPM in the regulation of transcription [79]. Indeed, RanBPM has been implicated in transcriptional regulation through its interaction with a number of transcription factors and transcriptional regulators. RanBPM directly interacts with steroid receptors and positively regulates their transcriptional activity in a ligand dependent manner. This has been demonstrated for the androgen receptor (AR), glucocorticoid receptor (GR) as well as the thyroid receptor (TR). However RanBPM did not affect the transcriptional activity of the estrogen receptor (ER) [17,39,83]. RanBPM has been shown to interact with TATA box binding protein-associated factor 4 (TAF4), a subunit of transcription factor II D (TFIID) which functions as a transcriptional co-activator for several classes of transcription factors [25]. In the context of the Epstein-Barr virus (EBV) lytic cycle activation, RanBPM increased the transcriptional activity of
both Rta and Zta through differential regulation of sumolyation of these proteins. Rta and Zta are transcription factors that activate the expression of key EBV lytic genes activating the EBV lytic cascade. RanBPM also mediates their interaction resulting in synergistic activation of their transcriptional activity [40,41].

RanBPM associated with minibrain-related kinase (Mirk) and inhibited its kinase activity as well as its transcriptional transactivator activity [42]. RanBPM has also been shown to regulate the transcriptional activation of the activator protein 1 (AP-1) transcription factor complex, through its synergistic association with Lymphocyte function-associated antigen-1 (LFA-1), a membrane integrin receptor [24]. RanBPM negatively regulates nerve growth factor (NGF) stimulated nuclear factor of activated T cells (NFAT) - dependent transcription through its association with the tropomyosin-related kinase (Trk) A receptor [43]. RanBPM also negatively regulated TNF receptor-associated factor 6 (TRAF6) activated nuclear factor kappa B (NF-κB) transcription by preventing the translocation of p65 by inhibiting the ubiquitination of TRAF6 [44]. Collectively, this data demonstrates that RanBPM both directly and indirectly regulates transcription, directly, through interaction with transcription factors and indirectly acting as a signaling molecule upstream of transcriptional regulation.

1.2.3.3 Roles in adhesion, morphology and migration

Through its interaction with different proteins, RanBPM has been implicated to have a role in cell adhesion, morphology and migration. Specifically, RanBPM associates with LFA-1, an integrin receptor with a role in cell adhesion. Integrins mediate the adhesive interactions between cells or between cells and the extracellular matrix. They act as signaling receptors transmitting information about extracellular environment inside the cells to affect the cell behavior [24]. Overexpression of RanBPM was shown to inhibit cell adhesion and delayed cell spreading and accordingly RanBPM knockdown promoted adhesion and spreading. RanBPM inhibited the localization of certain cell adhesion proteins at focal adhesions, thereby inhibiting focal adhesion assembly. In addition, RanBPM accelerated the endocytotic internalization of β1-integrin resulting in reduced levels at the cell surface and again these effects were reversed when RanBPM was knocked down [82]. Similarly, RanBPM was shown to inhibit cell adhesion and cell
morphology within the female germline stem cell niche in *Drosophila*. However, in the follicle RanBPM was a positive regulator of adhesion [84]. RanBPM associates with the PlexinA1 receptor, which is essential for the regulation of cell morphology. In the presence of PlexinA1, overexpression of RanBPM resulted in cell contraction [45]. In addition, RanBPM has been identified to associate with muskelin and knockdown of either muskelin or RanBPM result in altered cell morphology including enlarged cell perimeter and altered actin distributions [46].

RanBPM was found to both negatively and positively affect cell motility. Through its interaction with MET proto-oncogene, receptor tyrosine kinase (MET), RanBPM activated the extra-cellular signal regulated kinase (ERK) pathway stimulating cell motility [18]. However, this study employed the use of a RanBPM-GFP-fusion construct and the effect of the addition of a large fluorescent group is unknown. As RanBPM was shown to dimerize, it is possible that the GFP tag could interfere with dimerization, therefore having adverse effects on RanBPM function [48]. Alternatively, RanBPM was found to inhibit chemotactic migration by associating with leukotriene B4 receptor 2 (BLT2). Downregulation of RanBPM resulted in increased chemotactic migration whereas overexpression of RanBPM was found to inhibit migration demonstrated by transwell migration assays [47]. Our lab also found that cells with stable downregulation of RanBPM had increased migration compared to control cells in a wound healing scratch assay indicating that RanBPM functions to inhibit cell migration [48]. Recently, a study performed in gastric cancer cells confirmed that downregulation of RanBPM resulted in decreased cell adhesion and increased cell motility [78]. Taken together, RanBPM has been shown to function in inhibiting cell adhesion, altering cell morphology and inhibiting cell migration.

These cellular functions of RanBPM have implicated a much broader role for RanBPM in cancer, development and Alzheimer’s disease, indicating that RanBPM has a role in both normal and pathological development.
1.2.3.4 RanBPM in cancer

Work in our laboratory has suggested a tumour suppressor role for RanBPM through an inhibition of ERK signaling. RanBPM was found to interact with c-Raf and downregulate c-Raf protein levels. This is turn downregulates anti-apoptotic Bcl-2 family proteins at both the transcriptional and protein level [13,48]. Accordingly, cells with downregulated RanBPM were able to evade the activation of apoptosis, a common characteristic of cancer cells [13]. Downregulation of RanBPM resulted in loss of growth factor dependence as RanBPM shRNA cells were shown to continue to survive and proliferate in the absence of growth serum. Downregulation of RanBPM also resulted in increased cell migration and cell proliferation, indicating that downregulation of RanBPM promotes cellular transformation [48]. In addition, microarray analysis performed in our lab showed that one third of differentially expressed genes of control and RanBPM shRNA are associated with cancer. Pathways affected by downregulation of RanBPM included ERK, Wnt, Notch and phosphoinositide-3 kinase (PI3K)/Akt pathways. These pathways regulate cell cycle progression, cell proliferation, cell growth, differentiation, migration, adhesion and cell survival and are commonly found deregulated in cancer [79]. In the context of gastric cancer, downregulation of RanBPM also resulted in increased cell proliferation and cell motility [78]. As previously described, RanBPM was shown to upregulate the mRNA levels of the pro-apoptotic transcription factor p73, thereby promoting the activation of apoptosis [80]. It was also shown to stabilize its protein expression by preventing ubiquitination and subsequent degradation [38]. Similarly, RanBPM was able to prevent the ubiquitination and subsequent degradation of mammalian homolog of Drosophila tumour suppressor protein lethal giant larvae (Mgl1), by mediating its interaction with USP11. This resulted in prolonged half life and enhanced the tumour suppressor properties of Mgl1, as Mgl1 was shown to inhibit cell migration and cell proliferation [49,85]. A study of gastric cancers identified lower levels of RanBPM transcripts in tumours with distant metastases than in tumours without distant metastases implying that loss of RanBPM expression may aid in the development of metastases [78]. Also, RanBPM expression was decreased or altered in lung, kidney and breast primary tumours, further lending to the idea that RanBPM functions as a tumour suppressor [24].
The Candidate Cancer Gene Database (CCGD) identified RanBPM as a cancer driver gene mutation using analysis of transposon mutagenesis in mice, with the highest rank of common insertion sites (CIS) being liver cancer [86]. Interestingly, the International Cancer Genome Consortium (ICGC) identifies skin, breast and liver as the primary sites harbouring the majority of RanBPM mutations [87] (Figure 1-3A). The Catalogue of Somatic Mutations in Cancer (COSMIC) database indicated that RanBPM mutations are mostly nucleotide substitutions and are distributed throughout the entire gene [88] (Figure 1-3B). It is worth noting that a large-scale analysis of SNPs associated with early breast cancer cases identified 41 new SNPs, one of them being upstream of RanBPM [89].

Collectively, these data suggests a strong tumour suppressor role of RanBPM. Therefore further understanding its regulation and function is crucial in order to be able to exploit its ability to inhibit cancer progression in a therapeutic setting.

1.2.3.5 RanBPM in Alzheimer’s Disease

RanBPM has been shown to play a pathogenic role in Alzheimer’s disease (AD) as it acts as a scaffold bringing together amyloid precursor protein (APP), low-density lipoprotein receptor-related protein (LRP) and β-site APP-cleaving enzyme 1 (BACE1). This complex promotes the cleavage of APP to Aβ, the toxic fragment resulting in Aβ pathological plaques, a major hallmark of AD, at the expense of the non-pathogenic APP processing by α-secretase cleavage. Knockdown of RanBPM results in a decreased secretion of Aβ. The overexpression of RanBPM decreased the amount of APP at the cell surface due to increased internalization, which is necessary for its pathogenic cleavage [50]. An in vivo mouse study mirrored these results further confirming that RanBPM overexpression significantly increased the generation of Aβ in an AD model [90]. A comparison of AD brains to those of age-matched controls found increased levels of a RanBPM proteolytic fragment, N60 RanBPM. This N60 fragment was actually found to be more potent than full-length RanBPM in enhancing β-secretase processing of APP. However, it remains unclear how this proteolytic fragment is generated, but it has been shown to be dependent on cell density, as less confluent cells express higher levels of the N60 fragment compared to cells at a higher confluency [91].
Figure 1-3 RanBPM mutational cancer data.

A) Tissue distribution of RanBPM mutations retrieved from the ICGC database. Figure adapted from the ICGC database [87]. B) Above, Relative abundance of substitution mutations in RanBPM. Below, Histogram indicating the position of somatic mutations in RanBPM. Figures adapted from the COSMIC database [88].
RanBPM also has been implicated in the regulation of synapses. Overexpression of RanBPM in an AD model resulted in reduced levels of pre- and post-synaptic proteins, suggesting a role for RanBPM in the cognitive impairment, which accompanies AD [90,92]. In a mouse model of AD, mice with overexpressed RanBPM had increased levels of neuroinflammation and increased synaptic functional impairment due to reduction of synaptic proteins because of the loss of dendritic intersections and spines. These mice exhibited deficiencies in spatial learning and memory, which was accelerated by RanBPM overexpression [82,93-96].

RanBPM overexpression was shown to promote activated cofilin, which normally functions as a regulator of actin dynamics, translocation to the mitochondria. This occurs through positive regulation of Slingshot homolog 1 (SSH1) expression, which dephosphorylates and activates cofilin. Translocation of cofilin to the mitochondria results in mitochondrial dysfunction and promotes apoptosis [97]. Neurons generated from AD mice overexpressing RanBPM demonstrated deficits in the ability to clear $\text{Ca}^{2+}$ to the mitochondria resulting in increased reactive oxygen species (ROS) production contributing to decreased synaptic function [81]. These mice also exhibited increased levels of $\text{A}\beta$ induced apoptosis dependent on cofilin expression [82]. Overexpression of RanBPM has been shown to induce apoptosis through the intrinsic mitochondria-mediated pathway in brain cells therefore likely contributing to neurodegeneration in Alzheimer’s disease [80].

Conversely, an AD mouse model with reduced expression of RanBPM had reduced levels of $\text{A}\beta$ accumulation, reduced neuroinflammation and reduced loss of post-synaptic protein expression [97].

Taken together these results indicate that RanBPM has a pathological role in the major hallmarks contributing to Alzheimer’s disease: $\text{A}\beta$ production and decreased synaptic function and density and neuronal death contributing to neurological defects and can even accelerate disease pathology indicating that targeting RanBPM maybe an excellent therapeutic target for AD [94].
1.2.3.6 RanBPM in development

Two different groups have generated RanBPM knockout (KO) mice. Both groups found Mendelian proportion of RanBPM KO embryos in utero, indicating that RanBPM is not required for embryonic development [98,99]. The first group, Puveral et al., found that less than the expected number of KO pups were born and many died immediately after birth [98]. Palavicini et al., found that very few mice survived past 24 hours citing a failure to latch and suckle milk resulting in a lack of nourishment and a loss of bodily homeostasis in the absence of liquid as cause of death [99]. Both groups reported however that surviving mice were significantly smaller than their wildtype litter mates [98,99].

Puveral et al., further noted that both male and female KO mice were sterile. Closer examination showed that RanBPM is essential for spermatogenesis and oogenesis. It was revealed that both genders have meiotic defect occurring around the late pachytene/diplotene stages therefore suggesting the presence of a common mechanism requiring RanBPM function occurring in both genders at the end of prophase I [98]. RanBPM was also shown to play a role in spermatogenesis in the new world common marmoset [100]. RanBPM was previously suggested to function in spermatogenesis, as it is able to associate with mouse vasa homolog (MVH), a vasa protein, which plays an essential role in the development of the male germ cell [51,98,101]. In addition, RanBPM was shown to regulate mRNA splicing imperative for normal spermatogenesis and male fertility. A conditional RanBPM KO mouse showed that many transcripts in the testes were aberrantly spliced contributing to sterility in male mice [76]. Association with human sperm membrane protein-1 (hSMP-1) implicated RanBPM to function in spermiogenesis [52]. RanBPM was found to be an essential gene in Drosophila where it has two isoforms, long and short RanBPM. Both isoforms contain the central domains, the long isoform also contains an unstructured N-terminal region that is glutamine rich. The long isoform of RanBPM plays an important role in the niche development of the female ovaries and the short isoform negative regulates the organization of the germ stem cell niche [84].
RanBPM was shown to have a role in the regulation of mitosis through its interaction with PLK1, citron kinase (CITK), Yippee like 5 (YPEL5), cycle exit and neuronal differentiation 1 (Cend1) and dual specificity tyrosine-phosphorylation regulated kinase 1B (Dyrk1B) [30,53-55]. PLK1 is crucial for M-phase progression having a role in centrosome maturation and bipolar spindle formation. However the functional relevance of its association with RanBPM remains to be determined [30]. CITK functions downstream of PLK1 and plays a role in mitosis and cytokinesis and downregulation of RanBPM resulting in an increase in mitotic cells due to slower entry into cytokinesis, suggesting RanBPM functions to promote progression of mitosis by promoting entry into cytokinesis [53]. YPEL5 is localized with the mitotic machinery throughout the cell cycle and is involved in cell cycle progression and knockdown of YPEL5 suppressed growth rates and prolonged G1, G2 and M phases. Nonetheless, the functional consequences of the association of RanBPM and YPEL5 on cell cycle progression were not investigated [54]. Individual associations of Cend1 and Dyrk1B with RanBPM were shown to stabilize cyclin D1 protein levels, therefore promoting progression of the cell cycle. However, exogenous expression of all three of these proteins was found to promote the degradation of cyclin D1, demonstrating that RanBPM dynamically regulates progression of the cell cycle [55]. These studies suggest that RanBPM has the potential to regulate various steps of the mitotic pathway. However, the mechanisms of these regulations remain to be fully elucidated.

Palavicini et al., went on to characterize a severe deficit in brain development in their RanBPM KO mice. KO mice had reduced brain volumes and enlarged lateral ventricles. They attributed the failure to suckle milk resulting in death to abnormalities in brain development including defects in somatosensory systems, neuromuscular or craniofacial development. They suggest that RanBPM acts as scaffold within a complex that regulates neurite outgrowth and neuronal migration which are imperative for proper brain growth and development [99]. Previous studies have implicated a role for RanBPM in neurite outgrowth because of its interaction with PlexinA1 receptor [45], L1 receptor [33], p42IP4/centaurin α-1 [56], TrkB [57], and Dyrk1B and Cend1 [55]. Through association with TAF4 RanBPM was shown to have a role in neural stem cells (NSC) differentiation and seems to play a role in the initiation of neurite processes [25]. Also, RanBPM
interacts with fragile X mental retardation protein (FMRP). Transcriptional silencing of this gene results in fragile X syndrome, the most common form of inherited mental retardation [15]. RanBPM function has been shown to be required in the nervous system of *Drosophila* for proper larval feeding behavior, response to light and for coordinated locomotion. RanBPM was highly expressed within the mushroom body of *Drosophila* and re-expression in neurons within the mushroom body was sufficient to rescue all behavioural phenotypes of mutant larvae [102]. Other examples of RanBPM’s role in the neuronal system include its interaction with calbindin D$_{28K}$, which has a role as a calcium buffer and calcium sensor important in neuronal function [58] and its interaction with all metabotropic glutamate receptors (mGlu) receptors except for mGlu6 and specifically mGlu2, suggesting a role for RanBPM as a scaffold in the neuronal system at synaptic sites [59]. RanBPM was shown to interact with obscurin and titin suggesting that RanBPM is involved in the development of Z-disks in skeletal muscle. However the function of RanBPM in this process remains to be fully elucidated [60].

Subsequently, microarray analysis performed by our lab showed that developmental genes were highest among those differentially expressed between control and RanBPM shRNA cells [79]. These findings suggest a role for RanBPM in normal processes associated with development in addition to disease progression.

1.2.4 CTLH complex

1.2.4.1 Overview

RanBPM is part of a large protein complex, the CTLH complex. The complex comprises of armadillo repeat containing 8 (ARMC8) α and β, required for meiotic nuclear division homolog A (Rmnd5A), macrophage erythroblast attacher (MAEA), Muskelin and two-hybrid-associated protein 1 with RanBPM (Twa1) in addition to RanBPM (Figure 1-4). Many of the yeast homologues of these proteins make up the GID complex, which functions as an E3 ubiquitin ligase [11]. Interestingly, phylogenic analysis has demonstrated that components of the CTLH complex are very well conserved throughout evolution [23]. Consistently, *Arabidopsis* RanBPM was found to be part of a large
Figure 1-4 Model of the mammalian CTLH complex.

The proposed model of the topology of the mammalian CTLH complex.
cytoplasmic complex with many homologues of the mammalian CTLH complex and the yeast GID complex [19].

1.2.4.2 Ubiquitin-Proteasome System

Ubiquitination is a post-translational modification that regulates not only protein signaling but also protein turnover. Multiple ubiquitin moieties are covalently linked to the target protein, marking the protein for degradation by the proteasome. This occurs through a signaling cascade, which involves the transfer of ubiquitin to ubiquitin activating enzymes (E1) followed by ubiquitin transfer to ubiquitin-conjugating enzymes (E2). Finally, the ubiquitin is transferred to E3 ubiquitin ligases, which mediate the attachment of ubiquitin to the substrate protein. This is a highly selective, highly regulated process and the specificity of protein targets to be degraded is determined by E3 substrate recognition [23,103].

1.2.4.3 Yeast GID Complex Activity

A key enzyme required for gluconeogenesis is fructose-1,6-bisphosphatase (FBPase). When yeast are grown on a non-glucose carbon source, they rely on gluconeogenesis for the production of glucose. Once shifted to a glucose rich medium, enzymes necessary for gluconeogenesis are no longer required and thus degraded [104]. If yeast cells have been starved of glucose for one day, FBPase is degraded by the proteasome. If cells have been starved for 3 days, FBPase is degraded in the vacuole [105]. The GID complex is an E3 ubiquitin ligase complex and functions in the proteasomal degradation of FBPase [11,20,22]. The vacuole-dependent degradation pathway in yeast is analogous to the macroautophagy pathway in mammalian cells. Vid30/Gid1 with Vid24 are required for the vacuole degradation of FBPase and play an important role promoting the association of Vid vesicles and actin patches thus integrating the Vid and endocytic pathways [105].

1.2.4.4 Mammalian CTLH Complex

Many human homologs of the GID complex were found to make up the CTLH complex, with the exception of Gid4 and Gid7 corresponding to mammalian c17orf39 and
WDR26, respectively, which were not identified to be part of the CTLH complex [22,23]. It has been suggested that muskelin functions to replace Gid7 in the mammalian complex [23]. Interestingly, in *Arabidopsis*, RanBPM was found to be part of a large complex with many homologues of the mammalian CTLH complex including WDR26 [19]. It has been hypothesized that Twa1, MAEA, RMND5A and RanBPM make up the core of the CLTH complex, while Armc8, muskelin and WDR26 are peripheral components [23]. In yeast, RanBPM and Twa1 make up the core of the GID complex [106]. The mammalian CTLH complex is present in both the nucleus and cytoplasm. However MAEA is exclusively nuclear whereas muskelin is exclusively cytoplasmic. Therefore components of the complex could differ in each cellular compartment. Overexpression of these exclusively nuclear or cytoplasmic proteins changed the localization of overexpressed nucleocytoplasmic members of the complex to their respective compartment [22].

The Really Interesting New Gene (RING) domains that confer E3 ubiquitin ligase activity of Gid2 and Gid9 are evolutionarily conserved in the human homologs RMND5A and MAEA, respectively [23]. RMND5A was shown to have E3 ubiquitin ligase activity in *Xenopus laevis* [107]. However, it has not yet been shown that the human CTLH complex retains E3 ubiquitin ligase activity.

Many of the studies referenced above have studied RanBPM in isolation, however whether RanBPM functions alone or as a part of the CTLH to fulfill the many roles attributed to RanBPM remains to be identified. As the substrates targeted by the CTLH complex remain to be investigated, it would be of interest to evaluate if the proteins identified to interact or associate with RanBPM are substrates of the CTLH complex.

### 1.3 Subcellular localization

#### 1.3.1 Overview of classical transport

Cellular functions are dependent on protein function and subcellular localization of proteins is a common mode of protein regulation [108]. The subcellular localization of proteins is regulated by nucleocytoplasmic transport. Molecules under 50kDa passively transport though the nuclear membrane, but larger molecules and proteins rely on active transport [109]. Transport is regulated by the nuclear pore complex (NPC), which is
comprised of approximately 30 nucleoporin proteins that form a channel in the nuclear membrane. Binding of cargo to nucleoporins expands the channel allowing faster transport [110]. Importins, which recognize and bind nuclear localization signals (NLS) and exportins, which recognize and bind nuclear export signals (NES) on cargo proteins, are classes of karyopherins-β, and are key participants in the import and export of proteins [110]. Transport occurs through a series of regulated steps, first importins recognize and bind the cargo and mediate its docking at the NPC followed by translocation. Binding of RanGTP in the nucleus causes dissociation of the cargo-importin complex. Importins are then cycled back to the cytoplasm. Conversely, exportins bind the cargo via its NES in the nucleus and form a trimeric complex with RanGTP, followed by translocation through the nuclear pore. Hydrolysis to RanGDP in the cytoplasm promotes dissociation of the complex [111] (Figure 1-5). The GTPase, Ran, governs directionality of the pore. Importins release their cargo when bound by RanGTP, occurring in the nucleus. Conversely, exportins bind their cargo in the nucleus when in complex with RanGTP [108].

1.3.1.1 Nuclear localization signals

Nuclear localization sequences fall into three classes: a short stretch of basic amino acids, a bipartite NLS consisting of two short stretches of basic residues separated by 10-12 amino acids and a combination of charged/polar and non polar residues flanked by proline and aspartic acid residues [112].

1.3.1.2 Nuclear export signals

The most common characterized nuclear export signal consists of a motif made up of hydrophobic residues and is leucine rich [110]. They loosely conform to the traditional consensus sequence of Φ1-X2,3-Φ2-X2,3-Φ3-X-Φ4, where Φ represents either leucine, valine, isoleucine, phenylalanine or methionine and X can be any amino acid [113]. Crystallization has shown that NESs bind directly to the exportin1 (also referred to as chromosome region maintenance (CRM) 1) and the hydrophobic residues of the NES dock into the hydrophobic pockets of CRM1 [113].
Figure 1-5 Model of subcellular localization.

A) Schematic representation of nuclear import. Importin binds the NLS of the cargo protein in the cytoplasm and mediates its interaction and transport through the NPC. Once in the nucleus the importin-cargo complex with RanGTP and the cargo is released in the nucleus. B) Schematic representation of nuclear export. Exportins bind to the NES of the cargo protein which complexes with RanGTP in the nucleus. The complex is transported through the NPC, once in the cytoplasm RanGTP is hydrolyzed to RanGDP resulting in dissociation of the cargo in the cytoplasm.
A Nuclear Import

- Cargo Protein
- NLS
- Importin
- NPC
- Ran GTP

B Nuclear Export

- Cargo Protein
- NES
- Exportin
- NPC
- Ran GTP
- Ran GDP
1.3.2 Regulation of transport

As translocation is a complex process, regulation of protein localization is achieved at different steps. At the specific protein level, the number of localization signals as well as the strength of each signal plays a part in regulation [108]. The signal strength is the binding affinity of the signal to the karyopherin. This can be modulated through post-translational modifications, most commonly phosphorylation, on or near the signal [108,112]. Another mechanism of regulation involves intermolecular or intramolecular masking of signal sequences. This occurs through protein-protein interactions and conformational changes, respectively, which prevent signal recognition by karyopherins [110,112]. The concentration of freely diffusing protein to be transported provides yet another mechanism of regulation. Sequestration of a protein within a compartment can reduce the mobility and thus rate of transport of that protein. Anchoring of a protein can reduce the amount of free protein available to be transported. Anchoring occurs when a protein is bound to an immobile cellular compartment, for example, plasma membrane, cytoskeleton or chromatin [108]. Finally, at a more global level of regulation, the concentration and activity of the karyopherins can be regulated as different karyopherins recognize different cargoes [108,110,112].

1.3.3 Non-classical transport

Calmodulin mediated nuclear import is an example of non-classical import that occurs independently of importins and Ran. However, it relies on and is regulated by intracellular calcium, as calmodulin is a calcium binding protein. Under levels of low calcium, classical transport is prevalent, however, under high levels of calcium, classical transport is inhibited and calmodulin mediated nuclear import is predominant. Some proteins, which rely on this method of transport, have been shown to have a specific calmodulin binding sequence to facilitate their calcium dependent import. However, exactly how calmodulin mediates passage through the NPC remains to be elucidated [111]. Another example of calcium-regulated translocation is calreticulin-mediated transport. Calreticulin-mediated transport provides an alternative to exportin-mediated nuclear export. Calreticulin directly binds its substrates to be exported, but like
calmodulin-mediated nuclear import described above, is dependent on intracellular calcium levels [111].

Another example of non-classical transport is glycol-dependent transport. Sugar binding proteins, such as the lectin family of proteins, some of which shuttle between the nucleus and the cytoplasm, are thought to interact and mediate transport of glycosylated proteins [111].

Alternatively, there are some proteins that are able to directly bind to the NPC and translocate without the need of any carrier molecules mediating the interaction [111]. In addition, localization signals on one protein are able to move bound protein partners through the NPC, thus allowing the transport of proteins without a localization signal, therefore there is indirect control of the protein localization [108]. Viruses, on the other hand, have been shown to gain entry directly into the nucleus bypassing the NPC [111].

Alternative transport pathways provide multiple conditions in which proteins can enter the nucleus even in situations when classical karyopherin transport is inhibited, which occurs when cells are depleted of ATP [111,114].

1.4 HDAC6

1.4.1 Overview

Initially discovered on histones, lysine acetylation is a post-translational modification that plays a role in signal transduction. This modification is reversible, added by histone acetyl transferases (HATs) and removed by histone deacetylases (HDACs) [115]. HDAC6 is a class IIb HDAC but unlike other HDAC enzymes, HDAC6 shows cytoplasmic localization. Cytoplasmic localization is achieved by two strong NESs. HDAC6 also contains eight serine glutamine containing tetradecapeptide (SE14) repeats, specific to human HDAC6, which also aid in its cytoplasmic retention [116-118]. In spite of its name, HDAC6 does not deacetylate histones in vivo. Knockdown or chemical inhibition of HDAC6 in cells does not have any effect on histone acetylation [119,120]. Also HDAC6 KO mice do not show any changes in histone acetylation [121].
HDAC6 uniquely has duplicate deacetylase domains as well as a C-terminal binder of ubiquitin zinc finger (BUZ) domain, which is able to bind ubiquitin, identifying HDAC6 as a potential link between acetylation and ubiquitination signaling [117,122] (Figure 1-6).

HDAC6 KO mice are viable and fertile and only show moderate affects in immune response and bone development. This indicates that HDAC6 is dispensable for normal development [121].

1.4.2 Substrates

The most well characterized substrate of HDAC6 is acetylated α-tubulin [117]. HDAC6 was initially identified as a microtubule (MT) associated deacetylase since it colocalized with p150\textsuperscript{glued}, a protein part of the dynein-dynactin MT motor complex [123]. Treatment with nocodazole, an inhibitor that collapses the MT network, resulted in HDAC6 redistribution, however disruption of the actin network did not alter HDAC6 localization, suggesting that HDAC6 specifically associates with MTs [119,123]. A series of experiments confirmed that HDAC6 catalytic activity is necessary for α-tubulin deacetylation. First, overexpression of HDAC6 dramatically reduced levels of acetylated α-tubulin; however overexpression of a catalytically inactive HDAC6 mutant did not change acetylated α-tubulin levels [123,124]. Second, treatment with specific pan-HDAC inhibitors, trapoxin-B and sodium butyrate, to which HDAC6 is resistant, did not affect levels of acetylated α-tubulin. Treatment with trichostatin A (TSA), a pan-HDAC inhibitor to which HDAC6 is sensitive, however, resulted in increased levels of acetylated α-tubulin [119,123]. Third, HDAC6 knockout and siRNA knockdown in cells increased levels of acetylated α-tubulin and HDAC6 knockout mice showed significantly increased levels of acetylated α-tubulin in most tissues [121,123]. Finally, HDAC6 was also shown to deacetylate MTs \textit{in vitro} [123,124]. Interestingly, HDAC6 was shown by yeast-two hybrid to associate with β-tubulin, suggesting that β-tubulin mediates HDAC6 interaction with α-tubulin. Co-immunoprecipitation demonstrated that HDAC6 associates with both α-tubulin and β-tubulin and TSA does not disrupt this association. Similarly, a
Figure 1-6 HDAC6 schematic.

Schematic representation of HDAC6 domains and motifs, which include NES (nuclear export signal), CAT1 (first catalytic domain), DMB (dynein binding domain), CAT2 (second catalytic domain) SE14 (serine glutamine containing tetradecapeptide) and BUZ (binder of ubiquitin zinc finger).
mutated catalytically inactive HDAC6 can still associate with β-tubulin [119]. Altogether, these experiments confirmed that HDAC6 is the deacetylase responsible for the deacetylation of α-tubulin and therefore suggested to play a role in MT dynamics and cell motility as described below.

Heat shock protein 90 (Hsp90) was identified as a substrate for HDAC6 as shown by mass spectrometry and co-immunoprecipitation [125]. Hsp90 is a chaperone with nearly 100 client proteins that is part of an ATP dependent molecular chaperone complex [126,127]. Depending on the conformation of Hsp90, it can either stabilize its client proteins aiding in correct folding and proper conformation or promote their degradation [127]. Similarly, to what was observed for α-tubulin, overexpression of wildtype HDAC6 but not a catalytically inactive HDAC6 mutant reduced levels of acetylated Hsp90 and HDAC6 siRNA increased levels of acetylated Hsp90. Again, TSA treatment resulted in increased Hsp90 acetylation but Hsp90 acetylation did not change upon treatment with trapoxin-B or sodium butyrate. It was shown that Hsp90 association with HDAC6 requires HDAC6 catalytic and ubiquitin binding abilities as inactivation, by mutation or inhibition, and deletion of the BUZ domain failed to associate with Hsp90 [125,128]. The hyperacetylation of Hsp90 observed in HDAC6 knockdown or inhibited cells was found to reduce the association of Hsp90 and one of its co-chaperones, p23. This in turn was shown to reduce the association of Hsp90 and its client protein, indicating that hyperacetylation of Hsp90 impairs its chaperone function [125]. In addition, hyperacetylated Hsp90 showed reduced ATP binding and reduced Hsp90-client association promoting client ubiquitination and subsequent proteasomal degradation [128]. These data indicate a key role for HDAC6 in promoting Hsp90 chaperone function through the regulation of protein folding through its deacetylation of Hsp90.

Cortactin was first identified as an HDAC6 substrate by mass spectrometry and association was confirmed by co-immunoprecipitation [129]. Using bacterially expressed proteins, this was found to be a direct interaction. Cortactin is found at the leading edge of cells where it interacts with F-actin to promote polymerization and branching and has a regulatory role in cell migration. Cortactin deacetylation by HDAC6 was confirmed using
inhibitor treatments and HDAC6 knockdown. Deacetylation of cortactin was found to increase association with F-actin therefore, resulting in increased cell migration [129].

Other HDAC6 substrates include peroxiredoxins, redox regulatory proteins [130], β-catenin, a key signal transducer of the Wnt pathway [131], and Ku70, a factor in the non-homologus end joining pathway that is also found in the cytoplasm associated with Bax, a Bcl-2 family protein [130]. Trans-Activator of Transcription (Tat), a transcriptional activator of human immunodeficiency virus (HIV) [130], survivin, an oncoprotein that is an inhibitor of apoptosis [132], TRIM50, an E3 ubiquitin ligase [133] and microtubule-associated protein 1 light chain 3 (LC3) B-II an autophagosome component [134] have also been identified as HDAC6 substrates. Recently, a large proteomic analyses screen identified 107 proteins that had increased acetylation in the liver of HDAC6 knockout mice. Three of these proteins, myosin heavy chain 9 (MYH9), heat shock cognate protein 70 (Hsc70) and dnaJ homolog subfamily A member 1 (DNAJA1), were confirmed to interact with and be deacetylated by HDAC6. This, therefore, suggests that HDAC6 could have many more substrate targets yet to be biochemically confirmed [135].

As previously mentioned, HDAC6 possesses two catalytic domains. These domains are highly homologous to each other and highly conserved compared to other HDAC proteins [136]. However, whether one or both domains are required for deacetylation of HDAC6 substrates has been a matter of debate.

Studies have reported contradictory results about whether both catalytic domains possess catalytic activity in vitro. Initially, in vitro assays showed that both catalytic domains had individual catalytic activity for acetylated histone substrates [136]. On the other hand, a later study showed that in vitro the first catalytic domain alone showed no catalytic activity toward either acetylated histones or acetylated α-tubulin substrates [137]. The second catalytic domain alone, however, did have deacetylase activity toward both these substrates, albeit, lower than the full length wildtype protein [137]. Analysis of the full length protein in vitro using acetylated α-tubulin as a substrate, showed that the second catalytic domain was responsible for the deacetylation of α-tubulin since a catalytically inactivating mutation in the first catalytic domain had no effect on the deacetylation of
acetylated α-tubulin [138]. Similarly, Zou et al., observed in vitro that mutation within the first catalytic domain resulted in wildtype enzyme catalytic kinetics, however mutation in the second catalytic domain severely impaired enzyme catalytic efficiency [137].

Conversely, Zhang et al., demonstrated that both domains were required for α-tubulin deacetylation because mutation in either catalytic domain failed to deacetylate acetylated α-tubulin in vitro and in vivo [119]. Zhang et al., later suggested that cooperation of both catalytic domains is needed for deacetylase activity, and it was shown that the first catalytic domain of HDAC6 has been suggested to mediate tubulin binding, though the two domains can selectively recognize and interact with different substrates [138,139].

Interestingly, treatment with tubacin, a specific HDAC6 inhibitor, did not have striking effects on Hsp90 or cortactin acetylation, as was shown with α-tubulin acetylation [120,128,140]. Haggarty et al., demonstrated that tubacin only inhibits the second catalytic domain of HDAC [138]. This suggests that Hsp90 and cortactin deacetylation occurs via the first catalytic domain and that tubulin deacetylation occurs via the second catalytic domain. Thus, the catalytic activity of HDAC6 appears to be conferred by both domains in a substrate-dependent manner.

1.4.3 HDAC6 regulation

HDAC6 deacetylation activity is both negatively and positively regulated by post-translational modifications. HDAC6 phosphorylation by protein kinase C (PKC) ζ, PKCα, G protein-coupled receptor kinase 2 (GRK2), glycogen synthase kinase 3 β (GSKβ), casein kinase 2 (CK2), ERK and Aurora A promote α-tubulin deacetylase activity [140-146]. Conversely, epidermal growth factor receptor (EGFR) phosphorylation of HDAC6 decreases tubulin deacetylase activity [147]. Acetylation of HDAC6 p300 also inhibits HDAC6 α-tubulin deacetylase activity [134,148]. Some of these modifications, phosphorylation by GRK2 and ERK, were shown to only modulate HDAC6 tubulin deacetylase activity and have no effect on cortactin acetylation [140,145]. This again supports the idea that cortactin deacetylation occurs in a different
catalytic domain than tubulin deacetylation and post translation modifications differentially regulate the two catalytic domains.

In addition to being regulated by post-translational modification, HDAC6 is also regulated by protein-protein interactions. HDAC6 association with dysferlin, p62, paxillin, tau and tubulin polymerization-promoting protein/p25 (TPPP/p25) result in decreased α-tubulin deacetylase activity [149-153].

1.4.4 Role in cell migration and MT stability

Cell migration is an essential, highly regulated, complex process. Beginning with protrusions from the leading edge, translocation of the cell body and finally retraction of the rear. Membrane protrusions that push the membrane forward rely on both actin and MTs. Microtubules also aid in the formation of new adhesion assembly at the leading edge and in the disassembly of adhesions at the trailing edge. However, most importantly, the microtubule network acts to guide cell migration as microtubule polarization defines the direction of migration [154-157].

HDAC6 has been shown to promote cell motility through deacetylation of α-tubulin and/or cortactin. Overexpression of HDAC6 results in increased cell motility, however overexpression of a catalytically inactive HDAC6 mutant shows motility similar to that of control cells [123]. Consistently, HDAC6 siRNA resulted in decreased cell migration when compared to control siRNA, [120,129,158], and this was also observed in HDAC6 knockout cells [159]. Treatment of cells with TSA or tubacin also resulted in decreased cell motility compared to vehicle control [120,138,158]. Altogether, this evidence clearly indicates a role for HDAC6 catalytic activity in promoting cell motility.

Cells expressing a GRK2 mutant, which, fails to phosphorylate HDAC6 thereby resulting in decreased HDAC6 deacetylase activity showed defective locomotion [140]. Overexpression of a cortactin mutant, which mimics HDAC6 deacetylated cortactin, enhanced cell migration after HDAC6 or cortactin knockdown [129,160]. Accordingly, expression of a cortactin mutant that mimics acetylated cortactin resulted in decreased cell migration [129]. Interestingly, three different ovarian cancer cell lines exhibiting
different levels of acetylated cortactin exhibited different cell motility rates. OV2008 and SW626 show higher levels of HDAC6 and therefore have lower levels of acetylated cortactin and exhibit increased migration compared to OVCAR3, which has lower HDAC6 expression and therefore higher levels of acetylated cortactin [129].

Whether acetylation stabilizes MTs and therefore whether HDAC6 plays a role in MT stability is still a matter of debate. Matsuyama et al., demonstrated that pretreatment of cells with TSA to induce MT acetylation resulted in slower depolymerization upon treatment with demecolcin, a MT depolymerizing agent, compared to cells pretreated with vehicle control. Correspondingly, cells transfected with HDAC6, resulting in severely decreased acetylated MTs, showed more sensitivity to demecolcin compared to untransfected cells. These results indicate that hyperacetylated MTs are more stable than unacetylated MTs [124]. Conversely, Haggarty et al., found that pretreatment with tubacin, resulting in highly acetylated MTs, did not render MTs more resistant to nocodazole or cold depolymerization [138]. Accordingly, isolated acetylated and unacetylated microtubules were demonstrated to have similar stabilities in vitro [161]. Palazzo et al., demonstrated that acetylation of MTs does not make them more stable, rather stable MTs accumulate more acetylation [162]. Consequently, deacetylation of α-tubulin by HDAC6 does not destabilize MTs.

1.4.5 Role in aggresome formation

1.4.5.1 Overview

HDAC6 has been shown to be an important factor in the formation of aggresomes [163]. Aggresomes are insoluble aggregates of misfolded, damaged and ubiquitinated proteins [164-167]. Protein misfolding may occur for a number of reasons including: genetic mutations and either errors in transcription, mRNA processing or translation [167]. Thermal, osmotic, oxidative and pH stress can also interfere with correct folding resulting in misfolded proteins [166,167]. Failure to correctly translocate and integrate proteins with hydrophobic surfaces, that are normally buried within the protein, at the interface of binding sites or embedded in the lipid bilayer, results in the exposure of these surfaces and subsequent inappropriate interaction of these proteins resulting in aggregate
formation [167]. These misfolded proteins and protein aggregates are usually cleared by chaperone mediated refolding or by the ubiquitin proteasome system [165,167,168], where proteins tagged with ubiquitin are marked for degradation by the proteasome [103]. However, when the proteasome is overwhelmed or inhibited, this raises a problem as these misfolded and protein aggregates are not cleared and can become pathogenic. This is a common feature of neurodegenerative diseases [167,169]. To deal with this, cells have acquired the aggresome formation pathway [167].

Ubiquitinated damaged or misfolded proteins associate with HDAC6 via the BUZ domain. HDAC6 also associates with the MT motor protein dynein, which transports the ubiquitinated proteins toward the nucleus [163,165,167] (Figure 1-7). Aggresomes are located in a single juxtanuclear region and are 1-3μM in size [165,167]. The golgi apparatus and lysosomes are also found in this region, however aggresomes are close in proximity but distinct from both of these cellular compartments. Aggresomes are however located at the microtubule organizing centre (MTOC). The presence of aggresomes results in complete redistribution of the intermediate filament vimentin to a “cage-like structure” around the aggresome. The aggresome is not membrane bound suggesting that the vimentin cage contributes to the stability of the aggresome. Aggresomes do not disrupt the MT cytoskeleton, in fact the MTs are required for aggresome formation and treatment with MT-disrupting agents abrogates aggresome formation resulting in misfolded protein aggregates throughout the cytoplasm. Interestingly, MT disruption did not affect pre-existing aggresomes. Similarly, the actin cytoskeleton is not disrupted by aggresome formation, however unlike the MT cytoskeleton, disruption of the actin cytoskeleton has no effect on aggresome formation [167].

A number of proteasomes and heat shock proteins were found to be recruited to the aggresome [166,170]. In addition, HDAC6 was found to localize to the aggresome. HDAC6 is able to associate with ubiquitinated proteins via the BUZ domain and with dynein via the region between the catalytic domains and this interaction increased with proteasome inhibitor treatment. These findings identified HDAC6 as the link between ubiquitinated proteins and the motor protein dynein, providing explanation of how
Figure 1-7 Model of aggresome formation.

A) Misfolded or damaged proteins are ubiquitininated and degraded by the proteasome. B) Under conditions of proteasome impairment or inhibition, aggregated poly-ubiquitinated proteins bind to HDAC6 via its BUZ domain and HDAC6, through its dynein binding domain, associates with the microtubule motor protein, dynein. Dynein transports HDAC6 with the associated aggregated proteins toward the microtubule-organizing center in the perinuclear region forming an aggresome.
Misfolded/Damaged Protein
Ubiquitin
Proteasome
Microtubule
Dynein
HDAC6
Aggresome
Nucleus
ubiquitinated aggregates were able to travel along the microtubules. HDAC6 knockout cells showed significantly fewer aggresomes and the aggresomes that did form were severely smaller in size, indicating an essential role for HDAC6 in aggresome formation. Aggresome formation could be rescued with the addition of WT HDAC6. However a catalytically inactive mutant and a mutant with a BUZ domain deletion could not rescue aggresome formation. This identified that both the catalytic activity and ubiquitin binding of HDAC6 are required for aggresome formation [163].

1.4.5.2 Aggresome clearance

Aggresome structures are reversible and can be eliminated from cells, first by chaperone-mediated and proteasomal degradation. When these processes are insufficient in clearing aggresomes they are cleared by autophagy. Autophagy consists of double membrane bound autophagosomes engulfing the aggresome followed by fusion with the lysosomes. Degradation in the lysosome occurs by hydrolases [165,171]. Similarly to the formation of aggresomes, autophagy mediated clearance also requires HDAC6 catalytic activity as HDAC6 is required for autophagosome and lysosome fusion [172,173].

1.4.5.3 Aggresomes and cell death

There is some debate whether aggresome formation is seen as a protective or detrimental process. Aggresome formation can be seen as a cytoprotective function, protecting cells from the harmful effects of misfolding proteins [169]. Cells with aggresomes were shown to perform many normal cellular processes such as normal protein synthesis and protein quality control as well as endoplasmic reticulum to golgi transport [166]. Therefore, aggresomes are not seen as toxic to the cells and in fact, inhibiting aggresome formation results in toxicity [169]. Formation of aggresomes actually decreases the half-life of misfolded, ubiquitinated proteins indicating that aggresomes function to facilitate protein degradation [169]. Proteasome inhibition was actually found to result in significantly increased apoptosis in cells not able to form aggresomes compared to those that did [163]. However, prolonged aggresome formation can result in cellular dysfunction and subsequent cell death [174].
Since aggresomes are commonly formed in disease states, for example Huntington’s disease, Parkinson’s disease and Alzheimer’s disease, they have been suggested to be linked to pathogenesis. Aggresomes can be seen as cytotoxic as they sequester machinery needed for proper protein folding and degradation therefore further promoting protein misfolding and aggregation [165]. Aggresomes also decrease the proteasome activity within cells [168,174]. In contrast to what was described above, it was demonstrated that inhibition of aggresome formation with MT inhibitors abrogated caspase activation and consequent cell death in neuronal cells indicating that aggresome formation is associated with caspase activation, specifically caspase 8 and caspase 3 [175,176]. Aggresomes could also result in toxicity as they may highjack dynein-mediated transport that is imperative for cellular function [165]. In addition, accumulation of misfolded proteins at the centrosome can impair their function interfering with proper cell division [168].

It was shown that inhibition of the proteasome could either enable or prevent activation of apoptosis. In pro-apoptotic conditions the proteasome degrades anti-apoptotic proteins [177]. Conversely, in pro-survival conditions the proteasome degrades pro-apoptotic proteins. However, it is clear that activation of apoptosis does result in inactivation of the proteasome due to caspase-3 dependent cleavage of the base 19S regulatory particle of the proteasome. This caspase-induced cleavage reduces the functional activity of the proteasome at an early stage of apoptosis resulting in the accumulation of ubiquitinated proteins [178]. This results in the accumulation of pro-apoptotic proteins and amplification of the apoptotic signal [177,178].

1.4.6 HDAC6 in Cilia

HDAC6 also plays a role in the regulation of cilia, promoting its disassembly [179]. Cilia are ‘antenna- like’ organelles that extend from most mammalian cells. Cilia mediate extracellular environmental signals and are a critical organelle as defects in cilia structure of function are associated with many diseases [179,180]. Through deacetylation of α-tubulin and cortactin, HDAC6 contributes to ciliary resorption. Overexpression of HDAC6 results in both reduced number of ciliated cells and significantly reduced ciliary length. Downregulation of HDAC6 or treatment with HDAC6-specific inhibitors
protects from ciliary disassembly indicating that HDAC6 catalytic activity promotes ciliary disassembly [146,179].

1.4.7 HDAC6 and cancer

HDAC6 has been implicated in cancer development and HDAC6-specific inhibitors have emerged as a chemotherapeutic agent to combat cancer. HDAC6 is required for in vitro oncogene-induced cell transformation. Transformation of primary cells by human telomerase, simian virus 40 (SV40) early region and oncogenic Ras resulted in increased HDAC6 protein levels. More convincingly, HDAC6 knockout mouse embryonic fibroblasts (MEF) transformed with SV40 and oncogenic Ras resulted in more than 10 fold fewer colonies than transformed wildtype MEFs [181]. In addition, HDAC6 knockdown or inhibition attenuated the effects of transforming growth factor (TGF) β1 induced epithelial-mesenchymal transition (EMT). TGF-β1 is a strong inducer of EMT resulting in decreased E-cadherin, increased expression of vimentin and the induction of stress fibers, however inhibition or knockdown of HDAC6 mitigated these effects [182]. HDAC6 expression is also required to maintain anchorage independent growth of established cancer cell lines as HDAC6 downregulation by shRNA severely impaired this oncogenic characteristic [181,183]. This indicates that HDAC6 contributes to anoikis resistance, which is a cause of cell death due to lack of adhesion. Resistance to anoikis is a hallmark of malignant transformation. These experiments indicate that HDAC6 is required to induce transformation at the cellular level [181].

In vivo, SKOV3, ovarian carcinoma, cells expressing control or HDAC6 shRNA were subcutaneously injected into severe combined immunodeficiency (SCID) -Beige mice. Tumour growth of HDAC6 shRNA cells was significantly slowed compared to control cells and also showed significantly reduced tumour volume. Reintroduction of wildtype HDAC6 resulted in tumour formation similar to that of control whereas reintroduction of catalytically inactive HDAC6 mirrored HDAC6 shRNA. Analysis of tumour samples showed HDAC6 shRNA tumours had a significantly reduced number of mitotic cells demonstrated by Ki-67 staining compared to control tumour samples. Interestingly, HDAC6 knockout mice also showed delayed tumour formation and smaller and fewer tumours than wildtype mice after application of a topical carcinogen [181]. These
experiments demonstrate that HDAC6 contributes to tumourigenesis in *in vivo* mouse models.

Upregulated HDAC6 levels have been observed in oral squamous cell carcinoma (OSCC), hepatocellular carcinoma (HCC) and acute myeloid leukemia (AML) cancer cell lines compared to normal matched cells [184-186]. Similarly, HDAC6 was upregulated in a cohort of both OSCC and HCC patients. In OSCC patients, HDAC6 was significantly upregulated in advanced stages compared to early stages [184]. In HCC, overexpression of HDAC6 was significantly correlated with high clinical stage, number of tumours, vascular invasion and intrahepatic metastasis [185].

Tumour cell motility plays a positive role in tumour invasiveness [185]. As described above, HDAC6 promotes cell motility through deacetylation of α-tubulin and/or cortactin and subsequently HDAC6 has been shown to stimulate tumour invasiveness. HDAC6 knockdown by siRNA or inhibition resulted in significantly reduced invasiveness demonstrated either by matrigel invasion assay or 3D type I collagen gel invasion assay in HCC, MDA-MB-231 as well as bladder cancer cells [183,185,187].

Increased expression of HDAC6 results in increased deacetylation of Hsp90, which subsequently enhances its chaperone activity. Hsp90 client proteins include breakpoint cluster/Abelson murine leukemia viral oncogene homolog 1 (Bcr/Abl), Raf, epidermal growth factor receptor 2 (ErbB2) among others. Many Hsp90 client proteins contribute to cell growth and survival pathways commonly exploited in cancer cells. In fact, Hsp90 inhibitors are also under investigation as chemotherapeutic agents [188,189].

HDAC6 has also been demonstrated to play a role in promoting angiogenesis, a hallmark of cancer [4]. HDAC6 knockdown inhibited tube formation in an endothelial tube formation assay, a widely performed *in vitro* angiogenesis assay [160,190]. *In vivo*, HDAC6 inhibition by tubacin reduced the growth of blood vessels in chick embryos and similarly, HDAC6 knockdown in zebrafish also negatively impacted vessel formation [160,190]. In addition, HDAC6 knockout mice showed reduced capillary density compared to wildtype mice 14 days after induction of hind limb ischemia, identifying that HDAC6 is necessary for *in vivo* angiogenesis [160].
1.4.7.1 HDAC6 inhibitors in cancer treatment

As HDAC6 is able to promote cell migration leading to invasion of tumour cells and angiogenesis, two oncogenic characteristics, it is not surprising that HDAC6 inhibitors are currently being investigated in clinical trials as chemotherapeutic agents.

Bortezomib is a proteasome inhibitor that has been used in clinical trials for patients with multiple myeloma (MM), however there were high rates of relapse resulting in the need for a combinatorial treatment. Inhibition of the proteasome results in the accumulation of ubiquitinated proteins, which in turn activate the aggresome pathway. However, inhibition of HDAC6 would inhibit the aggresome pathway, resulting in the activation of apoptosis due to cell stress from the accumulation of ubiquitinated proteins [191,192].

A preclinical evaluation of the combination of bortezomib and ACY-1215, an orally bioavailable HDAC6 specific inhibitor demonstrated synergistic activation of apoptosis and promising outcomes in MM models. ACY-1215 exhibited specific inhibition of HDAC6 in MM cell lines and primary cells. ACY-1215 showed less cytotoxic effects in peripheral blood mononuclear cells (PBMC) from healthy donors compared to a pan HDAC inhibitor however, resulted in a dose dependent decrease in viability in MM cells, even in a bortezomib resistant cell line. Bortezomib, in combination with ACY-1215, resulted in synergistic apoptotic activation in MM cell lines and significantly increased the accumulation of ubiquitinated proteins. Combination treatment of bortezomib and ACY-1215 considerably delayed tumour growth and prolonged overall survival in a human MM xenograft mouse model compared to individual treatments. In addition, combination treatment was well tolerated by the animals, as it did not significantly affect their body weight. These results showed synergistic results and provided excellent grounds for combinational treatment for MM in a clinical setting. This phase I and II clinical trial was set to be completed in June 2016, however, to date no results have been released [192].

Similarly, ACY-1215, in combination with the proteasome inhibitor carfilzomib, was evaluated in non-Hodgkin lymphoma (NHL) models. Synergistic apoptotic activation was observed in combination treatment of NHL cellular models. Combined treatment
resulted in significantly reduced tumour volumes and prolonged survival compared to individual treatments in an *in vivo* xenograft mouse model. Once again, the combination treatment was well tolerated by animals, as there was minimal change in body weight. Currently, ACY-1215 for the treatment of lymphoma is under investigation in phase I and II clinical trials [193].

Currently, a clinical study is recruiting participants to evaluate the efficacy of ACY-1215 in metastatic breast cancer. A genome wide screen found that the viability of inflammatory breast cancer (IBC) cells depends on HDAC6 function. HDAC6 shRNA knockdown in IBC cell lines showed increased apoptotic activation and reduced proliferation when compared to HDAC6 knockdown in non-IBC cells. Results were mirrored with ACY-1215 treatment. *In vivo* IBC xenograft mouse models also demonstrated that ACY-1215 treatment significantly reduces tumour growth [194].

Taken together these preclinical results establish a strong rationale for the use of ACY-1215 in clinical trials. There are currently ten clinical trials in progress using ACY-1215 alone or in combination with other agents. The results from these trials will set the stage for targeting HDAC6 in the future.

### 1.5 Hypothesis

I hypothesize that the subcellular localization of RanBPM regulates its function.

### 1.6 Scope of thesis

The work presented in this thesis aims to characterize the regulation of RanBPM subcellular localization, its function in aggresome formation and the characterization of its association and inhibition of HDAC6.

Our lab previously described that RanBPM relocalized to the cytoplasm following exposure to IR. However, the regulation of the subcellular localization of RanBPM had previously not been investigated. The first objective of this thesis is to determine the domains and motifs that regulate RanBPM nuclear and cytoplasmic localization (Chapter
2). We also identify structures within both the nucleus and cytoplasm with which RanBPM associates that may function to retain RanBPM in these compartments.

The second objective is to illustrate that RanBPM relocalizes to the cytoplasm after IR to form perinuclear foci, which we identify as aggresomes. We demonstrate that aggresomes form as a result of exposure to IR. In addition, we found that RanBPM is essential to aggresome formation following both IR treatment and proteasome inhibition (Chapter 3). We proceed to demonstrate an interaction between RanBPM and HDAC6, a well characterized aggresome component, and reveal that RanBPM inhibits HDAC6 activity.

Subsequently, the data presented in chapter 4 further describes the association of RanBPM and HDAC6, identifying the domains required for interaction and demonstrating that RanBPM is dependent on the expression of HDAC6 to colocalize with the microtubules, specifically α-tubulin. We also demonstrate that components of the CTLH complex associate with both microtubules and HDAC6 mediating their interaction with RanBPM. Lastly, RanBPM was found to inhibit HDAC6 mediated cell migration. This suggests that the tumour suppressor functions of RanBPM stem, in part, from an inhibition of the oncogenic activities of HDAC6.
1.7 References


Chapter 2

2 Characterization of RanBPM molecular determinants that control its subcellular localization

RanBPM/RanBP9 is a ubiquitous, nucleocytoplasmic protein that is part of an evolutionary conserved E3 ubiquitin ligase complex whose function and targets in mammals are still unknown. RanBPM itself has been implicated in various cellular processes that involve both nuclear and cytoplasmic functions. However, to date, little is known about how RanBPM subcellular localization is regulated. We have conducted a systematic analysis of RanBPM regions that control its subcellular localization using RanBPM shRNA cells to examine ectopic RanBPM mutant subcellular localization without interference from the endogenously expressed protein. We show that several domains and motifs regulate RanBPM nuclear and cytoplasmic localization. In particular, RanBPM comprises two motifs that can confer nuclear localization, one proline/glutamine-rich motif in the extreme N-terminus which has a dominant effect on RanBPM localization, and a second motif in the C-terminus which minimally contributes to RanBPM nuclear targeting. We also identified a nuclear export signal (NES) which mutation prevented RanBPM accumulation in the cytoplasm. Likewise, deletion of the central RanBPM conserved domains (SPRY and LisH/CTLH) resulted in the relocalization of RanBPM to the nucleus, suggesting that RanBPM cytoplasmic localization is also conferred by protein-protein interactions that promote its cytoplasmic retention. Indeed we found that in the cytoplasm, RanBPM partially colocalizes with microtubules and associates with α-tubulin. Finally, in the nucleus, a significant fraction of RanBPM is associated with chromatin. Altogether, these analyses reveal that RanBPM subcellular localization results from the combined effects of several elements that either confer direct transport through the nucleocytoplasmic transport machinery or regulate it indirectly, likely through interactions with other proteins and by intramolecular folding.

2.1 Introduction

Transport in and out of the nucleus of proteins above 50KDa is an active process that requires the nucleocytoplasmic transport machinery [1]. Import to the nucleus is mediated
by a nuclear localization signal (NLS) that is recognized by an import receptor (importin) which transports its cargo through the nuclear membrane in an energy-dependent process [2]. Conversely, nuclear export is dependent on a nuclear export sequence (NES) that is recognized by exportins, which transport the protein out of the nucleus. Nuclear localization sequences fall into three classes: a short stretch of basic amino acids, a bipartite NLS consisting of two short stretches of basic residues separated by 10-12 amino acids and a combination of charged/polar and non-polar residues flanked by proline and aspartic acid residues [3,4]. The most common characterized NES consists of a non-conserved motif made up of hydrophobic residues and is leucine-rich [2]. Nucleocytoplasmic transport is a tightly monitored process regulated at many different stages [2,3]. One mechanism of regulation includes importin protein expression, as different importins recognize different cargoes. Another mechanism of regulation involves alteration of sequence affinity to karyopherins, for example by phosphorylation of the signal sequence. A third mechanism of regulation involves intermolecular or intramolecular masking of signal sequences. This occurs through protein-protein interactions and conformational changes, respectively, which prevent signal recognition by karyopherins [2,3]. In addition, non-conventional mechanisms exist which do not rely on importins/karyopherins but on interaction with other transporters, or through direct binding to nuclear pore complex components [5].

Ran binding protein M (RanBPM, also referred to as RanBP9), is a ubiquitous, nucleocytoplasmic 90kDa protein whose function is poorly understood. RanBPM contains three conserved domains (Figure 2-1A), none of which confers enzymatic activity or is indicative of any specific function, apart from protein interactions. The SPRY (SpLA and Ryanodine receptor) domain is a protein interaction domain present in protein families regulating a wide range of functions, including regulation of cytokine signaling, RNA metabolism and protein degradation [6]. The LisH/CTLH (LIS1-homology motif/C-terminal to LisH) domain is found in proteins associated with microtubule dynamics, cell migration and chromosome segregation, and mediates dimerization [7-9]. The CRA (CT11-RanBPM) domain is an α-helical structure of unknown function but is structurally reminiscent of the death domain superfamily [10].
RanBPM has been shown to interact with numerous proteins, implicating it in a variety of cellular processes including cell adhesion, migration, microtubule dynamics, and gene transcription [11-19]. It has been hypothesized that RanBPM functions as a scaffolding protein that may be part of a large complex [20-22]. RanBPM has been identified as a phosphoprotein and its phosphorylation is increased in response to stress stimuli, such as osmotic shock, ultraviolet light (UV), and ionizing radiation (IR) [23,24]. Thus, RanBPM is involved in both nuclear and cytoplasmic processes, but how its subcellular localization is regulated has not been characterized.

RanBPM is well conserved in mammals, in fact the mouse and human proteins are over 90% identical and their differences fall within the N-terminus [20]. The yeast homolog of RanBPM, called Gid1 (glucose-induced degradation-deficient 1) or Vid30 (vacuole import and degradation 30) was found to be part of an E3 ubiquitin ligase complex that functions to ubiquitinate fructose-1,6-bisphosphatase (FBPase), a key enzyme in the gluconeogenesis pathway [25-27]. Phylogenetic and sequence analyses revealed that the components of the Gid complex are conserved in eukaryotic genomes, suggesting an ancient and conserved function for this ubiquitin ligase complex in eukaryotes, with RanBPM being one of the most conserved proteins in the complex [28]. In mammalian cells, RanBPM was found in a large cytoplasmic complex together with the mammalian counterparts of all Gid proteins (except Gid4) [22]. This complex was named CTLH complex [22], but is also referred to as the muskelin/RanBPM/CTLH complex (MRCTLH) [28]. The subunits of the complex are present to different extents in both the cytoplasm and the nucleus, yet how their subcellular localization is regulated is still poorly understood [15,21,22,28]. Domain deletion analyses of RanBPM and complex members Twa1, MAEA and RMND5a revealed that several domains in each protein contribute differentially to their localization [28]. Previous investigations showed that the muskelin C-terminal domain is important for both RanBPM interaction and cytoplasmic localization, suggesting that RanBPM regulates the subcellular localization of muskelin [15]. However, how the nucleocytoplasmic localization of RanBPM itself is regulated is still largely unknown.
Here we have carried out a systematic analysis of RanBPM deletion mutants to investigate the determinants of RanBPM subcellular localization. Our results establish that RanBPM subcellular localization is dependent on several domains/motifs, relying on NLS and NES for direct transport by nucleocytoplasmic transport machinery and on protein domains which may function to retain RanBPM to specific subcellular compartments through interaction with other proteins.

2.2 Materials and methods

2.2.1 Plasmid expression constructs

pCMV-HA-RanBPM shRNA mutant construct (HA-RanBPM si-mt), pCMV-HA-RanBPM-ΔN (ΔN1), pCMV-HA-RanBPM-ΔN2 (ΔN2) and pCMV-HA-RanBPM-ΔC (ΔC1) were previously described [29]. pCMV-HA-RanBPM-Δ212 (Δ212), pCMV-HA-RanBPM-Δ360 (Δ360) and pCMV-HA-RanBPM-ΔC4 (ΔC4) were previously described [30]. pCMV-HA-RanBPM-ΔC2 (ΔC2), pCMV-HA-RanBPM-ΔC3 (ΔC3), pCMV-HA-RanBPM-ΔN3 (ΔN3), pCMV-HA-RanBPM-ΔN4 (ΔN4), pCMV-HA-RanBPM-Δ1-66 (Δ1-66), pCMV-HA-RanBPM-Δ1-25 (Δ1-25) mutant constructs were generated by polymerase chain reaction (PCR) amplification of RanBPM and cloned into digested pCMV-HA-RanBPM si-mt. pCMV-HA-RanBPM-ΔLisH (ΔLisH) and pCMV-HA-RanBPM-ΔCTLH (ΔCTLH) mutant constructs were generated in pCMV-HA-RanBPM si-mt using inverse PCR using tail-to-tail primers on each side of the region to be deleted (367-393 and 393-460, respectively). pCMV-HA-RanBPM-NLS1/NES, pHM830-WT NLS2, pHM830-MUT NES, pHM830-WT NLS2, pHM830-MUT NLS2, pHM840-1-25, pHM840-NLS1/NES, pHM840-MUT NES, pHM840-WT NLS2, pHM840-MUT NLS2, and pHM840-1-25 were produced using annealed oligos that generated overhangs that could be ligated with digested pHM830 and pHM840 ([31], obtained from Addgene). pHM830-LisH/CTLH and pHM840-LisH/CTLH was generated by PCR amplification of RanBPM (aa 360-460) digested and ligated with digested pHM830 and pHM840. All PCR reactions were done using PfuTurbo from Agilent Technologies (Mississauga, ON, Canada) or KOD polymerase
and primers from Sigma-Aldrich (Oakville, ON, Canada), BioCorp UWO Oligo Factory (London, ON, Canada) and Integrated DNA Technologies (Coralville, Iowa, USA).

2.2.2 Cell culture, transfections and treatments

Hela control shRNA and RanBPM shRNA stable cell lines (2-7 and 2-6) were described previously [29,32] and were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.35 mg/ml G418 (Geneticin, Bioshop Canada, Burlington, ON, Canada) at 37°C in 5% CO₂. 3T3 mouse embryonic fibroblasts (MEFs) were cultured in high-glucose DMEM supplemented with 10% FBS. Plasmid transfections were carried out with ExGen500 (Fermentas, Burlington, ON Canada), TurboFect Transfection Reagent (Thermo Fisher Scientific, Burlington, ON, Canada) or jetPRIME (Polypus Transfection) according to the manufacturer’s protocol. Leptomycin B (LMB, Bioshop Canada, Burlington ON, Canada) was added to the cells’ media at 20nM concentration for the times indicated in the figure legends. Nocodazole (Abcam) was added to the cell’s media at 10µM for 4 hours as previously described [33,34].

2.2.3 Extract preparation, subcellular fractionation, western blot and immunoprecipitations

Whole cell extracts were prepared as described [29] and resolved by SDS-PAGE (between 8% and 12%). Subcellular fractionation and chromatin extractions were adapted from [35]. Briefly, cells were washed twice in PBS, and lysed in buffer A (10 mM HEPES buffer (pH 7.9), 5 mM MgCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml of aprotinin, 10 µg/ml of pepstatin, 1 µg/ ml of leupeptin and 10 mM KCl), for 25 min and centrifuged at 1000 rpm for 5 min at 4°C and the supernatant (cytosolic fraction) was collected. The pellet was washed in PBS, lysed in buffer B (20 mM HEPES buffer (pH 7.9), 25% glycerol, 5 mM MgCl, 0.2% NP-40, 1 mM DTT, 1 mM PMSF, 1 µg/ml of aprotinin, 10 µg/ml of pepstatin, 1 µg/ ml of leupeptin and 150 mM KCl) for 15 min and centrifuged at 3000 rpm for 5 min at 4°C to collect the supernatant (soluble nuclear fraction). The resulting pellet was washed in PBS and lysed
in buffer C (20 mM HEPES buffer (pH 7.9), 25% glycerol, 5 mM MgCl, 0.2% NP-40, 1 mM DTT, 1 mM PMSF, 1 µg/ml of aprotinin, 10 µg/ml of peptatin, 1 µg/ml of leupeptin and 420 mM KCl) for 15 min and centrifuged at 14000 rpm for 10 min at 4°C, to yield the supernatant which is the chromatin-associated fraction. Samples were resolved on 10% SDS-PAGE and transferred on polyvinylidene difluoride (PVDF) membranes. Samples were analyzed with the following antibodies: HA (HA-7, Sigma-Aldrich), β-actin (I-19, Santa Cruz, Santa Cruz, CA, USA), RanBPM (5M, Bioacademia, Japan), α-tubulin (Sigma-Aldrich), α-tubulin (ab15246, Abcam) and Ku70 (N3H10, Santa Cruz, CA USA). Quantifications were done using ImageJ software. Co-immunoprecipitation experiments were performed in 0.5% NP-40 and 100mM KCl lysis buffer and were carried out overnight at 4°C with α-tubulin (Sigma-Aldrich). Immunoprecipitates were isolated with Dynabeads® protein G (Invitrogen, Life Technologies, Burlington, ON, Canada).

### 2.2.4 Immunofluorescence

Cells were plated on coverslips and transfected following overnight incubation. Cells transfected with pHM830 and pHM840 vectors were fixed with 3% paraformaldehyde and mounted with DAPI (Invitrogen). Cells transfected with pCMV-HA-RanBPM constructs were fixed with 3% paraformaldehyde, permeabilized in 0.5% Triton-X100 for 10 min and pre-blocked in 5% FBS diluted in PBS. Coverslips were incubated overnight with primary antibodies (see below), washed in PBS and incubated with secondary antibodies: anti-goat Alexa Fluor 488, anti-mouse Alexa Fluor 488, anti-mouse Alexa Fluor 555, anti-rabbit Alexa Fluor 647 or anti-mouse Alexa Fluor 647 (Invitrogen). Cells were mounted with ProLong® Gold antifade with DAPI (Invitrogen). Visualization was done using an Olympus BX51 microscope with a 40x objective and images were captured with the Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). Primary antibodies used in immunofluorescence: RanBPM (Ab5295, Abcam and K-12, Santa Cruz), HA (HA-7, Sigma-Aldrich), Cyclin B1 (Cell Signaling) and α-tubulin (Sigma-Aldrich). For quantification analysis, images were blinded by a third party and coded images were scored independently by two individuals. For each treatment, at least 100 cells (for HA-RanBPM mutant analyses) or 50 cells (for pHM830/pHM840 analyses)
per sample were scored by each individual and results were averaged from at least three separate experiments. Quantitative subcellular localization was performed using ImageJ. Whole cell and nuclear fluorescence signal intensity was measured and subtracted to calculate cytoplasmic fluorescence signal intensity. Nuclear and cytoplasmic intensity was calculated as a percent of whole cell intensity. Confocal images were acquired using an inverted IX51 Olympus microscope equipped with a Perkin Elmer Spinning Disk Confocal attachment with a 60x objective using Velocity software and image analyses were done using Imaris software (Bitplane, Zurich, Switzerland).

2.2.5 Statistical analyses

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

2.3 Results

2.3.1 Analysis of RanBPM deletion mutants

To start evaluating the regions of RanBPM that regulate its subcellular localization, we engineered a series of deletion mutants lacking N-terminal, C-terminal and internal domain regions (Figures 2-1 to 2-3). All constructs contained an N-terminal HA tag to assess expression and subcellular localization by indirect immunofluorescence. Mutants were transiently transfected in Hela cells stably expressing a RanBPM shRNA (clone 2-7), in which we have previously shown that RanBPM expression is effectively downregulated to near undetectable levels [29,32,36]. The design of this strategy was prompted by the fact that previous studies have documented that the LisH domain can mediate protein dimer and tetramer formation [7-9]. Indeed a report suggested that RanBPM is able to form homo-dimeric or -multimeric complexes [37]. We reasoned that if this were to occur, the RanBPM mutants that retain the LisH domain would not show a substantial change in subcellular localization upon transfection in normal Hela cells as they would be localized based on their interaction with endogenous RanBPM. Thus, expressing the RanBPM mutants in cells lacking endogenous RanBPM would circumvent this possible limitation and also minimize potential artefacts arising from overexpression
of the RanBPM protein. To prevent degradation of the transfected constructs by the RanBPM siRNA (which targets a specific sequence located in the extreme C-terminal region, Figure 2-1A), all mutants containing the C-terminal region comprised a point mutation in the sequence targeted by the siRNA, as previously described [29].

To quantify RanBPM localization through indirect immunofluorescence, we employed a localization scoring protocol that we have described previously [29]. Using this approach, RanBPM full-length (wild-type, WT) expressed in RanBPM shRNA cells was determined to present nuclear and cytoplasmic distribution similar to what has been reported for endogenous RanBPM (Figure 2-1) [29]. To verify the accuracy of our scoring evaluation, we repeated our measurements of nucleocytoplasmic distribution by quantifying the signal intensity of the whole cell and the nuclear compartment and calculating the resulting cytoplasmic intensity (Supplementary Figure 2-1A). This yielded a similar distribution for WT RanBPM and two RanBPM mutants (described later in Figures 2-2 and 2-4).

Progressive deletion of the C-terminal region (ΔC1-C4) did not alter this nuclear and cytoplasmic distribution, suggesting that the C-terminal region does not primarily contribute to RanBPM subcellular localization (Figure 2-1).

N-terminal deletions revealed to have a much greater impact on regulating RanBPM localization. First, a large (251 aa) N-terminal deletion (ΔN1) resulted in a near complete relocalization of RanBPM to the nucleus (Figure 2-2B,C). Smaller deletions resulting in truncation of the first 138 and 209 aa (ΔN3 and ΔN4) had a similar effect. However, deletion of the first 101 aa (ΔN2) had a completely reverse effect, resulting in near complete cytoplasmic localization (Figure 2-2B,C). This suggested that while the first 101 aa contain sequences required for RanBPM nuclear localization, elements in RanBPM sequences C-terminal to aa 251 can also promote nuclear localization. Interestingly, the region included between aa 102 and aa 138 appeared to contain elements that retain or promote RanBPM localization to the cytoplasm, since its deletion elicited RanBPM relocalization to the nucleus.
Figure 2-1 Deletion of RanBPM C-terminus does not alter its subcellular localization.

A) Schematic diagram of full length wild-type (WT) human RanBPM. The conserved domains are indicated. The red asterisk represents the point mutations conferring siRNA resistance. B) Schematic diagram of C-terminal mutant RanBPM constructs. C) Analysis of RanBPM deletion mutant subcellular localization. Hela RanBPM shRNA cells fixed 24h after transfection were incubated with an HA antibody and then with an Alexa Fluor 555 secondary antibody. Nuclei were stained with DAPI. Subcellular localization was scored as either, N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent standard deviation (SD). Mutant RanBPM constructs versus WT, ***, P<0.001; **, P<0.01; *, P<0.05. D) Representative images of transfected mutant RanBPM localization. Scale bar: 10µm.
**Figure 2-2 RanBPM N-terminal region contains several determinants that regulate its subcellular localization.**

A) Schematic diagram of N-terminal mutant RanBPM constructs. B) Mutant RanBPM (ΔN1-4) were transfected and scored as described in Figure 2-1. Data represent averages from three separate experiments, each assessing a minimum of 100 cells. Error bars represent SD. Mutant RanBPM constructs versus WT, ***, P<0.001; **, P<0.01; *, P<0.05. C) Representative images of transfected mutant RanBPM localization quantified in B. D) Identification of a nuclear targeting sequence in the extreme N-terminal region. Mutants with various deletions of N-terminal sequences (as indicated) were transfected and processed as above. E) Representative images of the localization of the transfected mutant RanBPM quantified in D. Scale bar: 10μm.
To further investigate which region between aa 1 and 102 is responsible for nuclear localization, smaller deletions within this region were generated (Figure 2-2). Deletion of the first 66 amino acids (Δ1-66) resulted in an increased cytoplasmic localization similar to that of RanBPM ΔN2, suggesting that nuclear localization determinants were located in the very N-terminal region of the protein (Figure 2-2D, E). A smaller deletion of the first 25 amino acids (Δ1-25) still resulted in decreased nuclear and increased cytoplasmic localization, suggesting that the first 25 amino acids contain determinants that direct or retain RanBPM in the nucleus.

We next examined the potential contribution of the SPRY and LisH/CTLH domains to RanBPM subcellular localization. Internal deletion of the SPRY domain (Δ212) resulted in increased nuclear localization compared to WT suggesting that this region plays a role in cytoplasmic targeting of the protein (Figure 2-3). The SPRY domain is known to mediate protein-protein interactions [6], suggesting that interaction of RanBPM through this domain with yet unidentified partner(s) may be responsible for cytoplasmic retention. In addition, and in contrast to full length RanBPM and all other mutants examined so far which showed diffuse staining in both the nucleus and cytoplasm, most cells expressing RanBPM Δ212 displayed a speckled nuclear staining, with small aggregates present throughout the nucleus. These aggregates could be the result of misfolding, indicating that this domain may also be needed for correct folding of the protein.

Deletion of the LisH/CTLH domains (Δ360) also resulted in predominant nuclear redistribution of RanBPM, suggesting that this region promotes cytoplasmic localization. Individual deletions of either the LisH or the CTLH domain did not significantly alter RanBPM nucleocytoplasmic distribution (Figure 2-3B,C). Altogether, it appears that disruption of both the LisH and CTLH domains perturbs RanBPM subcellular localization by promoting its recruitment to the nucleus.

To confirm that the localization of the RanBPM mutants was not influenced by cell-specific properties of the clonal cell line expressing RanBPM shRNA, the subcellular localization of RanBPM WT, ΔN2 and Δ360 was assayed in another RanBPM shRNA cell line (RanBPM shRNA 2-6). The results revealed no differences in the subcellular localization.
Figure 2-3 Deletion of RanBPM SPRY and LisH/CTLH domains promotes RanBPM nuclear localization.

A) Schematic diagram of internal deletion mutant RanBPM constructs. B) Cells were fixed 24h after transfection of the RanBPM mutants indicated and incubated with an HA antibody and then with an Alexa Fluor 555 secondary antibody. Nuclei were stained with DAPI. Subcellular localization was scored as either, N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. Mutant RanBPM constructs versus WT, ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$. C) Representative images of transfected mutant RanBPM localization. Scale bar: 10µm.
localization of these mutants between the two clonal cell lines (Supplementary Figure 2-1B).

2.3.2 Identification of RanBPM NLS and NES motifs

RanBPM deletion mutant analyses indicated that several regions of RanBPM are involved in regulating its subcellular localization. The RanBPM C-terminal deletions did not affect RanBPM nucleocytoplasmic distribution, which initially suggested that this region did not contain elements contributing to the localization of the protein. However, intriguingly, various deletions of the N-terminal region (ΔN1, ΔN3, ΔN4) caused the relocalization of RanBPM to the nuclear compartment (Figure 2-2). This suggested that the C-terminal region contains elements directing RanBPM in the nucleus, even though these may be subordinate to primary nuclear localization signals present in the N-terminus. A search for clusters of basic residues (which are typical of NLS) revealed two potential NLS for RanBPM, in the N-terminus (NLS1, 140-155) and in the C-terminus (NLS2, 635-649) (Figure 2-4A). Interestingly, the N-terminal potential NLS1 also features characteristics of a leucine-rich NES, suggesting that this element could be conferring both import and export properties, which has been previously reported for similar sequences [38,39].

Point mutations of residues assumed to confer either nuclear (K/R) or cytoplasmic (L) localization in the putative NES/NLS1 (NLS1 mut and NES mut) in the N-terminal region of the protein resulted in both cases in near complete relocalization of RanBPM to the nucleus, suggesting that this element functions as a NES (Figure 2-4B,C). Introduction of point mutations in the putative C-terminal NLS (NLS2 mut) resulted in a slight, albeit significant decrease in nuclear localization (Figure 2-4B,C). However this mutant still displayed predominant nucleocytoplasmic localization, suggesting that this element only partly contributes to RanBPM nuclear targeting. To further characterize these motifs, we investigated whether they were able to confer specific localization to a Green Fluorescent Protein-β-galactosidase (GFP-β-gal) fusion protein. For these experiments, we subcloned RanBPM motifs in the pHM830 and pHM840 vectors which encode a GFP-β-gal fusion protein. When expressed from pHM830 (830 EV), GFP-β-gal is cytoplasmic as its size precludes passive diffusion to the nucleus, but addition of a
**Figure 2-4 Identification of RanBPM NLS and NES.**

**A)** Schematic diagram indicating the position of RanBPM putative NLS and NES which are represented by green and yellow flags, respectively. The amino acid sequence of these elements is indicated below, with the WT sequences on the left, and the mutated sequences on the right. Conserved basic residues are underlined, the leucines present in the putative NES are boxed and the alanine point mutations are marked in red. The asterisk represents the point mutations conferring siRNA resistance. **B)** Cells were transfected with the RanBPM mutants indicated and immunofluorescence and scoring was performed as described in Figure 2-1. Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. Mutant RanBPM constructs versus WT, ***, P<0.001; **, P<0.01; *, P<0.05. **C)** Representative images of transfected mutant RanBPM localization. Scale bar: 10µm.
Simian virus 40 (SV40) NLS sequence in the pHM840 (840 EV) vector promotes its localization to the nucleus [31] (Figure 2-5A). Fusion of the RanBPM NLS1/NES sequence in pHM830 (830 WT NLS1/NES) did not affect GFP-β-gal cytoplasmic localization (Figure 2-5B). However, NLS1/NES was able to promote cytoplasmic export of the nuclear GFP-β-gal expressed from pHM840 (840 WT NLS1/NES), and this was prevented by point mutations in this element (MUT NES, Figure 2-5C), confirming that this element functions as a NES. Interestingly, the NLS2 sequence in the C-terminal region of RanBPM was able to promote nuclear localization of cytoplasmic GFP-β-gal (Figure 2-5D), and this was prevented by point mutations of three basic residues (MUT NLS2, Figure 2-5E). This suggested that this element can function as a NLS, even though its deletion or mutation in the context of the RanBPM protein only mildly affects RanBPM recruitment to the nucleus.

Consistent with the results of the RanBPM deletion mutant experiments, RanBPM 1-25 was able to direct GFP-β-gal to the nucleus, thus suggesting the presence of a nuclear targeting element in this region (Figure 2-5F). Altogether, these analyses suggest that RanBPM possesses two elements capable of conferring nuclear localization. However, only the 1-25 NLS appears to efficiently function to direct RanBPM in the nucleus. While NLS2 has the properties of a NLS, it appears to be marginally functional in the context of the full-length RanBPM protein.

Finally, the RanBPM LisH/CTLH domain was able to prevent nuclear localization of the nuclear GFP-β-gal expressed from pHM840 (840 LisH/CTLH), suggesting that the LisH/CTLH domain promotes localization to the cytoplasm, possibly by conferring cytoplasmic retention (Figure 2-5G).

These results suggested a very complex regulation of RanBPM cytoplasmic localization since deletions of the SPRY and the LisH/CTLH domains as well as mutation of the NES sequence all resulted individually to a relocalization of RanBPM to the nucleus. To determine whether RanBPM cytoplasmic localization was subject to a CRM1-export dependent regulation, we treated cells with leptomycin B (LMB) to inhibit CRM1-dependent nuclear export. Surprisingly, standard LMB treatment (20nM, 3h) had no
Figure 2-5 Characterization of RanBPM motifs that confer nuclear or cytoplasmic localization of GFP-β-gal.

A-G) RanBPM shRNA Hela cells were transfected with either pHM830 (830) or pHM840 (840) empty vectors (EV) or vectors containing various motifs or domains of RanBPM fused to GFP-β-gal. The identity of the RanBPM motif/domain fused to GFP-β-gal is indicated above each panel. Left, pHM830 fusion constructs, right, pHM840 fusion constructs. Cells were fixed 24 hours after transfection and nuclei stained with DAPI. GFP-β-gal subcellular localization was scored as either N>>C (completely nuclear), N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), C>N (cytoplasmic greater than nuclear, or C>>N (completely cytoplasmic). Data represent averages from three separate experiments, each assessing a minimum of 50 cells. Error bars represent SD. RanBPM motifs versus EV, ***, P<0.001; **, P<0.01; *, P<0.05. Inset, representative images of transfected pHM830 or 840 fusion constructs alone (EV) or subcloned with RanBPM motifs. Scale bar: 10µm.
effect on RanBPM localization, while it significantly affected cyclin B1, which undergoes LMB-sensitive nuclear export [40] and accumulated in the nucleus under the same conditions (Supplementary Figure 2-2). However, treatment of 840 WT NLS1/NES with LMB prevented cytoplasmic accumulation of GFP-β-gal and resulted in its nuclear localization, which suggested that the RanBPM NES activity in this context is sensitive to CRM1 inhibition (Figure 2-6A). One possibility to explain these seemingly contradictory results is that only a small fraction of RanBPM is shuttling and thus longer treatment would be needed to reach a detectable accumulation in the nucleus. Thus, we tested the effect of prolonged LMB treatment on endogenous RanBPM localization and found that 16h incubation with LMB induced a significant nuclear accumulation of RanBPM (74.1%) when compared to vehicle treatment (42.6%) (Figure 2-6B). These results suggest that RanBPM undergoes CRM1-dependent nuclear export but that the activity of the NES is limited in the context of the RanBPM protein possibly due to the fact that a large proportion of the RanBPM cellular pool is not actively shuttling. Altogether, it appears that RanBPM cytoplasmic localization is dependent on the integrated action of several domains and sequences and that the function of these regulatory regions may be modulated by specific RanBPM protein folding which remains uncharacterized.

2.3.3 Effect of RanBPM deletions on protein expression

To assess the effect of RanBPM deletions on protein expression, we analyzed the levels of expression of all mutants by western blot. As previously reported, endogenous RanBPM expression is severely reduced in RanBPM shRNA cells (Figure 2-7A) [29,32,36]. RanBPM mutants bearing either C-terminal deletions or internal deletions of the SPRY and LisH/CTLH domains were found expressed at levels similar to WT (Figure 2-7B,D,E). However, N-terminal mutants showed significantly reduced expression levels, particularly the ΔN1, ΔN3 and ΔN4 deletion mutants, while ΔN2 expression was somewhat decreased (Figure 2-7C). It should be noted that, as reported previously for ΔN1 and ΔN2 [29], the expression levels of the ΔN1-4 mutants in individual cells was not noticeably different than WT RanBPM or any other mutant and this is reflected by the fact that exposure times for image capture was similar for all
Figure 2-6 Effect of Leptomycin B (LMB) treatment on RanBPM nuclear export.

A) RanBPM shRNA Hela cells transfected with 840 WT NLS1/NES were treated with ethanol (EtOH) or 20nM LMB, fixed following 3h of treatment and stained with DAPI. Subcellular localization was scored as either N>>C (completely nuclear), N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), C>N (cytoplasmic greater than nuclear, or C>>N (completely cytoplasmic). Data represent averages from three separate experiments, each assessing approximately 50 cells. Error bars represent SD. ***，P<0.001; **，P<0.01; *，P<0.05. B) Hela cells were treated with EtOH or 20nM LMB and incubated for 16h. Cells were fixed and processed for immunostaining with antibodies to RanBPM and cyclin B1 and nuclei stained with DAPI. At least 100 cells were scored as N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. Scale bar: 10µm.
Figure 2-7 Representative Western blot analysis of endogenous RanBPM in control and RanBPM shRNA cell lines and RanBPM protein expression.

A) Left, Whole cell extracts prepared from control and RanBPM shRNA cells were analyzed by western blot with a RanBPM antibody. Right, quantification of endogenous RanBPM normalized to β-actin loading control. Data represent averages from three experiments. Error bars represent standard deviation. B) Expression of wild-type (WT) and ΔC deletion constructs. Left, whole cell extracts were prepared from RanBPM shRNA Hela cells transfected with pCMV-HA-RanBPM WT and mutant constructs 24h after transfection. An HA antibody was used to detect HA-RanBPM and a β-actin antibody was used as a loading control. Right, quantification of pCMV-HA-RanBPM constructs normalized to β-actin loading control. Data represent averages from three experiments. Error bars represent standard deviation. Mutant RanBPM constructs versus WT, ***, P<0.001; **, P<0.01; *, P<0.05. C) Expression of RanBPM ΔN deletion constructs. Analysis and quantifications are as described above. D) Expression of RanBPM Δ1-66, Δ1-25, ΔLisH and ΔCTLH. Analysis and quantifications as described in B. E) Expression of Δ212, Δ360 and point mutation constructs. Analysis and quantifications as described in B.
A

Control shRNA | RanBPM shRNA

RanBPM

43-

β-Actin

B

WT ΔC1 ΔC2 ΔC3 ΔC4

HA-RanBPM

95-

72-

55-

43-

β-Actin

C

WT ΔN1 ΔN2 ΔN3 ΔN4

HA-RanBPM

95-

72-

55-

43-

β-Actin

D

WT ΔN2 Δ1-66 Δ1-25 WT ΔLisH ΔCTLH

HA-RanBPM

95-

72-

55-

43-

β-Actin

E

WT ΔA21 ΔA25 ΔLisH ΔCTLH

HA-RanBPM

95-

43-

β-Actin

Normalized RanBPM Expression

Control shRNA RanBPM shRNA

WT ΔN1 ΔN2 ΔN3 ΔN4

HA-RanBPM mutant expression

WT Δ1-66 Δ1-25 ΔLisH ΔCTLH

HA-RanBPM mutant expression

WT ΔA21 ΔA25 ΔLisH ΔCTLH

HA-RanBPM mutant expression

0.0 0.5 1.0 1.5

0 1 2 3 4

* * * * * *
mutants. However, we consistently obtained a lower number of cells transfected with the ΔN1-4 mutants compared to the other mutants, which explains the lower level of expression of these mutants observed by western blot analysis, although the reason for this phenomenon remains unclear. As all mutants are ectopically expressed from the same promoter and the extreme N-terminal deletions (Δ1-66, Δ1-25 Figure 2-7D) resulted in protein levels comparable to WT, this suggests that sequences between 102 and 139 are particularly important for this effect. Also, while all the low expressing mutants are predominantly nuclear, localization of RanBPM in the nucleus is likely not the cause of the reduced expression, as other mutants comprising domain deletions that re-localize RanBPM to the nucleus, such as Δ212 and Δ360 are expressed at levels comparable to WT (Figure 2-7E). Finally, point mutations in either NES or NLS2 did not affect expression (Figure 2-7E). A summary of level of expression and subcellular localization of the mutants tested in this study is shown in Table 2-1.

Examination of the N-terminal region revealed the presence of two putative USP7 (ubiquitin-specific protease 7) binding sites at residues 39-42 and 125-128. USP7, also known as HAUSP (herpes virus associated ubiquitin-specific protease), is a member of the superfamily of deubiquitinating enzymes that are responsible for the removal of ubiquitin from their target proteins [41]. USP7 was previously shown to interact with and stabilize p53 and Mdm2 [42]. USP7 interacts with p53 at two closely spaced USP7 binding sites and both sites are needed for USP7 binding [43]. Therefore USP7 N-terminal sites appeared to be good candidates to regulate RanBPM stability. One or both USP7 sites were mutated by site-directed mutagenesis resulting in point mutations or deletion of the USP7 binding consensus sequences, and protein expression was assessed by western blot analysis (Supplementary Figure 2-3). Since all resulting mutants were expressed at levels similar to that of wild-type, we concluded that the USP7 sites do not regulate RanBPM protein expression.

Next, we examined WW binding motifs present at positions 17-22 and 117-122 of RanBPM. Binding motifs of class IV WW domains such as the ones found at these positions are characterized by two proline residues and a phosphorylated serine/threonine
Table 2-1 Summary of protein expression and subcellular localization for RanBPM mutants.

<table>
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<tr>
<th>Mutant</th>
<th>Expression</th>
<th>Subcellular Localization</th>
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<tr>
<td>WT</td>
<td>***</td>
<td>N=C</td>
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<tr>
<td>ΔC1</td>
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<td>ΔC2</td>
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<td>ΔN1</td>
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<td>N&gt;C</td>
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<tr>
<td>ΔN2</td>
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<td>ΔN4</td>
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<tr>
<td>Δ1-66</td>
<td>***</td>
<td>C&gt;N</td>
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<td>Δ1-25</td>
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<td>Δ360</td>
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<td>ΔLisH</td>
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<td>N=C</td>
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<tr>
<td>ΔCTLH</td>
<td>***</td>
<td>N&gt;C</td>
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<tr>
<td>NLS1 Mut (150, 152, 153)</td>
<td>***</td>
<td>N&gt;C</td>
</tr>
<tr>
<td>NES Mut (147, 151)</td>
<td>***</td>
<td>N&gt;C</td>
</tr>
<tr>
<td>NLS2 Mut (635, 636, 640)</td>
<td>***</td>
<td>N=C</td>
</tr>
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</table>

*low, **medium ***high expression, N=C nucleocytoplasmic, N>C mostly nuclear, C>N mostly cytoplasmic
residue [44]. This particular motif was chosen because class IV WW motifs have been shown to be a target of the peptidyl-prolyl cis-trans isomerase Pin-1 and isomerization of these WW motifs is key as kinases, phosphatases and ubiquitin ligases specifically recognize the cis or trans conformation of the prolyl peptide bond of their substrate, ultimately resulting in modulation of protein stability [45,46]. Mutation of critical residues within both WW motifs resulted in protein expression similar to that of WT RanBPM (Supplementary Figure 2-3), suggesting that these WW motifs do not affect RanBPM protein expression.

2.3.4 RanBPM is associated with microtubules in the cytoplasm and with chromatin in the nucleus

We next investigated whether RanBPM is associated with particular structures in the nucleus and in the cytoplasm. Several lines of evidence suggest that RanBPM may be associated with microtubules. First, the LisH/CTLH domain is a domain present in proteins that are associated with microtubules [8,9]. In addition, RanBPM was reported to cofractionate with components of microtubules, such as dynactin and dynein [47]. Moreover, our findings that the LisH/CTLH domain can promote cytoplasmic localization of a nuclear protein and that deletion of the RanBPM LisH/CTLH domain results in its nuclear accumulation suggested that this domain could be mediating cytoplasmic retention through microtubule binding. Thus we investigated whether RanBPM colocalizes with α-tubulin, a main component of microtubules [48] using confocal microscopy analyses of endogenous RanBPM and α-tubulin (Figure 2-8). RanBPM displayed a punctate pattern throughout the cytoplasm which indeed revealed partial colocalization with α-tubulin at specific microtubule structures in both Hela (Figure 2-8A) and 3T3 mouse embryonic fibroblasts (MEFs, Figure 2-8B). To substantiate the colocalization of RanBPM with α-tubulin, we performed co-immunoprecipitation experiments which revealed that endogenous RanBPM co-immunoprecipitated with endogenous α-tubulin (Figure 2-8C). To determine whether microtubule depolymerization would affect RanBPM subcellular localization, we analyzed the effect of microtubule disruption on RanBPM subcellular localization. Treatment with nocodazole, which interferes with microtubule polymerization,
Figure 2-8 RanBPM is associated with microtubules.

A) Hela and B) 3T3 MEFs were fixed and incubated with antibodies against RanBPM and α-tubulin. Shown are single plane confocal images. Insets are enlarged images of the boxed regions from the above panels and arrows indicate areas of colocalization. The right panels show merged images (RanBPM, green; α-tubulin, red). Scale bar: 10µm. C) Hela whole cell extracts were incubated with either an α-tubulin antibody or mouse IgG control. Immunoprecipitates were analyzed by western blot using RanBPM and α-tubulin antibodies and compared with 5% of input proteins.
surprisingly did not alter the subcellular localization of RanBPM (Appendix A), indicating that RanBPM cytoplasmic localization is not sensitive to microtubule depolymerization. Overall, these data show that cytoplasmic RanBPM is partially associated with microtubules and suggest that RanBPM cytoplasmic localization could be conferred, at least in part, through retention of RanBPM via microtubule interaction.

To assess the status of RanBPM in the nucleus, we performed subcellular fractionations. Consistent with our immunofluorescence evaluations and previous analyses [22,29], quantification of endogenous RanBPM in Hela cytoplasmic and nuclear fractions showed that about 70% of RanBPM was present in the nucleus versus 30% in the cytoplasm (Figure 2-9A). While most of nuclear RanBPM was present in the nuclear soluble fraction, approximately 20% of RanBPM was detected in the chromatin fraction, suggesting its association with DNA. We obtained identical results with ectopically expressed HA-RanBPM in Hela RanBPM shRNA cells (Figure 2-9B). Overall, these results suggest that RanBPM can associate with microtubules in the cytoplasm and with chromatin in the nucleus.

2.4 Discussion

Determining the physiological role played by RanBPM and its associated CTLH complex requires a detailed understanding of how its subcellular localization is regulated and contributes to its activity. In this study, we have investigated the contribution of various RanBPM protein regions to its subcellular localization using RanBPM shRNA cells, which allowed the analysis of ectopically expressed RanBPM mutants without interference from the endogenously expressed protein. Our results reveal that multiple regions and motifs regulate RanBPM nuclear and cytoplasmic localization and that in particular the RanBPM N-terminal domain is critical for both localization and protein stability.

2.4.1 RanBPM determinants of nuclear localization

We determined that RanBPM possesses two motifs that confer nuclear localization: one sequence present at the extreme N-terminus (aa 1-25, hereafter called 1-25 poly-P/Q motif), and a non-canonical NLS located near the C-terminus (NLS2, Figure 2-4). The 1-
Figure 2-9 Quantitative Analysis of RanBPM in cytoplasmic and nuclear fractions and its association with chromatin.

A) Hela cell extracts were partitioned in cytoplasmic, nuclear soluble and chromatin fractions as described in Materials and Methods. Proportional amounts of each fraction were analyzed by western blot with the indicated antibodies. *Left*, representative western blot, with the percentage of each fraction loaded indicated above each lane. *Right*, bar graph representing the percentage of RanBPM protein present in each fraction. Data represent averages from three separate experiments with error bar representing SD. B) Hela RanBPM shRNA cells were transfected with pCMV-HA-WT-RanBPM and processed as in A.
25 poly-P/Q motif functions as a NLS as it was able to promote nuclear localization of a cytoplasmic GFP-β-gal fusion construct. This region contains two proline stretches flanking a series of glutamines (Supplementary Figure 2-4), but, to our knowledge, does not contain any sequence matching previously characterized NLS consensus. The most likely possibility is that this region interacts with a partner protein that promotes its translocation to the nucleus. A search for conserved motifs indicates that the 1-25 poly-P/Q motif contains four non-canonical class I-type SH3 binding motifs, and one overlapping WW class IV Peptidyl-Prolyl Isomerase (Pin1) binding motif. Future investigations will be needed to identify protein(s) interacting with this region. It is worth noting that Pin1 has been shown to promote nuclear localization of Rel proteins [49] and the adenosine deaminase ADAR2 [50], however how this is achieved remains unknown.

The RanBPM aa 635-649 sequence (NLS2) appeared to present some of the characteristics of a bi-partite NLS, with two clusters of basic amino acids. However, while mutations in the first cluster (R635A, R656A and K640A) did inhibit its ability to direct cytoplasmic GFP-β-gal to the nucleus, surprisingly, alanine substitutions of the three lysines in the second cluster of the motif (K645A, K646A and K649V) did not prevent NLS activity (Appendix B). This suggests that NLS2 activity is dependent on determinants present in the first cluster of basic residues, in the sequence “RRDCGK”. Interestingly, this motif has similarities to that of a non-canonical NLS identified in a BRCA1 splice variant (KRAAER) [51], with two basic residues separated from a third basic residue by three amino acids.

Both the 1-25 poly-P/Q motif and NLS2 elements were able to direct a cytoplasmic GFP-β-gal construct to the nucleus. However, our results suggest that RanBPM nuclear localization is primarily dependent on the 1-25 poly-P/Q motif and that the C-terminal NLS2 does not appreciably contribute to RanBPM localization in normally cycling cells, conditions used in our experiments. This conclusion is supported by two observations: first, deletion of the C-terminal region containing NLS2 and mutation of the NLS2 sequence had little effect on RanBPM subcellular localization, and second, deletion of aa 1-25 poly-P/Q motif prevented RanBPM nuclear localization. One possibility to explain why NLS2 is not functional in the context of the full length RanBPM protein is that it is
masked by protein folding. Indeed, we observed a dramatic shift in RanBPM localization from cytoplasmic to nuclear upon deletion of the 102-138 region (in the ΔN3 mutant) compared to the ΔN2 mutant. Thus, we postulate that this region normally folds over RanBPM C-terminus and masks NLS2, and that its deletion relieves the inhibition on NLS2. This folding could serve to modulate the activity of sequences such as NLS2 that are present in the C-terminal region in response to stress or physiological stimuli. A previous study identified this element as a putative NLS [37]. This study also reported that an N-terminal proteolytic fragment of RanBPM (aa 1-392) displayed cytoplasmic localization and this was attributed to the loss of that element in the C-terminal region of the protein. Since we have demonstrated that NLS2 is not imperative for nuclear localization, this is unlikely to be the case, however the reason for the cytoplasmic localization of this proteolytic fragment remains to be elucidated.

Our analysis showed that about 20% of total RanBPM, which represents about a third of the RanBPM nuclear pool, is associated with chromatin. RanBPM does not appear to comprise sequences conferring DNA binding properties, thus we postulate that its association with chromatin is mediated through interaction(s) with chromatin-associated partners. RanBPM was previously reported to interact with the Transcription Factor IID (TFIID) subunit TAF4 and with the glucocorticoid, androgen and thyroid receptors [11,13,14]. The thyroid receptor was suggested to interact with the C-terminal region of RanBPM [13], but the localization of the complex was not evaluated. Interestingly, the interaction of p73 with RanBPM was shown to promote RanBPM localization to the nucleus [12]. Whether and how RanBPM affects genomic regulations and DNA metabolism and whether this is in the context of the CTLH complex or in association with other proteins will need to be investigated.

2.4.2 RanBPM in the cytoplasm

We have identified three motifs or domains that promote cytoplasmic localization: a bona fide NES, the SPRY domain and the LisH/CTLH domain. RanBPM NES presents the characteristics of a typical leucine-rich motif [52], but we also noted the presence of basic amino acids which led us to hypothesize that it may also function as a NLS. However, this element was only able to direct cytoplasmic localization of a nuclear GFP-β-gal
fusion construct, suggesting that it only functions as a NES. We showed that this NES is readily sensitive to LMB in isolation (GFP-β-gal) which is further typical of leucine-rich NES which are CRM1/Exportin1-dependent [2,52]. But, while mutation of the NES promoted RanBPM nuclear accumulation, endogenous RanBPM was only sensitive to LMB treatment when subjected to LMB for longer periods of time, suggesting that only a small fraction of RanBPM is actively shuttling. In parallel, we found that deletion of both SPRY and LisH/CTLH domains promoted RanBPM nuclear accumulation. Since neither domain contains any identifiable NES, cytoplasmic localization through these domains most likely occurs through protein-protein interactions. As we have shown that RanBPM is associated with microtubules, it is possible that the LisH/CTLH domain could mediate RanBPM recruitment to microtubules and that this serves to retain RanBPM in the cytoplasm. As for SPRY domains, they are found in a wide array of proteins and are known to engage in protein-protein interactions. SPRY domain-containing proteins have been suggested to function as adaptors and play roles as scaffold proteins in a variety of signaling pathways [6]. Several proteins have been shown to interact with RanBPM through the SPRY domain in both the nuclear and cytoplasmic compartments. In the nucleus RanBPM has been shown to interact with cyclin-dependent kinase 11 CDK11(p46) [53], the immediate-early protein Rta of Epstein-Barr virus [54], and the ubiquitin-specific protease USP11 [55]. The SPRY domain of RanBPM has been demonstrated to interact with cytoplasmic or membrane bound proteins such as the TNF receptor associated factor TRAF6 [56], the receptor tyrosine kinase MET [57], the neural cell adhesion molecule L1 [58], and the neurotrophin receptor TrkA [59]. However, the contribution of these interactions to RanBPM subcellular localization was not investigated. Our results imply that the SPRY domain functions as a cytoplasmic restraint, suggesting that it mediates interaction of RanBPM with cytoplasmic protein(s), although this remains to be confirmed.

RanBPM has long been suspected to associate with microtubules, and was previously reported to cofractionate with components of the microtubules dynein and dyactin and dynamitin [47]. We show here that RanBPM indeed colocalizes and associates with α-tubulin. However, it is not clear whether this reflects a direct interaction with microtubule components, or if it is due to RanBPM association with microtubule-interacting proteins.
Studies of the microtubule motor-regulating protein LIS1, the most extensively studied LisH-containing protein, suggest that the N-terminal LisH domain of LIS1, which is necessary for microtubule association, is not involved in dynein binding (which occurs through the C-terminal of LIS1), but that dimerization of LIS1 through the LisH domain is essential for dynein motility [60]. Therefore, we speculate that RanBPM is associating with microtubules through the LisH domain, however this remains to be determined. Interestingly, we recently reported that RanBPM forms a complex with the histone deacetylase HDAC6 [30]. HDAC6 is a microtubule-associated deacetylase that regulates α-tubulin acetylation and participates in microtubule metabolism [61-63], so the possibility exists that RanBPM is recruited to microtubules through HDAC6.

### 2.4.3 Regulatory function of the N-terminal domain

RanBPM N-terminal region has a dual function in regulating RanBPM subcellular localization as it harbours both nuclear targeting and nuclear export sequences. Interestingly, in addition to modulating subcellular localization, the N-terminal 102-139 region also appears to affect protein stability. Progressive deletion of the N-terminal sequences resulted in a gradual decrease in protein expression, most notably when deleting the 102-139 region (ΔN3). Mutations in this region (WW and USP7 motifs) did not affect protein stability, suggesting that it is not the sequences per se that are important but that the region may function intramolecularly to regulate stability and localization.

One possibility to explain the changes in protein stability and subcellular localization observed with N-terminal deletions is that the RanBPM C-terminus is unstable/unfolded in the absence of the N-terminal region. In support of this, a previous study documented that C-terminal fragments of RanBPM (corresponding to the C-terminal 350 aa) were very weakly expressed when transfected in mammalian cells [15]. The RanBPM N-terminal region contains several amino acid repeats (proline, glutamine, alanine) that are characteristic of a low complexity regions (LCR) predicted to be unstructured [64]. LCRs are often found in ‘hub’ proteins and C-terminal LCRs have been predicted to have high levels of connectivity and are enriched in stress-response proteins [65]. While the function of the RanBPM LCR is unknown, our data suggest that it is critical for RanBPM stability and subcellular localization.
It is puzzling that the deletion of the 102-139 (ΔN3) results in significant accumulation of RanBPM in the nucleus, despite the presence of the NES and the central domains, SPRY and LisH/CTLH, which would be expected to collectively allow cytoplasmic localization. Moreover, removal of the NES in the ΔN4 mutant and further deletion encompassing part of the SPRY domain (ΔN1) only marginally increased nuclear localization. Since this prominent nuclear localization occurs concurrently with the decrease in the level of protein expression, it is possible that the deletion of the N-terminal domain promotes the degradation of the cytoplasmic pool of these mutants, leaving the nuclear fraction somewhat stable.

RanBPM is well conserved between eukaryotic species, with the SPRY, LisH, CTLH and CRA domains being conserved throughout eukaryotes [28]. Both the NES identified within residues 140 and 155 and NLS2 comprising residues 635 and 649 are well conserved in mammals and in chordates in general, while in arthropods, such as Drosophila, these elements are partially conserved. The N-terminal domain however is the least conserved region of the protein. In particular, the poly-Q and poly-P repeats present in the human RanBPM N-terminus are not found in most homologs. Therefore the RanBPM 1-25 poly-P/Q motif is not present in other species, except in the mouse homolog where it is partially conserved [66]. Thus the regulations conferred by the 1-25 poly-P/Q region and the N-terminal region in general may only occur in human and possibly in mouse RanBPM, perhaps allowing NLS2 to be the predominant NLS in other species. In the S. cerevisiae RanBPM homolog Gid1, the domains are conserved however the NLS/NES motifs that we identified are not conserved.

Finally, one major element that may be contributing to RanBPM localization is its interaction with members of the CTLH complex. All components of the CTLH complex have been shown to be present within both the nuclear and cytoplasmic compartments with the exception of MAEA (Macrophage Erythroblast Attacher, also called p48EMLP, or EMP), which is only present in the nucleus and muskelin, which is mostly cytoplasmic [15,22]. These two proteins have been demonstrated to influence the localization of the other complex components. Ectopic expression of MAEA was shown to trigger increased recruitment of Twa1, the armadillo-repeat protein ARMC8α and RanBPM to the nucleus
MAEA has been shown to contain a putative NLS between aa 110-113, however whether this element is a functional NLS that imports MAEA to the nucleus has not been determined [67]. Conversely, overexpression of muskelin resulted in cytoplasmic localization of Twa1, ARMC8α and RanBPM [22]. Previous analyses suggested that the subcellular distribution of muskelin is also modulated by several domains, a C-terminal domain that restrains it in the cytoplasm and a LisH domain that, contrary to that of RanBPM, has nuclear targeting activity [15]. However, the details of the CTLH complex formation remain unclear and the effect of RanBPM localization on the other members of the CTLH complex remains to be elucidated.

In yeast, RanBPM (Gid1) was found to be a crucial component for the architecture of the Gid complex as any alteration in this protein was found to disrupt the complex [25]. Given the high conservation of the members of the complex between yeast and mammals, it will be interesting to determine how the RanBPM mutations that affect its subcellular localization influence the CTLH complex formation and localization.
2.5 References


Regulated by the Met Adaptor Ran-binding Protein M. J Biol Chem 278: 49573-49581.


2.6 Supplementary material

Supplementary Figure 2-1 Validation of subcellular localization scoring protocol.

A) Cells were fixed 24h after transfection of the RanBPM mutants indicated and incubated with an HA antibody and then with an Alexa Fluor 555 secondary antibody. Nuclei were stained with DAPI. Subcellular localization was quantified with ImageJ as described in materials and methods. Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent standard deviation. Mutant RanBPM constructs versus WT, ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

B) Cells from two clonal derivatives, Hela RanBPM shRNA 2-7 (employed throughout the study) or Hela RanBPM shRNA 2-6 were fixed 24h after transfection of the RanBPM mutants indicated and incubated with an HA antibody and then with an Alexa Fluor 555 secondary antibody. Nuclei were stained with DAPI. Subcellular localization was scored as either, N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. Statistical analysis was performed to compare RanBPM shRNA clone 2-7 versus RanBPM shRNA clone 2-6 for each RanBPM mutant construct.
Supplementary Figure 2-2 Effect of short LMB treatment on RanBPM nuclear export.

A) Hela cells treated with EtOH or 20nM LMB were fixed 3h after treatment. Cells were processed for immunostaining with antibodies to RanBPM and cyclin B1 and nuclei stained with DAPI. At least 100 cells were scored as N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. B) RanBPM shRNA Hela cells transfected with pCMV-HA-WT-RanBPM were incubated O/N and treated with EtOH or 20nM LMB for 3h. Cells were analyzed as described above. Scale bar: 10µm.
Supplementary Figure 2-3 Mutations of N-terminal USP and WW motifs do not affect RanBPM expression.

A) Amino acid sequence and position of the USP and WW domains in RanBPM. Mutations are indicated to the right. The predicted motifs are underlined and mutations are marked in red and deletions are represented by a dash (-). USP7 1Δ and 2Δ mutant is comprised of both 1Δ and 2Δ mutations. B) Whole cell extracts were prepared from RanBPM shRNA Hela cells transfected with pCMV-HA-RanBPM mutant constructs 24h after transfection. An HA antibody was used to detect HA-RanBPM and β-actin was used as a loading control. Western blots show expression of WT and USP mutant constructs. C) Expression of WT and WW mutant constructs as described in B.
Supplementary Figure 2-4 Amino acid 1-25 of RanBPM.

The sequence of the first 25 amino acids of human RanBPM is shown.
Chapter 3

3 Aggresome formation is regulated by RanBPM through an interaction with HDAC6

In conditions of proteasomal impairment, the buildup of damaged or misfolded proteins activates a cellular response leading to the recruitment of damaged proteins into perinuclear aggregates called aggresomes. Aggresome formation involves the retrograde transport of cargo proteins along the microtubule network and is dependent on the histone deacetylase HDAC6. Here we show that ionizing radiation (IR) promotes Ran-Binding Protein M (RanBPM) relocalization into discrete perinuclear foci where it co-localizes with aggresome components ubiquitin, dynein and HDAC6, suggesting that the RanBPM perinuclear clusters correspond to aggresomes. RanBPM was also recruited to aggresomes following treatment with the proteasome inhibitor MG132 and the DNA-damaging agent etoposide. Strikingly, aggresome formation by HDAC6 was markedly impaired in RanBPM shRNA cells, but was restored by re-expression of RanBPM. RanBPM was found to interact with HDAC6 and to inhibit its deacetylase activity. This interaction was abrogated by a RanBPM deletion of its LisH/CTLH domain which also prevented aggresome formation, suggesting that RanBPM promotes aggresome formation through an association with HDAC6. Our results suggest that RanBPM regulates HDAC6 activity and is a central regulator of aggresome formation.

3.1 Introduction

Misfolded proteins are generally processed by chaperone-mediated refolding or by proteasomal degradation through the ubiquitin-proteasome system (UPS) [1,2]. In conditions where these systems are impaired or overwhelmed, misfolded proteins accumulate in perinuclear structures called aggresomes [2-4]. Unfolded/misfolded proteins are transported from throughout the cell to the aggresome via a dynein-dependent retrograde transport along the microtubule network. The formation of aggresomes can be induced by proteasome inhibitors (such as MG132) and also by overexpression of various proteins [3,5,6]. In addition to aggregated proteins, aggresomes recruit several other components, including chaperones, for instance heat shock protein
70 (Hsp70), ubiquitin and ubiquitination enzymes such as ataxin 3 (AT3) and carboxy terminus of Hsp70-interacting protein (CHIP), as well as proteasome components and motor proteins such as dynein and dynamitin [3,7-9,10]. Recently, the histone deacetylase HDAC6 has been shown to be an essential component of the aggresome pathway, by functioning as a key factor recruiting protein cargo to the dynein motor for transport into the aggresome and by regulating a cell response pathway involving the activation of a heat-shock response that helps the clearance of the aggregates [11,12]. Other components that appear to be essential to aggresome formation include the chaperones CHIP and Hsp70, as well as protein kinase CK2 which has recently been shown to regulate HDAC6 activity through phosphorylation [9,13,14].

The relationship between protein aggregation and cell death is still a matter of debate, as both protective and death-inducing functions have been suggested for aggresome-like structures [3,4]. Aggresome formation is generally recognized as a protective response from the cell to an otherwise toxic build-up of abnormal/unfolded proteins. However, it has also been concluded that aggresomes can be toxic and induce apoptosis if the aggregated substrates cannot be processed [2,3,15-19]. Finally, while aggresomes have raised considerable interest as a hallmark of neurodegenerative diseases, they have also more recently attracted attention in the cancer field because of the link between aggresomes and the UPS [8,20]. Proteasome inhibitors (such as Bortezomib) have recently emerged as promising therapeutic agents in the treatment of some cancers [21]. However, our understanding of aggresome formation and regulation as well as their role in regulating cell viability, which is crucial to understand how these drugs function, remains limited.

RanBPM (Ran-binding protein M, also called RanBP9) is a ubiquitous, nucleocytoplasmic and evolutionary conserved protein whose function is largely unknown. RanBPM contains several conserved domains including a SP1a/Ryanodine receptor (SPRY), a protein interaction module [22], a lissencephaly type-1-like homology (LisH) motif suggested to function as a dimerization domain and a microtubule-binding domain [23,24], and a carboxy-terminal to LisH (CTLH) domain of unknown function [23]. RanBPM was originally identified in a yeast two-hybrid screen as a protein
interacting with the transport protein Ran [25]. However, the interaction was not confirmed and its involvement in nucleocytoplasmic transport was not substantiated [26]. Subsequently, RanBPM was reported to interact with various proteins, including cytoplasmic kinases, steroid receptors and transcription factors, and was suggested to participate in various cellular processes such as cell growth and cell migration signaling [27-29], neuronal morphogenesis [30,31,32] and the regulation of gene transcription [31,33]. Several studies have also suggested RanBPM to be present in a large multiprotein complex and to function as an adaptor or a scaffolding protein [26,34,35]. Additionally, a function for RanBPM in regulating apoptosis has been suggested based on its interaction with factors implicated in apoptotic pathways [36-39]. The generation of RanBPM-deficient mice has recently revealed a role for RanBPM in male and female gametogenesis, however, additional defects resulting from RanBPM deficiency remain to be investigated [40].

Our previous investigations have provided evidence that RanBPM functions as an activator of apoptotic pathways and regulates the activation of apoptosis induced by DNA damage [41]. We showed that siRNA-directed knockdown of RanBPM prevented DNA damage-induced apoptosis and promoted cell survival in response to ionizing radiation (IR). RanBPM shRNA cells displayed a sharp decrease of mitochondria-associated Bax protein levels, whereas Bcl-2 levels were dramatically up-regulated, providing a novel function for RanBPM in the regulation of DNA damage-induced apoptosis through regulation of the mitochondrial apoptotic pathway. In addition, following IR treatment, we observed the relocalization of RanBPM from the nucleus to the cytoplasm, suggesting that the activation of apoptotic pathways by RanBPM in response to DNA damage may be regulated by nucleocytoplasmic trafficking [41].

In follow-up studies aimed at characterizing IR-induced RanBPM relocalization, we found that RanBPM clustered into discrete perinuclear foci where it co-localized with ubiquitin, dynein and HDAC6, revealing that these RanBPM aggregates correspond to aggresomes. We show that RanBPM is also recruited to aggresomes in response to the proteasome inhibitor MG132 and the DNA-damaging agent etoposide. In addition we show that RanBPM is essential for aggresome formation and that this function is
dependent on the RanBPM LisH/CTLH domain which mediates its interaction with HDAC6. Our work suggests that RanBPM regulates HDAC6 activity and is a central regulator of aggresome formation.

3.2 Materials and methods

3.2.1 Plasmid expression constructs

pCMV-HA-RanBPM shRNA mutant construct (HA-RanBPM si-mt) and pCMV-HA-RanBPM-ΔN2 (ΔN2) were previously described [41]. pCMV-HA-RanBPM-Δ212 (Δ212) and pCMV-HA-RanBPM-Δ360 (Δ360) mutant constructs were generated in pCMV-HA-RanBPM shRNA si-mt using inverse polymerase chain reaction (PCR) using tail-to-tail primers on each side of the region to be deleted. pCMV-HA-RanBPM-ΔC4 (ΔC4) was generated by PCR amplification of RanBPM (aa 1-471) and cloning into pCMV-HA digested with XhoI and SalI. PCR reactions were done using PfuTurbo from Agilent Technologies (Mississauga, ON, Canada) and primers from Sigma-Aldrich (Oakville, ON, Canada).

3.2.2 Cell culture and treatments

Hela and HEK293 control shRNA and RanBPM shRNA stable cell lines were described previously [41,43] and were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.35 mg/ml G418 (Hela) or 0.45 mg/ml G418 (HEK293) (Geneticin, Bioshop Canada, Burlington, ON, Canada) at 37°C in 5% CO2. Ionizing radiation (IR) treatments (10 Gy) were performed with a Faxitron RX-650 at a dose rate of 1.42 Gy/min on cells plated the night before irradiation at 50–60% confluency. For MG132 treatment, Hela and HEK293 cells were incubated with 10 µM or 5 µM MG132 (EMD-CalBiochem, San Diego, CA) respectively, for 16 hours. For etoposide treatment, cells were incubated in media containing 2 µM etoposide (Sigma-Aldrich) for 1 hour, washed and changed to regular media and incubated for 72 hours before analysis. Cycloheximide (CHX) treatments were performed by incubating cells in media containing 25µg/ml CHX (Sigma-Aldrich) for the indicated times (6-24 hours).
3.2.3 Transfection assays

Plasmid transfections were carried out with TurboFect Transfection Reagent (Thermo Fischer Scientific, Burlington, ON, Canada) according to the manufacturer’s protocol. siRNA transfections were carried out as previously described [41,81].

3.2.4 Extract preparation, western blot and immunoprecipitations

Whole cell extracts were prepared as described [41] and resolved by SDS-PAGE (between 8% and 12%). Preparation of soluble/insoluble fractions was adapted from [5]. Briefly, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 1% NP-40, 0.05% deoxycholate, 0.1% SDS, and 150 mM NaCl) supplemented with protease inhibitors. Lysates were then passed 10 times through a 25-gauge needle. Insoluble material was collected after centrifugation and pellets were resuspended in 1% SDS in PBS. Equal volumes of pellets and supernatants were resolved by SDS-PAGE (between 8% and 12%). For western blot analyses, 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), HA (HA-7, Sigma-Aldrich), HDAC6 (D-11 and H-300, Santa Cruz), acetylated α-tubulin (6-11B-1, Santa Cruz), α-tubulin (Sigma-Aldrich), Mcl-1 (S-19, Santa Cruz). The blots were developed using the Western Lightning® Enhanced Chemiluminescence Reagent (Perkin Elmer, Waltham, MA, USA). Quantifications were done using ImageJ software and Image Lab (Bio-Rad, Hercules, CA).

For co-immunoprecipitation experiments, extracts were adjusted to 0.25% NP-40 and 100 mM KCl, immunoprecipitations were carried out overnight at 4°C with antibodies to RanBPM (F-1, Santa Cruz) or HDAC6 (D-11, Santa Cruz). Immunoprecipitates were isolated with Dynabeads® protein G (Invitrogen, Life Technologies, Burlington, ON, Canada).
3.2.5 Immunofluorescence

Cells were plated on coverslips and incubated overnight and treated as described in figure legends. Cells were fixed with 3% paraformaldehyde, permeabilized in 0.5% Triton-X100 for 10 minutes and pre-blocked in 5% FBS diluted in PBS. Coverslips were incubated overnight with primary antibodies (see below), washed in PBS and incubated with secondary antibodies: anti-rabbit Alexa Fluor 488, anti-goat Alexa Fluor 488 and anti-mouse Alexa Flour 488, anti-goat Alexa Fluor 594, anti-rabbit and anti-mouse Alexa Fluor 647 (Invitrogen). Cells were mounted with ProLong® Gold antifade with DAPI (Invitrogen). Visualization was done with an Olympus BX51 microscope with a 40x objective and images were captured with the Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). Primary antibodies used in immunofluorescence: RanBPM (Ab5295, Abcam and K-12, Santa Cruz), HA (HA-7, Sigma-Aldrich), HDAC6 (H-300, Santa Cruz), ubiquitin (Sigma-Aldrich), Dynein (clone 70.1, Sigma-Aldrich.) and Golgin-97 (Thermo Scientific). For quantification analysis, images were blinded by a third party and coded images were scored independently by two individuals. Aggresomes were scored on the criteria of size (min. 1 µm), signal intensity and perinuclear localization. For each treatment, at least 100 cells per sample were scored by each individual and results were averaged from at least three separate experiments. Confocal images were acquired using an inverted IX51 Olympus microscope equipped with a Perkin Elmer Spinning Disk Confocal attachment with a 60x objective. Image deconvolution was done with AutoQuant software (AutoQuant Imaging, Burnbury, Ontario, Canada) and co-localization analyses were done using Imaris software (Bitplane, Zurich, Switzerland).

3.2.6 HDAC assay

HDAC6 was immunoprecipitated as described above and immunoprecipitates were resuspended in assay buffer (1 mM KCl, 10 mM HEPES (pH 7.4), 1.5 mM MgCl2, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin 2 μg/mL aprotinin and 10% glycerol). HDAC activity was measured using a HDAC Fluorometric Activity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol. Briefly, immunoprecipitates with and without HDAC inhibitor (1 μM TSA) in
duplicate wells, were incubated with an HDAC substrate (200 \( \mu \text{M} \)). Deacetylated substrate was measured at 450 nm using a SpectraMax M5 fluorimeter. Average fluorescence of TSA treated samples was subtracted from the average of untreated corresponding samples. HDAC Activity was determined using the deacetylated product concentration obtained using the deacetylated standard curve. HDAC activity is represented as fold activation of HDAC activity in Hela RanBPM shRNA extracts normalized to Hela control shRNA extracts.

3.2.7 Statistical analyses

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

3.3 Results

3.3.1 RanBPM is recruited to aggresomes in response to IR and proteasome inhibition

We previously reported that IR treatment induces RanBPM relocalization from the nucleus to the cytoplasm [41]. This relocalization was initiated within 24 hours following IR treatment (10 Gy), but intensified and persisted up to 72 hours. In examining more closely RanBPM staining in Hela cells following IR exposure 72 hours post IR treatment, we noticed that a large proportion of cells displayed perinuclear dots or foci often located close to an invagination of the nucleus. We initially considered the possibility that these foci could be associated with the Golgi apparatus. Co-staining of RanBPM with a Golgi marker (golgin-97) showed a consistent immunostaining of RanBPM foci in the vicinity of the Golgi complex, however, the two signals did not co-localize (Figure 3-1A). One particular type of structure that has been reported to localize in the region occupied by the Golgi is the aggresome [2-4]. We found that the RanBPM foci formed in our Hela cells bore striking resemblance with previously documented aggresomes in Hela cells [19]. Thus, the general features of these IR-induced RanBPM foci, including their localization with respect to the Golgi apparatus suggested that RanBPM may be recruited to aggresomes in response to IR.
Figure 3-1 RanBPM is recruited to aggresomes in response to IR and MG132.

(A) Hela cells either untreated or 72h after IR treatment (10 Gy) were immunostained with RanBPM and Golgin-97 antibodies and DAPI. Inset shows close proximity but no colocalization between Golgin-97 and RanBPM. (B) Hela cells were analyzed as described for panel A with RanBPM and ubiquitin antibodies. Inset shows aggresome with colocalization of RanBPM and ubiquitin. (C) Hela cells were analyzed as described above with HDAC6 and RanBPM antibodies. Inset shows aggresome with colocalization of RanBPM and HDAC6. (D) Hela cells were analyzed as described above with Dynein and RanBPM antibodies. Inset shows aggresome with colocalization of RanBPM and Dynein. (E) Hela cells treated either with DMSO or 10 µM MG132 (16h) were immunostained with RanBPM and HDAC6 antibodies and DAPI. Inset shows aggresome with colocalization of RanBPM and HDAC6. (F) Hela cells were analyzed as described for panel E with RanBPM and Dynein antibodies. Inset shows aggresome with colocalization of RanBPM and Dynein. Scale bars, 10 µm.
To assess whether RanBPM IR-induced foci were indeed aggresomes, we co-stained Hela cells following IR treatment with an antibody directed against ubiquitin, a well-established marker for aggresomes [4,12,15,19,42]. Ubiquitin clearly co-localized to the same aggregates as RanBPM in IR-treated cells (Figure 3-1B). To further confirm that these foci were aggresomes, we assessed co-localization of RanBPM with HDAC6 and the molecular motor dynein, both of which are known to be recruited to aggresomes and required for aggresome formation [7,11,12]. Both dynein and HDAC6 were found co-localized with RanBPM in aggresome-like structures (Figure 3-1C,D). We obtained similar results in HEK293 cells (Appendix C). Altogether, these results suggested that IR treatment triggers the formation of aggresomes, and that RanBPM is recruited to aggresomes.

Since aggresomes have been documented to form in response to proteasome inhibitors, such as MG132, we conducted experiments to determine whether RanBPM was recruited to aggresomes in response to MG132 in Hela cells. Following MG132 treatment, we observed the relocalization of both HDAC6 and dynein with RanBPM to perinuclear aggregates (Figure 3-1E,F). While the timeline of aggresome formation was faster in response to proteasomal inhibition (MG132, 16 hours as previously reported [19]) than in response to IR (72 hours), both treatments triggered morphologically similar structures. Again, we confirmed that this was not a Hela cell-specific response as we also observed RanBPM recruitment to HDAC6-containing aggresomes in HEK293 cells (Supplementary Figure 3-1).

### 3.3.2 RanBPM down-regulation impairs aggresome formation

RanBPM expression is not affected either by IR [41] or MG132 treatment (see Figure 3-6A). In addition, overexpression of RanBPM by transient transfection did not trigger aggresome formation (data not shown). We sought to determine whether RanBPM was simply recruited to aggresomes or if it could play a specific function in the aggresome pathway, in which case its impairment or down-regulation would affect aggresome formation. We previously engineered Hela cells where RanBPM is effectively down-regulated through stable expression of a RanBPM shRNA [41]. Thus, we assessed
aggresome formation by HDAC6 in RanBPM shRNA Hela cells treated with MG132 or IR. Quantification of aggresome formation in RanBPM shRNA and control shRNA Hela cells revealed that HDAC6 aggresome formation was noticeably impaired in RanBPM shRNA cells both in response to MG132 and IR (Figure 3-2A,C). Upon IR treatment, we found that 20.1% of Hela control cells formed RanBPM aggresomes, versus only 4.9% of RanBPM shRNA cells (Figure 3-2A). These numbers were similar for HDAC6 (17.4% versus 3.8% respectively). In the conditions used, MG132 treatment (10 µM, 16 hours) was slightly more efficient than IR (10 Gy) at inducing aggresome formation, with 29.0% of control cells displaying RanBPM aggresomes and 26.6% forming HDAC6 aggresomes. MG132 treatment of RanBPM-depleted cells also revealed a drastic reduction of HDAC6 aggresomes (9.5%) compared to control cells (26.6%) (Figure 3-2A,C). We obtained similar results in conditions where RanBPM was transiently downregulated via siRNA transfections (Supplementary Figure 3-2). To verify that this effect was not cell type-specific, we repeated these experiments in HEK293 cells, in which we previously generated RanBPM shRNA stably expressing cells where RanBPM expression is reduced [43]. Compared to Hela cells, control HEK293 cells displayed a higher percentage of aggresome-bearing cells upon similar MG132 and IR exposure (Figure 3-2B, Supplementary Figure 3-1). The increased propensity of HEK293 cells to form aggresomes was previously suggested to be due to the expression of the adenoviral protein E1B55K in these cells [44]. In control HEK293 cells, IR treatment induced RanBPM and HDAC6 aggresomes in 44.2% and 40.6% of cells respectively, whereas MG132 treatment triggered aggresome formation in 81.6% (RanBPM) and 75.0% (HDAC6) of control HEK293 cells. Downregulation of RanBPM also significantly reduced aggresome formation by HDAC6 in response to MG132 (48.7%) and IR (19.3%), albeit to a lower extent than in Hela shRNA cells, likely because the HEK293 RanBPM shRNA cells express higher levels of residual RanBPM protein (Figure 3-2B, see inset). To ensure that the effect of RanBPM downregulation was not specific to HDAC6, we quantified aggresome formation by dynein in Hela control and RanBPM shRNA cells in response to IR and MG132 (Figure 3-2D,E). Similarly to what was observed with HDAC6, aggresome formation by dynein was significantly reduced in MG132-treated Hela RanBPM shRNA cells (18.5%) compared to control cells (38.3%).
Figure 3-2 RanBPM downregulation impairs aggresome formation.

(A) Hela cells stably expressing control shRNA or RanBPM shRNA were subjected to IR treatment (10 Gy) or left untreated, or treated with MG132 (10 µM) or vehicle (DMSO) and fixed 72h following IR or 16h following MG132. Cells were processed for immunostaining with antibodies to RanBPM and HDAC6 and mounted with DAPI. At least 150 cells were scored per experiment for the presence of RanBPM and HDAC6 aggresomes and the results (IR treatment, left graph, and MG132 treatment, right graph) are expressed as percentage of cells containing RanBPM aggresomes (solid bars) or HDAC6 aggresomes (open bars). Results are averaged from three different experiments, with error bars indicating SD. Asterisks indicate statistical significance for the differences in percentage of aggresomes obtained in control versus RanBPM shRNA cells, \( P < 0.005 \) (**); \( P < 0.05 \) (*). Inset, western blot analysis of extracts from Hela control and RanBPM shRNA cells, showing RanBPM expression with respect to a β-actin loading control. (B) HEK293 cells expressing control or RanBPM shRNA were processed and analyzed as described in panel A. Results are expressed as in A. Inset, western blot analysis of extracts from HEK293 control and RanBPM shRNA cells, showing the levels of expression of RanBPM versus β-actin used as a loading control. (C) Representative images of Hela cells stably expressing control shRNA or RanBPM shRNA treated with 10 µM MG132 and processed with antibodies to RanBPM and HDAC6 as described in A. (D) Hela cells stably expressing control shRNA or RanBPM shRNA were processed with antibodies against RanBPM and Dynein and analyzed as described in panel A. Results are expressed as in A. (E) Representative images of Hela cells stably expressing control shRNA or RanBPM shRNA treated with 10 µM MG132 and processed with antibodies to RanBPM and Dynein as described in A.
Likewise, IR treatment triggered 31% of cells to form dynein-containing aggresomes in control cells, whereas only 22% were observed in RanBPM shRNA (Figure 3-2D). Altogether, these results suggest that aggresome formation upon MG132 and IR treatment is dependent on RanBPM expression.

3.3.3 RanBPM is recruited to aggresomes in response to DNA damage

Since aggresomes are formed in response to UPS defects, we thought it was important to determine whether the induction of aggresomes by IR is triggered specifically through signaling by the DNA damage response to the UPS, or is due to secondary effects of IR, such as protein oxidation. We used Hela cells to assess aggresome formation by RanBPM/HDAC6 in response to etoposide, which specifically causes double-stranded breaks (DSBs) through inhibition of topoisomerase II [45]. We treated cells with 2 µM etoposide, a dose previously shown to instigate comparable DNA damage as 10 Gy of IR [46] and processed samples for HDAC6 and RanBPM immunofluorescence 72 hours following treatment. Similarly to IR, etoposide treatment triggered the formation of co-localized RanBPM and HDAC6 perinuclear aggregates (Figure 3-3A). Quantifications indicated that 19.1% of control cells displayed HDAC6 aggresomes (Figure 3-3B), which closely matched the number obtained in response to IR (17.4%, see Figure 3-2A) and this number was significantly reduced in RanBPM shRNA cells (6.5%). Quantification of RanBPM aggresomes yielded similar numbers (data not shown). This confirmed that DSBs can elicit a response leading to the formation of aggresomes.

3.3.4 RanBPM forms a complex with HDAC6

Confocal microscopy analyses conducted on both IR and MG132-treated cells revealed that RanBPM and HDAC6 co-localized within the aggresome (as evidence by the white co-localized voxels, Figure 3-4A), suggesting that the two proteins interact. To corroborate these findings, we performed co-immunoprecipitation analysis to determine a potential association of RanBPM with HDAC6. Endogenous HDAC6 was indeed co-immunoprecipitated with endogenous RanBPM in untreated Hela cells, suggesting that the two proteins form a complex (Figure 3-4B). Reversely, immunoprecipitation of
Figure 3-3 RanBPM localizes to aggresomes with HDAC6 in response to DNA damage.

Hela cells stably expressing control shRNA or RanBPM shRNA were fixed 72h following 1h treatment with etoposide (2 µM) or vehicle (DMSO). Cells were processed for immunostaining with antibodies to RanBPM and HDAC6 and mounted with DAPI. (A) Representative images of etoposide-treated Hela control cell showing colocalization of RanBPM and HDAC6 in perinuclear aggresome. (B) Quantification of aggresome formation in response to etoposide. At least 100 cells per experiment were scored for HDAC6-containing aggresomes. Results are averaged from three different experiments, with error bars indicating SD. Asterisks indicate statistical significance between treatments and cell lines, $P < 0.005$ (**); $P < 0.05$ (*).
Figure 3-4 RanBPM forms a complex with HDAC6.

(A) IR (10 Gy, 72h) and MG132 (10 μM, 16h) treated Hela cells were analyzed using confocal microscopy, and colocalization of HDAC6 and RanBPM analyzed using Imaris software. White signal represents RanBPM and HDAC6 colocalization. Scale bar, 10 μm. (B) Control shRNA Hela whole cell extracts were incubated with either a RanBPM antibody or mouse IgG control. Immunoprecipitates were analyzed by western blot using HDAC6 and RanBPM antibodies and compared with 5% of input proteins. (C) Whole cell extracts of RanBPM shRNA Hela cells transfected with pCMV-HA-RanBPM si-mt were incubated with an HDAC6 antibody or mouse IgG control. Immunoprecipitates were analyzed by western blot with HA and HDAC6 antibodies and compared to 5% input extracts.
endogenous HDAC6 was found to co-immunoprecipitate HA-RanBPM expressed in Hela RanBPM shRNA cells, which confirmed complex formation between RanBPM and HDAC6 (Figure 3-4C). Altogether, these analyses suggest that RanBPM associates with HDAC6 both in untreated cells and within aggresome structures.

### 3.3.5 RanBPM expression inhibits HDAC6 activity

To start investigating the effect of RanBPM interaction with HDAC6, we first determined whether RanBPM expression affected HDAC6 protein levels and activity towards its substrate α-tubulin. A comparison of HDAC6 protein levels in Hela and HEK293 cells expressing control or RanBPM shRNA did not reveal any obvious effect of RanBPM on HDAC6 protein expression (Figure 3-5A). In addition, treatment with the protein synthesis inhibitor cycloheximide (CHX) revealed no change and no difference in HDAC6 levels between RanBPM shRNA cells and control cells, inferring that RanBPM does not affect HDAC6 protein stability (Figure 3-5B). However, the levels of acetylated α-tubulin were found significantly decreased in RanBPM shRNA cells, suggesting that RanBPM downregulation resulted in enhanced HDAC6 deacetylase activity (Figure 3-5A). Quantification indicated at least 2-fold reduction of acetylated α-tubulin in RanBPM shRNA cells compared to control cells (Figure 3-5A, lower panel). Re-expression of RanBPM in RanBPM shRNA cells restored α-tubulin acetylation, thus confirming that this effect was specific to RanBPM expression (Figure 3-5C). To substantiate that α-tubulin decrease in acetylation was due to an increase in HDAC6 deacetylase activity in RanBPM shRNA cells, we conducted deacetylation assays using a fluorometric HDAC activity assay. We compared deacetylase activity of HDAC6 immunoprecipitates prepared from control and RanBPM shRNA cells. RanBPM shRNA cells showed a 2.5 fold higher HDAC6 activity compared to control cells, indicating that the downregulation of RanBPM increases HDAC6 activity (Figure 3-5D). Altogether, these experiments suggest that RanBPM exerts an inhibitory effect on HDAC6 activity.

We then assessed a potential effect of RanBPM on HDAC6 levels and activity following proteasome inhibition by MG132. RanBPM protein levels were unaffected upon MG132 treatment (Figure 3-6A), whereas those of the anti-apoptotic factor Mcl-1 used as a
Figure 3-5 RanBPM inhibits HDAC6 activity.

(A) Top, Hela and HEK293 control shRNA (C) and RanBPM shRNA whole cell extracts were analyzed by western blotting and hybridized with the indicated antibodies. Bottom, quantification of relative amounts of acetylated α-tubulin was normalized to total α-tubulin levels. Results are averaged from three different experiments, with error bars indicating SD. $P < 0.005$ (**); $P < 0.05$ (*).  

(B) Control shRNA (C) and RanBPM shRNA Hela cells were treated with either DMSO or 25µg/ml CHX, for the times indicated and whole cell extracts were analyzed by western blotting and hybridized with the antibodies indicated.  

(C) Top, Control shRNA (C) and RanBPM shRNA Hela cells were left untransfected (-) or transfected with pCMV-HA-RanBPM si-mt and whole cell extracts were prepared and analyzed by western blotting and hybridized with the antibodies indicated. Bottom, quantification of relative amounts of acetylated α-tubulin was normalized to total α-tubulin levels. Results are averaged from three different experiments, with error bars indicating SD. $P < 0.005$ (**).  

(D) The activity of HDAC6 immunoprecipitates from Hela control shRNA and RanBPM shRNA was measured using a deacetylation assay. Shown is the RanBPM shRNA HDAC6 immunoprecipitate activity normalized that of control shRNA. Results are averaged from three different experiments, with error bars indicating SD. $P < 0.05$ (*).
Figure 3-6 Effect of RanBPM expression and MG132 treatment on tubulin acetylation and HDAC6 levels and solubility.

(A) Control shRNA Hela whole cell extracts either untreated or treated with DMSO or 10 µM MG132 for 16h were analyzed by western blotting and hybridized with the antibodies indicated. (B) Control shRNA and RanBPM shRNA Hela and HEK293 cells were treated with either DMSO or 10 µM or 5 µM MG132, respectively for 16h and whole cell extracts were analyzed by western blotting and hybridized with the antibodies indicated. (C) Top, Hela control shRNA or RanBPM shRNA cells were treated with either DMSO or 10 µM MG132 for 16h were fractionated into soluble (Sol) or insoluble (Insol) fractions and analyzed by western blotting with antibodies to HDAC6, RanBPM and β-actin. Bottom, quantification of HDAC6, and RanBPM in soluble and insoluble fractions. Graphs show the percentage of protein present in each fraction for each treatment condition. Results are averaged from three different experiments, with error bars indicating SD. $P < 0.005$ (**); $P < 0.05$ (*).
control increased significantly, as expected [47]. By contrast, HDAC6 protein levels were surprisingly found to decline in conditions of proteasomal impairment (Figure 3-6B). Previous studies found that MG132 treatment results in the redistribution of HDAC6 (and other aggresome proteins) into insoluble fractions [48-50], so we reasoned that the MG132-dependent decrease in HDAC6 protein levels detected by analysis of whole cell extracts may be reflecting an increase in detergent-insoluble HDAC6. Thus, we tested the effect of RanBPM expression on the distribution of HDAC6 in soluble and insoluble fractions following MG132 treatment (Figure 3-6C). In untreated cells, 68% of HDAC6 was present in the soluble fraction, versus 32% in the insoluble fraction. However, this distribution was reversed in MG132-treated cells, with 53% of HDAC6 found in the insoluble fraction. In the same fractions, RanBPM was mostly soluble (91%) in untreated cells, but MG132 treatment caused a significant accumulation (38%) in the insoluble fraction. However, we did not detect any significant change in HDAC6 redistribution in insoluble versus soluble fractions in RanBPM shRNA cells in response to MG132 compared to control cells, suggesting that RanBPM expression does not affect HDAC6 solubility. Thus, RanBPM does not appear to affect HDAC6 expression or solubility in normal and MG132-treated cells.

To identify a potential effect of RanBPM on HDAC6 activity in conditions of proteasome inhibition, we analyzed acetylated α-tubulin in whole cell extracts (Figure 3-6B). MG132 treatment elicited a marked increase in acetylated α-tubulin in both Hela and HEK293 control cells. In RanBPM down-regulated cells, MG132 treatment also triggered an increase in acetylated α-tubulin. While this increase appeared slightly dampened by the lack of RanBPM, quantifications did not reveal this decrease to be significant (Appendix D) suggesting that MG132 effect on α-tubulin acetylation is independent of RanBPM expression. Altogether, these results suggest that RanBPM inhibits HDAC6 activity and that proteasome inhibition affects HDAC6 solubility and α-tubulin acetylation through mechanisms that appear to be independent of RanBPM expression.
3.3.6 Deletion of the RanBPM LisH/CTLH domain prevents HDAC6 interaction and aggresome formation

Since RanBPM was found to form a complex with HDAC6 and was required for aggresome formation, we investigated whether the interaction of RanBPM with HDAC6 was a prerequisite for aggresome formation. HA-RanBPM wild-type (WT) or deletion mutants lacking either the N-terminus (ΔN2), the C-terminus (ΔC4), the SPRY domain (Δ212) or the LisH/CTLH domain (Δ360) (Figure 3-7A) were transiently expressed in Hela RanBPM shRNA cells, and assessed for their ability to form aggresomes in response to MG132 treatment. All HA-RanBPM constructs contain a point mutation in the sequence targeted by the RanBPM siRNA (except for ΔC4 which lacks the targeted sequence), as previously described [41]. Aggresome formation by HA-RanBPM WT and mutants was assessed by quantifying the number of aggresomes where RanBPM was found to co-localize with endogenous HDAC6 (Figure 3-7B,C). Re-introduction of WT RanBPM re-established aggresome formation, as 38% of transfected cells showed aggresomes positive for HDAC6 and RanBPM, similar to that found in control Hela cells (Figure 3-2A). Similarly, RanBPM mutants ΔN2, ΔC4 and Δ212 were able to rescue aggresome formation to levels comparable to WT RanBPM. However, the RanBPM Δ360 mutant was unable to induce aggresome formation to the expected level, suggesting that the LisH/CTLH domain is involved in mediating aggresome formation. To determine whether this domain was involved in complex formation with HDAC6, we performed immunoprecipitations of HA-RanBPM WT, Δ360 and Δ212 and assessed endogenous HDAC6 association. Co-immunoprecipitation of HDAC6 with RanBPM Δ360 was markedly reduced compared to RanBPM WT and Δ212, suggesting that deletion of the LisH/CTLH domain severely impairs the interaction of RanBPM with HDAC6 (Figure 3-7D). These results indicate that the RanBPM LisH/CTLH domain is involved in both HDAC6 interaction and aggresome formation, suggesting that complex formation of HDAC6 with RanBPM functions to regulate aggresome formation.
Figure 3-7 The RanBPM LisH/CTLH domain is required for aggresome formation and HDAC6 interaction.

(A) Schematic representations of HA-tagged RanBPM wildtype (WT) and deletion mutant constructs ΔN2, Δ212, Δ360 and ΔC4. The RanBPM conserved domains are indicated. (B) Hela cells stably expressing RanBPM shRNA were transfected with HA-WT, HA-ΔN2, HA-Δ212, HA-Δ360 and HA-ΔC4 and were fixed 16h following 10 µM MG132 treatment. Cells were processed for immunostaining with antibodies to HA and HDAC6 and mounted with DAPI. At least 100 transfected cells were scored per experiment for the presence of aggresomes and the results are expressed as percentage of cells containing aggresomes showing colocalization of RanBPM and HDAC6. Results are averaged from four different experiments, with error bars indicating SD. P < 0.05 (*).

(C) Representative images of HA-tagged RanBPM constructs transfected into RanBPM shRNA Hela cells and treated with 10 µM MG132, processed as described above. (D) The RanBPM LisH/CTLH domain is necessary for interaction with HDAC6. Right, whole cell extracts were prepared from RanBPM shRNA Hela cells untransfected (-) or transfected with HA-WT-, HA-Δ360 or HA-Δ212 constructs. RanBPM was immunoprecipitated with a RanBPM antibody, and immunoprecipitates analyzed by western blot with an HDAC6 antibody. RanBPM WT and deletion mutant immunoprecipitation was verified using an HA antibody. Input, 5% input extract. Left, quantification of relative amounts of co-immunoprecipitated HDAC6 normalized to immunoprecipitated RanBPM. Results are averaged from three different experiments with error bars indicating SD. P < 0.05 (*).
3.4 Discussion

The regulatory mechanisms underlying aggresome formation and the key proteins involved in this process remain poorly understood. Here we report that aggresome formation can be elicited by exposure to DNA damaging agents and that the protein RanBPM plays an essential role in the aggresome pathway. We present evidence that RanBPM forms a complex with HDAC6 and inhibits HDAC6 activity and that RanBPM function in aggresome formation is dependent on its association with HDAC6.

HDAC6 is a critical regulator of aggresome formation and cells deficient in HDAC6 cannot form aggresomes [12]. HDAC6 interacts with the microtubule-associated motor dynein and with polyubiquitinated misfolded proteins, functioning to recruit protein cargo to dynein motors for transport to the aggresome [12]. We have shown here that RanBPM associates with HDAC6 and that downregulation of RanBPM results in a strong reduction in aggresome formation, suggesting that the lack of RanBPM causes a severe disruption in the aggresome pathway. Expression of a RanBPM mutant (Δ360) that impaired complex formation with HDAC6 elicited a modest, but significant reduction in aggresome formation by HDAC6. This suggests that the association of RanBPM with HDAC6 promotes HDAC6 function in the aggresome pathway. Yet, whether this interaction is direct or mediated by other proteins remains to be determined. Interestingly, the RanBPM region identified as interacting with HDAC6 is a LisH/CTLH domain, which is found in proteins that interact with microtubules [23]. Functional studies of LisH motif-containing proteins suggest that LisH motifs mediate microtubule binding and/or metabolism [23]. For instance, Lissencephaly (LIS1), the best characterized LisH-containing protein, functions in microtubule organization and homeostasis through binding to dynein and regulation of dynein motor function [51]. The LisH motif of LIS1 is not involved in dynein binding, but mediates LIS1 dimerization, which is essential for its regulatory function of dynein motility [52]. HDAC6 interacts with microtubules and dynein and also co-localizes with p150glued, a subunit of the dynactin complex [12,53]. Thus RanBPM association with HDAC6 could be functioning to regulate HDAC6 function in microtubule-based cargo transport to the aggresome. It was previously suggested that RanBPM interacted with microtubules, but this observation was later
dismissed as the original study used an antibody that did not recognize RanBPM [25,26]. Some studies have subsequently suggested a potential role for RanBPM in microtubule regulation [30,54], although a direct association of RanBPM with microtubules remains to be confirmed. Interestingly, the highly similar protein RanBP10, whose expression is restricted to hematopoietic cell lineages, has been shown to function in platelet microtubule organization through an interaction with β1-tubulin [55,56]. RanBPM and RanBP10 display 67% amino acid sequence identity and, while having divergent N-terminal domains, share a SPRY, LisH, and CTLH domains [57,58]. Thus, microtubule association and regulation may be a common feature of both proteins, however their functions at microtubules appear to be distinct since the RanBP10 knockout mouse platelet microtubule defects are not compensated for by RanBPM [56,59]. Interestingly, HDAC6 was recently shown to regulate tubulin deacetylation during platelet activation, raising the intriguing possibility of a potential interplay between HDAC6 and RanBP10 in platelet activation [60].

In addition to HDAC6, several proteins have been shown to regulate aggresome formation. Several of these factors are either chaperones or part of ubiquitin/deubiquitin complexes, such as Hsp70, the ubiquitin ligase CHIP and the deubiquitinating enzyme AT-3. In yeast, RanBPM has been found to be associated with a multi-subunit ubiquitin ligase complex called the Vid or Gid complex [61]. In mammalian cells RanBPM has been shown to be part of large cytosolic complex called the CTLH complex [34,35]. The mammalian homologs of several Gid/Vid proteins have been found to be part of the CTLH complex and/or interact with RanBPM, suggesting that the CTLH complex may be the mammalian ortholog of the Gid/Vid complex [34,35,61]. This raises the possibility that RanBPM may be functioning in the aggresome pathway as part of this complex, but whether it is associated with ubiquitin ligase activity remains to be demonstrated.

We found RanBPM to have a negative effect on HDAC6 activity. The levels of acetylated α-tubulin, a known substrate of HDAC6 were found reduced in both Hela and HEK293 RanBPM shRNA cells. Using a deacetylation assay, we detected an increased deacetylation activity in RanBPM Hela shRNA cells, further suggesting that RanBPM functions as an HDAC6 inhibitor. Interestingly, two other HDAC6 inhibitors identified
so far, Tau and TPPP/p25, are microtubule-interacting proteins that are recruited to aggresomes in response to proteasome inhibition [6,49,62,63]. How these two proteins inhibit HDAC6 remains to be detailed, but this suggests that HDAC6 activity at microtubules is subjected to multiple regulations.

It is unclear at present whether the inhibitory effect of RanBPM on HDAC6 deacetylase activity is linked to its function in aggresome formation. HDAC6 deacetylase activity has been demonstrated to be essential for aggresome formation [12,14]. Thus, relieving an inhibitory effect on HDAC6 deacetylase activity through RanBPM downregulation would have been expected to facilitate aggresome formation, however, the opposite effect was observed. On the other hand, the possibility exists that HDAC6 hyperactivity resulting from RanBPM downregulation could be detrimental to aggresome formation. Notwithstanding, the regulation of HDAC6 deacetylase activity may not be the mechanism through which RanBPM functions to regulate aggresome formation. In support of this, we observed increased α-tubulin acetylation upon MG132 treatment (and in response to IR, Appendix E) and this occurred in both control and RanBPM shRNA cells and thus appeared to be independent of RanBPM. Hyperacetylation of α-tubulin in response to proteasome inhibitors was previously noted by others, however, the mechanism by which this hyperacetylation occurs has not been elucidated [64,65]. Tubulin acetylation has been linked to increased microtubule transport and was shown to promote the recruitment of dynein to microtubules [66]. Consistent with a previous report [48], our results show that HDAC6 insolubility is increased upon MG132 treatment, however, this was not affected by RanBPM either. This effect could also be linked to tubulin hyperacetylation, since increased acetylation of tubulin has been linked to its insolubility [67,68]. Thus, tubulin acetylation may be a prerequisite for transport of cargo to the aggresome but RanBPM does not appear to be involved in this regulation in conditions of proteasome impairment. Hence, the role of RanBPM in aggresome formation may be to promote the processivity of HDAC6 along microtubules but how this is achieved remains to be determined.

The inhibitory role of RanBPM on HDAC6 activity could nonetheless have important consequences on other cellular processes. Increased HDAC6 activity is known to be
associated with increased cell motility, in part through deacetylation of α-tubulin, but also due to the increased chaperone function of deacetylated Hsp90 towards oncogenic proteins such as Akt/PKB, ErbB2 and c-Raf [69,70]. Increased HDAC6 levels have been noted in certain tumor types, such as oral squamous cell cancer, ovarian cancer and glioma [70-72]. Interestingly, our previous studies showed that RanBPM expression inhibits the ERK pathway through a regulation of c-Raf stability and also restricts cell proliferation and mobility [43]. We previously attributed the effect of RanBPM on cell motility to its negative regulation of the ERK pathway, however, in light of the results of this study, it is possible that inhibition of HDAC6 activity could account, at least in part, for the inhibitory effect of RanBPM on cell migration.

To our knowledge, this is the first report documenting aggresome formation in response to IR or other DNA damaging agents. We have shown here that both IR and etoposide that specifically induce DSBs result in the formation of aggresomes. Intriguingly however, we only observed aggresomes in response to high doses of IR (10 Gy) which induce massive cell death [41]. Also, aggresome formation did not occur as an immediate response to the DNA injury but appeared to be a delayed consequence of the DNA damage. Aggresomes were first noticed around 60 hours following IR treatment (data not shown), and their accumulation appeared maximal at 72 hours following IR treatment. We previously determined that this correlates with the onset of apoptosis in these cells [41], suggesting a link between aggresome formation and apoptosis. Previous studies have reported that the proteasome is inhibited through caspase-mediated cleavage following the activation of apoptosis in response to various apoptotic stimuli, including DNA damage [73]. The inactivation of the proteasome after the initiation of apoptosis was suggested to facilitate and amplify the apoptotic cascade [73,74]. Therefore, aggresome formation may be a consequence of the loss of proteasome function in the early stages of apoptosis. In RanBPM shRNA cells, decreased aggresome formation could therefore be the combined result of reduced apoptosis activation and impaired aggresome formation, both of which occurring due to the loss of RanBPM.

This study has uncovered a new role for RanBPM in aggresome formation and as an HDAC6 inhibitor. These findings have important consequences for cellular processes.
related to cancer and neurodegenerative pathologies, in which both RanBPM and HDAC6 have previously been implicated. HDAC6 overexpression has been linked to cancer development in several tissues, cancer cell lines and tumour mouse models [72]. Conversely, several studies, including ours, have suggested that RanBPM has tumour suppressor functions by promoting apoptosis and inhibiting cell proliferation and migration [39,41,43,75]. Thus, tumour suppressive functions of RanBPM may be at least in part linked to its ability to repress the oncogenic effects of HDAC6 activity. HDAC6 has also been implicated as a key player in axonal transport and protein aggregation in several neurodegenerative processes including Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS) [76,77]. In turn, RanBPM has been implicated in the pathogenesis of AD, in part through the potentiation of amyloid-β peptide generation [78-80]. Thus the interplay between RanBPM and HDAC6 that we have uncovered in this study may also help understand the cellular pathology underlying protein aggregation in neurodegenerative diseases.
3.5 References


3.6 Supplementary materials

Supplementary Figure 3-1 RanBPM downregulation impairs aggresome formation after MG132 in HEK293 cells.

Representative images of HEK293 cells stably expressing control shRNA or RanBPM shRNA treated with 5 µM MG132 fixed 16h following MG132. Cells were processed for immunostaining with antibodies to RanBPM and HDAC6 and mounted with DAPI.
Supplementary Figure 3-2 Transient down regulation of RanBPM impairs MG132-induced aggresome formation.

A) Hela cells transfected with RanBPM siRNA or left untransfected were treated with either 10 µM MG132 or DMSO and fixed 16h following MG132. Cells were processed for immunostaining with antibodies to RanBPM and HDAC6 and mounted with DAPI. At least 50 cells were scored per experiment for the presence of RanBPM and HDAC6 aggresomes and the results are expressed as percentage of cells containing RanBPM and HDAC6 aggresomes. Results are averaged from three different experiments, with error bars indicating SD. $P < 0.005$ (**); $P < 0.05$ (*). B) Representative images of Hela cells transfected with RanBPM siRNA treated with 10 µM MG132 and processed for immunostaining as described above.
Chapter 4

4 Interaction of RanBPM with HDAC6 through the CTLH complex inhibits HDAC6 activity and regulates cell migration

Ran-binding protein M (RanBPM) has been shown to interact with numerous proteins, implicating it in a variety of cellular processes including apoptosis, cell adhesion, migration, and gene transcription. RanBPM is part of a large protein complex, termed the C-terminal to Lissencephaly type-1 like Homology (LisH) (CTLH) complex. Components of the complex are conserved from the homologous yeast complex, the glucose-induced degradation (Gid) complex, which functions as an E3 ubiquitin ligase. RanBPM is a nucleocytoplasmic protein that partially colocalizes with microtubules and associates with α-tubulin. Our studies have previously suggested a tumour suppressor role for RanBPM, as its downregulation disrupted apoptotic activation, led to loss of growth factor dependence and increased cell migration. Recently, we characterized an interaction between RanBPM and Histone Deacetylase 6 (HDAC6), a cytoplasmic deacetylase that localizes at microtubules and increases cell migration, and found that RanBPM expression inhibits HDAC6 activity. We demonstrate that RanBPM-mediated inhibition of HDAC6 is dependent on its association with HDAC6. We show that, while HDAC6 does not require RanBPM to associate with microtubules, RanBPM association with microtubules requires HDAC6. Additionally, we show that Twa1 (Two-hybrid-associated protein 1 with RanBPM) and MAEA (Macrophage erythroblast attacher), two CTLH complex members, also associate with α-tubulin and that muskelin, another component of the CTLH complex, is able to associate with HDAC6. Finally, we demonstrate that the increased cell migration resulting from downregulation of RanBPM is due to the relief in inhibition of HDAC6 α-tubulin deacetylase activity. Overall, our results suggest that the tumour suppressor functions of RanBPM could stem, at least in part from an inhibition of the oncogenic activities of HDAC6.
4.1 Introduction

Ran binding protein M (RanBPM), also referred to as RanBP9, is a 90kDa ubiquitous protein localized both in the nucleus and the cytoplasm, which has been implicated in various cellular functions but has no intrinsic enzymatic activity. RanBPM contains a Spla kinase and ryanodine receptor (SPRY) domain, known to mediate protein-protein interactions. It also contains a lissencephaly type-1 like homology (LisH) domain, known to mediate protein dimerization and tetramer formation and a C-terminal to LisH (CTLH) domain, whose function remains unknown [1-3]. The C terminal CT-11-RanBPM (CRA) domain is made up of six helices that resemble the death domain superfamily [4]. RanBPM has been shown to interact with numerous proteins, implicating RanBPM to function in a variety of cellular processes including cell adhesion, migration, microtubule dynamics, and gene transcription [5-7]. It has been hypothesized that RanBPM functions as a scaffolding protein that is part of a large complex [5,6,8,9]. RanBPM is well conserved in eukaryotes and a RanBPM counterpart, vacuolar import and degradation 30 (Vid30) or glucose induced degradation 1 (Gid1), has been identified in yeast [10,11]. Gid1 also contains SPRY, LisH, CTLH and CRA domains [11]. Gid1 is a component of a large protein complex made up of several other Gid proteins [5,10,11]. The mammalian homologs of almost all of these proteins have also been found in a large complex, which has been called the CTLH complex [12]. The yeast complex, functions as an E3 ubiquitin ligase complex in the degradation of fructose-1,6-bisphosphatase (FBPase), a gluconeogenic enzyme required when yeast is growing in a carbon-poor medium [5,10,11]. The Really Interesting New Gene (RING) domains of Gid2 and Gid9 which confer the E3 ubiquitin ligase activity in the Gid complex are conserved in the human homologs required for meiotic nuclear division homolog A (RMND5A) and macrophage erythroblast attacher (MAEA), respectively [12]. This suggests that the human CTLH complex may also have E3 ubiquitin ligase activity, however this remains to be confirmed.

Our previous studies have suggested that RanBPM has tumour-suppressive activities. Downregulation of RanBPM resulted in decreased apoptotic activation in response to IR as well as disrupted localization of Bax, a pro-apoptotic Bcl-2 family member, and
resulted in increased expression of Bcl-2, an anti-apoptotic Bcl-2 family member [2]. Downregulation of RanBPM also resulted in loss of growth factor dependence, and resulted in a significant increase in cell motility compared to control shRNA cells, suggesting that RanBPM expression confers activities that restrict cell growth and cell migration [13].

Histone deacetylase 6 (HDAC6) is a class IIb HDAC and unlike other HDAC enzymes, HDAC6 shows cytoplasmic localization. HDAC6 uniquely has duplicate deacetylase domains as well as a C-terminal binder of ubiquitin zinc finger (BUZ) domain, which is able to bind ubiquitin [14]. Although named HDAC6, HDAC6 does not have detectable deacetylase activity toward histones in vivo [15,16]. Its most characterized substrates include α-tubulin, heat shock protein 90 (Hsp90) and cortactin [15]. HDAC6 deacetylation activity is both negatively and positively regulated by post-translational modifications. HDAC6 phosphorylation by protein kinase C (PKC) ζ, PKCa, G protein-coupled receptor kinase 2 (GRK2), glycogen synthase kinase 3 β (GSKβ), casein kinase 2 (CK2), ERK and Aurora A promote α-tubulin deacetylase activity [17-23]. Conversely, epidermal growth factor receptor (EGFR) phosphorylation of HDAC6 decreases tubulin deacetylase activity [24]. Acetylation of HDAC6 by p300 also inhibits HDAC6 α-tubulin deacetylase activity [25]. In addition to being regulated by post-translational modification, HDAC6 is also regulated by protein-protein interactions. HDAC6 association with dysferlin, p62, paxillin, tau and tubulin polymerization-promoting protein/p25 (TPPP/p25) result in decreased α-tubulin deacetylase activity [26-30].

HDAC6 has been shown to promote cell motility through deacetylation of α-tubulin and/or cortactin. Overexpression of HDAC6 results in increased cell motility, however knockout, downregulation or inhibition of HDAC6 by trichostatin A (TSA) or tubacin results in severely reduced cell migration [16,31-35]. This clearly indicates a role for HDAC6 catalytic activity in promoting cell motility.

HDAC6 has been implicated in cancer development and HDAC6-specific inhibitors have emerged as a chemotherapeutic agent to combat cancer. HDAC6 is required for in vitro oncogene-induced cell transformation and transforming growth factor (TGF) β1 induced
epithelial-mesenchymal transition (EMT) [36,37]. HDAC6 expression is also required to maintain anchorage-independent growth of established cancer cell lines [36]. HDAC6 has been shown to promote tumour formation in mouse models [36]. Upregulated HDAC6 levels have been observed in many cancer cell lines and in cohorts of oral squamous cell carcinoma (OSCC) and hepatocellular carcinoma (HCC) patients [38,39]. HDAC6 has also been demonstrated to play a role in promoting angiogenesis [40,41]. Several clinical trials are in progress using ACY-1215, an HDAC6 specific inhibitor alone or in combination with other agents. Preclinical studies for ACY-1215 have shown very promising results for the treatment of multiple myeloma, non-Hodgkin lymphoma and inflammatory breast cancer and the treatment was well tolerated in animals [42-44].

We have previously demonstrated that RanBPM is able to form a complex with HDAC6 [45]. The LisH/CTLH domains of RanBPM were found necessary for association with HDAC6, as deletion of these domains resulted in loss of interaction [45]. We also reported that RanBPM is able to inhibit HDAC6 activity using an in vitro HDAC6 activity assay. Consistent with this, levels of acetylated α-tubulin, a specific HDAC6 substrate were found significantly reduced in cells stably expressing RanBPM shRNA compared to those expressing a control shRNA [45]. Interestingly, we also reported that RanBPM partially co-localizes with microtubules in both Hela and 3T3 mouse embryonic fibroblasts (MEFs) and that RanBPM associates with α-tubulin using co-immunoprecipitation [46].

In this study, we show that RanBPM-mediated inhibition of HDAC6 α-tubulin deacetylase activity is on dependent its association with HDAC6. The RanBPM-HDAC6 interaction requires the second catalytic domain of HDAC6 and the LisH domain of RanBPM. We show that HDAC6 does not require RanBPM to associate with microtubules, but that RanBPM colocalization to microtubules requires HDAC6. Furthermore, we demonstrate that components of the CTLH complex associate with both microtubules and HDAC6. Lastly, RanBPM was found to inhibit HDAC6 mediated cell migration. Our work suggests that RanBPM associates with HDAC6 via the CTLH complex and RanBPM inhibits oncogenic activities of HDAC6.
4.2 Materials and methods

4.2.1 Plasmid expression constructs

pCMV-HA RanBPM shRNA mutant construct (WT RanBPM) and pCMV-HA-RanBPM-Δ360 (Δ360), pCMV-HA-RanBPM-ΔLisH (ΔLisH) and pCMV-HA-RanBPM-ΔCTLH (ΔCTLH) were previously described [2, 45, 46]. pcDNA-HDAC6-FL-FLAG (FL) (Addgene Plasmid #30482), pcDNA-HDAC6-DC-FLAG (DC) (Addgene Plasmid #30483) were obtained from Addgene and pcDNA-HDAC6-1-840-FLAG (1-840), pcDNA-HDAC6-1-503-FLAG (1-503) and pcDNA-HDAC6-ΔN-439-1215-FLAG (ΔN-439) were a gift from Tso-Pang Yao [47]. pcDNA-HA-HDAC6-1-840-FLAG (HA-1-840) and pcDNA-HA-HDAC6-1-503-FLAG (HA-1-503) constructs were produced using annealed HA tag oligos that generated overhangs that could be ligated with digested pcDNA-HDAC6-1-840-FLAG (1-840) and pcDNA-HDAC6-1-503-FLAG (1-503), respectively. pGEX4T1-GST-WT-RanBPM was generated by PCR amplification of full length RanBPM from pCMV-HA-RanBPM and cloned into digested pGEX4T1. pET28a-HDAC6-catalytic domain 2 (CAT2) was generated by PCR amplification of the second catalytic domain of HDAC6 from pcDNA-HDAC6-FL-FLAG and cloned into digested pET28a. All PCR reactions were done using KOD polymerase (Novagen, Germany) and primers from Integrated DNA Technologies (Coralville, Iowa, USA).

4.2.2 Cell culture, transfections and treatments

Hela, Hela control and RanBPM shRNA cells, HEK293, HEK293 control and RanBPM shRNA cells were previously described [2,13]. Wildtype (WT) and HDAC6 knockout (KO) MEFs were a gift from Tso-Pang Yao [34]. All cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (10%) at 37°C in 5% CO₂. Control and RanBPM shRNA stable Hela and HEK cells were maintained in media supplemented with 0.35mg/ml and 0.45mg/ml G418, respectively (Geneticin, Bioshop Canada, Burlington, ON Canada). Tubacin (Cayman Chemical) was added to the cell media at the concentrations and durations indicated in the figure legends.
4.2.3 Transfection assays

Plasmid transfections were carried out with jetPRIME (Polypus Transfection) according to the manufacturer’s protocol or by calcium phosphate transfection according to standard protocols.

4.2.4 Extract preparation, western blot and immunoprecipitation

Whole cell extracts were prepared as described [2] and resolved by SDS-PAGE (between 8% and 12%) and transferred to polyvinylidene difluoride (PVDF) membranes. Samples were analyzed with the following antibodies: HDAC6 (H-300, Santa Cruz, Santa Cruz, CA, USA), HA (HA-7, Sigma–Aldrich), Acetylated α-tubulin (6-11B-1, Santa Cruz, Santa Cruz, CA, USA), α-tubulin (T5168, Sigma–Aldrich), β-Actin (I-19, Santa Cruz, Santa Cruz, CA, USA), RanBPM (5M, Bioacademia, Japan) and muskelin (C-12, Santa Cruz, Santa Cruz, CA, USA). The blots were developed using Clarity ECL Western Blotting Substrate (BioRad, Hercules, CA). Quantifications were done using Image Lab (BioRad, Hercules, CA) and ImageJ software. Co-immunoprecipitation experiments were performed in 0.25% NP-40 and 100mM KCl lysis buffer and were carried out overnight at 4°C with antibodies to HA (HA-7, Sigma–Aldrich), OctA-Probe (D-8 Santa Cruz, Santa Cruz, CA, USA), HDAC6 (D-11 Santa Cruz, Santa Cruz, CA, USA) and RanBPM (F1 Santa Cruz, Santa Cruz, CA, USA). Immunoprecipitates were isolated with PureProteome Protein G Magnetic Beads (EMD Millipore, Billerica, Massachusetts) or Dynabeads Protein G (Invitrogen, Life Technologies, Burlington ON, Canada).

4.2.5 Immunofluorescence and confocal microscopy

Cells were plated on coverslips and following overnight incubation were either fixed or transfected and incubated for 24 hours. Cells were fixed with 3% paraformaldehyde, permeabilized in 0.5% Triton-X100 for 10 minutes and pre-blocked in 5% FBS diluted in PBS. Coverslips were incubated overnight with primary antibodies (see below), washed in PBS and incubated with secondary antibodies: anti-rabbit Alexa Fluor 488, anti-goat Alexa Fluor 488, anti-mouse Alexa Fluor 488, anti-mouse Alexa Fluor 647 and anti-
rabbit Alexa Fluor 647. Cells were mounted with ProLong Gold antifade with DAPI (Invitrogen). Primary antibodies used in immunofluorescence: HDAC6 (H-300, Santa Cruz, Santa Cruz, CA, USA), α-tubulin (T5168, Sigma–Aldrich), RanBPM (K12, Santa Cruz, Santa Cruz, CA, USA), HA (HA-7, Sigma–Aldrich), α-tubulin (ab15246, Abcam), MAEA (ab151304, Abcam) and Twa (ab97653, Abcam). Confocal images were acquired using an inverted IX51 Olympus microscope equipped with a Perkin Elmer Spinning Disk confocal attachment with a 60x objective using Velocity software (Improvision). Z-stack image deconvolution was done using AutoQuant (Media Cybernetics, Rockville, MD, USA) software and image analyses for both plane and Z-stacks images were done using Imaris software (Bitplane, Zurich, Switzerland). Colocalization analyses were done using Imaris software using the top 2% of colocalized voxels.

4.2.6 Scratch assay

Scratch assay experiments were performed as described [13]. Briefly, control and RanBPM shRNA HEK cells were plated and following overnight incubation were transfected with pCMV empty vector (EV), pCMV-HA-RanBPM-WT (WT) or pCMV-HA-RanBPM-Δ360 (Δ360). Cell monolayers were incubated in the presence of 2 mM hydroxyurea (Sigma-Aldrich) for 24 hours to prevent cell proliferation. Cells were scratched using a sterile 200 µL pipette tip following a 4 hour pretreatment with either DMSO or tubacin. Wound closure was assessed at 0 hour and 24 hour using a fluorescent microscope (IX70, Olympus) and images were captured using a charge-coupled device camera (Q-imaging). Samples were performed in triplicate, three pictures were taken per sample, and the wound width was measured using ImageJ software using an average of three width measurements per picture. Fold migration was calculated by normalizing the average wound width at 24 hr to the average wound width at 0 hr.

4.2.7 Statistical analyses

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

4.3.1 HDAC6 and RanBPM interaction

We previously documented that RanBPM shRNA cells displayed decreased levels of acetylated α-tubulin compared to control shRNA cells, and that these levels could be restored to that of control cells upon re-expression of RanBPM, demonstrating that RanBPM expression inhibits HDAC6 activity [45]. To determine if RanBPM association with HDAC6 is necessary for its inhibition, we assayed levels of acetylated α-tubulin in RanBPM shRNA cells re-expressing either wildtype RanBPM and LisH/CTLH RanBPM deletion mutant (Δ360), which we had previously demonstrated has significantly impaired association with HDAC6 [45]. We found that cells transfected with the Δ360 RanBPM mutant had levels of acetylated α-tubulin similar to that of RanBPM shRNA cells and therefore this mutant was not able to restore acetylated α-tubulin to the same level as wildtype RanBPM (Figure 4-1). This indicates that association of RanBPM through the LisH and CTLH domains is required for inhibition of HDAC6.

Since complex formation between RanBPM and HDAC6 had an important effect on HDAC6 activity, we decided to investigate in more detail the specifics of this interaction. To determine more precisely the RanBPM domain responsible for association with HDAC6, we generated individual deletions of the LisH and CTLH domains and by co-immunoprecipitation analysis we assayed their ability to associate with HDAC6. Deletion of the LisH domain resulted in significantly reduced co-immunoprecipitation of HDAC6, whereas deletion of the CTLH did not affect HDAC6 association (Figure 4-2A and B). These results indicate that the LisH domain is necessary for the interaction of RanBPM with HDAC6.

To evaluate which region of HDAC6 is required for association with RanBPM, we transfected HA and/or FLAG tagged HDAC6 deletion constructs (Figure 4-2C) [47] into HDAC6 knockout (KO) mouse embryonic fibroblasts (MEFs) and performed co-immunoprecipitation analyses. Deletion of the N-terminal region of HDAC6 (ΔN439) did not affect HDAC6 association with RanBPM (Figure 4-2D), indicating that the N-terminal region of HDAC6 is not required for association with RanBPM. HDAC6
Figure 4-1 RanBPM association with HDAC6 is required for inhibition of HDAC6 deacetylase activity.

Left, whole cell extracts from RanBPM shRNA Hela cells either left untransfected or transfected with HA-RanBPM-WT or HA-RanBPM-Δ360 were analyzed by western blot with the antibodies indicated. Right, quantification of relative amounts of acetylated α-tubulin was normalized to total α-tubulin levels. Results are averaged from three different experiments, with error bars indicating SEM. P<0.05 (*).
Figure 4-2 Identification of the RanBPM and HDAC6 domains that mediate their association.

A) Schematic representation of HA-tagged RanBPM wildtype (WT) and deletion mutant constructs ΔLisH and ΔCTLH. B) Left, whole cell extracts were prepared from RanBPM shRNA Hela cells untransfected (-) or transfected with HA-RanBPM-WT, HA-RanBPM-ΔLisH, or HA-RanBPM-ΔCTLH constructs. RanBPM was immunoprecipitated with an HA antibody and immunoprecipitates were analyzed by western blot with an HDAC6 antibody. RanBPM mutant immunoprecipitation was verified using an HA antibody. Input, 5% input extract. Right, quantification of relative amounts of co-immunoprecipitated HDAC6 normalized to immunoprecipitated RanBPM. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*). C) Schematic representation of HDAC6 full length (FL) and deletion mutant constructs. D) Left, whole cell extracts were prepared from HDAC6 knockout MEFs untransfected (-) or transfected with full length (FL) or ΔN439 HDAC6 constructs. HDAC6 was immunoprecipitated with a FLAG antibody and immunoprecipitates were analyzed by western blot with a RanBPM antibody. HDAC6 mutant immunoprecipitation was verified using an HDAC6 antibody. Input, 5% input extract. Right, quantification of relative amounts of co-immunoprecipitated RanBPM normalized to immunoprecipitated HDAC6. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*). E) Left, whole cell extracts were prepared from HDAC6 knockout MEFs untransfected (-) or transfected with HA-1-503 or HA-1-840 HDAC6 constructs and immunoprecipitation and analysis was performed as described above except that HDAC6 mutant immunoprecipitation was verified using an HA antibody. The arrow indicates RanBPM signal from previous hybridization. Right, quantification of relative amounts of co-immunoprecipitated RanBPM normalized to immunoprecipitated HDAC6. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*).
deletion mutant 1-840, which lacks HDAC6 C-terminal domain but contains both catalytic domains, retained its ability to interact with RanBPM. However deletion mutant 1-503, which only contains the first catalytic domain, was no longer able to associate with RanBPM (Figure 4-2E). This indicates that the second catalytic domain of HDAC6 is the region responsible for the interaction with RanBPM.

We then wanted to determine if the association of RanBPM and HDAC6 was direct. We generated constructs to bacterially express full length RanBPM and an HDAC6 peptide corresponding to the second catalytic domain of HDAC6. We performed GST-pull-down assays where GST-tagged RanBPM was incubated with T7-tagged HDAC6 catalytic domain 2 (CAT2). However, we were unable to detect HDAC6 CAT2 in the GST-RanBPM pull-downs, suggesting that the interaction of HDAC6 and RanBPM is not direct (Appendix F).

4.3.2 RanBPM association with α-tubulin

We have previously established that RanBPM colocalizes with microtubules and associates with α-tubulin [46]. Since HDAC6 associates with α-tubulin [15], we wanted to determine whether RanBPM was necessary for HDAC6 association with α-tubulin and vice versa. First we assayed whether HDAC6 could associate with α-tubulin independently of RanBPM using quantitative measurements of confocal microscopy analysis of HDAC6 colocalization with α-tubulin in Control or RanBPM shRNA cells (Figure 4-3A). We found no significant difference in the amount HDAC6 colocalized with α-tubulin in Control and RanBPM shRNA cells, indicating that HDAC6 is able to associate with α-tubulin independent of RanBPM. Next, we performed the reciprocal experiment to determine if RanBPM colocalization with α-tubulin was dependent on HDAC6 using wildtype (WT) and HDAC6 knockout (KO) MEFs. For this experiment, colocalization analysis indicated that there was a significant decrease in RanBPM colocalized with α-tubulin in HDAC6 KO cells (Figure 4-3B), denoting that RanBPM requires HDAC6 for association with α-tubulin. Altogether, this suggests that RanBPM does not directly interact with microtubules and that HDAC6 is required to mediate RanBPM association with microtubules.
Figure 4-3 RanBPM requires HDAC6 for association with α-tubulin.

A) Control or RanBPM shRNA Hela cells were fixed and incubated with antibodies against HDAC6 (green) and α-tubulin (red). Colocalization of HDAC6 and α-tubulin was analyzed using Imaris software. Top, representative images where white signal represents the top 2% of HDAC6 and α-tubulin colocalization. Bottom left, quantification of the top 2% of pixels representing HDAC6 colocalized with α-tubulin. Data are representative of a minimum of 60 cells from three experiments. Error bars represent SEM. P<0.05 (*). Bottom right, western blot analysis of extracts from Hela control and RanBPM shRNA cells, showing RanBPM expression with respect to β-actin loading control.

B) Wildtype (WT) or HDAC6 knockout (KO) MEFs were fixed and incubated with antibodies against RanBPM (green) and α-tubulin (red). Colocalization of RanBPM and α-tubulin was analyzed using Imaris software. Top, representative images where white signal represents the top 2% of RanBPM and α-tubulin colocalization. Bottom left, quantification of the top 2% of pixels representing RanBPM colocalized with α-tubulin. Data are representative of a minimum of 60 cells from three experiments. Error bars represent SEM. P<0.05 (*).
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<td>RanBPM shRNA</td>
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![Bar graph](Image7) | ![Bar graph](Image8) |

B

<table>
<thead>
<tr>
<th>RanBPM</th>
<th>α-tubulin</th>
<th>Merge</th>
<th>Colocalization</th>
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![Bar graph](Image15) | ![Bar graph](Image16) |
The observation that RanBPM colocalization with microtubules was dependent on HDAC6 expression suggested that RanBPM association with HDAC6 was necessary for its colocalization with microtubules, specifically α-tubulin. To assess this, we transfected HA-tagged WT, Δ360, ΔLisH, ΔCTLH RanBPM constructs into RanBPM shRNA cells and assessed colocalization of the WT and RanBPM mutants and α-tubulin using confocal microscopy analysis. Intriguingly, we found significantly reduced association of all three RanBPM mutants with α-tubulin when compared to wildtype (Figure 4-4). As we have previously shown that the RanBPM LisH domain is required for association with HDAC6 and that HDAC6 is necessary for RanBPM to associate with α-tubulin, it was expected that Δ360 and ΔLisH would have reduced association with α-tubulin. However, since the ΔCTLH HA-tagged RanBPM also demonstrated significantly reduced colocalization with α-tubulin, this suggests that the CTLH domain is also involved in RanBPM’s association with α-tubulin independent of HDAC6 association.

4.3.3 HDAC association with the CTLH complex

As RanBPM is known to be a component of the CTLH complex, our next objective was to determine whether other members of the CTLH complex could also associate with HDAC6. We first evaluated whether components of the CTLH complex were able to colocalize with α-tubulin as we have previously shown for RanBPM. In Hela cells, we established that both endogenous MAEA and Twa1, two members of the CTLH complex, colocalized with α-tubulin by confocal microscopy analysis (Figure 4-5). This experiment strongly suggests that the CTLH complex as a whole is present at microtubules.

As we could not identify a direct interaction between HDAC6 CAT2 and RanBPM in our in vitro binding assays (Appendix F), we speculated that another member of the CTLH complex could be directly associating with HDAC6 to mediate the interaction between HDAC6 and RanBPM. It came to our attention that muskelin, a component of the CTLH complex has been shown to interact with HDAC6 in a large proteomic screen [48]. Muskelin interacts with RanBPM [6,49] and interestingly, muskelin, through its LisH and CTLH domains, has previously been shown to interact directly with the retrograde microtubule motor protein dynein [50]. Thus we employed a co-immunoprecipitation analysis to evaluate if muskelin could associate with HDAC6 and whether this
Figure 4-4 RanBPM requires the LisH and CTLH domains to associate with $\alpha$-tubulin.

Hela RanBPM shRNA cells were transfected with the indicated HA-RanBPM deletion constructs. Cells were fixed and incubated with antibodies against HA (green) and $\alpha$-tubulin (red). Colocalization of HA-RanBPM and $\alpha$-tubulin was analyzed using Imaris software. *Top*, representative images where white signal represents the top 2% of HA-RanBPM and $\alpha$-tubulin colocalization. Scale bar: 10 $\mu$m. *Bottom*, Quantification of the top 2% of pixels representing HA-RanBPM colocalized with $\alpha$-tubulin. Data are representative of a minimum of 10 transfected cells from three experiments. Error bars represent SEM. P<0.05 (*).
**% HA-RanBPM colocalized with α-tubulin**

- **HA-WT** RayBPM α-Tubulin Merge Colocalization
- **HA-Δ360** RayBPM α-Tubulin Merge Colocalization
- **HA-ΔLisH** RayBPM α-Tubulin Merge Colocalization
- **HA-ΔCTLH** RayBPM α-Tubulin Merge Colocalization

**Graph:**
- x-axis: HA-RanBPM Mutants
- y-axis: % HA-RanBPM colocalized with α-tubulin

Legend:
- HA-WT
- HA-Δ360
- HA-ΔLisH
- HA-ΔCTLH
Figure 4-5 CTLH components associate with microtubules.

A) Hela cells were fixed and incubated with antibodies against MAEA and α-tubulin. Shown are single plane confocal images. Insets are enlarged images of the boxed regions from the above panels and arrows indicate areas of colocalization. The right panels show merged images (MAEA, green; α-tubulin, red) Scale bar: 10 µm. B) Hela cells were fixed and incubated with antibodies against Twa1 and α-tubulin and analysis was performed as described above (Twa1, green; α-tubulin, red).
association was dependent on the expression of RanBPM. HDAC6 was immunoprecipitated from both Control and RanBPM shRNA Hela and HEK293 cells and the association of muskelin was evaluated by western blot. Muskelin was co-immunoprecipitated with HDAC6 in both control and RanBPM shRNA cells (Figure 4-6), indicating that muskelin does not require RanBPM to associate with HDAC6. Thus, muskelin associates with HDAC6 independent of RanBPM, suggesting that it could bridge RanBPM to HDAC6.

4.3.4 RanBPM inhibits HDAC6 mediated cell migration

To evaluate the functional effects of RanBPM inhibition of HDAC6 on cell migration, we performed a wound healing assay, also called scratch assay, a commonly used method to analyze the migration of cells. A ‘wound’ is induced by scraping a confluent monolayer of cells with a pipette tip, creating a void in the monolayer of cells. Cell movement is calculated by measuring the decreasing cell-free void size over specified timepoints [51]. A confluent monolayer of control shRNA cells transfected with empty vector (EV) or RanBPM shRNA cells transfected with either EV, WT or Δ360 RanBPM were incubated for 24 hours with hydroxyurea to prevent cell proliferation. To confirm constant expression of mutant RanBPM constructs throughout the assay, an extract was prepared 24 hours after transfection and at the end of the assay (24 hours after the scratch) (Figure 4-7A). Prior to the scratch, cells were pretreated for 4 hours with either DMSO or tubacin, a specific HDAC6 inhibitor. Four-hour treatment with tubacin was shown to result in increased acetylated α-tubulin which persisted up to 28 hours post treatment, indicating that HDAC6 α-tubulin deacetylase activity is inhibited throughout the duration of the assay (Figure 4-7B) [33]. Following the scratch, cells were imaged and the width of the scratch was measured at time zero and again twenty-four hours later. As previously reported [13], RanBPM shRNA cells showed significantly increased cell migration compared to control shRNA (Figure 4-7C). Reintroduction of WT RanBPM was able to decrease cell migration to that of control cells. However, transfection of Δ360 RanBPM, which does not associate with HDAC6 and is unable to inhibit HDAC6 α-tubulin deacetylase activity, was unable to decrease cell migration (Figure 4-7C and D). Tubacin treatment of all conditions resulted in cell migration comparable to that of control.
Figure 4-6 HDAC6 associates with Muskelin independently of RanBPM.

A) Left, whole cell extracts were prepared from Control or RanBPM shRNA Hela cells and immunoprecipitated with either HDAC6 or IgG. Immunoprecipitates were analyzed by western blot with a muskelin antibody. HDAC6 immunoprecipitation was verified using an HDAC6 antibody. Input, 5% input extract. Right, quantification of relative amounts of co-immunoprecipitated muskelin normalized to immunoprecipitated HDAC6. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*). B) Whole cell extracts were prepared from Control or RanBPM shRNA HEK cells and analyzed as described above.
Figure 4-7 RanBPM inhibits HDAC6 mediated cell migration.

A) Control or RanBPM HEK cells transfected with either empty vector (EV), WT-RanBPM (WT) or Δ360 RanBPM (Δ360) were harvested 30 hr before the scratch or 24 hr after the scratch. Whole cell extracts were analyzed by western blot and hybridized with the antibodies indicated. B) Control shRNA HEK cells were treated with either DMSO or 2 µM Tubacin for the indicated time points. Whole cell extracts were analyzed by western blot and hybridized with the antibodies indicated. C) Confluent monolayers of Control or RanBPM shRNA HEK cells were transfected with either EV, WT or Δ360 RanBPM and were cultured in the presence of 2 mM hydroxyurea for 24 hr, and pretreated with either DMSO (black bars) or 2 µM Tubacin (white bars) for 4 hr. Cells were then scratched and width of the wound was assessed at the time of the scratch and 24 hr later using a microscope at 4x magnification. Fold migration was calculated by normalizing the average wound width at 24 hr to the average wound width at 0 hr and each sample was normalized to Control shRNA EV transfected, DMSO treated samples. Results are averaged from three independent experiments, with error bars indicating SEM. P<0.05 (*). D) Representative images of Control or RanBPM shRNA HEK cells transfected with either EV, WT or Δ360 cultured in the presence of 2 mM hydroxyurea for 24 hr, and pretreated with either DMSO or 2 µM Tubacin for 4 hr. Cells were scratched at 0 hr and assessed again at 24 hr.
A  

30 hr before scratch  
24 hr after scratch  

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<th>RanBPM shRNA + EV (DMSO)</th>
<th>RanBPM shRNA + WT (DMSO)</th>
<th>RanBPM shRNA + Δ360 (DMSO)</th>
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Tubacin  

DMSO 4 hr 28 hr  

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C  

Fold Migration  

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<th>RanBPM shRNA + EV</th>
<th>RanBPM shRNA + WT</th>
<th>RanBPM shRNA + Δ360</th>
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D  

Control shRNA + EV (DMSO)  
2 μM Tubacin  

0hr  
24hr  

RanBPM shRNA + WT (DMSO)  
RanBPM shRNA + Δ360 (DMSO)  

Control shRNA + EV (Tubacin)  

0hr  
24hr
shRNA, indicating that increased cell migration in RanBPM shRNA cells is due to the relief of RanBPM-mediated inhibition of HDAC6 α-tubulin deacetylase activity.

4.4 Discussion

Expanding on our previous findings that RanBPM is able to inhibit HDAC6 deacetylase activity, our study demonstrates a functional effect of RanBPM on HDAC6 activity in cell migration and reveals the involvement of the CTLH complex in this regulation. Investigation of the specific domains of RanBPM and HDAC6 that mediate their interaction revealed that, while their interaction does not appear to be direct, the LisH domain of RanBPM is required for association with HDAC6, while HDAC6 interaction with RanBPM requires its second catalytic domain. We show that RanBPM colocalization with α-tubulin is dependent on HDAC6 and that both the LisH and CTLH domains are required for this association. We demonstrate that members of the CTLH complex are able to colocalize with microtubules and that muskelin is able to associate with HDAC6 independently of RanBPM, suggesting that muskelin may bridge RanBPM to HDAC6. Finally, we show that RanBPM’s association with HDAC6 inhibits HDAC6 activity and prevents HDAC6-mediated cell migration.

A large proteomic screen previously demonstrated that muskelin associates with HDAC6 [48]. We confirmed here that muskelin does form a complex with HDAC6 through co-immunoprecipitation analysis and showed that the HDAC6-muskelin association occurs independently of RanBPM, as muskelin and HDAC6 still associated in RanBPM shRNA cells. We previously showed that RanBPM associates with both HDAC6 and the microtubules, specifically α-tubulin [46], and RanBPM and muskelin have been shown to be associated and be a part of the CTLH complex [5,12,49]. Multiple domains of RanBPM are required for the interaction with muskelin, including the LisH domain [49]. In this study we demonstrated other members of the CTLH complex, namely Twa1 and MAEA, are also localized at microtubules. This suggests that the entire CTLH complex is present at microtubules and associates with HDAC6 through muskelin. Interestingly, there is evidence in the literature of the LisH domain mediating interactions with HDAC proteins. Transducing β-like 1 (TBL1) and transducing β-like 1 receptor (TBLR1), LisH domain-containing proteins, were shown to be part of a large protein complex
encompassing HDAC3, that functions as a transcriptional repressor. Similar to how the RanBPM association with HDAC6 was significantly reduced upon deletion of the LisH domain, both TBL1 and TBLR1 lose their ability to associate with HDAC3 when the LisH domain is removed [52] (Figure 4-1). Therefore, it is possible that the LisH domain of muskelin mediates its association with HDAC6, bringing the entire CTLH complex, including RanBPM, in proximity to HDAC6.

Our data demonstrates that RanBPM association with microtubules is mediated through its LisH and CTLH domains. LisH domains have been shown to be important for dimerization and also binding to microtubules [3], however the role of the CTLH domain in microtubule interaction is still unclear. It would be of interest to evaluate if muskelin is also required for RanBPM to associate with microtubules, via α-tubulin or if this association is dependent on another protein, which would bridge RanBPM and α-tubulin. As all members of the CTLH complex, with the exception of Armc8, have LisH and CTLH domains [12], it is also possible that other CTLH complex members may function to recruit the CTLH complex to microtubules [46].

It would be of interest to determine if HDAC6 regulation occurs through RanBPM independently, or if the CTLH complex modulates HDAC6 activity. The obvious conclusion would be that the CTLH complex, which is hypothesized to have E3 ubiquitin ligase activity, inhibits HDAC6 by targeting it for degradation by the proteasome through the addition of ubiquitin. Recently, RMND5A from Xenopus laevis was demonstrated to have E3 ubiquitin ligase activity [53], however, whether the human CTLH complex retains E3 ubiquitin ligase activity remains to be verified. We have previously demonstrated however, that HDAC6 protein levels remain unchanged in control and RanBPM shRNA cells [45], indicating that RanBPM is not inhibiting HDAC6 activity through modulation of its protein levels. However, the CTLH complex activity could affect an HDAC6 regulator, as HDAC6 activity is positively and negatively modulated through many post-translational modification events and interactions with several proteins. HDAC6 activity is positively regulated by phosphorylation by PKCζ, PKCα, GRK2, GSKβ, CK2, ERK and Aurora A and negatively regulated by phosphorylation by EGFR and acetylation by p300 [17-25]. Therefore we speculate that RanBPM’s
inhibitory effect on HDAC6 could be mediated by the activity of the CTLH complex, which could target an activator of HDAC6 activity for proteasomal degradation.

HDAC6 activity is also modulated through its interaction with other proteins. Association with dysferlin, p62, paxillin, tau and TPPP/p25 have all been reported to result in decreased HDAC6 α-tubulin deacetylase activity [26-30]. Similar to RanBPM, p62 associates with HDAC6 via the second catalytic domain and inhibits its tubulin deacetylase activity, but the precise mechanism of p62 inhibition of HDAC6 has not yet been elucidated [27]. The interaction of TPPP/p25 and HDAC6 was reduced by the presence of tubulin [30], suggesting that HDAC6 associates with TPPP/p25 and tubulin via the same domain. Similarly, dysferlin, a transmembrane protein, inhibits HDAC6 deacetylation of α-tubulin by associated with HDAC6 preventing it from interacting with α-tubulin [26]. However, it does not appear that this is the mechanism through which RanBPM inhibits HDAC6 α-tubulin deacetylase activity, as we observed no significant decrease in the colocalization of HDAC6 and α-tubulin in cells expressing RanBPM shRNA.

HDAC6 retains the ability to associate with α-tubulin even when its catalytic activity is inhibited, either by mutagenesis of critical residues within the catalytic domain or by treatment with an inhibitor [15]. RanBPM is still able to associate with HDAC6 even when HDAC6 is inactivated by chemical inhibition, tubacin treatment or by mutagenesis rendering it catalytically incompetent (Supplementary Figure 4-1). This indicates that the catalytic activity of HDAC6 is not required for its association with RanBPM.

HDAC6 has been shown to promote cell motility through deacetylation of α-tubulin and/or cortactin. Overexpression of HDAC6 results in increased cell motility, however, overexpression of a catalytically inactive HDAC6 mutant shows motility similar to that of control cells [31]. Consistently, downregulation, knockdown or chemical inhibition of HDAC6 results in reduced cell migration [16,32-35]. This evidence clearly indicates a role for HDAC6 catalytic activity in promoting cell motility. Interestingly, RanBPM has previously been shown to have a role in inhibiting cell migration. RanBPM was found to inhibit chemotactic migration by associating with leukotriene B4 receptor 2 (BLT2) [54].
Recently, a study performed in gastric cancer cells also reported that downregulation of RanBPM resulted in decreased cell adhesion and increased cell motility [55]. Our own studies also uncovered that cells with stable downregulation of RanBPM had increased migration compared to control cells in a wound healing assay, indicating that RanBPM functions to inhibit cell migration [13]. This led us to evaluate the effect of RanBPM inhibition of HDAC6 on cell migration. RanBPM shRNA cells exhibited increased cell migration when compared to control shRNA and expression of WT RanBPM but not a LisH/CTLH domain RanBPM deletion mutant, which no longer associates with HDAC6, was able to restore cell migration activity to that of control cells. We also demonstrated here that inhibition of HDAC6 with tubacin restored cell migration to that of control cells, indicating that the loss RanBPM modulation of HDAC6 activity is responsible for the increased migration observed in RanBPM shRNA cells. Interestingly, muskelin siRNA resulted in significantly increased cell migration compared to control siRNA [56], similar to what we observed with stable downregulation of RanBPM. This suggests that the CTLH complex functions to inhibit cell migration and disruption of the complex by downregulation of its components relieves the inhibition of cell migration.

As RanBPM is able to inhibit HDAC6 mediated cell migration, which promotes tumour invasiveness an oncogenic characteristic, this identifies a tumour suppressive function for RanBPM. RanBPM has been identified to have other tumour suppressor functions. We previously showed that RanBPM through its inhibition of ERK signaling promotes apoptosis by preventing expression of pro-apoptotic Bcl-2 family proteins at both the transcriptional and protein level [2,13]. Similarly, RanBPM upregulates the mRNA levels of the pro-apoptotic transcription factor p73, contributing to the activation of apoptosis [57]. Furthermore, downregulation of RanBPM resulted in loss of growth factor dependence as RanBPM shRNA cells were shown to continue to survive and proliferate in the absence of growth serum [13].

Aside from promoting cell migration and consequently tumour invasiveness, HDAC6 contributes to other oncogenic activities. Increased expression of HDAC6 results in deacetylation of Hsp90, enhancing its chaperone activity. Hsp90 client proteins include breakpoint cluster/Abelson murine leukemia viral oncogene homolog 1 (Bcr/Abl), Raf,
epidermal growth factor receptor 2 (ErbB2) among others. Many Hsp90 client proteins contribute to cell growth and survival pathways commonly exploited in cancer cells. In fact, Hsp90 inhibitors are also under investigation as chemotherapeutic agents [58]. HDAC6 has also been demonstrated to play a role in promoting angiogenesis, a hallmark of cancer [40,41,59]. It would be of interest to evaluate whether RanBPM and its associated CTLH complex are also able to restrict these other oncogenic activities of HDAC6.

As HDAC6 has been demonstrated to have a causal role in the development of cancer, specific inhibitors have emerged as a promising target for cancer treatment and have been shown to result in decrease cell growth and decreased tumour formation in preclinical studies [42-44]. Since RanBPM has been shown to have tumour suppressor functions and has been demonstrated to inhibit HDAC6 activity, this study provides mechanistic insights to understand the function of RanBPM in prevention of tumourigenesis and cellular transformation through regulation of HDAC6. Understanding the mechanism by which RanBPM is able to inhibit HDAC6 could provide insight on the development of small molecule HDAC6 inhibitors that could be used in chemotherapy treatment.
4.5 References


4.6 Supplementary materials

Supplementary Figure 4-1 RanBPM associates with catalytically inactive or Tubacin-inhibited HDAC6.

A) Left, whole cell extracts were prepared from HEK cells treated with either DMSO or 10µM Tubacin for 16 hours. Extracts were then immunoprecipitated with either RanBPM or IgG. Immunoprecipitates were analyzed by western blot with an HDAC6 antibody. RanBPM immunoprecipitation was verified using a RanBPM antibody. Input, 5% input extract. Right, quantification of relative amounts of co-immunoprecipitated HDAC6 normalized to immunoprecipitated RanBPM. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*).

B) Left, whole cell extracts were prepared from HDAC6 knockout MEFs untransfected (-) or transfected with full length (FL) or mutated catalytically inactive (DC) HDAC6 constructs. HDAC6 was immunoprecipitated with a FLAG antibody and immunoprecipitates were analyzed by western blot with a RanBPM antibody. HDAC6 mutant immunoprecipitation was verified using an HDAC6 antibody. Input, 5% input extract. Right, quantification of relative amounts of co-immunoprecipitated RanBPM normalized to immunoprecipitated HDAC6. Results are averaged from three different experiments with error bars indicating SEM. P<0.05 (*).
Chapter 5

5  Discussion

5.1  Summary of findings

Previous to this investigation, Ran binding protein M (RanBPM) was shown to be nucleocytoplasmic and function in both nuclear and cytoplasmic processes. However its regulation of localization had not yet been determined. In Chapter 2 we demonstrate that RanBPM possesses two sequences that confer nuclear localization, one in the extreme N-terminus consisting of the first 25 amino acids and a nuclear localization signal (NLS) in the C-terminal region, which only partly contributes to nuclear localization. We postulate that residues 102-138 of RanBPM fold over the C-terminus therefore masking the NLS2 in the context of the full-length protein and deletion of this region relieves the inhibition on NLS2 (Figure 5-1). We identify the presence of a nuclear export signal (NES) in the N-terminal region and demonstrate that the Spla kinase and ryanodine receptor (SPRY), lissencephaly type-1 like homology (LisH) and C-terminal to LisH (CTLH) domains contribute to cytoplasmic localization likely through protein-protein interactions. We also demonstrate that RanBPM associates with both microtubules (MTs) and chromatin, a function that we speculate retains RanBPM in the cytoplasmic and nuclear compartments respectively. Our lab previously established that RanBPM relocalizes to the cytoplasm in response to ionizing radiation (IR) [1]. In Chapter 3 we demonstrate that RanBPM localizes to aggresome structures both after IR and proteasome inhibition. We identify RanBPM as an essential factor in aggresome formation and establish an association between RanBPM and Histone deacetylase 6 (HDAC6), a critical component in aggresome formation. We demonstrate that RanBPM inhibits HDAC6 deacetylase activity and in Chapter 4 we further investigate the relationship between HDAC6 and RanBPM, identifying HDAC6 as mediating the colocalization of RanBPM and microtubules. Our lab has previously identified tumour suppressive functions of RanBPM including the activation of apoptosis and inhibition of the extra-cellular signal regulated kinase (ERK) cascade through destabilization of c-Raf. Loss of this inhibition resulted in increased cell proliferation, loss of growth factor dependence as well as increased cell
Figure 5-1 Model of RanBPM subcellular localization.

A) Summary of domains and motifs that regulate RanBPM subcellular localization. Putative NLS and NES are represented by green and yellow flags, respectively. The first 25 amino acids promote nuclear localization. The putative N-terminal NLS/NES functions as an NES. The SPRY, LisH and CTLH domains promote cytoplasmic localization. The putative C-terminal NLS functions as an NLS. B) Model depicting the masking of C-terminal NLS, denoted by green flag, by amino acids 102-139. Identified through analysis of the subcellular localization of the N-terminal deletion mutant constructs.
A

Promotes nuclear localization

Promotes cytoplasmic localization

Functions as a NES

Functions as a NLS

B

CRA

SPRY

LisH

CTLH

NLS2 mutant (635, 636, 640)

NLS1/NES

NLS1 mutant (150, 152, 153)

NES mutant (147, 151)

LNEQE

ELQ

RR

LKR

LY

140  145  155
migration [2]. We demonstrate here that RanBPM inhibits HDAC6 mediated cell motility indicating that RanBPM exerts some of its tumour suppressive function through inhibition of HDAC6.

5.2 RanBPM localization and its implications in apoptotic activation and CTLH complex localization

In Chapter 2, we identify that several domains and motifs control the subcellular localization of RanBPM. To identify regions of RanBPM that regulated its subcellular localization, we generated many RanBPM mutant constructs. Some RanBPM constructs consisted of a series of point mutations within a putative NLS or NES motif to determine if it was a functional localization signal. However, some RanBPM mutant constructs consisted of large deletions encompassing entire domains. As the crystal structure of RanBPM remains to be solved, we do not know how these large deletions affect the protein structure and consequently protein function.

In this study, we characterized the localization of RanBPM in isolation, but RanBPM has been demonstrated to be apart of a large protein complex, the CTLH complex [3-5]. Similar to RanBPM, required for meiotic nuclear division homology A (RMND5A), two-hybrid-associated protein 1 with RanBPM (Twa1) and armadillo repeat containing 8 (Armc8α) of the CTLH complex have been shown to be both nuclear and cytoplasmic. However macrophage erythroblast attacher (MAEA) is exclusively nuclear and muskelin is exclusively cytoplasmic but both have still been demonstrated to be part of the CTLH complex. Therefore, it has been hypothesized that there is a nuclear and a cytoplasmic CTLH complex [5]. There is evidence that the localization of some members of the complex can regulate the localization of others. Ectopic expression of muskelin resulted in the cytoplasmic accumulation of exogenous RanBPM, Twa1 and Armc8α. However transfection of MAEA resulted in their nuclear accumulation [5]. The topology of the yeast CTLH complex, the glucose-induced degradation (Gid) complex, has been elucidated, and RanBPM (Gid1/ vacuolar import and degradation (Vid) 30) has been identified as forming part of the core of the complex. Our lab is currently evaluating how RanBPM functions in the mammalian CTLH complex by assessing the effect of RanBPM localization on other members of the CTLH complex as well as investigating the
architecture of the complex. The localization of complex members will be evaluated in the presence and downregulation of RanBPM to determine if RanBPM plays a role in regulating the localization of the CTLH complex. The architecture of the human CTLH complex will be investigated using RanBPM deletion mutants as well as transient siRNA knockdowns of complex components and co-immunoprecipitation analysis to determine which components are necessary for CTLH complex members to associate.

In this study we identified regions of RanBPM that regulate its localization in a resting state. Previously, our lab showed that upon exposure to IR, RanBPM localization changes, becoming more cytoplasmic [1]. This cytoplasmic shift correlated with an increase in the activation of apoptosis [1]. Protein localization is regulated by a number of factors, including post-translational modifications, which can result in the alteration of the signal strength, or signal sequence masking or unmasking due to conformational changes resulting in a change in subcellular localization [6-8]. Ataxia telangiectasia mutated (ATM) is a kinase, which is a key regulator in the DNA damage response pathway. ATM phosphorylates RanBPM at serine 181 and 603 after IR [9, 10]. It would be of interest to examine if phosphorylation by ATM initiates the relocalization of RanBPM after IR. This could be uncovered with the addition of an ATM inhibitor after exposure to IR. Also, generating a non-phosphorylatable mutant or creating a phosphomimetic mutant through site directed mutagenesis would determine whether phosphorylation of this residue is responsible for the cytoplasmic export of RanBPM observed in response to IR. We have yet to determine if RanBPM’s relocalization to the cytoplasm is a cause or a consequence of apoptotic activation. This could be uncovered by using a mutant that fails to relocalize to the cytoplasm following IR followed by quantification of apoptotic induction. If apoptotic activation is dampened due to failed relocalization, this would indicate that RanBPM relocalization or the increased presence in the cytoplasm is required for the induction of apoptosis. Translocation of proteins from the nucleus to the cytoplasm to trigger apoptotic activation after IR-induced DNA double strand breaks is not an uncommon occurrence. Although the insult occurs in the nucleus, the intrinsic apoptotic pathway is activated through mitochondria depolarization and thus occurs in the cytoplasm. Therefore nucleocytoplasmic trafficking is crucial for apoptotic signaling cascades [11-13]. For example, nuclear hormone receptor/orphan nuclear
receptor (Nur77/TR3) is a protein that translocates from the nucleus to the mitochondria to activate apoptosis. Nur77/TR3 is a member of the orphan steroid receptor family. Once out of the nucleus Nur77/TR3 interacts with B-cell lymphoma 2 (Bcl-2) inducing a conformational change, which contributes to the mitochondrial release of cytochrome c [14]. Other examples of proteins that translocate as a result of apoptosis or in order to activate apoptosis include breast cancer 1 (BRCA1) and death-domain associated protein (DAXX) [11, 14]. To determine whether the act of relocalization to the cytoplasm or simply the presence within the cytoplasm is required for the activation of apoptosis, apoptotic activation could be assayed following IR of a predominantly cytoplasmic RanBPM mutant such as the ΔN2 or Δ1-25 RanBPM deletion constructs.

### 5.3 RanBPM in aggresome formation

In addition to the relocalization of RanBPM to the cytoplasm potentially having a role in the activation of apoptosis, we demonstrate in Chapter 3 that upon exposure to IR RanBPM relocalizes to discrete, perinuclear foci or aggresomes. Previously, aggresomes have been documented to form in conditions of proteasomal impairment and the overexpression of certain proteins [15, 16]. However this is the first report that documents the formation of aggresomes after IR. As the formation of aggresomes in response to IR corresponds to the activation of apoptosis [1], we speculate that the aggresomes form as a result of apoptotic activation due to DNA damage induced by IR. It has been demonstrated that during apoptosis, the proteasome is impaired due to caspase-mediated cleavage, therefore resulting in the accumulation of ubiquitinated proteins and thus this may explain the IR-induced aggresome formation that we have reported [17, 18]. RanBPM was found to be essential for the formation of aggresomes, both after IR and as a result of proteasome inhibition, as downregulation of RanBPM severely reduced the number of aggresomes. Another essential regulator of aggresome formation is HDAC6 [19]. We found that RanBPM associated with HDAC6 and inhibited HDAC6 α-tubulin deacetylase activity. Knockout or knockdown of HDAC6 severely impairs aggresome formation [19]. Re-introduction of wildtype (WT) HDAC6, but not a catalytically inactive HDAC6 is able to restore aggresome formation [19]. In addition, treatment with trichostatin A (TSA) and tubacin which both inhibit HDAC6 prevent the
formation of aggresomes [19-21]. This demonstrates that the catalytic activity of HDAC6 is required for aggresome formation. However, intriguingly, we demonstrate that RanBPM inhibits HDAC6 activity, which inhibits aggresomes formation and that RanBPM is required for aggresomes formation. Similarly, tau was found to associate with and inhibit HDAC6 tubulin deacetylase activity [22]. Tau was also found to relocalize to aggresomes structures when overexpressed and in conditions of proteasomal impairment in an HDAC6 dependent manner [23, 24]. Therefore, it was demonstrated that tau can inhibit HDAC6 tubulin deacetylase activity without inhibiting its role in aggresome formation, similarly, we observed that RanBPM was able to inhibit HDAC6 without inhibiting the formation of aggresomes.

Interestingly, it was demonstrated that increased α-tubulin acetylation promotes recruitment of MT motors, kinesin and dynein, therefore stimulating both anterograde and retrograde transport [25-27]. Specifically, HDAC6 inhibition increases the association of motor proteins to microtubules and significantly increases the rate of transport of dynein cargo and these effects were specifically a result of increased α-tubulin acetylation [26]. Retrograde transport of ubiquitinated proteins by dynein, mediated by HDAC6, is an essential part of aggresome formation [15, 28]. In fact, dynamitin-mediated disruption of dynein severely impaired aggresome formation [28]. However, this is counterintuitive since HDAC6 activity is required for aggresome formation. Yet, HDAC6 deacetylates α-tubulin resulting in unfavourable conditions for dynein motor protein binding to microtubules [19, 26]. Therefore, it is possible that dynamic regulation of deacetylation activity of HDAC6 is required for aggresome formation. We suggest that RanBPM functions to inhibit HDAC6 which results in increased acetylated α-tubulin thereby facilitating dynein mediated transport of ubiquitinated proteins resulting in aggresome formation. Consequently, in conditions where we observed significantly reduced aggresomes with downregulated RanBPM, dynein was not effectively recruited to MTs. However, the effect of RanBPM inhibition of HDAC6 on dynein MT recruitment and transport remains to be evaluated.

Aggresomes, when not cleared by chaperone-mediated refolding or proteasomal degradation are cleared by autophagy [16, 29]. The RanBPM yeast homologue, Vid30
has been shown to be required in the vacuole degradation, the homologous autophagy pathway in yeast. One of the proteins targeted through this pathway is fructose-1,6-bisphosphatase (FBPase), an enzyme required for gluconeogenesis for yeast grown in a non-glucose medium. Vid30 acts by mediating the association of the Vid vesicles with actin patches, integrating the Vid and endocytic pathways [30]. Therefore, in addition to being required for aggresome formation, RanBPM could also function in mediating the activation of autophagy to clear aggresomes, however this remains to be evaluated.

5.4 RanBPM and HDAC6 interaction

In Chapter 4 of this thesis we further investigate the association of RanBPM and HDAC6. We determined that RanBPM does not directly interact with HDAC6 (Appendix F). Nevertheless RanBPM requires HDAC6 to associate with α-tubulin. We speculate that muskelin mediates the interaction of HDAC6 and RanBPM since muskelin has been demonstrated to directly interact with HDAC6 [31]. We suggest that the muskelin and HDAC6 interaction is mediated through the muskelin LisH domain as the LisH domain of transducing β-like 1 (TBL1) and transducing β-like 1 receptor (TBLR1) were identified to mediate its association with HDAC3 [32]. Previous studies have shown that multiple domains of RanBPM are required for association with muskelin and deletion of the LisH domain impairs RanBPM’s ability to associate with muskelin [33]. Therefore deletion of the RanBPM LisH domain would impact its ability to interact with muskelin and subsequently HDAC6. It would be of interest to evaluate if muskelin is also required for RanBPM to associate with microtubules, via α-tubulin or if this association is dependent on another protein, potentially another protein within the CTLH complex that associates with RanBPM through the CTLH domain, as this domain was required for RanBPM association with α-tubulin. Accordingly, the LisH and CTLH domains acted together in retaining RanBPM in the cytoplasm. Although individual deletions of either the LisH or CTLH domain had no significant effect on RanBPM localization, the deletion of both these domains resulted in significantly increased nuclear localization. This indicates that both of these domains are required for RanBPM to associate with microtubules which functions to retain RanBPM in the cytoplasm. RanBPM cytoplasmic localization is necessary for association and subsequent inhibition of HDAC6, an
exclusively cytoplasmic protein. Therefore, the subcellular localization of RanBPM regulates its function.

We have not yet determined the mechanism of RanBPM mediated inhibition of HDAC6. However, we speculate that RanBPM through the putative E3 ubiquitin ligase activity of the CTLH complex targets an activator of HDAC6 activity for degradation therefore inhibiting HDAC6 deacetylase activity.

As RanBPM was shown to associate with the second catalytic domain of HDAC6, which shares high homology with the other HDAC catalytic domains [34], it would be of interest to evaluate if RanBPM is able to associate with and inhibit nuclear HDACs. We speculate that muskelin mediates the association between RanBPM and HDAC6, since muskelin is an exclusively cytoplasmic protein it is unlikely that it could mediate this association in the nucleus. However, it is possible that another member of the CLTH complex is responsible for mediating an interaction of RanBPM with nuclear HDACs since almost all components (except Armc8) contain a LisH domain [5, 35]. However this has not yet been explored.

5.5 RanBPM and HDAC6 in cilia

As we have demonstrated a relationship between RanBPM and the CTLH complex with HDAC6 at microtubules it is interesting to speculate that this association could also occur in other specialized regions within the cell. Cilia are microtubule-rich organelles where HDAC6 has been shown to be present and where it functions to promote ciliary disassembly through deacetylation of α-tubulin and cortactin [36-38]. Lebercilin is a ciliary protein that was shown to associate with microtubules and the intraflagellar transport machinery, including the motor protein dynein, thus having an integral role in ciliary transport [39-41]. Interestingly, RanBPM and most of the proteins comprised in the CTLH complex have been demonstrated to associate with the lebercilin complex [39, 40, 42]. Yippie like (YPEL) 5, a RanBPM-interacting protein involved in cell cycle progression and shown to localize with mitotic machinery throughout the cell cycle, was also found to be a component of this complex [40, 42, 43]. The function of RanBPM and
the CTLH complex in cilia regulation is currently unknown, however their presence in cilia suggests that they could regulate HDAC6 activity in ciliogenesis.

5.6 RanBPM and HDAC6 in neurodegenerative diseases

One of the hallmarks of Alzheimer’s Disease (AD) in addition to β-amyloid plaques includes neurofibrillary tau tangles. Interestingly tau association with HDAC6 was shown to inhibit HDAC6 activity [22]. However, HDAC6 expression was found to be upregulated in AD. Conversely increased levels of α-tubulin are also observed in AD [23, 44]. It has been hypothesized that neurons increase HDAC6 expression to combat the accumulation of amyloid-β and tau, but tau then inhibits HDAC6 activity resulting in increased α-tubulin acetylation [44]. RanBPM is also overexpressed in AD brains and has a pathological role in disease progression [45, 46]. Therefore, it is possible that RanBPM, in addition to tau, inhibits HDAC6 activity consequently resulting in the observed increase of α-tubulin acetylation. HDAC6 has been implicated in other neurodegenerative diseases such as Parkinson’s Disease, Huntington’s Disease and Amyotrophic lateral sclerosis [44]. However RanBPM has only been studied in AD. Therefore it would be of interest to uncover if RanBPM also plays a role in these diseases regulating HDAC6 activity. This is particularly significant since we have identified RanBPM as an essential component in aggresome formation and a common characteristic of these neurodegenerative diseases is aggregated proteins.

5.7 RanBPM functions as a tumour suppressor

Thus far, our lab’s published studies and the ones presented in this thesis have identified tumour suppressor functions of RanBPM in cellular in vitro models. The next step would be to validate that RanBPM could exert these tumour suppressor functions in an in vivo animal model. A commonly used in vivo tumour formation is the ectopic model where cells are injected subcutaneously in the flank of the mice to assess potential for promoting or repressing tumour formation by evaluating tumour growth and size [47, 48]. Tail vein injection of immunodeficient mice is a mouse model that evaluates metastasis [49]. Additionally, gene specific knockout mice can evaluate if knockout of a gene results in higher susceptibility of cancer development accelerating by exposure to carcinogens, for
examples a topical carcinogen applying to both wildtype and knockout mice [50]. Experiments in our lab have evaluated the tumour suppressor functions of RanBPM in vivo. NOD scid gamma (NSG) mice were injected, via flank subcutaneous injections, with either control or RanBPM shRNA HEK cells. Mice injected with RanBPM shRNA human embryonic kidney (HEK) cells developed significantly larger tumours, with much earlier tumour onset. These experiments were also repeated using HEK RanBPM shRNA cells stably expressing a Tetracyclin (Tet)-off RanBPM vector where RanBPM is re-expressed upon the removal of Tet/Doxycycline (Dox) to demonstrate that these effects were not due to off-target effects of the RanBPM shRNA. Mice fed Dox-containing chow developed tumours whereas those fed regular chow did not, indicating that RanBPM re-expression prevents tumour formation. We also performed tail vein injections and observed similar results. Six out of seven mice injected with RanBPM shRNA cells developed tumours, while no tumours were observed in the mice injected with control shRNA cells (unpublished data, Schild-Poulter Lab). To confirm that RanBPM exerts tumour suppressor functions through inhibition of HDAC6 activity, mice could be treated with an HDAC6-specific inhibitor once tumours are established, to determine whether inhibiting HDAC6 can reduce tumour development. We hypothesize that treatment with an HDAC6 inhibitor will result in decreased tumour burden. In addition, treatment with an HDAC6-specific inhibitor can be tested in a tail vein injection model. RanBPM shRNA cells will be engineered with a luciferase gene and injected into the tail vein of mice and treated with an HDAC6 inhibitor and tumour growth will be monitored via non-invasive bioluminescent imaging. These experiments would confirm in vivo that RanBPM has tumour suppressor proprieties through inhibition of HDAC6 oncogenic functions.

5.8 Conclusion

In summary, this work has contributed to the understanding of RanBPM localization and in particular identified that RanBPM associated with microtubules. We found that RanBPM is an essential regulator of aggresome formation and interacts with and inhibits HDAC6 deacetylase activity. As HDAC6 is an important regulator of microtubules we further characterized the role of RanBPM at microtubules with HDAC6. We demonstrate
that RanBPM can inhibit cell motility through inhibition of HDAC6 deacetylase activity, one of the oncogenic properties of HDAC6. Studies in our lab have previously demonstrated that RanBPM possesses tumour suppressor functions and here we propose that some of these functions are due to RanBPM inhibition of HDAC6.

HDAC6 has been shown to have a causative role in cancer development, as it is required for cell transformation and through its role in cell motility and subsequent tumour invasion and angiogenesis [50-52]. HDAC6 small molecule inhibitors are currently being tested for use as chemotherapeutic agents in clinical trials. Therefore a thorough understanding of how RanBPM is able to elicit its tumour suppressor functions, specifically through inhibition of HDAC6 will provide insight into improving or designing new HDAC6 inhibitors.
5.9 References

### Appendices

**Appendix Table 1 Chemical inhibitor treatments.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Purpose</th>
<th>Company</th>
<th>Cell Type</th>
<th>Concentration</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Cyclohexamide</td>
<td>Inhibits protein synthesis</td>
<td>Sigma</td>
<td>HeLa cells</td>
<td>25ug/mL</td>
<td>6-24 hours</td>
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<tr>
<td>Etoposide</td>
<td>Inhibits topoisomerase (resulting in DNA DSBs)</td>
<td>Sigma</td>
<td>HeLa cells</td>
<td>2uM</td>
<td>1 hour</td>
</tr>
<tr>
<td>Hydroxyurea (HU)</td>
<td>Inhibits DNA synthesis</td>
<td>Sigma</td>
<td>HEK cells</td>
<td>2mM</td>
<td>24+ hours</td>
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<tr>
<td>Leptomycin B (LMB)</td>
<td>Inhibits nuclear export</td>
<td>Bioshop</td>
<td>HeLa cells</td>
<td>20nM</td>
<td>3 hours, 16 hours</td>
</tr>
<tr>
<td>MG132</td>
<td>Inhibits proteasome</td>
<td>EMD-Cal Biochem</td>
<td>HeLa cells</td>
<td>10uM</td>
<td>16 hours</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Inhibits microtubules polymerization</td>
<td>Abcam (ab120630)</td>
<td>HeLa cells</td>
<td>10uM</td>
<td>4 hours</td>
</tr>
<tr>
<td>Tubacin</td>
<td>Inhibits HDAC6 deacetylase activity</td>
<td>Cayman Chemical</td>
<td>HEK cells</td>
<td>2uM</td>
<td>4-28 hours</td>
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Appendix A Nocodazole treatment does not affect RanBPM subcellular localization.

RanBPM shRNA Hela cells transfected with WT RanBPM were either untreated or treated with 10μM Nocodazole and fixed 4h after treatment and incubated with an HA antibody and then with an Alexa Fluor 555 antibody. Nuclei were stained with DAPI. Subcellular localization was scored as either, N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), or C>N (cytoplasmic greater than nuclear). Data represent averages from three separate experiments, each assessing approximately 100 cells. Error bars represent SD. Nocodazole treatment versus untreated, *, P<0.05.
Appendix B Mutations in the second cluster of basic residues of NLS2 do not affect its ability to direct nuclear localization.

RanBPM shRNA cells were transfected with either pHM830 (left) or pHM840 (right) containing mutations in the second cluster of basic residues of NLS2 fused to GFP-β-gal. Cells were fixed 24 hours after transfection and nuclei stained with DAPI. Subcellular localization was scored as either N>>C (completely nuclear), N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), C>N (cytoplasmic greater than nuclear), C>>N (completely cytoplasmic). Data represent averages from three separate experiments, each assessing a minimum of 50 cells. Error bars represent SD. RanBPM motifs versus EV, ***, P<0.001; **, P<0.01; *, P<0.05.
Appendix C RanBPM colocalized with HDAC6 after IR in HEK cells.

HEK cells either untreated or 72h after IR treatment (10 Gy) were immunostained with RanBPM and HDAC6 antibodies and DAPI.
Appendix D MG132-induced increased in acetylated α-tubulin occurs independently of RanBPM expression.

Control and RanBPM shRNA Hela cells were treated with 10uM MG132 for 16h and whole cell extracts were analyzed by western blot. Quantification of relative amounts of acetylated α-tubulin was normalized to total α-tubulin levels. Results are averaged from three different experiments, with error bars indicating SEM. P<0.05 (*).
Control shRNA +MG132

RanBPM shRNA +MG132
Appendix E IR treatment results in increased acetylated α-tubulin.

Whole cell extracts were prepared from control and RanBPM shRNA Hela cells following IR treatment (10 Gy) and were analyzed by western blot and hybridized with the indicated antibodies.
Appendix F RanBPM does not directly interact with HDAC6 CAT 2.

GST pull-down assays were performed using GST and GST-WT-RanBPM and T7-HDAC6 CAT2 E. coli extracts. Pull-downs were analyzed by Western blot.
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• Robarts Molecular Medicine, Student Executive Committee; organized and hosted the inaugural (2014) and second annual (2015) Robarts Research Retreat with over 100 oral and poster presentations and over 200 attendees

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