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Regulation of Beta-Catenin by the CTLH Complex

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Abstract

RanBPM is a highly conserved protein that has been shown to impact numerous cellular processes including migration, proliferation, and apoptosis. RanBPM exists in a multi-protein complex called the CTLH complex, which functions as an E3 ubiquitin ligase. A putative member of the CTLH complex, WDR26, has been implicated in the Wnt pathway through regulating β-catenin protein levels. This study investigated whether β-catenin regulation is performed by the CTLH complex. WDR26 was confirmed as part of the CTLH complex, and this complex is interacting with Axin1. CTLH members RanBPM and RMND5A both regulate beta-catenin levels in a proteasome dependent manner. Additionally, this regulation affected downstream expression of β-catenin target genes. Together these results have revealed a potential target of the CTLH complex and suggests a novel mechanism that regulates Wnt signalling. This study has revealed new insights into the CTLH complex functioning as a novel tumour suppressor.

Keywords

RanBPM, RMND5A, CTLH Complex, Ubiquitination, Protein Regulation, Degradation, Wnt Pathway, β-catenin.

Co-Authorship Statement

This thesis was written entirely by Christopher Chiasson and was edited by Caroline Schild-Poulter. All experiments contributing to this thesis were performed by Christopher Chiasson except for the following;

- HeLa cell lines stabling expression RanBPM shRNA and non-targeting control shRNA were generated by Dawn Bryce.
- Immortalized mouse embryonic fibroblast cell lines either wild-type or RanBPM deficient were generated by Xu Wang.
- HeLa RMND5A and non-targeting control CRISPR-Cas9 Knockout cell lines were generated by Xu Wang, Matthew Maitland and Katrina Cirone.
- Proteasomal inhibition of RanBPM shRNA and control shRNA HeLa cell lines were conducted by Christina McTavish.

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List of Abbreviations

ANOVA	one-way analysis of variance
APC	adenomatous polyposis coli
AR	androgen receptor
ARMc8	armadillo repeat containing 8
ATCC	American type culture collection
ATP	adenosine triphosphate
Aβ	amyloid-beta
BARL	β-catenin activated reporter luciferase
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
Bcl-X_L	B cell lymphoma extra large
BLT2	leukotriene B4 receptor 2
β-catenin	beta-catenin
β-Gal	beta-galactosidase
β-Me	beta-mercaptoethanol
β-TrCP	beta-transducin repeat containing E3 ubiquitin protein ligase
CDK11p^{46}	caspase-processed C-terminal domain of cyclin-dependent kinase 11
cDNA	complementary DNA
CK1	casein kinase 1
CRA	CT11-RanBPM
CRISPR	clustered regularly interspaced short palindromic repeats
CTLH	C-terminal to LisH
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

Dvl	dishevelled
ECM	extracellular matrix
EDD	E3 ubiquitin ligase identified by differential display
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-related kinase
FAP	familial adenomatous polyposis
FBPase	fructose-1,6-bisphophatase
FBS	fetal bovine serum
FOX	Forkhead box
Fz	Frizzled receptor
GID	glucose induced degradation deficient
GR	glucocorticoid receptor
Grb2	growth factor receptor bound protein 2
GSK3β	glycogen synthase kinase 3-beta
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	human influenza hemagglutinin
HAT	histone acetyltransferase
HBP1	HMG box containing protein 1
HDAC6	histone deacetylase 6
HEPES	hydroxyethyl piperazineethanesulfonic acid
HMG	high mobility group
HSP90	heat shock protein 90
ICGC	international cancer genome consortium
ICL1	isocitrate lyase
IR	ionizing radiation

JADE-1	gene for apoptosis and differentiation in epithelia 1
JNK2	Jun N-terminal kinase 2
KDM2a/b	lysine demethylase 2a/b
LEF	lymphoid enhancer factor
LFA-1	lymphocyte function-associated antigen-1
LisH	lissencephaly type-1-like homology
LRP5/6	LDL receptor-related proteins 5 and 6
MAEA	macrophage erythroblast attacher
MDH2	malate dehydrogenase
MEF	mouse embryonic fibroblast
MET	mesenchymal epithelial transition factor
MG132	N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal
NFAT	nuclear factors of activated T-cells
NF-κB	nuclear factor kappa B
NLS	nuclear localization signal
NP-40	Nonidet P-40
P75NTR	p75 neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PARylate	poly-ADP-ribosylate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide-3-kinase
PKCδ	protein kinase C-delta
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
RAC1	Ras-related C3 botulinum toxin substrate 1

RAF	rapidly accelerated fibrosarcoma
Ran	Ras related nuclear
RanBP9	Ran binding protein 9
RanBPM	Ran binding protein microtubule organization center
RAS	rat sarcoma
RING	really interesting new gene
RIPA	radioimmunoprecipitation assay
RIPK4	receptor-interaction protein kinase 4
RMND5A	required for meiotic nuclear division 5 homolog A
RYR	ryanodine receptor calcium-release channel
SDS	sodium dodecyl sulphate
sgRNA	single-guide RNA
shRNA	short hairpin RNA
SIAH-1	Seven in Absentia homolog-1
siRNA	short interfering RNA
SplA	spore lysis A
SPRY	spore lysis A and ryanodine receptor
Src	sarcoma
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TAF4	TATA box binding protein-associated 4
TBST	Tris-buffered saline with Tween-20
TCF	T-cell factor
TFBS	transcription factor binding site
TFIID	transcription factor II D
TLE1	transducin like enhancer-1
TNF	tumour necrosis factor
TP53	tumour protein p53

TNKS2	Tankyrase-2
TR	thyroid hormone receptor
TRAF6	tumour necrosis factor receptor-associated factor 6
TRIM33	tripartite motif-containing protein 33
TRIS	Tris-hydroxymethyl amino methane
TrkA	tropomyosin kinase A
TUBE	tandem ubiquitin binding entities
TWA1	two hybrid-associated protein 1 with RanBPM
TX100	Triton X-100
WDR26	WD repeat-containing protein 26
Wnt	wingless-integrated
WRE	Wnt responsive element

Chapter 1

1 Introduction

1.1 Cancer

Cancer is one of the leading causes of death in Canada, and it is estimated that one in two Canadians will develop cancer within their lifetime with one of four dying of the disease (1). Over the past three decades, the advancement in technology and scientific research of cancer has decreased the mortality rates for all forms of cancer (1). In spite of decreasing mortality rates, cancer-related deaths have been increasing and are expected to continue as our population grows in number and age (1). To combat this increased incidence, further advancements must be made into understanding the complex disease of cancer to provide more effective diagnoses and treatments.

Initially, tumorigenesis was only linked to genomic alterations such as single deoxyribonucleic acid (DNA) mutations or the complete loss or duplication of genetic material (2). However, recent discoveries in the growing field of epigenomics has revealed that epigenetic changes such as DNA methylation, histone acetylation, or chromosomal rearrangement also promote tumorigenesis (3). It is the multi-step accumulation of these genomic and epigenomic changes that promotes the transformation of a normal human cell to a cancerous cell (3). During carcinogenesis, there are six targeted processes commonly altered presenting what we know as the six hallmarks of cancer (4). These hallmarks consist of: the abilities to evade growth suppression, resist cell death, enable replicative immortality, provide sustained proliferative signalling, induce angiogenesis and promote invasion and metastasis (4). While each hallmark may individually contribute to cancer progression, not all are required for the transformation of a normal cell to cancerous cell (4). Different hallmarks may be displayed within a heterogeneous collection of cancer cells, while the same cell may display different hallmarks at different times of cancer progression (4). Thus, each cancer may display a unique and changing pattern of hallmarks and genomic alterations increasing the complexity of, and difficulty in treating cancer. To effectively treat cancer, we need to understand the underlying mechanism of how a cell activates these hallmark pathways.

While many proteins and targets have already been identified and used to design new strategies for cancer therapy, there is still much to discover. This study investigates the role that the Ran-binding protein microtubule organization center (RanBPM) plays in the regulation of the signal transduction molecule beta-catenin (β-catenin) of the wingless-integrated (Wnt) pathway.

1.2 RanBPM

1.2.1 Overview

RanBPM, also known as RanBP9, was originally identified in 1998 through a yeast two-hybrid assay as a 55 kDa protein and the ninth member of the Ras-related nuclear (Ran) protein family (5). Ran proteins are guanosine triphosphatases (GTPases) of the rat sarcoma (Ras) superfamily and are involved in nuclear transport during interphase, mitotic spindle formation during mitosis, and are co-activators of the androgen receptor (6). RanBPM was first reported to interact with Ran, localize to the centrosome and was believed to assist in microtubule nucleation (5).

These findings were later dismissed as it was discovered the 55 kDa protein was a truncated version of the full length 90 kDa RanBPM (7). The full length RanBPM only weakly interacted with Ran and no longer localized to the centrosome (7). Instead, the full length RanBPM was found to be a ubiquitously expressed nucleocytoplasmic protein possessing a number of highly conserved domains, however these did not confer any enzymatic activity (8). Since its discovery, RanBPM has been demonstrated to influence several cellular processes including apoptosis, cell adhesion and migration, gene transcription and metastasis (9-23). Although it has no enzymatic activity, RanBPM instead functions as a scaffold protein and impacts these pathways through mediating protein-protein interactions (22, 24).

1.2.2 Conserved Domains and Structure

RanBPM has five conserved domains that contribute to its protein-protein interactions; the N-terminal proline-glutamine domain, the spore lysis A and ryanodine receptor domain (SPRY), the lissencephaly type-1-like homology domain (LisH), the C-Terminal

to LisH domain (CTLH) and the C-terminal CT11-RanBPM domain (CRA) (Figure 1.1) (25). The N-terminal domain of RanBPM consists of a highly disordered proline and glutamine rich region (7). Polyglutamine proteins have previously been described to be pathogenic and form aggresomes around the centromere and attributed to neurodegenerative disease (26, 27). While RanBPM does contribute to aggresome formation, it is known that it achieves this by interacting with histone deacetylase 6 (HDAC6) via the LisH/CTLH domains (28).

Additionally, the proline rich region of the N-terminal domain contains six SH3-binding domains, which are predicted to have high affinity for the sarcoma (Src) and growth factor receptor bound protein 2 (Grb2) proteins (29, 30). The SPRY domain was first identified as being homologous to regions within the *Dictyostelium* discoideum, spore lysis A (SplA) kinase and the mammalian ryanodine receptor calcium-release channels (RyR) (31). The function of the SPRY domain is poorly understood, however many SPRY containing proteins harbour a really interesting new gene (RING) domain, which is characteristic of an E3 ubiquitin ligase (31). The LisH and CTLH domains are to the C-terminus of the SPRY domain. Both domains are predicted to have α-helical topology and together may form a unique globular domain responsible for regulating protein-protein interactions and microtubule dynamics (32-34). Lastly, the C-Terminal CRA domain is predicted to have six α-helices which is characteristic of the death domain superfamily (32).

1.2.3 Role in Transcriptional Regulation

Through its protein interacting domains, RanBPM has been demonstrated to influence several cellular processes, including transcriptional regulation. Performing a microarray analysis on cells stably expressing RanBPM shRNA or non-targeting control shRNA revealed a widespread change in gene expression upon downregulation of RanBPM (19). It was found that many genes affected had similar transcription factor binding sites in their promoter regions. The High Mobility Group (HMG), Forkhead (FOX) and Homeobox families of transcription factors were overrepresented among the promoters of genes affected by RanBPM downregulation (19). These families are known to have in important role in both development and tumorigenesis (19).

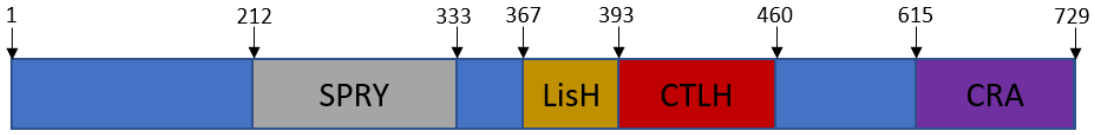

Figure 1.1. Schematic Diagram of RanBPM. The conserved domains of RanBPM are shown: The SPRY (spore lysis A and ryanodine receptor) domain; the LisH (lissencephaly type-1-like homology) domain; the CTLH (C-terminal to LisH) domain; and the CRA (CT11-RanBPM) domain. Figure is adapted from (22).

Additionally, RanBPM has been shown to be a potent transcriptional co-activator for the glucocorticoid (GR), androgen (AR), and thyroid hormone (TR) nuclear receptors (21,35). RanBPM is also a cofactor for the TATA box binding protein-associated factor 4 (TAF4), a subunit of transcription factor II D (TFIID), a coactivator of several transcription factors and nuclear receptors (36).

RanBPM was shown to interact with tropomyosin kinase A (TrkA) and negatively regulate nerve growth factor activated nuclear factors of activated T-cells (NFAT)-dependent transcription (37). Additionally, RanBPM is a negative regulator of the nuclear factor kappa B (NF-kB) pathway. RanBPM has been reported to bind to TND receptor-associated factor 6 (TRAF6) to inhibit auto-ubiquitination and activation of NF-kB signalling (38). Together, these exemplify the role RanBPM plays as both a positive and negative regulator of transcriptional regulation through the direct interaction with transcription factors or the indirect targeting of upstream trans-regulatory elements. However, the exact mechanism of how RanBPM achieves this transcriptional regulation remains unknown.

1.2.4 Role in Apoptosis

RanBPM has also been demonstrated to play a role in the activation of apoptosis. RanBPM promotes cell death through caspase-3 activation mediated by the CRA domain, which as previously mentioned resembles the death domain superfamily (10, 32). Caspase-3 activation is regulated by B cell lymphoma 2 (Bcl-2) family proteins. These proteins function to regulate the intrinsic apoptotic pathway through the interaction of both inhibitors and activators of cell death (39). In response to DNA damage, pro-apoptotic Bcl-2 family proteins function to release cytochrome *c* from the mitochondrial membrane in order to promote caspase activation and trigger cell death (39). It is through this intrinsic pathway that RanBPM regulates apoptosis. Cells with stably downregulated RanBPM showed increased resistance to cell death following treatment with ionizing radiation (IR) (10). Similarly, reduced RanBPM provides resistance to chemotherapeutic agents in gastric cancer cells (23). It was found the downregulated RanBPM cells had an increase in expression of anti-apoptotic proteins Bcl-2 and B cell lymphoma extra large (Bcl-X_L) while there was a decrease in mitochondria-associated Bcl-2-associated X

protein (Bax), a pro-apoptotic Bcl-2 family protein (10). Together these lead to a significantly reduced induction of caspase-3 and apoptosis. Conversely, overexpression of RanBPM has been shown to decrease Bcl-2 protein levels, increase Bax oligomerization at the mitochondrial membrane increasing the permeability and release of cytochrome *c* to activate apoptosis (11, 22). Lastly, overexpression of RanBPM in transgenic mice resulted in impaired mitochondrial function, and potentiated amyloid-β (Aβ)-induced apoptosis (40).

RanBPM's regulation of the intrinsic apoptotic pathway may be mediated through its protein-protein interactions with factors that regulate apoptotic activation. For instance, RanBPM interacts with p75 neurotrophic receptor (p75NTR), a member of the tumour necrosis factor (TNF) receptor superfamily (41). While p75NTR has a dichotomous role in promoting both cell survival and apoptosis, it is suggested that RanBPM may act as an adaptor protein that mediates p75NTR-induced apoptosis as it interacts with p75NTR's cytoplasmic death domain (41). Additionally, RanBPM is known to interact with the C-terminal domain of cyclin-dependent kinase 11 ($CDK11^{p46}$) (42). $CDK11^{p46}$ is the caspase-processed product of the larger $CDK11^{p110}$ isoform, and is known to promote apoptosis, however the mechanism of $CDK11^{p46}$ apoptotic activation is unclear (42). However, in these studies, the functional outcome of RanBPM's interaction with p75NTR and $CDK11^{p46}$ was not investigated, thus its role in influencing the apoptotic process through these two pathways is unclear. Lastly, RanBPM interacts with and stabilizes the tumour suppressor p73 by preventing its ubiquitination and subsequent degradation (12). The overexpression of RanBPM was shown to increase p73 mRNA levels and loss of RanBPM blocked p73-induced apoptosis (11). While the mechanism may not be fully understood, these studies indicate RanBPM has a pro-apoptotic affect and may regulate the apoptotic pathway in multiple ways.

1.2.5 RanBPM in Migration, Adhesion and Morphology

RanBPM has also been reported to regulate cellular adhesion, morphology and migration. One way this is accomplished is through its direct interaction with the β-integrin lymphocyte function-associated antigen-1 (LFA-1) receptor (18). Integrins have an important role in cell adhesion and migration where they are responsible for attaching the

cytoskeleton to the extracellular matrix (ECM) (43). They also function to transmit information from the ECM into the cell and control the activation of several signalling pathways (43). Overexpression of RanBPM was shown to increase endocytosis and internalization of β-integrin proteins resulting in decreased cell adhesion and spreading (44). Alternatively, upon knockdown of RanBPM, β-integrin levels increased at the cell surface promoting cell adhesion and spreading (44). RanBPM also prevents focal adhesion signalling and assembly by preventing the localization of cell adhesion proteins to focal adhesion points (44). Similarly, RanBPM was shown to prevent cell adhesion and affected cell morphology in female germline stem cell niche in *Drosophila* (13). RanBPM also interacts with Plexin-A1 receptor, and when RanBPM was overexpressed, cell spreading was reduced, and cells appeared irregularly contracted (45). Lastly, recent studies have shown RanBPM to form a complex with Muskelin, and the knockdown of either RanBPM or Muskelin resulted in an enlarged and protrusive cell perimeter (46).

RanBPM has been shown to both positively and negatively affect cell migration. RanBPM is known to interact with the mesenchymal epithelial transition factor tyrosine kinase (MET) and activate the extracellular signal regulated kinase (ERK) pathway, which in turn promotes cell migration (16). On the other hand, RanBPM has been shown to inhibit leukotriene B4 receptor 2 (BLT2)-mediated migration, where overexpression of RanBPM inhibited chemotactic migration (47). Our laboratory has demonstrated that downregulation of RanBPM led to increased cell migration in a wound healing scratch assay (20). A recent study in gastric cancer cells has shown that the downregulation of RanBPM led to a decrease in cell adhesion, increased cell migration and was commonly found downregulated in metastatic gastric tumours compared to non-metastatic tumours (23). Together, the function of RanBPM in maintaining adhesion and morphology and its opposing role in migration indicate its importance for normal physiological development as well as pathogenesis.

1.2.6 RanBPM in Cancer

As previously described, cancer progression is linked to six hallmarks including but not limited to proliferative signalling, evasion of apoptosis, invasion and metastasis (4). Previous studies have suggested that the dysregulation of RanBPM may promote these

three hallmarks of cancer (9-23). The microarray analysis on cells with stably downregulated RanBPM showed 167 differentially expressed genes, with over one third of these genes being associated with cancer (19). Notably, pathways affected by RanBPM downregulation included the ERK, wingless-integrated (Wnt), Notch and phosphoinositide-3 kinase (PI3K) pathways (19). These pathways are known to regulate proliferation, cell cycle progression, growth, survival and are commonly dysregulated in cancer (48-51).

Further characterization of RanBPM in our laboratory has revealed a tumour suppressive role for RanBPM through the inhibition of ERK signalling (20). RanBPM was shown to interact with and down regulate rapidly accelerating fibrosarcoma (RAF) protein levels, preventing its binding to heat shock protein 90 (Hsp90), subsequently preventing the activation of ERK1 (20). This in turn downregulated anti-apoptotic BCL-2 family proteins both at the transcriptional and protein level (10, 20). As such, downregulation of RanBPM caused an increase in anti-apoptotic proteins, decreasing mitochondrial permeabilization, and provided these cells the ability to evade apoptosis (10).

Additionally, studies have implicated a role for RanBPM in regulating cellular migration and metastasis. RanBPM has been shown to interact with histone deacetylase 6 (HDAC6) through its LisH and CTLH domains and to negatively regulate its deacetylase activity (28). HDAC6 is a cytoplasmic deacetylase necessary to the formations of aggresomes and when overexpressed is known to promote cellular transformation and metastasis (28, 52). Similarly, in gastric cancer cells, the downregulation of RanBPM was associated with an increase in cell proliferation and motility (23). Additionally, metastatic gastric cancers were shown to have lower expression of RanBPM in distant metastatic tumours compared to non-distant metastatic tumours, further implicating RanBPM in the regulation of metastases (23). While many reports suggest RanBPM to play a tumour suppressive role, there have been contrasting studies suggesting it to function as an activator of ERK signalling (16). RanBPM has been shown to interact with the tyrosine-protein kinase MET mediated its SPRY domain, inducing the guanosine triphosphate (GTP) transfer to rat sarcoma (RAS), leading to the phosphorylation and activation of

ERK1 (16). This indicates a more complex role of RanBPM in tumorigenesis that may be cell-type or cancer stage dependent.

1.3 The CTLH Complex

While previous studies have looked at RanBPM in isolation, when first characterized it was found to exist in a 670 kDa protein complex (7). This was later identified to be a seven-member complex comprised of two-hybrid-associated protein 1 with RanBPM (TWA1), Muskelin, armadillo repeat containing 8 (ARMC8) α and β, required for meiotic nuclear division homologue A (RMND5A), macrophage erythroblast attacher (MAEA) and RanBPM (Figure 1.2) (53). It was given the name the C-terminal to LisH (CTLH) complex as each member, other than ARMC8α/β, contains a CTLH and LisH domain (53). Apart from Muskelin, each member of the CTLH complex has a homologous protein in *Saccharomyces cerevisiae* (54). Together, in *S. cerevisiae* these form the glucose induced degradation deficient (GID) complex which contains WD repeat-containing protein 26 (WDR26) or GID7, in the place of Muskelin (54). While the initial characterization of the CTLH complex did not find WDR26, more recent studies suggest it exists in the CTLH complex (55, 56).

The yeast GID complex is a E3 ubiquitin ligase with a role in regulating glucose metabolism (54). When yeast cells are switched from an ethanol carbon source to a glucose media, the GID complex becomes activated and targets fructose-1-6-bisphosphatase (FBPase), a key enzyme in the gluconeogenic pathway, for ubiquitination and subsequent proteasomal degradation (54, 57). Targeting FBPase upon reintroduction of glucose allows yeast to utilize glycolysis over gluconeogenesis. Interestingly, three members of the GID complex, GID1, GID4 and GID5, also participate in the vacuolar proteolytic system, an alternative pathway which also triggers the degradation of FBPase (58).

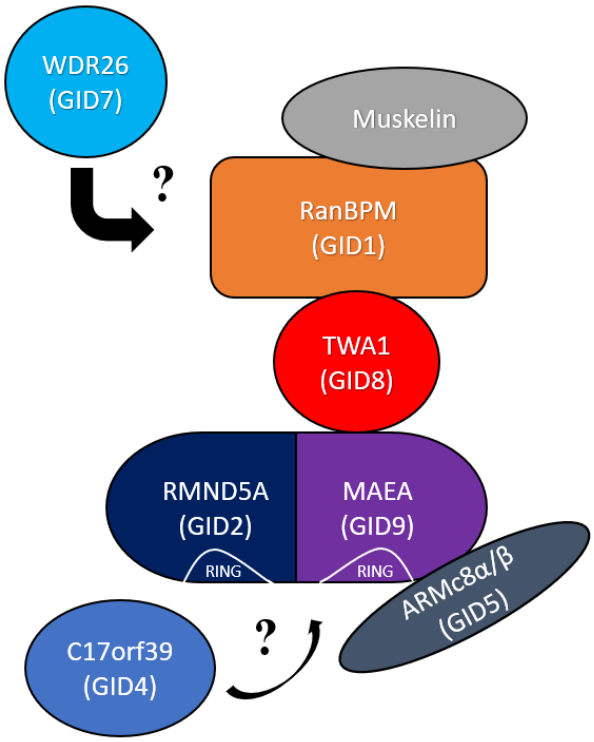

Figure 1.2 Proposed topology of the CTLH complex. The CTLH complex consists of the Scaffolding protein RanBPM (Ran binding protein microtubule organization center), with required for meiotic nuclear division homologue A (RMND5A), macrophage erythroblast attacher (MAEA), two-hybrid-associated protein 1 with RanBPM (TWA1), Muskelin, armadillo repeat containing 8 α and β (ARMC8α/β), and Muskelin. Each protein is labelled with its corresponding *S. cerevisiae* homologue (indicated in parentheses). It is hypothesized to also comprise of WD repeat 26 (WDR26) and C17orf39, the mammalian homologue to the *S. cerevisiae* GID4, however these have yet to be confirmed as part of the complex. Figure adapted from (22).

A recent study has revealed one GID member, GID4, to be the recognition component of the GID complex (59). GID4 is absent from yeast cells when grown in ethanol, but upon introduction of glucose it is rapidly synthesized which coincides with the degradation of FBPase (59). It was determined that GID4 specifically recognizes the N-terminal proline of FBPase, as well as recognizing and targeting isocitrate lyase (Icl1) and malate dehydrogenase (Mdh2), two additional gluconeogenic enzymes, for ubiquitination (59).

Two members of the GID complex, GID2 (RMND5A) and GID9 (MAEA), contain RING zinc finger domains, which is the functional domain conferring E3 ubiquitin ligase activity (54, 60, 61). RING-type E3 ligases function together with an E2 ubiquitin-conjugating enzyme to mediate the transfer of a ubiquitin onto a target substrate (61). While both GID subunits contain RING domains, only GID2 has successfully been shown to have *in vitro* ubiquitin ligase activity (54, 60). However, GID9 is required for GID2 ubiquitination activity *in vivo*, as mutations made to the RING domain of GID9 abolishes the FBPase ubiquitination by GID2 following a switch to a glucose-rich media (60).

While the GID complex appears to be conserved in the mammalian CTLH complex, which also contains two RING domain containing proteins, RMND5A (GID2) and MAEA (GID9), the mammalian complex has yet to be shown to contain ubiquitination activity *in vivo* (25). However, recently through reconstituting each member, the CTLH complex ubiquitination activity has been demonstrated *in vitro* with HMG box-containing protein 1 (Hbp1) as a substrate (62).

A number of studies have also implicated components of the CTLH complex in the regulation of β-catenin (13, 19, 56, 63, 64). The putative CTLH member, WDR26, has previously been shown to interact with Axin1 and regulate β-catenin protein levels (63). A separate interactome study has revealed that WDR26, and all CTLH members to be an interacting partner with Axin1 (56). As previously mentioned, our laboratory has demonstrated that the downregulation of RanBPM results in dysregulation of genes within the Wnt signalling pathway (13). RanBPM has been shown to regulate β-catenin

protein levels in *Drosophila*, and ARMc8α has been shown to interact with β-catenin and regulate α-catenin protein levels (19, 64). These previous studies implicating the CTLH complex with β-catenin regulation raises the question whether it could be a novel target of CTLH ubiquitination.

1.4 Ubiquitination Overview

The ubiquitination pathway provides an essential post-translational modification that regulates cellular signalling and protein turnover (65). Ubiquitin is a small 8.5 kDa protein that can be covalently linked to lysine residues of a protein substrate including itself (66). The addition of a single ubiquitin (mono-ubiquitination) can affect protein stability, cellular localization and protein-protein interactions (67, 68). Addition of multiple, or polyubiquitination, serves as a signal to target proteins for degradation by the 26S proteasome (69). This process requires three enzymes: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin-protein ligases (E3) (70). The E1 activating enzyme uses adenosine triphosphate (ATP) to form a thiol-ester bond with the ubiquitin protein at its carboxyl terminus activating the ubiquitin. The activated ubiquitin is transferred to a E2 conjugating enzyme which transiently carries the ubiquitin and recruits an E3 ubiquitin ligase. The E3 ubiquitin ligase then transfers the ubiquitin from the E2 enzyme onto a specific lysine residue of a target substrate (70). The specificity of ubiquitination is driven by E3 ubiquitin ligases, of which over 600 are encoded in the human genome giving a diverse range of target substrates influencing a number of processes including protein trafficking, DNA repair and cell signalling (65). One pathway that is regulated by ubiquitination is the canonical Wnt signalling pathway.

1.5 The Wnt Signalling Pathway

1.5.1 β-Catenin Overview

β-catenin is a 90 kDa, multi-functioning protein that is involved in both the regulation of gene transcription and cellular adhesion (71, 72). β-catenin was first discovered as an interacting partner to the cadherin family of proteins (73). Cadherins are a family of cell adhesion molecules that promote cell-cell adhesion through the formation of adherens junctions (74). β-catenin has been shown localize to adherens junctions and interact with

the cytoplasmic tail of E-cadherin forming a Cadherin-β-catenin complex (75, 76). It is thought that β-catenin indirectly mediates the interaction of the actin cytoskeleton with E-cadherin to promote cell-cell contact, and the loss of E-cadherin-β-catenin complex formation has been associated with increased epithelial-to-mesenchymal transition (EMT) (77).

Since its discovery, β-catenin has also been shown to play a crucial role in the Wnt signalling pathway (78). The Wnt signalling pathway is a key regulator of cellular proliferation, differentiation and is critical for embryonic development (72). β-catenin is required for the activation of Wnt targeted gene expression, and the dysregulation of β-catenin/Wnt signalling has been closely linked to the progression of Familial Adenomatous Polyposis (FAP), among various forms of cancer (79). See figure 1.3 for an overview of Wnt signalling and the regulation of β-catenin.

1.5.2 β-catenin regulation

In the absence of Wnt ligand, or in the Wnt "off" state, β-catenin is under strict regulation by the Axin degradation complex consisting of the proteins Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase 3-beta (GSK3β) and Casein Kinase 1 (CK1) all bound to the Axin1 scaffolding protein (80). Together, this protein complex works to sequentially phosphorylate β-catenin at serine-45 (S45) by CK1 followed by GSK3β phosphorylation at threonine-41 (T41) and serine-33 and -37 (80, 81). This phosphorylation pattern is recognized by the beta-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) which ubiquitinates and promotes β-catenin for degradation by the proteasome (82). While β-TrCP is the most common E3 ubiquitin ligase associated with β-catenin regulation, additional E3 ubiquitin ligases including Seven in Absentia Homologue 1 (SIAH-1) and the E3 ubiquitin ligase identified by Differential Display (EDD), have been shown to ubiquitinate β-catenin, revealing a more diverse mechanism to β-catenin regulation (83, 84).

1.5.3 Activation of Wnt signalling

The activation of the canonical Wnt signalling pathway occurs by one of six Wnt ligands (Wnt3, Wnt4, Wnt5b, Wnt7a, Wnt10a and Wnt10b) and requires both the Frizzled (Fz)

and the LDL receptor-related proteins 5 and 6 (LRP5/6) receptors (72, 85, 86). Stimulation by Wnt ligands causes these receptors to form a Wnt-Fz-LRP5/6 complex and in turn recruits the intracellular scaffolding protein Dishevelled (Dvl) (87, 88). Dvl promotes the oligomerization of individual Wnt-Fz-LRP5/6 complexes at the cell membrane (88). LRP5/6 contains five PPPSPxS (P: Proline; S: Serine or Threonine; x: variable) phosphorylation motifs mimicking that of β-catenin (89). When these receptors are aggregated, the Axin degradation complex has a high affinity for these PPPSPxS motifs and will move to the cell membrane to phosphorylate LRP5/6. This essentially sequesters the Axin degradation complex at the cell membrane, allowing for β-catenin to accumulate (81, 90).

While the stabilization of β-catenin protein levels coincides with an increased nuclear presence, the exact mechanism for β-catenin translocation into the nucleus is not fully understood. It has been suggested that β-catenin contains a nuclear localization signal (NLS) and thus enters directly into the nucleus through nuclear pore proteins while β-catenin co-activators increase its retention in the nucleus (91, 92). More recent studies show that in addition to stabilization of β-catenin protein levels, activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) is also required for β-catenin nuclear import (93). It is suggested that Rac1 forms a cytoplasmic complex with Jun N-terminal kinase 2 (JNK2) and β-catenin, and JNK2 phosphorylates β-catenin at serine 191 and 605 (S191/S605) promoting its nuclear import (93).

Upon nuclear translocation, β-catenin interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors (94). TCF/LEF proteins are HMG DNA-binding factors that target and bind to Wnt responsive element (WRE) DNA sequences (95). In the absence of β-catenin, the TCF/LEF proteins interact with transducin-like enhancer 1 (TLE1) which functions to promote histone deacetylation of WREs, causing chromatin compaction and inhibiting gene expression (96). When interacting with TCF/LEF proteins, β-catenin directly displaces TLE1 and recruits a host of co-activators, including chromatin remodeling proteins and histone acetyltransferases (HAT), to enable gene expression (97, 98).

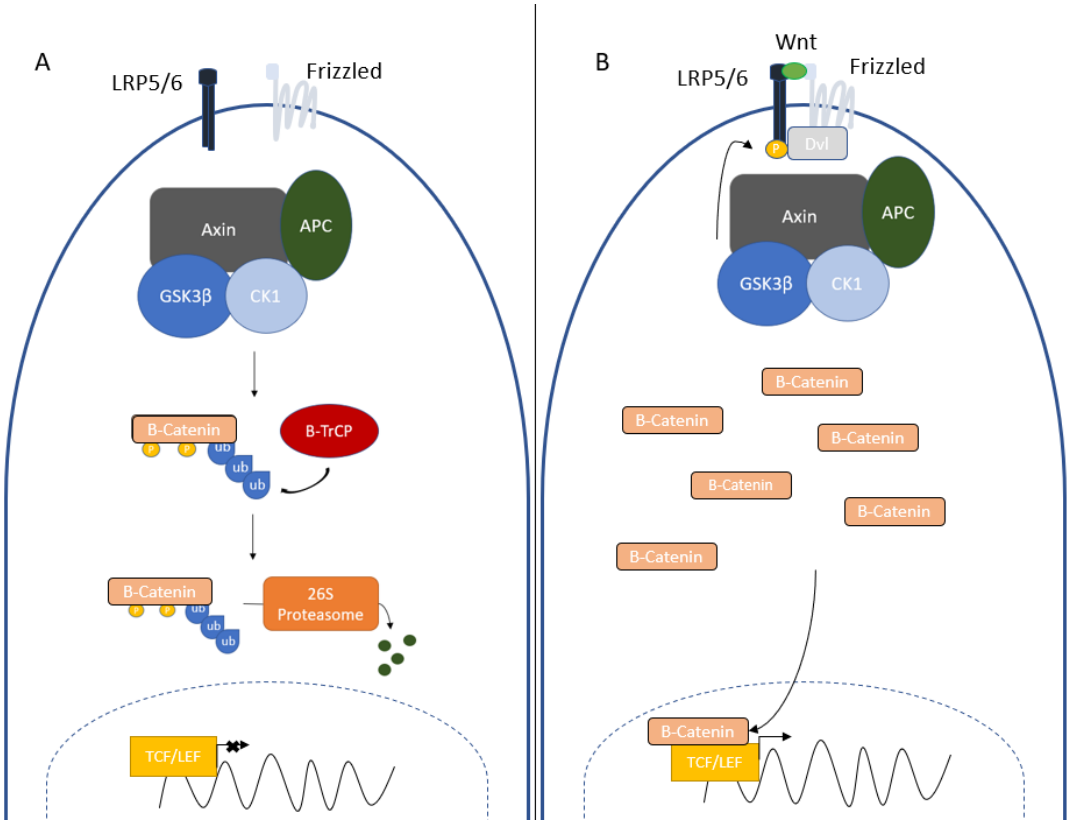

Figure 1.3 Overview of β-catenin regulation. A) In the absence of a Wnt signal, β-catenin is under strict regulation by the Axin degradation complex, which consists of the scaffolding protein Axin along with APC (adenomatous polyposis coli) protein, CK1 (casein kinase 1) and GSK3β (glycogen synthase kinase 3-beta). The Axin degradation complex functions to sequentially phosphorylate β-catenin. This phosphorylation is identified by the E3 ubiquitin ligase β-TrCP (beta-transducin repeat containing E3 ubiquitin protein ligase), which polyubiquitinates β-catenin promoting it for degradation by the 26S proteasome. B) In the presence of a Wnt signal, the receptors LRP5/6 (LDL receptor-related proteins 5 and 6) and Frizzled dimerize and recruit the protein Dvl (Dishevelled). Dvl functions to recruit and sequester the Axin degradation complex at the cell membrane, allowing for β-catenin to accumulate, translocate into the nucleus where it activated Wnt target gene expression. Figure adapted from (72).

The dysregulation of β-catenin signalling has been associated with numerous forms of cancer including but not limited to colorectal cancer, hepatocellular carcinoma, ovarian cancer and melanoma (72, 79, 85). The oncogenes MYC and CCND1 contain WREs and have been identified as targets of β-catenin signalling (99, 100). C-Myc is a key regulator for both cellular proliferation and metabolism while cyclin D1 is a key regulator of cell cycle progression, and individually can promote cancer progression when dysregulated (100, 101). Given β-catenin's role in regulating these oncogenic pathways, it is crucial to identify novel therapeutic mechanisms to potentially downregulate β-catenin.

1.6 Hypothesis and Objectives

Several studies have revealed CTLH members to interact with components of the Wnt signalling pathway and to regulate β-catenin levels, however how this regulation is achieved has yet been investigated. Thus, I hypothesize that the CTLH complex is an additional E3 ubiquitin ligase regulating the Wnt signalling pathway through the targeting of β-catenin for degradation. The outcome of this study is to address the following objectives:

1) To show that the putative member, WDR26, is part of the CTLH complex and this complex is interacting and/or co-localizing with Axin

2) To determine whether the CTLH complex regulates β-catenin protein levels

3) To determine whether the CTLH complex regulates Wnt/β-catenin target-gene expression

This work could potentially provide a novel way to target β-catenin for regulation, revealing a potential new therapeutic strategy in treating cancers characterized with oncogenic mutations in β-catenin/components of the Wnt pathway. The research performed in this study will examine the possible tumour suppressor role that the CTLH complex plays in negatively regulating Wnt/β-catenin signalling.

Chapter 2

2 Materials and Methods

2.1 Chemical Reagents

2.1.1 DNA Constructs and Cloning

pCMV-HA was purchased from ClonTech Laboratories Inc. (Currently known as Takara Bio USA, Inc.), pCMV-HA-RanBPM was provided by Dr. Mark Nelson (University of Arizona, Tucson, AZ, USA). pCDNA3.1-HIS-FLAG-WDR26 was provided by Dr. Songhai Chen (University of Iowa, Iowa City, IA, USA). A pGL3 luciferase vector containing a concatemer of 12 consecutive TCF response elements upstream of Promega's MinP minimal promoter, designated β-catenin Activate Reporter Luciferase (pBARL) was provided by Dr. Greg Kelly (University of Western Ontario, London, ON, Canada). pSV40-Renilla was purchased from Promega. The pCGN-HA-RMND5A was prepared by a previous Schild-Poulter Laboratory member by polymerase chain reaction (PCR) amplifying RMND5A from a complementary DNA (cDNA) library. RMND5A was ligated into a pCGN-HA vector using the enzymes *BamH1* and *Xba*. A pCMV-HA-Ubiquitin was received from Dr. Lina Dagnino (University of Western Ontario, London, ON, Canada).

2.1.2 In vitro assays

Sodium chloride (NaCl), potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), hydroxyethyl piperazineethanesulfonic acid (HEPES), Tris-hydroxymethyl amino methane (Tris), hydrochloric acid (HCl), Tween-20 and sodium dodecyl sulphate (SDS) were purchased from Wisent Inc. (St. Bruno, QC, Canada). Nonidet P-40 (NP-40), TritonX-100 (TX100), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), leupeptin, aprotinin, pepstatin, sodium fluoride (NaF) and sodium orthovanadate (Na_3VO_4) were purchased from Bioshop Inc. (Burlington, ON, Canada). Glycerol was purchased from Caledon Laboratory Chemicals Ltd. (Georgetown, ON, Canada).

2.2 Cell Culture, Transfections and Treatments

HeLa and HCT 116 cells were purchased from the American Type Culture Collection (ATCC). HeLa and HCT 116 cell lines stabling expressing RanBPM short-hairpin (shRNA) or non-targeting control shRNA were generated as previously described in (10, 20). The cell lines were grown in high-glucose DMEM (DMEM) (Wisent Inc.) supplemented with 8% fetal bovine serum (FBS) (Wisent Inc.), 1% sodium pyruvate (Wisent Inc.), 1% L-glutamine (Wisent Inc.) and 4.5 g/L glucose and incubated at 37^{O} C and 5% CO_2. To maintain stable selection the HeLa and HCT 116 shRNA cells were grown in 0.35 g/L G418 sulphate (Bioshop Inc). Immortalized mouse embryonic fibroblasts (MEFs) deficient for RanBPM were generated by Schild-Poulter laboratory member Xu Wang using methods previously described (102). Mouse embryonic fibroblasts were grown in high-glucose DMEM with the same supplementation as above, with G418 sulphate being replaced with 0.1 mM of beta-mercaptoethanol (βME). CaCo2 cells were generously provided by Dr. Tirona Rommel (University of Western Ontario, London, ON, Canada) and were grown in before mentioned high-glucose conditions supplemented with 1% penicillin-streptomycin (450-201-EL; Wisent Inc.). For a full list of cell lines used refer to Table 2.1.

Cells were cultured onto 6 or 10 cm plates for transfection and protein isolation. Plasmid transfections were performed using JetPRIME® Transfection Reagent (PolyPlus Transfection) according to the manufacturer's protocol. The amount of DNA used for each transfection is provided in figure legends. pBS-SK (Agilent Technologies) was used to supplement transfected DNA to the minimum amount if required.

CaCo2 cells were treated using RanBPM short-interfering RNA (siRNA) (AM16708A; Ambion) or control siRNA (4390843; Ambion) at a concentration of 10 nM. N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal (MG-132) (Bioshop Inc.) treatments were performed using MG-132 or dimethyl sulfoxide (DMSO) (Bioshop Inc.) as a vehicle at 10 μM for 6-24 h.

Table 2.1 List of cell lines used with cell type of origin and gene status of CTLH members, β-catenin and APC. B-catenin and APC status adapted from (112).

Cell Type	Origin of Cell	CTLH Member Status	B-catenin Status	APC Status
HeLa	Cervical Carcinoma	Wild-Type RanBPM downregulated RMND5A -/-	Wild-type	Wild-Type
HCT116	Colorectal Carcinoma	Wild-Type RanBPM Downregulated	Wild-Type	Mutant
CaCo-2	Colorectal Carcinoma	Wild-Type	Mutant	Mutant
Mouse Embryonic Fibroblast	Fibroblast	Wild-Type RanBPM -/-	Wild-Type	Wild-Type

2.3 HeLa RMND5A CRISPR Knockout Clones

Required For Meiotic Nuclear Division 5 Homolog A (RMND5A) CRISPR knockouts were generated by Schild-Poulter laboratory members Xu Wang and Matthew Maitland. Single-guide RNA (sgRNA) were designed against exon 3 of RMND5A using www.benchling.com (On-target score = 65.7, Off-target score = 86.0). Top and bottom oligomers (5' – CACCGTGGAGCACTTCTTTCGACA – 3'; 5' – AAACTGTCGAAAGAAGTGCTCCAC – 3') were annealed together and ligated with pSpCas9(BB)-2A-Puro (PX459) V2.0 that was digested with *BpiI*. Early passage wild-type HeLa cells were seeded on 12-well dishes and transfected with 800 ng of plasmid DNA. Forty-eight hours post-transfection, cells were put under puromycin selection for seven days. Afterwards, remaining single cells were transferred to a 48-well dish and expanded. Loss of RMND5A protein was confirmed by Western blot and DNA mutation was confirmed and the top off-target sites were checked for absence of mutations through sequencing.

2.4 Preparation of Cell Extracts

HeLa, HCT 116, MEF and CaCo2 cells were grown in 6 or 10 cm culture dishes to approximately 80% confluency. Briefly, the cells were washed thrice with cold phosphate buffered saline (PBS) (Wisent Inc.) and were scraped into a small amount of the final wash for collection. Cells were pelleted by centrifugation for 5 minutes at 8000rpm. The supernatant was removed, and the cell pellets were lysed on ice for 25 minutes in whole cell extract (WCE) buffer (150 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 7.4, 10% glycerol, 0.5% NP-40, and 0.5% TX100), or radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCL pH 8.0, 1% TX100, 0.1% sodium deoxycholate, and 0.1% SDS) supplemented with 0.1 mM PMSF, 1 mM DTT, 2 μg/mL leupeptin, 10 μg/mL aprotinin, 2.5 μg/mL pepstatin, 2 mM NaF and 2 mM Na_3VO_4. After lysis the cells were centrifuged for 20 minutes at 13,000 rpm at 4^{O} C and the resulting supernatant was collected. Protein concentration was then quantified using a Bradford Protein Assay (Bio-Rad) according to the manufacturer's protocol on an LKB Ultraspec 4050 (Biochrom).

2.5 Western Blot Analysis

Up to 50 μg of protein was resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following separations, gels were transferred to polyvinylidene fluoride (PVDF) membranes using a semi-dry Trans-Blot® Turbo (Bio-Rad) transfer system at 25V for 25 minutes. Membranes were blocked in 5% skim milk dissolved in Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.05% Tween-20) at room temperature for 1 h or overnight at 4^{O} C. Primary antibodies were hybridized overnight at 4^{O} C, or 2 h at room temperature. After primary hybridization, the membranes were washed three times in TBS-T for 10 minutes. Secondary antibodies were incubated at room temperature for 1 h, followed by another three 10-minute TBS-T washes. Membranes were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories Inc.) and imaged using a ChemiDoc MP (Bio-Rad Laboratories Inc.). Resulting images were analyzed using Image Lab software (Bio-Rad Laboratories Inc.).

2.6 Antibodies

2.6.1 Primary Antibodies

Primary antibodies used were β-Catenin (610154; BD Transduction Laboratories), Axin1 (C76H11; Cell Signaling Technology), Vinculin (E1E9V; Cell Signaling Technology), Beta-Actin (I-9; sc-1616-R; Santa Cruz), Human influenza hemagglutinin (HA; H9658; Sigma-Aldrich), FLAG (M2; F1804; Sigma-Aldrich), RanBPM (71-001; BioAcademia), RMND5A (NBP1-92337; Novus Biologicals), MAEA (AF7288; R&D Systems), ArmC8 (E-1; sc-365307; Santa-Cruz), Muskelin (C-12; sc-398956; Santa-Cruz), and WDR26 (ab85962; Abcam). Antibody concentrations can be found in Table 1.1.

2.6.2 Secondary Antibodies

Secondary antibodies used were peroxidase-conjugated AffiniPure Goat anti-Mouse IgG (H+L; Jackson ImmunoResearch Laboratories Inc.), Blotting Grade Goat anti-Rabbit IgG Horseradish Peroxidase Conjugate (H+L; Bio-Rad Laboratories Inc.), and Rabbit Anti-Sheep IgG Horseradish Peroxidase Conjugate (H+L; Bio-rad Laboratories Inc.). All secondary antibodies were used at a 1:5000 dilution.

Table 2.2 Concentrations used for primary antibodies in Western Blot Analysis

Antibody	Species	Dilution
β-Catenin	Mouse	1:5000
Axin1	Rabbit	1:2000
B-Actin	Mouse	1:5000
Vinculin	Rabbit	1:10,000
HA	Rabbit	1:2000
FLAG	Mouse	1:2000
RanBPM	Rabbit	1:2000
RMND5A	Rabbit	1:100
MAEA	Sheep	1:250
Armc8	Mouse	1:100
Muskelin	Mouse	1:250
WDR26	Rabbit	1:1000

2.7 *In vitro* Experiments

2.7.1 Immunoprecipitation Experiments

For co-immunoprecipitation experiments, 1 mg of total protein was used and immunoprecipitates were isolated with Dynabeads Protein G (Invitrogen). Every 500 µL of protein lysate was diluted with 800 µL of binding buffer (150 mM NaCl, 25 mM HEPES pH 7.9, 0.5 mM EDTA, 12% glycerol and 0.1% NP-40) supplemented with 0.1 mM PMSF, 1 mM DTT, 2 µg/mL leupeptin, 10 µg/mL aprotinin, 2.5 µg/mL pepstatin, 2 mM NaF and 2 mM Na_3VO_4. Samples were pre-cleared for one hour using one-eighth of total Dynabead Protein G volume. Pre-clear beads were removed, and the samples were incubated with up to 7 µg of FLAG (M2; F1804; Sigma-Aldrich) or RanBPM (F-1; sc-271727; Santa-Cruz) antibodies overnight at 4^{O} C. Beads were washed three times with an immunoprecipitation (IP) wash buffer (25 mM HEPES pH 7.9, 60 mM KCl, 0.5 mM EDTA, 0.25% NP-40 and 2% glycerol). Beads were resuspended in SDS loading dye (0.105 g/mL SDS, 0.093 g/mL DTT, 0.35 M Tris-HCl pH 6.8, and 30% glycerol). The samples were then resolved and analyzed through western blot methods.

For *in vitro* ubiquitin assays, wild-type and RMND5A CRISPR knockout cells were transfected with a pMT123-HA-Ubiquitin plasmid. Twenty-hour hours after transfection, cells were incubated with 10 µM MG132 for 6 hours. Cells were lysed in denaturing buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1% Triton-X, 1% SDS,10 mM NaF, 1 mM Na_3VO_4 and 25 mM NEM (N-Ethylamalide, Bioshop Canada, Burlington, ON, Canada)), and passed through a 23G needle ten times and incubated on ice for 30 minutes. Lysates were diluted 1:10 in buffer A (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1% Triton-X, 10 mM NaF, 1 mM Na_3VO_4 and 25 mM NEM) and incubated in primary β-catenin antibody overnight at 4 ^{O}C, followed by incubation with Dynabeads for 1 hour. Beads were washed 3 times with buffer A, resuspended and bolding in SDS loading dye and separated through SGS-Page and analyzed through western blot methods.

2.7.2 Immunofluorescence

Cells were seeded onto coverslips in a 24-well plate and incubated overnight. Cells were washed three times in cold PBS then fixed using 4% paraformaldehyde for 15 minutes.

Cells were permeabilized in 0.5% Triton-X100 for 10 minutes then blocked in 5% FBS diluted in PBS for 1 h at room temperature. Coverslips were incubated with β-Catenin (610154; BD Transduction Laboratories) antibody overnight at 4^{O} C with agitation. Coverslips were washed three times with cold PBS then incubated in the dark with anti-mouse Alexa Fluor 488 (Invitrogen) for 1 hour. Cells were washed again three times with cold PBS then mounted using ProLong® Gold antifade with DAPI (Invitrogen). Visualization was performed using an Olympus BX51 microscope with a 40x objective and images were taken using Image-Pro Plus software (Media Cybernetics Inc.). For each experiment, at least 50 cells analyzed and quantified using ImageJ software.

2.7.3 Luciferase Assay

5×10^4 cells were seeded into a 6-well plate and incubated overnight. Cells were co-transfected with pGL3-BARL and either pCMV-HA-Empty Vector or pCGN-HA-RMND5A. Cells were also transfected with either pSV40-Renilla or pCMV-β-Galactosidase as internal controls to normalize luciferase levels. Luciferase activity was measured using the Luciferase Assay System (E1500; Promega). Cells were collected 48 h after transfection and lysed in 1X Cell Culture Lysis Reagent for 15 minutes at room temperature. Immediately following lysis, 40 µL of cell lysates were added to 50 µL of Luciferase Assay Reagent in a 96-well micro-plate and activity was quantified using an Orion Microplate Luminometer (Berthold Detection Systems). Beta-galactosidase activity was measured using a Beta-Glo Assay System (E4270; Promega). Separately, 40 µL of cell lysate was added to 50 µL of Beta-Glo® Reagent and incubated for 30 minutes. Beta-galactosidase activity was quantified using an Orion Microplate Luminometer. pBARL luciferase activity was normalized to the β-galactosidase levels.

2.8 Statistical Analysis

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

Chapter 3

3 Results

3.1 Overview

The GID homologue of the CTLH complex in *S. cerevisiae* is a well-documented E3 ubiquitin ligase that is responsible for promoting the polyubiquitination and proteasomal degradation of multiple gluconeogenic enzymes including FBPase, Icl1 and Mdh2 (54, 57, 59). GID1, the yeast counterpart of RanBPM, was shown to be crucial for this ubiquitination, as altering either the SPRY, LisH or CTLH domain of GID1 impaired the ubiquitination of FBPase. In addition to GID1, the RING domain containing subunits GID2 and GID9 were also required and conferred the enzymatic activity of the complex (54). Since these components are highly conserved to the mammalian CTLH complex, it is theorized that the CTLH complex may also function similarly as an E3 ubiquitin ligase (25). Despite being highly conserved, the CTLH complex has only recently been demonstrated to contain ubiquitination activity, and thus far only Hbp1 has been identified as a substrate (62). Hbp1 is not a gluconeogenic enzyme, so this suggests the CTLH complex may regulate different pathways than its *S. cerevisiae* homologue.

Previous studies have implicated multiple members of the CTLH complex to regulate β-catenin, the signal transducer of the Wnt signalling pathway. As mentioned, the putative CTLH member WDR26 has been shown to interact with Axin1, and over expression of WDR26 caused a decrease in β-catenin protein levels (63). A separate study also showed that RanBPM regulates β-catenin in *Drosophila* where an increase in β-catenin protein levels were observed in RanBPM mutant cells (13). Together, this suggests that β-catenin may be regulated by the CTLH complex. However, we must first ascertain that WDR26 is part of the CTLH complex.

3.2 WDR26 is part of the CTLH complex

3.2.1 Disrupted WDR26 and core CTLH members show similar pattern of target tissues

Proper regulation of cell growth is maintained by cross-talk of intra- and extracellular signalling pathways including the Tumor protein 53 (p53), ERK and Wnt signalling pathways (103). Each has multiple components responsible for regulating pathway activation, but in many cases only one component is needed to be dysregulated for pathogenesis to occur. In context of the Wnt pathway, pathogenesis occurs through the activation of β-catenin, however this can occur through multiple mechanisms. Wnt pathway activation has been reported though activating mutations in β-catenin as well as dysregulation of components in the Axin degradation complex (112). Separate cases have demonstrated that deletion of Axin, GSK3β or APC causes the upregulation of β-catenin (112). Furthermore, dysregulation of Axin, GSK3β and APC commonly result in the development and progression of hepatocellular and colorectal carcinoma's (117-119). This suggests that proteins within one pathway, or complex, when dysregulated lead to a similar pathogenic outcome. Thus, we sought to determine the pathogenic outcome of dysregulated WDR26 in comparison to CTLH complex members to support the premise that WDR26 exists within the CTLH complex.

To achieve this, analysis of a large-scale cancer genomics data set was performed to assess the tissue distribution of mutations in WDR26 in comparison to other core CTLH members. Using the International Cancer Genome Consortium (ICGC), data was collected assessing the number tissues harbouring a point mutation in WDR26, RanBPM, and Core CTLH members, MAEA and RMND5A. The number of nonsynonymous mutations with a predicted functional impact found in these genes in primary tumours were 94, 87, 83, and 10 respectively. All four members showed mutations occurring in liver, colorectal or stomach tissue at the highest frequency, accounting for 40% to 70% of total mutations (Figure 3.1). Having such a highly correlated pattern in targeted tissues suggest that these proteins may act within the same pathway, or complex. As a control, these were compared to the tumour suppressors p53 and phosphatase and tensin homolog

(PTEN). PTEN displayed mutations commonly occurring in breast, stomach and esophageal tissue while p53 was commonly mutated in uterine, brain and breast tissue, and contained 651 and 3966 mutations respectively (Figure 3.1). Having a unique distribution of mutations shared by all core CTLH members that differs from two independent proteins suggests that WDR26 may share a similar function to the CTLH complex and that this result is not due to a sampling bias if liver, colorectal and stomach were to be the largest data sets in the ICGC.

3.2.2 WDR26 is localized to the cytoplasm and interacts with the CTLH complex

A recent study using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was performed assessing functional protein associations and determined that WDR26 was part of an interacting network with RanBPM and other CTLH members (56). However, in addition to known interactions, the STRING database uses computational methods to predict protein interactions, thus WDR26 interaction with the CTLH complex still needed to be confirmed. Previous studies investigating the cellular localization of RanBPM determined it was a nucleocytoplasmic protein and was found to localize with chromatin in the nucleus, as well as the plasma membrane (8, 22). The localization of other CTLH members has yet to be reported, however the LisH domain has been reported to be an important factor in determining localization and was shown to promote localization to actin fibers in the cytoplasm (106). Preliminary immunofluorescence experiments in our laboratory has indicated that all CTLH members are nucleocytoplasmic with a higher tendency to reside in the nucleus, with the exception of Muskelin, which was primarily cytoplasmic (120).

To determine the localization of WDR26, immunofluorescence experiments were performed by imaging ectopically expressed WDR26 in wild-type HeLa cells. A FLAG-tagged WDR26 was transfected into wild-type HeLa cells and its subcellular localization evaluated 48h post-transfection. WDR26 was found to be primarily cytoplasmic, with up to 10 % imaged cells showing equal localization to the nucleus and cytoplasm (Figure

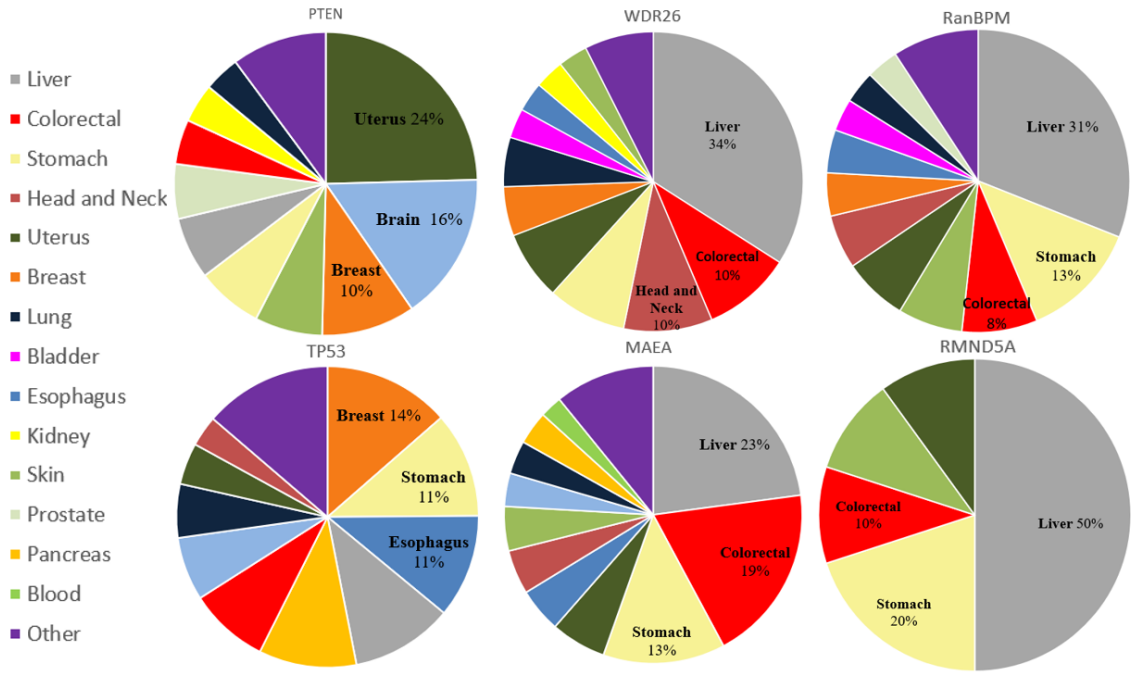

Figure 3.1. Analysis of tissues affected from alterations of WDR26 and core CTLH members as determined by the international cancer genome consortium. Using the International Cancer Genome Consortium (ICGC), the number of primary tumours, per tissue type containing nonsynonymous point mutations in either *WDR26* (94) or *RanBPM* (87) and the Core CTLH members *RMND5A* (10) and *MAEA* (83) were scored and arranged in a pie chart with the percentage of tissues containing mutations ordered from most to least prevalent. Primary tumours, per tissue type, containing nonsynonymous point mutations in *PTEN* (651) and *TP53* (3966) were collected and compared to as controls.

3.2A, B). This suggests WDR26 is a nucleocytoplasmic protein and localizes to the same compartments as core CTLH members, thus could interact with the CTLH complex.

To determine if WDR26 associates with the CTLH complex, co-immunoprecipitation (Co-IP) experiments were carried out. FLAG-tagged WDR26 transiently transfected into wild-type HeLa cells was immunoprecipitated (IP) and the samples were analyzed using Western blot to evaluate the Co-IP of CTLH members. These immunoprecipitation experiments were compared to a control which consisted of un-transfected wild-type HeLa cells subjected to the same FLAG IP. All CTLH members tested, RanBPM, MAEA, RMND5A and Muskelin co-immunoprecipitated with FLAG-WDR26 while none of them were found in the un-transfected sample (Figure 3.2C). These results suggest that WDR26 associates with the CTLH complex *ex vivo*.

3.3 Endogenous Axin Co-Immunoprecipitates with CTLH members

Previously, a study identified that mammalian WDR26 interacts with Axin1 through a co-immunoprecipitation of ectopically expressed WDR26 and Axin in HEK 293 cells (63). In addition to this, STRING analysis of Axin immunocomplexes predicted the interaction of WDR26 with the CTLH complex, also predicted all CTLH complex members to be an interacting partner with Axin1 (56). To confirm this, additional Co-IP experiments were performed to IP endogenous RanBPM from wild-type HeLa cells, followed by Western blot analysis. In the first instance, endogenous RanBPM immunoprecipitates were analyzed to test the presence of Axin. RanBPM was successfully immunoprecipitated and showed endogenous Axin to Co-IP (Figure 3.3A). This IP was repeated and endogenous Axin again co-immunoprecipitated, along with CTLH members ARMC8α and MAEA (Figure 3.3B). These results suggest that not only WDR26 but the whole CTLH complex is associating with Axin.

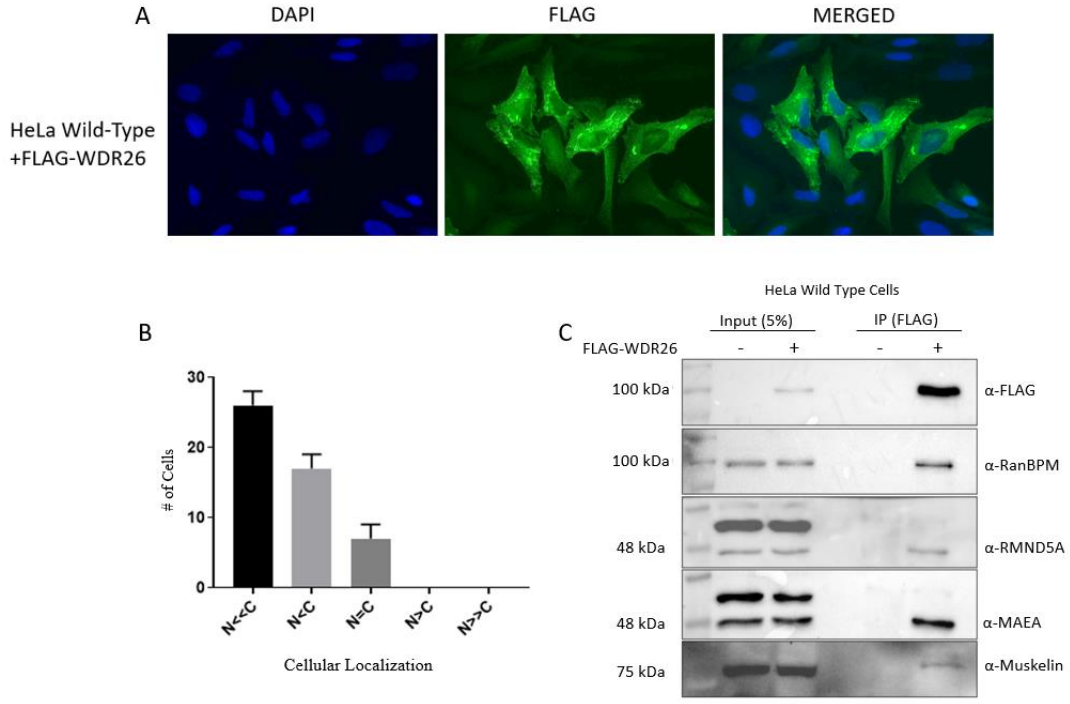

Figure 3.2 WDR26 localizes to the cytoplasm and interacts with the CTLH complex. (A) Representative images of ectopically expressed WDR26 in HeLa wild-type cells. pCMV-FLAG-WDR26 was transfected in wild-type HeLa cells. Cells were fixed 24 h after transfection and incubated using a FLAG antibody and then an Alexa Fluor 488 secondary antibody (green). Nuclei were stained with DAPI (blue). (B) Subcellular localization was scored as either N>>C (nuclear much greater than cytoplasmic), N>C (nuclear greater than cytoplasmic), N=C (nuclear equal to cytoplasmic), N<C (cytoplasmic greater than nuclear) and N<<C (cytoplasmic much greater than nuclear). Data represents averages from three separate experiments each assessing a total of 50 cells. (C) Western blot analysis of co-immunoprecipitation of CTLH members following immunoprecipitation of FLAG tagged WDR26. pCMV-FLAG-WDR26 was transfected into HeLa wild-type cells and harvested after 24 hours. pCMV-FLAG-WDR26 transfected (+) or untransfected (-) extracts were subjected to immunoprecipitation with a FLAG antibody. Immunoprecipitates were analyzed by western blot.

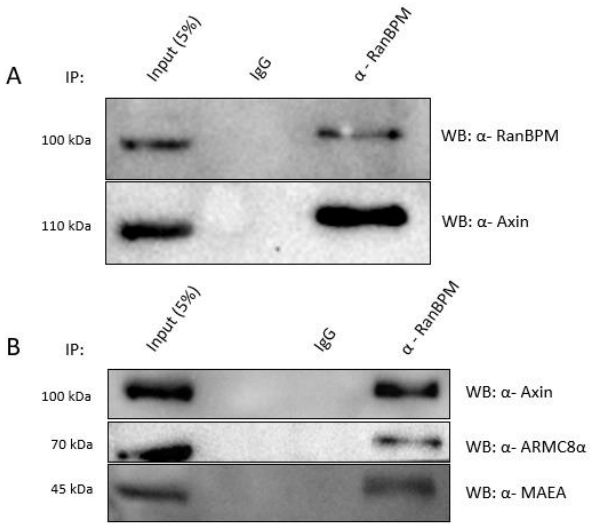

Figure 3.3 Endogenous Axin Co-Immunoprecipitates with RanBPM and core CTLH members. (A) Western blot analysis was performed to assess the co-immunoprecipitation of Axin following the immunoprecipitation of endogenous RanBPM. (B) A second immunoprecipitation of endogenous RanBPM was performed to confirm Axin Co-IP and assess the co-immunoprecipitation with core CTLH complex members. All immunoprecipitations were performed using 1mg of protein while 5% (50µg) was loaded for the input and were analyzed using primary Axin, RanBPM, MAEA and ARMC8α/β antibodies.

3.4 RanBPM negatively regulates β-catenin protein levels

3.4.1 β-catenin is elevated in RanBPM downregulated cells

Our laboratory previously performed micro-array analysis on HeLa and HCT116 cells stably expressing RanBPM shRNA and found that downregulation of RanBPM resulted in dysregulation of genes within the Wnt pathway (19). A previous study has also shown that downregulation of RanBPM results in an increase in the β-catenin homologue in *Drosophila*, suggesting that the dysregulation of Wnt genes was due to alteration of β-catenin levels (13). To determine the effect of RanBPM expression on β-catenin protein levels, extracts from various cell lines with stably downregulated RanBPM were analyzed by Western blot. Using RanBPM shRNA, or control shRNA HeLa and HCT 116 cell lines, a modest, yet significant increase in β-catenin was found in RanBPM shRNA cells compared to the control cells (Figure 3.4A, B). Additionally, 3T3 mouse embryonic fibroblasts (MEFs) from RanBPM knockout mice displayed a similar significant increase of β-catenin compared to wild-type MEFs (Figure 3.4C). Lastly, RanBPM siRNA was used to transiently knock down RanBPM expression in CaCo-2 cells. Again, there was a significant increase in β-catenin protein levels in the RanBPM downregulated CaCo-2 cells compared to cells transfected with scrambled siRNA (Figure 3.4D). To confirm the specificity of the effect on β-catenin, RanBPM expression was restored in HeLa RanBPM shRNA cells. Increasing amounts of HA-tagged RanBPM, (from 1 – 3 μg), was transfected into the RanBPM shRNA cells. Upon overexpression of RanBPM, there was a significant decrease, up to 50%, in β-catenin levels (Figure 3.5). Together these results suggest RanBPM functions as a negative regulator of β-catenin.

3.4.2 RanBPM may regulate β-catenin indirectly through the proteasome

Negative regulation of the canonical Wnt signalling pathway involves the polyubiquitination of β-catenin and its subsequent degradation by the proteasome (82). While this ubiquitination is most commonly performed by the E3 ubiquitin ligase β-TrCP, additional ligases have been shown to target β-catenin for ubiquitination (82-84).

To determine whether RanBPM is regulating β-catenin levels through the ubiquitin-mediated proteasomal degradation pathway, similar to the regulation of gluconeogenic enzymes by the GID complex, RanBPM shRNA HeLa cells were used to assess β-catenin protein levels under conditions of proteasomal inhibition to determine if the loss of RanBPM affected β-catenin degradation. To accomplish this, RanBPM shRNA and non-targeting control shRNA HeLa cells were treated with MG132, a proteasomal inhibitor, or with the vehicle, DMSO. Under normal conditions, MG132 inhibition of the proteasome will prevent the degradation of ubiquitinated proteins leading to their accumulation in the cell. However, cells lacking E3 ubiquitin ligase activity, due to the loss of RanBPM, would lack this accumulation. Cells were treated with 10 μM of MG132 or DMSO and were harvested 16 h after treatment and analyzed through Western blot. HeLa cells expressing RanBPM shRNA showed a minimal, and non-significant increase in β-catenin protein levels under proteasomal inhibition, whereas the control shRNA cells showed over a 2-fold increase in β-catenin protein levels upon proteasomal inhibition, compared to vehicle treated cells (Figure 3.6). This suggests β-catenin may be regulated by the proteasome indirectly through RanBPM.

3.5 CTLH member RMND5A negatively regulates β-catenin protein levels

Thus far, evidence that the CTLH complex regulates β-catenin has been limited to RanBPM. To support that this regulation occurs through the CTLH complex activity, additional CTLH members must demonstrate a similar effect on β-catenin protein levels. Our laboratory utilized CRISPR-Cas9 to target and knockout *RMND5A* in HeLa wild-type cells. RMND5A is the mammalian homologue of GID2, the RING domain containing subunit conferring the E3 ubiquitin ligase activity of the GID complex which is essential for *in vivo* ubiquitination of FBPase by the GID complex (57, 58). The high conservation of its mammalian homologue, RMND5A, also containing a RING domain, suggests that it functions as the E3 ubiquitin ligase of the CTLH complex. While RMND5A has demonstrated ubiquitination activity in *Xenopus laevis*, it has yet to be confirmed as the source of E3 ubiquitination activity of the CTLH complex (104).

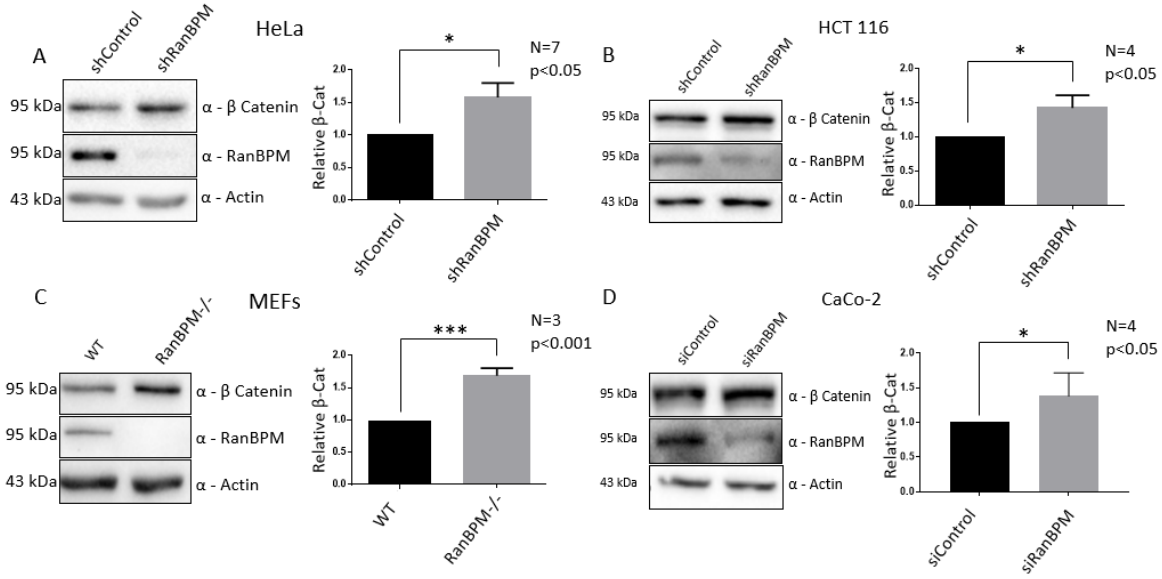

Figure 3.4 β-Catenin protein levels are elevated in RanBPM downregulated cell lines. Western blot analysis of β-catenin protein levels in HeLa (A) and HCT 116 (B) cell lines stably expressing RanBPM shRNA or control shRNA. (C) Similar analysis was made using immortalized mouse embryonic fibroblasts (MEFs) that are RanBPM deficient. (D) CaCo-2 cells were transfected with 10 nM of RanBPM targeting siRNA or scrambled non-targeting siRNA and were harvested 24 h post-transfection and β-catenin protein levels were assessed through Western blot. All western blot analyses were performed using up to a total of 50 μg of protein and using primary β-catenin, RanBPM and Actin antibodies. Relative β-catenin protein levels were quantified by normalizing β-catenin to actin and expressing values relative to control (or wild-type) cells which were given a value of 1. Quantifications are shown with error bars indicating SD. A two-sample t-test, assuming equal variance, was used to determine significance. ** $p<0.05$; *** $p<0.001$*

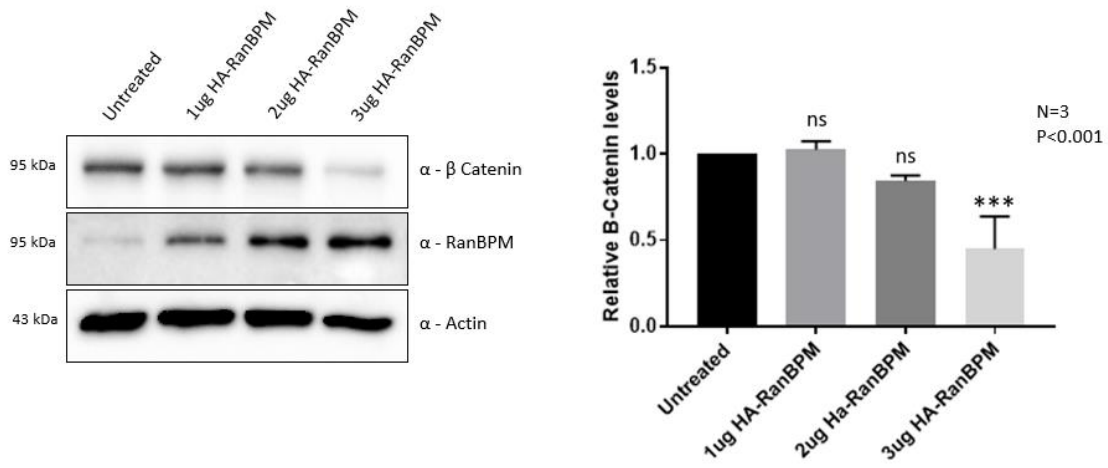

Figure 3.5 Overexpression of RanBPM decreases β-catenin protein levels. (Left) Western blot analysis of β-catenin protein levels in HeLa cells stably expressing RanBPM shRNA following the re-expression of RanBPM. An increasing amount, to 3 μg, of pCMV-HA-RanBPM was transfected into HeLa shRanBPM cells and were harvested 24 h post-transfection. Up to 50 μg of protein was used for analysis and blots were analyzed using primary β-catenin, RanBPM and Actin antibodies. Relative β-catenin protein levels were quantified by normalizing β-catenin to actin and expressing values relative to untreated cells which were given a value of 1. Quantifications are shown with error bars indicating SD. A two-sample t-test, assuming equal variance, was used to determine significance. *** $p<0.001$; ns not significant.

To determine the effect of the knockout of RMND5A on β-catenin expression, a cohort of monoclonal RMND5A knockout cell lines were generated and screened for β-catenin protein levels through Western blot. The initial screen revealed a ubiquitous increase in endogenous β-catenin levels in all RMND5A knockout clones compared to parental HeLa cells (Figure 3.7). To confirm this result, two clones, HeLa RMND5A $^{-/-}$ clones 16 and 18 (RMND5A$^{-/-}$ C16 and RMND5A$^{-/-}$ C18), were selected for further analysis and confirmed a consistent and significant increase in β-catenin protein levels compared to HeLa CRISPR control cells (Figure 8A, B). β-catenin protein regulation was rescued by ectopically expressing HA-RMND5A in RMND5A$^{-/-}$ C16 cells for 24 h, decreasing β-catenin back to levels observed in HeLa CRISPR control cells (Figure 8C, D). These results demonstrate that endogenous β-catenin levels are downregulated by RMND5A expression suggesting RMND5A plays a key role in regulating β-catenin.

3.6 CTLH complex members affect β-catenin target gene expression

3.6.1 Downregulation of RanBPM leads to increased β-catenin nuclear localization

In the canonical Wnt signalling pathway, the cellular levels of β-catenin are primarily controlled by ubiquitination and proteasomal degradation (82). Only when β-catenin can escape this regulation will it begin to accumulate (90). Once cytoplasmic levels have increased, β-catenin will translocate into the nucleus and upregulate its target genes (91-93). To determine whether the CTLH complex affects β-catenin target gene expression, we sought to test whether the increased β-catenin levels observed in RanBPM/RMND5A downregulated cells coincided with an increase in nuclear localization. To test this, immunofluorescence experiments were performed imaging endogenous β-catenin in RanBPM shRNA and control shRNA HeLa cells (Figure 3.9A). These analyses revealed a significant increase in β-catenin nuclear localization in the RanBPM downregulated cells compared to control cells (Figure 3.9B). This demonstrates that the loss of β-catenin regulation by RanBPM results in increased nuclear translocation.

3.6.2 RMND5A regulates β-catenin target gene expression

β-catenin target genes are regulated by the TCF/LEF transcription factors, which under normal conditions interact with TLE1/Groucho proteins and recruit transcriptional repressors to Wnt responsive elements and inhibit gene expression (94, 96). When β-catenin enters the nucleus, it will displace TLE1/Groucho from the TCF/LEF transcription factors, recruit transcriptional co-activators and enable WRE target gene expression (97, 98). Given that the loss of RanBPM and CTLH member RMND5A have been demonstrated to increase β-catenin protein levels and its nuclear localization, it is hypothesized that β-catenin target gene expression will be altered upon dysregulation of the CTLH complex.

To determine if the regulation of β-catenin by the CTLH complex translated into a change in target gene expression, a luciferase assay was performed using RMND5A$^{-/-}$ C16 HeLa cell line along with a β-catenin activated reporter luciferase (pBARL), which contains 12 consecutive TCF/LEF response elements, the target motif of β-catenin upstream of a Promega MinP minimal promoter (94). The pBARL plasmid, a β-galactosidase internal control and either pCMV-Empty Vector or HA-RMND5A were co-transfected into RMND5A$^{-/-}$ C16, and cells were harvested 48 h after transfection. Under these conditions, the luciferase activity provides a trackable change in TCF/LEF mediated gene expression in response to RMND5A. Upon reintroduction of RMND5A, there was a significant decrease, up to 70%, in relative fluorescence (Figure 3.10). This result suggests that the regulation of the CTLH complex on β-catenin negatively affects its downstream target gene expression.

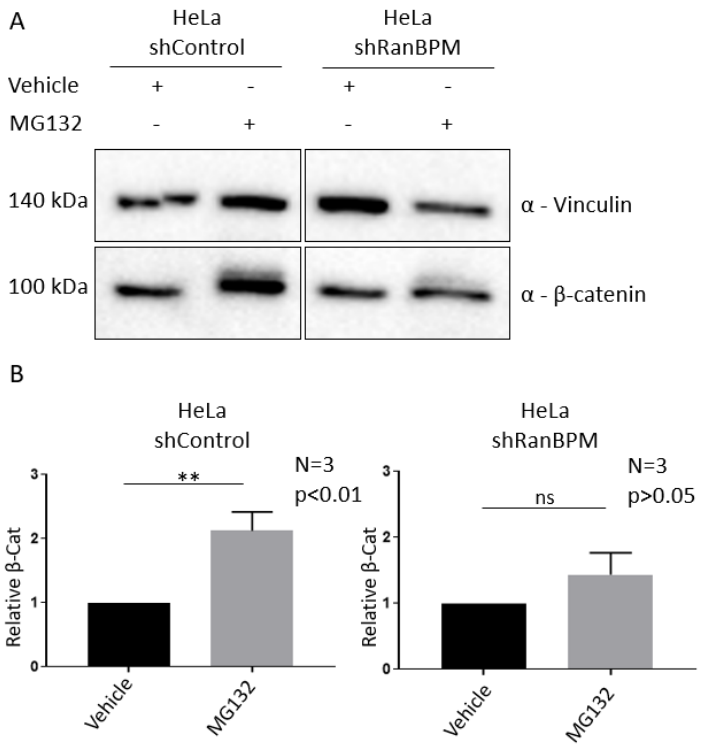

Figure 3.6 β-Catenin is regulated by the proteasome through RanBPM. A) Control shRNA (shControl) and RanBPM shRNA (shRanBPM) HeLa cells were treated with 10 μM of MG132 or DMSO, as a vehicle, for 24 h. Cells were harvested in whole cell extract buffer and up to 50 μg of protein was used for Western blot analysis and blots were hybridized with β-catenin, RanBPM and Actin antibodies. B) Relative β-catenin protein levels were quantified by normalizing β-catenin to actin and expressing values relative to DMSO treated cells which were given a value of 1. Quantifications are shown with error bars indicating SD. A two-sample t-test, assuming equal variance, was used to determine significance. ** *p<0.01; ns* not significant.

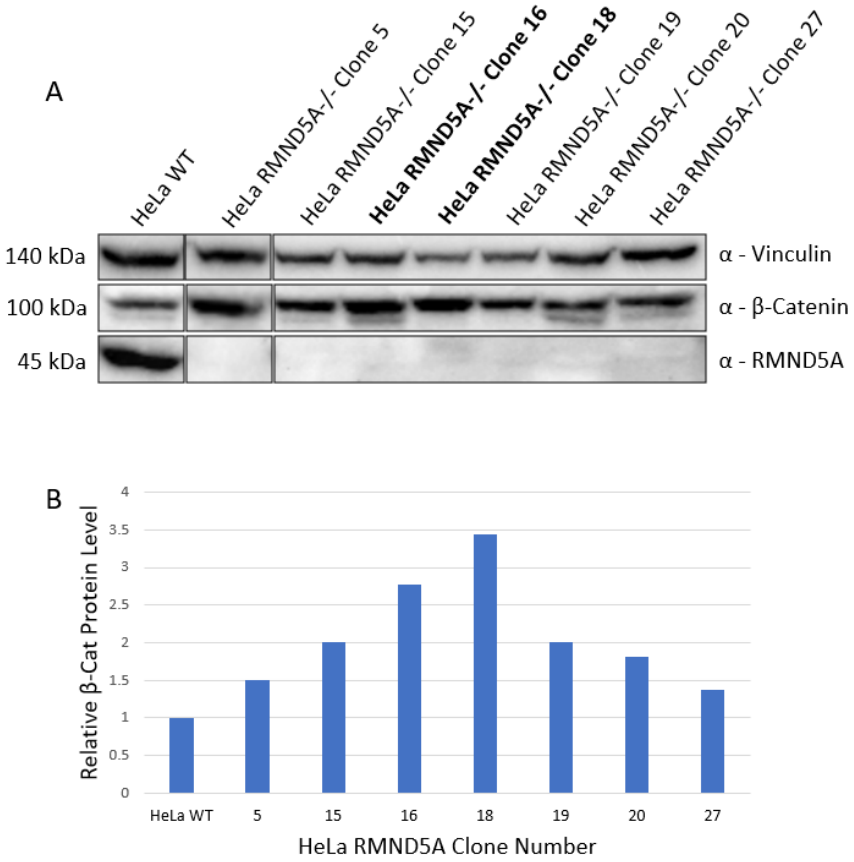

Figure 3.7 RMND5A regulates β-catenin protein levels. A) CRISPR-Cas9 was used to create monoclonal *RMND5A* knockouts in HeLa cells. A cohort of RMND5A knockout clones were harvested in whole cell extract, and up to 50 µg of protein was prepared and analyzed by Western blot. Proteins were probed for using primary β-catenin, vinculin and RMND5A antibodies. In bold are the two HeLa RMND5A knockout clones selected for further experimentation B) Relative β-catenin protein levels were quantified by normalizing β-catenin to vinculin and expressing values relative to wild-type cells which were given a value of 1. Significance was not determined.

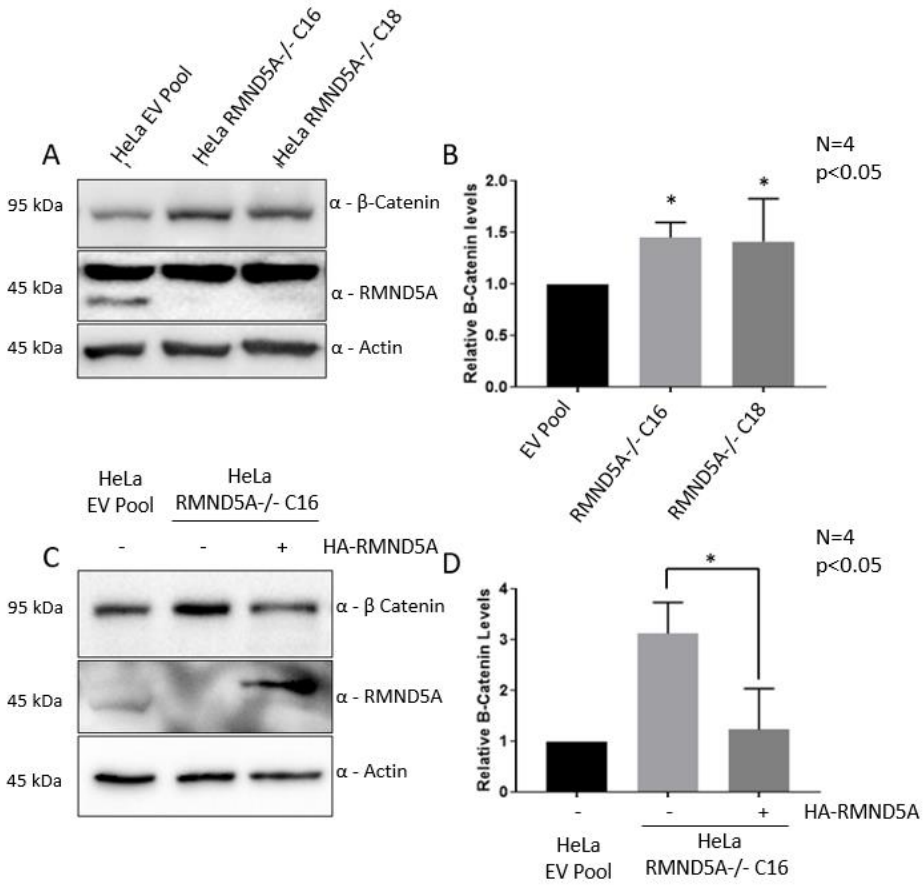

Figure 3.8 Rescue of RMND5A restores β-catenin Regulation. (A)Whole cell extracts of two HeLa RMND5A CRISPR-Cas9 clones (Clones 16 and 18; C16 and C18) and a pool of HeLa cells that underwent the same CRISPR-Cas9 selection process absent guide RNA (HeLa EV Pool) were prepared and analyzed by Western blot using primary β-catenin, Actin and RMND5A antibodies. (B) Relative β-catenin protein levels were quantified by normalizing β-catenin to actin and expressing values relative to HeLa EV Pool, which were given a value of 1. (C) pCGN-HA-RMND5A was transfected into HeLa RMND5A-/- clone 16 cells to rescue the effect on β-catenin protein levels. These cells were harvest and analyzed by Western blot using primary β-catenin, Actin and RMND5A antibodies (D) Relative β-catenin protein levels were quantified by normalizing β-catenin to actin and expressing values relative to HeLa Empty Vector Pool, which were given a value of 1. A two-sample t-test, assuming equal variance, was used to determine significance. *$* p < 0.05$*

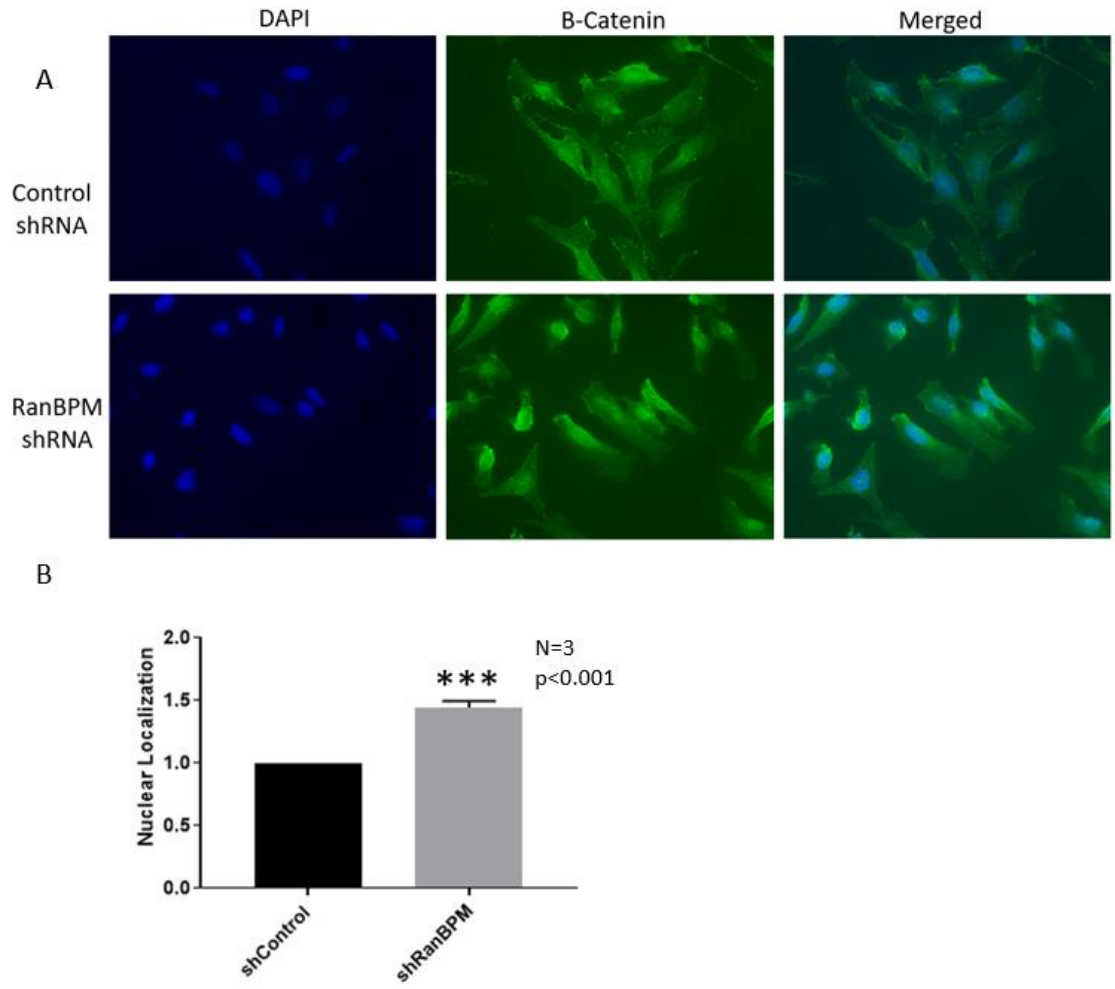

Figure 3.9 Downregulation of RanBPM causes increase nuclear localization of β-catenin. A) Representative images of nuclear (DAPI) and endogenous β-catenin staining in control shRNA and RanBPM shRNA HeLa cells. Nuclear staining was performed using DAPI (blue). β-catenin staining was performed by using a primary mouse- β-catenin antibody and a secondary mouse antibody conjugated to Alexa Fluor 488 (green). (B) Quantification of the HeLa nuclear staining was performed by counting 50 cells and determining the relative total cell and nuclear intensity using ImageJ. After accounting for background signal intensity, the nuclear intensity was compared to the total intensity to determine the relative percentage of nuclear β-catenin levels. The percent of nuclear β-catenin levels were expressed relative to control shRNA cells, which were given a value of 1. A two-sample t-test, assuming equal variance, was used to determine significance. *** $p < 0.001$

3.7 The CTLH complex may promote β-catenin ubiquitination

Canonical ubiquitination of β-catenin involves the poly-ubiquitination of lysine 19 (K19) and lysine 49 (K49) by the E3 ubiquitin ligase β-TrCP, marking it for proteasomal degradation (105). Since the CTLH complex is an E3 ubiquitin ligase, it is hypothesized that the regulation of β-catenin by the CTLH complex is through ubiquitination. To determine whether the CTLH complex is targeting β-catenin for degradation, *in vitro* experiments were performed to assess β-catenin ubiquitination by the CTLH complex. To accomplish this, an HA-tagged ubiquitin expression plasmid was transfected into wild-type and RMND5A$^{-/-}$ C16 HeLa cells. In addition, these cells were treated with the proteasomal inhibitor, MG132. Using a tagged ubiquitin molecule in combination with a proteasomal inhibitor allows for the visualization of ubiquitination of a protein. Through inhibition of the proteasome, ubiquitinated forms of β-catenin would begin to accumulate, whereas cells lacking CTLH complex activity due to the loss of RMND5A, would not show the ubiquitinated forms of β-catenin.

These cells were treated with 10 μM of MG132 for 8 hours and harvested 24 hours post-transfection. Endogenous β-catenin was immunoprecipitated and analyzed through Western blot. In the wild-type HeLa cells expressing ubiquitin, after hybridization with a primary HA antibody a laddering, or smear characteristic of ubiquitination was revealed above 100 kDa, the approximate molecular weight of β-catenin (Figure 3.11). This pattern was also visible in the immunoprecipitate of the RMND5A knockout cells expressing ubiquitin, however the ubiquitin smear was greatly reduced. These were compared to parental cells not transfected with HA-ubiquitin in which there was no laddering present. The reduced ubiquitin laddering in the RMND5A knockout cell line suggests RMND5A and the CTLH complex functions to promote the ubiquitination of β-catenin.

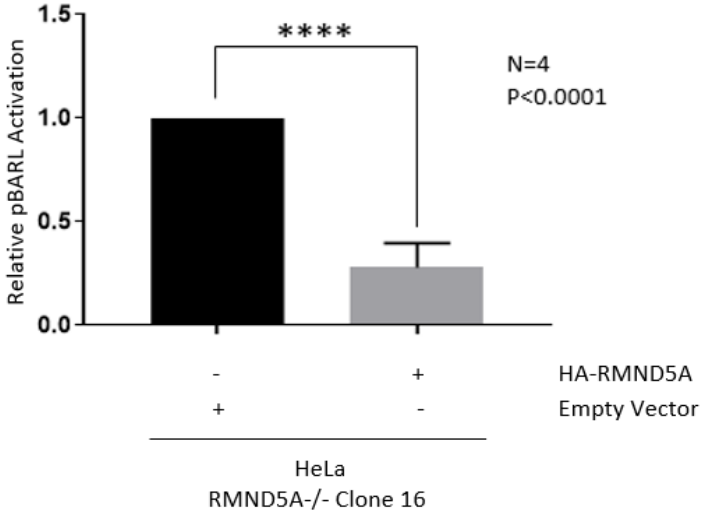

Figure 3.10 RMND5A regulates β-catenin target gene expression. HeLa RMND5A knockout clone 16 cells were transfected with a luciferase plasmid containing a β-catenin target motif (TCF/LEF promoter), a β-galactosidase internal control, and either pCMV-Empty Vector or pCGN-HA-RMND5A. Cells were harvested and analyzed 48 h post-transfection using a Promega Luciferase Assay System and Promega Beta-Glo on an Orion II Microplate Luminometer. Relative β-catenin gene expression was normalized to the β-galactosidase internal control and expressed relative to the RMND5A knockout cells transfected with pCMV-Empty Vector, which was given a value of 1. **** *p<0.0001*.

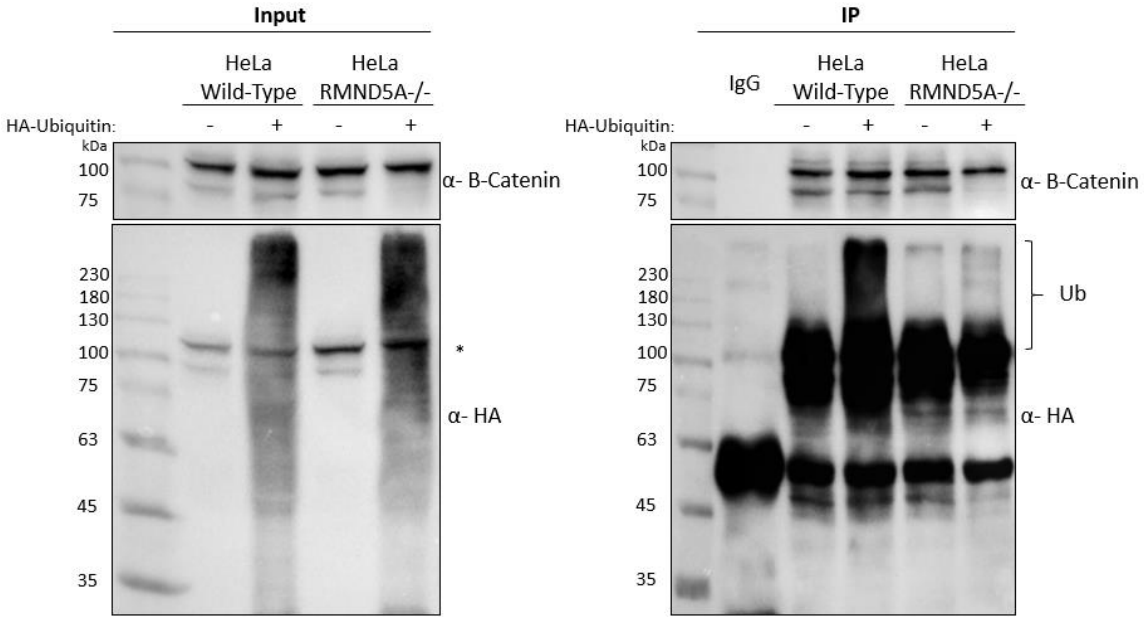

Figure 3.11 The CTLH complex may promote ubiquitination of β-catenin. Western blot analysis of the immunoprecipitation of ubiquitinated β-catenin. An HA-tagged Ubiquitin plasmid was transfected into HeLa wild-type cells and HeLa RMND5A knockout clone 16. These cells were treated with 10 μM of MG132 and harvested 24 h post-transfection. Endogenous β-catenin was immunoprecipitated from 500 μg of untransfected (-) and transfected (+) cell lysates and analyzed by western blot. The input (5% total protein) and immunoprecipitates were analyzed by western blot to assess immunoprecipitation using a β-catenin antibody and an HA antibody to assess ubiquitination of β-catenin.

Chapter 4

4 Discussion

4.1 Summary of findings

The goal of the study was to characterize the regulation of β-catenin protein levels and downstream target gene expression by the CTLH complex. It was hypothesized that the CTLH complex is regulating the Wnt signalling pathway through the targeting of β-catenin for degradation. Specifically, we sought to confirm that the putative complex member, WDR26, was in fact part of the CTLH complex, to determine the effect of the CTLH complex on β-catenin protein levels, and to investigate whether this effect translated to a change in β-catenin target gene expression. In summary, we found that WDR26 interacts with RanBPM and core CTLH members, and that the CTLH complex forms a complex with Axin. We determined that RanBPM and RMND5A negatively regulate β-catenin protein levels, and that this was mediated through the proteasome. Additionally, it was shown that loss of RanBPM results in an increase in β-catenin nuclear localization. Lastly, it was found that RMND5A regulates β-catenin target gene expression. Whether this regulation is direct or indirect is still unknown, but preliminary data suggests the CTLH complex may promote the ubiquitination of β-catenin.

4.2 WDR26 interacts with the CTLH complex

While the CTLH complex has long been thought to function as an E3 ubiquitin ligase, similar to its yeast counterpart, the GID complex, it has only very recently been demonstrated to contain E3 ubiquitin ligase activity (62). In this discovery its first confirmed substrate was also identified as Hbp1, however previous studies have implicated it in the regulation of β-catenin (62-64). A recent study has demonstrated that WDR26, a putative member of the CTLH complex downregulates β-catenin when overexpressed (63). Although interactome studies have predicted WDR26 to exist as part of the CTLH complex, this needed to be confirmed (56). In Chapter 3.2.1, we investigated the tissue distribution of mutations in WDR26 in comparison to core CTLH members and found that WDR26 mutations have a similar tissue distribution as those of other CTLH complex members RanBPM, RMND5A and MAEA. Proteins within the

same pathway, when dysregulated in theory could promote tumour development at the same primary site, thus this highly correlated pattern of mutation distribution suggests WDR26 may be part of the CTLH complex. In Chapter 3.2.2, we investigated the interaction of WDR26 with the CTLH complex *ex vivo*. WDR26 was found to interact with multiple members of the CTLH complex, including Muskelin. While it was initially thought that Muskelin replaced WDR26 in the CTLH complex compared to the yeast GID complex (54), these results suggest WDR26 does interact with the CTLH complex.

4.3 The CTLH complex regulates the canonical Wnt signalling pathway through proteasomal degradation

While the main mechanisms of regulation of β-catenin is through the Axin degradation complex in the canonical Wnt pathway, additional means to regulate β-catenin have been uncovered. As previously stated, the canonical regulation of β-catenin involves the sequential phosphorylation by CK1 and GSK3β, which serves as a signal to recruit β-TrCP promoting the ubiquitination and degradation of β-catenin by the proteasome (80-82). While initially it was thought that nuclear β-catenin was transported back into the cytosol to be targeted by the Axin degradation complex, there have been studies identifying nuclear components to additionally target β-catenin (107). Nuclear β-catenin was shown to be regulated by a separate phosphorylation mediated ubiquitination event performed by protein kinase C delta type (PKCδ) and tripartite motif-containing protein 33 (TRIM33) respectively (108). In a similar mechanism as the Axin degradation complex, PKCδ directly phosphorylates nuclear β-catenin at serine 715 (S715), which is required for the subsequent ubiquitination by TRIM33 (108). Additionally, TRIM33 ubiquitination of β-catenin was shown to be independent of GSK3β and β-TrCP activity (108). A separate study has identified nuclear β-catenin to be regulated through methylation (109). In *Xenopus*, it was demonstrated that lysine demethylase 2a and lysine demethylase 2b (Kdm2a/b) were also required for regulation of nuclear β-catenin (109). Lysine demethylase 2a/b were shown to interact with the fourth and fifth armadillo repeats of β-catenin and were responsible for demethylating specific lysine residues in this region that were targets of ubiquitination (109). Additional studies have revealed other cytoplasmic E3 ubiquitin ligases to target and regulate β-catenin, including Seven

in Absentia Homologue 1 (SIAH-1), the E3 ubiquitin ligase identified by Differential Display (EDD), and Gene for Apoptosis and Differentiation in Epithelia 1 (JADE-1) (83, 84, 110). SIAH-1 and JADE-1 have both been shown to directly ubiquitinate β-catenin promoting it for degradation, however EDD ubiquitination has been shown to increase the stability of β-catenin (83, 84, 110). Having multiple ubiquitination pathways regulating β-catenin stability implies its physiological importance, and further understanding these pathways is important to understand the relation between β-catenin and oncogenesis.

In Chapters 3.4 and 3.5, we investigated the role of CTLH members RanBPM and RMND5A and their relation to β-catenin. Individually, the loss of these two members showed a significant increase in β-catenin protein levels, and this affect was rescued when these proteins were re-expressed. As these two members serve as the scaffold to the CTLH complex and the RING domain subunit responsible for E3 ligase activity respectively, the loss of either would result in less complex formation, less active complex, allowing β-catenin to escape regulation and accumulate. Additionally, a previous study has demonstrated that the overexpression of WDR26 promoted degradation of β-catenin (63). Together, RanBPM, RMND5A and WDR26 individually regulating β-catenin levels strongly suggests this regulation is being performed by the CTLH complex.

In Chapter 3.4 we also investigated whether the loss of RanBPM would affect β-catenin degradation under conditions of proteasomal inhibition. Treating control cells expressing RanBPM with the proteasomal inhibitor MG132 showed a significant increase in β-catenin protein levels. This is in contrast to cells with stably downregulated RanBPM, which did not show a similar increase in β-catenin upon proteasomal inhibition. This likely means that downregulation of RanBPM resulted in reduced ubiquitination of β-catenin, thus decreasing the effect observed in under proteasomal impairment. This suggests RanBPM may function to promote the ubiquitination of β-catenin promoting it for degradation by the proteasome.

While dysregulation of CTLH complex members has been shown to alter β-catenin protein levels, this on its own does not confirm its role in the Wnt signalling pathway. To address this, in Chapter 3.6.1 we investigated the localization of β-catenin upon RanBPM loss of expression. It was found that decreased RanBPM expression resulted in an increased nuclear localization of β-catenin compared to control cells. Beta-catenin fulfills two separate functions, one in the Wnt signalling pathway, the other in adherens junction formation. (71, 72). These regulations involve two different pools of β-catenin localized in different cellular compartments. Its role in the Wnt signalling pathway involves the migration of β-catenin to the nucleus to regulate gene transcription, while adherens junctions are localized to the cell membrane (111). Thus, our results demonstrating an increased nuclear localization of β-catenin upon RanBPM downregulation suggests it affects its role in the Wnt signalling pathway.

Therefore, in Chapter 3.6.2, we investigated whether RMND5A, the subunit of the CTLH complex conferring E3 ubiquitination enzymatic activity, affects the target gene expression of β-catenin. Using a luciferase plasmid responsive to the β-catenin target TCF/LEF, the downstream activation of β-catenin target genes were assessed in response to re-expression of RMND5A in CRISPR-Cas9 RMND5A knockout HeLa cells. Upon the re-expression of RMND5A in these cells there was a drastic decrease in the β-catenin reporter activation, indicating RMND5A is involved in regulating β-catenin target gene expression.

In Chapter 3.7 we sought to determine whether the CTLH complex was affecting the ubiquitination of β-catenin through *in vitro* ubiquitination assays. Endogenous β-catenin was immunoprecipitated from extracts of wild-type and RMND5A CRISPR-Cas9 knockout HeLa cells transfected with a tagged-ubiquitin following proteasomal inhibition. Treating these cells with MG132 would lead to the accumulation of ubiquitinated products that would be visible as a laddering or smear through western blot. It was found that there was a drastic decrease in visible ubiquitinated β-catenin in the RMND5A knockout cells compared to wild-type HeLa cells. There was still ubiquitination of β-catenin in the RMND5A knockout HeLa cells, however this is expected as additional E3 ubiquitin ligases are known to target β-catenin for

ubiquitination (83, 84, 109). The appreciable decrease in ubiquitinated β-catenin upon the loss of RMND5A suggests that the CTLH complex is enhancing the ubiquitination of β-catenin, however whether this is a direct or indirect function of the CTLH complex has yet to be determined.

4.4 Regulation of β-catenin is independent of the Axin degradation complex

In Chapter 3.3 we demonstrated that endogenous Axin co-immunoprecipitates with RanBPM and core CTLH complex members. Although this suggests that the complex interacts with Axin, it is suspected that the regulation of β-catenin by the CTLH complex is independent of the Axin degradation complex. A previous study has shown that the regulation of β-catenin by WDR26 was independent of GSK3β activity (63). WDR26 was overexpressed in the presence and absence of a GSK3β inhibitor, and it was found that WDR26 decreased β-catenin under both conditions. If WDR26 activity was dependent on GSK3β phosphorylation, then abolishing GSK3β activity would eliminate the ability for WDR26 to regulate β-catenin. Additionally, in chapter 3.4.1 while investigating the effect of RanBPM on β-catenin, we used two cell lines (HCT 116 and CaCo-2) that have previously been characterized to contain mutations in β-catenin or components of the Axin degradation complex (112, 113). HCT 116 contains a heterozygous deletion of codon 45 in exon 3, which results in a deletion of serine 45 (112). Serine 45 is one of the phosphorylation sites of CK1 of the Axin complex and is required for GSK3β phosphorylation. CaCo-2 cells are characterized as containing two mutations. First, there is a guanine to cytosine transversion resulting in a change from glycine to alanine in codon 245, within the *Arm* repeat region of exon 5 (112). Additionally, CaCo-2 cells have a truncating mutation in APC in exon 16 in addition to loss of heterozygosity (113). The mutation in β-catenin occurs at the site of APC binding, and while it has not been fully characterized, the structural change is theorized to decrease APC binding to β-catenin (112). More significantly, the loss of APC due to truncation would result in loss of control of normal β-catenin signalling (114). The mutations characterized in both HCT 116 and CaCo-2 cells cause a functional loss of β-catenin regulation mediated by the Axin degradation complex. If the effect of RanBPM

on β-catenin was mediated through the Axin degradation complex, then this would be abolished in these cell lines. The change in β-catenin in these cell lines in response to RanBPM suggests it is regulating β-catenin in a novel mechanism, different than the canonical pathway.

Disruption of the canonical Wnt signalling pathway is often associated with colorectal carcinoma resulting from loss of function mutations in APC (117). This is consistent with the results presented in Chapter 3.2.1, where the tissue distribution of mutations displayed colorectal tissue to contain a high number of mutations in WDR26, RanBPM, MAEA andRMND5A. Interestingly, these CTLH complex members were also found mutated in liver tumours at a higher rate. While Wnt signalling in carcinogenesis is most commonly associated with colorectal cancer, previous studies have showed activating mutations in β-catenin to contribute to the pathogenesis of hepatocellular carcinoma among many cancer types (118, 119). While many components of the Wnt signalling pathway may contribute to various forms of cancer, mutations in CTLH complex members leading to loss of CTLH regulation may contribute to Wnt signalling hyperactivation and the progression of these cancers.

4.5 Limitation of the study and future work

Previous studies along with the results presented here suggest that both WDR26 and Muskelin may exist in the CTLH complex, despite initial reports suggesting Muskelin replaces WDR26. While the exact topology of the CTLH complex is not fully understood, previous work in our laboratory has identified CTLH complex members as nucleocytoplasmic proteins (120). Interestingly however, RanBPM, RMND5A and MAEA were primarily nuclear, while Muskelin and WDR26 were primarily cytoplasmic (120). It is possible that there are multiple forms of the CTLH complex, containing either WDR26, Muskelin, or both depending on the compartmentalization of the complex as well as temporal expression of these members. To fully understand the function of the CTLH complex, further studies should focus on determining where these proteins colocalize within the cell, and whether WDR26 and Muskelin localize to the CTLH complex simultaneously or whether these interactions are mutually exclusive.

When performing the β-catenin activated luciferase assay, the reporter activation was normalized to a β-galactosidase (β-Gal) reporter that was co-transfected to control for transfection efficiency. Initially this experiment was performed using RanBPM shRNA HeLa cells co-transfected with the β-catenin reporter as well as a *Renilla* reporter (pRL). Repeatedly, it was found that the re-expression of RanBPM was altering the pRL activation making it unreliable to use to normalize the samples (Supplemental Figure 1). To avoid this, the experiment was changed to use RMND5A CRISPR-Cas9 knockout HeLa cells instead, however the same effect on pRL was observed upon re-expression of RMND5A. This suggests the CTLH complex may alter pRL expression, and an alternative control was needed. While β-Gal is a less commonly used reporter for luciferase assays compared to a dual-luciferase system, it did provide consistent results that were not influenced by RMND5A. However, using β-Gal as a control, we were unable to simultaneously measure β-catenin activation and the negative control in the same sample through a dual luciferase system. The measurements had to be taken in parallel with separate sample preparations measured at the same time. Although this was not the ideal system, the results were consistent between replicates.

The data presented in Chapters 3.4 and 3.5 strongly implicates RanBPM and RMND5A in the regulation of β-catenin protein levels, however the mechanism behind this regulation is still unknown. In Chapter 3.7 we determined RMND5A affects β-catenin ubiquitination by visualizing ectopically expressed HA-ubiquitination of endogenous β-catenin that was immunoprecipitated under proteasomal inhibition. Our results displayed a decreased presence of HA-ubiquitin upon the knockout of RMND5A. While these results suggest RMND5A functions to enhance the ubiquitination of β-catenin, whether this effect is direct or indirect is uncertain. To determine whether the CTLH complex is directly ubiquitinating β-catenin, an *in vitro* ubiquitination assay could be performed where purified members of the CTLH complex are immunoprecipitated and incubated with purified β-catenin along with all required components of the ubiquitination reaction. If ubiquitinated under these conditions, it would suggest the CTLH complex is directly ubiquitinating β-catenin.

Another possibility is that the CTLH complex is regulating β-catenin through an intermediate protein or complex that regulates elements upstream of β-catenin. Recently, a member of our laboratory performed a large-scale proteomic analysis to assess global changes in ubiquitination upon the loss of CTLH members. Initial results have identified a few potential targets for ubiquitination that have been shown to interact with components of the Wnt pathway (Maitland, unpublished). The first target identified is tankyrase-2 (Tnks2) which functions to poly-ADP-ribosylate (PARylate) Axin, promoting it for degradation (115). An upregulation of Tnks2 due to loss of RanBPM theoretically would result in a decrease in Axin allowing for β-catenin to accumulate. A second potential target identified for CTLH ubiquitination was the receptor-interaction protein kinase 4 (RIPK4). RIPK4 has been shown to promote Wnt signalling by phosphorylating dishevelled and promoting Wnt receptor activation (116). Upregulation of RIPK4 due to loss of RanBPM would stabilize β-catenin mediated through Wnt receptor activation, however whether this activation occurs independent of the Axin degradation is unknown.

A final option is that the CTLH complex is targeting an interacting partner with Axin. Although our data suggests that this regulation is independent of the Axin degradation complex, it was shown that the regulation of β-catenin by WDR26 required interaction with Axin1 (63). In this case, the CTLH complex may be regulating an interacting partner with Axin, different than those in the canonical degradation complex, that also influence β-catenin stability. See figure 4.1 below for an overview of possible targets of CTLH ubiquitination that mediate the regulation of β-catenin. Further studies should be performed to identify and validate additional targets of CTLH ubiquitination that may mediate the regulation of β-catenin.

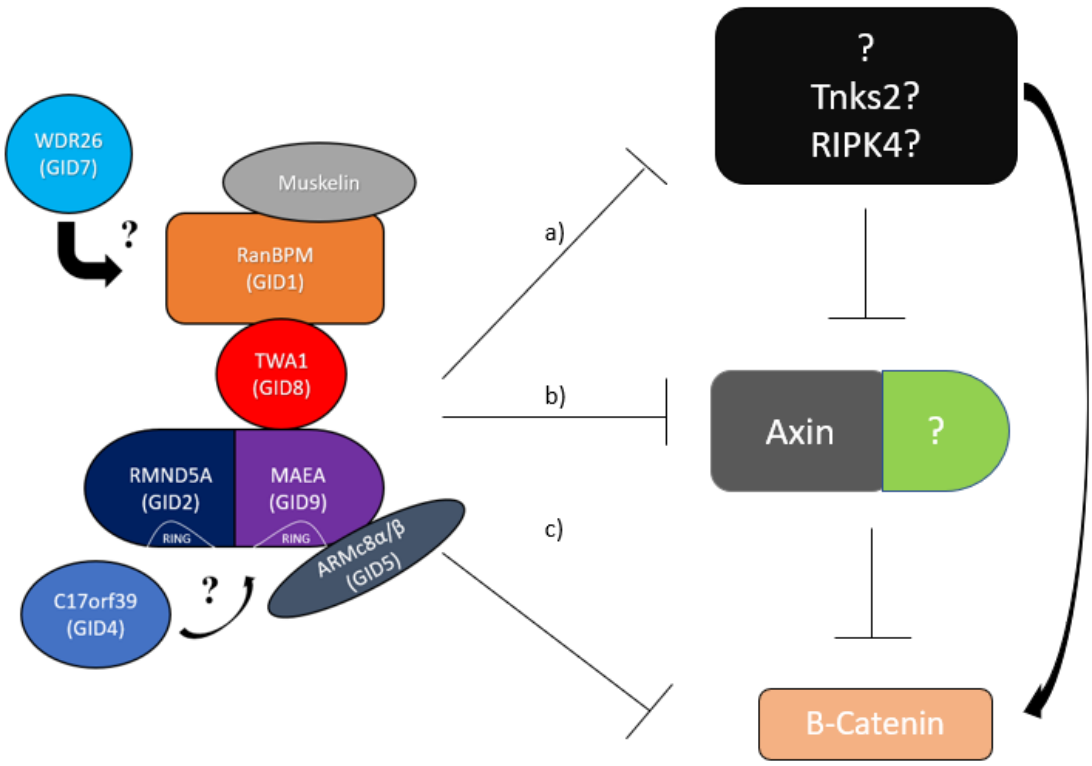

Figure 4.1 Potential mechanisms of CTLH mediated β-catenin regulation. A) The CTLH complex may indirectly regulate β-catenin protein levels by targeting Tnks2 (Tankyrase-2) or RIPK4 (receptor interaction protein kinase 4) which are known to regulate components of the Axin degradation complex. Alternatively, the CTLH complex may regulate a currently unidentified target that may directly regulate β-catenin. B) The CTLH complex may target a component within the Wnt signalling pathway that is upstream to β-catenin, including an interacting partner with Axin apart from CK1 (casein kinase 1), APC (adenomatous polyposis coli) or GSK3β (glycogen synthase kinase 3-beta). C) The CTLH complex may directly target β-catenin for degradation.

4.6 Conclusion

Overall, this study was performed to investigate the regulation of β-catenin and the Wnt signalling pathway by the CTLH complex. This study has further implicated the CTLH complex to regulate β-catenin, as well as Wnt target gene expression. While further studies need to be performed to elucidate the mechanism behind the regulation of β-catenin, the results demonstrated here suggest a novel tumour suppressive role of the CTLH complex as a negative regulator of the Wnt signalling pathway.

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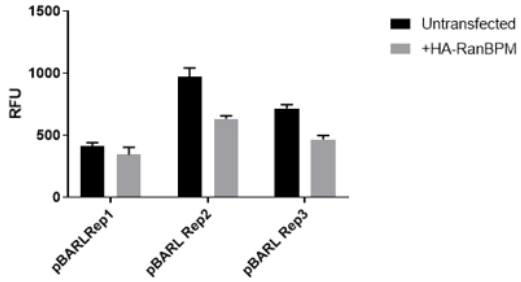
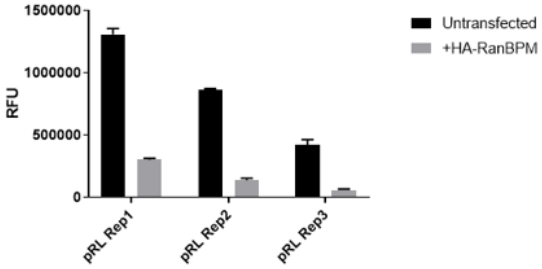
6 Appendices

Appendix 1 Raw pBARL and pRL data obtained from luciferase assay performed in HeLa RanBPM downregulated transfected with HA-RanBPM.

	HeLa shRanBPM	
	pBARL Relative Fluorescence	
Replicate	Untransfected	Transfected
R1	414.5	341.0
R2	967.9	635.1
R3	716.6	466.2

	HeLa shRanBPM	
	pRL Relative Fluorescence	
Replicate	Untransfected	Transfected
R1	1309167	303669.0
R2	866889	137757
R3	422027	59291





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Abstracts:
Chiasson, C and Schild-Poulter, C. Regulation of β-catenin signalling by CTLH complex.
14th annual Oncology Research and Education Day
London, ON
June 16th, 2017

Chiasson, C and Schild-Poulter, C. Regulation of β-catenin signalling by CTLH complex.
5th annual Robarts Research Retreat
London, ON
June 20th, 2017

Chiasson, C and Schild-Poulter, C. Regulation of β-catenin signalling by CTLH complex.
15th annual Oncology Research and Education Day
London, ON
June 8th, 2017