Uncovering the ubiquitin ligase activity and substrates of the human C-terminal to LisH (CTLH) complex

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Abstract

Ubiquitination is the transfer of a ubiquitin molecule to protein substrates by the sequential actions of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases. It is a post-translational modification that controls the fate and function of the substrate protein. Substrate specificity in the ubiquitination reaction is conferred by the E3 ligases. Sequence homology suggests the human C-terminal to LisH (CTLH) complex could be an E3 ligase; however, very little is known about this complex. In this thesis, I characterize the human CTLH complex as a multi-subunit E3 ligase and define its activity, structure, and substrates. I demonstrate that the CTLH complex is comprised of several interdependent subunits localizing to the nucleus and cytoplasm. I determine that the complex has E3 ligase activity which is dependent on its two Really Interesting New Gene (RING) domain subunits, RMND5A and MAEA. I found that the complex controls ubiquitination and degradation of muskelin. Since muskelin is a subunit of the human CTLH complex and likely serves as a substrate receptor, this finding revealed a potential autoregulation mechanism. Residues critical for controlling muskelin protein levels in the RMND5A RING domain were characterized. This demonstrated which residues are required for zinc coordination, E2 conjugating enzyme binding, and stimulation of the ubiquitination reaction. I mapped the subunit arrangement of the endogenous complex using cross-linking mass spectrometry and immunoprecipitations, which provided a clearer depiction of complex architecture. Finally, quantitative analyses of global proteomes and ubiquitin-enriched proteomes in various complex-depleted HeLa cells and affinity purification mass spectrometry of endogenous RanBPM (a core complex member) were conducted and compared to identify CTLH complex ubiquitination targets. I focused on an emerging theme from the datasets of glucose
metabolism and show that the CTLH complex controls ubiquitination and inhibits activity levels of multiple glycolysis enzymes. In addition, the loss of RanBPM results in increased glycolysis and deregulated central carbon metabolism. Overall, this thesis establishes the human CTLH complex as a multi-subunit E3 ligase that regulates glucose metabolism. It also provides critical and fundamental insights into the structure, E3 ligase activity, and possible ubiquitination targets of the human CTLH complex.

**Keywords**

Ubiquitination, proteomics, E3 ligase, CTLH complex, glycolysis, RanBPM, muskelin, mass spectrometry, RING domains, RMND5A, MAEA, cancer
Summary for Lay Audience

Cells execute timed biological processes and adapt to changes in their environment by controlling signal transduction pathways. To do so, they have evolved a system to regulate proteins by quickly adding or removing chemical tags. Ubiquitination is one important example of this. It is a reaction that occurs frequently inside a cell where a ubiquitin molecule is specifically added to a protein (a substrate). Once ubiquitinated, the substrate is either eliminated from the cell or its function is changed.

E3 ligases are proteins that determine which substrates are ubiquitinated. In this thesis, I discover that a particular group of proteins that interact continuously with each other, called the ‘CTLH complex’, is an E3 ligase in human cells. I also characterize its structure and function.

At the start of this thesis, very little was known about this complex. Therefore, a first step was developing tools to study the human CTLH complex. By doing so, I characterized basic properties of the CTLH complex, such as its composition, stability determinants, and architecture. Importantly, I demonstrated using biochemical assays that the CTLH complex has E3 ligase activity and revealed a potential autoregulation mechanism via ubiquitination of one of its components.

It was previously found that loss of the CTLH complex resulted in increased growth of cancer cells. To reveal mechanisms that regulate cancer development by the CTLH complex, I used a technique that can analyze thousands of proteins at once (mass spectrometry-based proteomics). This was done to identify which proteins are ubiquitinated by the CTLH complex. It uncovered that the human CTLH complex has several substrates, including proteins in glucose metabolism. Following up on this, I found that inactivation of
the CTLH complex increased the speed of glucose metabolism in cancer cells. This helps explain why mutation of the CTLH complex can cause cancer cells to be more aggressive. Overall, this thesis sets the foundation for the study of the CTLH complex in human cells and establishes it as an E3 ligase that controls essential biological pathways via ubiquitination.
Co-Authorship Statement

All chapters of this thesis were written by Matthew Maitland and edited by Drs. Caroline Schild-Poulter and Gilles Lajoie. Chapters 2-4 are manuscripts that have been published or are in preparation to be submitted to a peer-reviewed journal. I participated in the design, execution and analysis of all experiments presented in this thesis with the following exceptions:

**Chapter 2:** Gabe Onea generated data for figures 2.1D, GID4 localization in 2.3, and 2.11A and C. Christopher Chiasson generated data for figures 2.1C and WDR26 localization in 2.3. Xu Wang helped generate GST-RMND5A expression in bacteria, performed the initial culturing and selection to generate CRISPR stable cell lines, and helped with the qPCR analysis. Jun Ma assisted with mass spectrometry work and performed the data analysis. Sarah Moor contributed data for figure 2.3. Kathryn Barber generated purified ubiquitin protein and E1 enzymes for *in vitro* ubiquitin assays. Caroline Schild-Poulter, Gary Shaw, and Gilles Lajoie contributed to experimental design, analyzed data, and provided reagents and analytical tools. Caroline Schild-Poulter wrote the discussion section.

**Chapter 3:** Caroline Schild-Poulter and Gilles Lajoie contributed to experimental design, analyzed data, and provided reagents and analytical tools.

**Chapter 4:** Miljan Kuljanin provided essential assistance and guidance for the initial development of the ubiquitin enrichment proteomic protocol and subsequent analysis. Xu Wang assisted with ubiquitination validations and cell culturing. Caroline Schild-Poulter and Gilles Lajoie contributed to experimental design, analyzed data, and provided reagents and analytical tools. Metabolite profiling was performed at the University of Victoria Genome BC Proteomics Centre.
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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>~</td>
<td>thioester</td>
</tr>
<tr>
<td>ABC</td>
<td>ammonium bicarbonate</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase promoting complex/cyclosome</td>
</tr>
<tr>
<td>ARM</td>
<td>armadillo</td>
</tr>
<tr>
<td>ARMC8</td>
<td>armadillo repeat-containing protein 8</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine triphosphate</td>
</tr>
<tr>
<td>atTPL</td>
<td><em>Arabidopsis thaliana</em> TOPESS</td>
</tr>
<tr>
<td>BS3</td>
<td>bis(sulfosuccinimidyl)suberate</td>
</tr>
<tr>
<td>C or Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>CHX</td>
<td>cyclohexamide</td>
</tr>
<tr>
<td>CLMS</td>
<td>cross-linking mass spectrometry</td>
</tr>
<tr>
<td>CRA</td>
<td>CT11-RanBPM</td>
</tr>
<tr>
<td>CRL</td>
<td>cullin RING ligase</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>cryo-electron microscopy</td>
</tr>
<tr>
<td>CTLH</td>
<td>C-terminal to LisH</td>
</tr>
<tr>
<td>diGLY</td>
<td>diglycine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMP</td>
<td>dimethyl pimelimidate dihydrochloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DUBs</td>
<td>deubiquitinases</td>
</tr>
<tr>
<td>E1</td>
<td>E1 activating enzymes</td>
</tr>
<tr>
<td>E2</td>
<td>E2 conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>E3 ligase</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein–Barr virus</td>
</tr>
<tr>
<td>ECAR</td>
<td>extracellular acidification rate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine</td>
</tr>
</tbody>
</table>
FA  formic acid
Fbp1  yeast Fructose 1,6-bisphosphatase
GABAA\(_R\)  GABA\(_A\) Receptor
Gid  glucose-induced degradation-deficient
GID4  glucose-induced degradation protein 4 homolog
GO  gene ontology
H or His  histidine
HBP1  HMG-Box Transcription Factor 1
HDAC6  histone deacetylase 6
HR  homologous recombination
HRS  hepatocyte growth factor-regulated tyrosine kinase substrate
HSV-1  herpes simplex virus 1
I  isoleucine
IP  immunoprecipitation
IR  ionizing radiation
K or Lys  lysine
kDa  kilodaltons
Khib  2-hydroxyisobutyrylation
KO  knockout
L  leucine
LC-MS/MS  Liquid chromatography-tandem mass spectrometry
LDH  lactate dehydrogenase
LDHA  L-lactate dehydrogenase A chain
LisH  lissencephaly type-1-like homology
MAEA  E3 ubiquitin-protein transferase macrophage erythroblast attacher
Mda  megadaltons
MEL  murine erythroleukemia
MKLN1  muskelin
mRNA  messenger RNA
MS  mass spectrometry
MZT  maternal-to-zygotic transition
NEM  N-ethylmaleimide
OCR  oxygen consumption rate
osTPR  rice TOPLESS-related
PEP  phosphoenolpyruvate
PIPE  protein-protein interaction prediction engine
PK  pyruvate kinase
PMSF  phenylmethylsulfonyl fluoride
PrPC  cellular prion protein
PTM  post-translational modification
PVDF  polyvinylidene difluoride
R  arginine
RanBP10  Ran-binding protein 10
RanBPM  Ran-binding protein 9
RING  really interesting new gene domain
RMND5A  E3 ubiquitin-protein transferase required for meiotic nuclear division 5 homolog A
RMND5B  E3 ubiquitin-protein transferase required for meiotic nuclear division 5 homolog B
S  serine
SCF  skp, cullin, f-box
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
sgRNA  single guide RNA
SPRY  SPla and the RYanodine Receptor
TCA  tricarboxylic acid
TFA  trifluoroacetic acid
TNF-α  tumor necrosis factor α
TWA1  glucose-induced degradation protein 8 homolog
Ub  ubiquitin
UBC  ubiquitin conjugating domain
Ubc8  ubiquitin-conjugating enzyme E2-24 kDa
UBE2D2  ubiquitin-conjugating enzyme E2 D2
UBE2H  ubiquitin Conjugating Enzyme E2 H
USP11  ubiquitin Specific Peptidase 11
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>VID</td>
<td>vacuolar import and degradation</td>
</tr>
<tr>
<td>WDR26</td>
<td>WD repeat-containing protein 26</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>YPEL5</td>
<td>yippee-like 5</td>
</tr>
</tbody>
</table>
Table of Contents

Abstract ............................................................................................................................... ii
Summary for Lay Audience ............................................................................................... iv
Co-Authorship Statement ................................................................................................. vi
Acknowledgments ............................................................................................................. vii
List of Abbreviations ........................................................................................................ ix
Table of Contents ............................................................................................................. xiii
List of Tables ..................................................................................................................... xvii
List of Figures .................................................................................................................. xviii
List of Appendices ............................................................................................................ xxii

Chapter 1 ......................................................................................................................... 1

1 Introduction .................................................................................................................. 1

1.1 Ubiquitination ........................................................................................................... 2

1.1.1 Ubiquitin and the ubiquitin code ....................................................................... 3

1.1.2 E1 and E2 enzymes ......................................................................................... 6

1.1.3 RING E3 ligases .............................................................................................. 8

1.1.4 RING heterodimers and multi-subunit E3 ligases ............................................ 10

1.2 The CTLH complex ............................................................................................... 12

1.2.1 The yeast Gid complex function in catabolite inactivation ............................... 14

1.2.2 Gid complex structure and topology ............................................................... 18

1.2.3 MAEA and RMND5A RING domains and E3 ligase activity .......................... 24

1.2.4 Subcellular localization of mammalian CTLH complex subunits ................. 25

1.2.5 Functions and ubiquitination targets of the CTLH complex ......................... 27

1.3 Scope of thesis ........................................................................................................... 43

1.4 Hypothesis & objectives ......................................................................................... 44
1.5 References................................................................................................................................. 46

Chapter 2........................................................................................................................................... 59

2 The mammalian CTLH complex is an E3 ubiquitin ligase that targets its subunit muskelin for degradation ................................................................. 59

2.1 Introduction.................................................................................................................................. 59

2.2 Results......................................................................................................................................... 62

  2.2.1 WDR26 and GID4 are CTLH complex members ............................................................... 62

  2.2.2 Interdependence of CTLH complex subunit stability ..................................................... 65

  2.2.3 Characterization of the CTLH complex E3 ligase activity ........................................... 70

  2.2.4 Characterization of E2 pairings and lysine linkage ..................................................... 76

  2.2.5 Muskelin is a target of the CTLH complex ................................................................. 76

2.3 Discussion.................................................................................................................................... 81

2.4 Methods....................................................................................................................................... 87

  2.4.1 Cell culture, transfections and treatments ..................................................................... 87

  2.4.2 Generation of CRISPR Knockout cells ........................................................................ 88

  2.4.3 Plasmid constructs ........................................................................................................... 89

  2.4.4 RNA extraction, reverse transcription and quantitative PCR ................................... 90

  2.4.5 Western blot ..................................................................................................................... 91

  2.4.6 Immunofluorescence microscopy ................................................................................. 92

  2.4.7 Immunoprecipitations .................................................................................................... 92

  2.4.8 In vitro ubiquitination assays ......................................................................................... 93

  2.4.9 Chain linkage analysis by Mass Spectrometry (MS) .................................................. 94

  2.4.10 Statistical analysis ......................................................................................................... 95

2.5 References.................................................................................................................................... 95

Chapter 3........................................................................................................................................... 100

3 Characterizing the structure of the human CTLH E3 ligase complex ..................... 100
3.1 Introduction .......................................................................................................................... 100
3.2 Results .................................................................................................................................. 104
  3.2.1 Analysis of MAEA and RMND5A RING domains .................................................. 104
  3.2.2 Mapping topology of the human CTLH complex ................................................. 110
  3.2.3 Domains of RanBPM required to mediate association in the complex ............. 114
  3.2.4 Cross-linking mass spectrometry of the endogenous human CTLH complex .............................................................. 116
3.3 Discussion ............................................................................................................................. 119
3.4 Methods .................................................................................................................................. 126
  3.4.1 Cell culture .................................................................................................................. 126
  3.4.2 Cloning ........................................................................................................................ 126
  3.4.3 Multiple sequence alignment and prediction of protein structure .......... ....... 127
  3.4.4 Extract preparation and immunoprecipitations for Western blot .................. 127
  3.4.5 Immunoprecipitation and sample prep for Cross-linking Mass spectrometry ........................................................................... 129
  3.4.6 Identification and analysis of cross-linked peptides ........................................ 132
  3.4.7 Statistics ....................................................................................................................... 133
3.5 References ............................................................................................................................ 133
Chapter 4 ...................................................................................................................................... 137
4 Proteomic-based identification of ubiquitination substrates reveals the CTLH E3 ligase complex regulates glycolysis ................................................. 137
  4.1 Introduction ....................................................................................................................... 137
  4.2 Results ............................................................................................................................... 140
    4.2.1 RanBPM-dependent global proteome ................................................................. 140
    4.2.2 RMND5A and MAEA-dependent global proteomes ....................................... 146
    4.2.3 Commonalities in the global proteomic screens .............................................. 154
    4.2.4 RanBPM and RMND5A-dependent ubiquitinomes ...................................... 154
4.2.5 The endogenous RanBPM interactome .................................................. 166
4.2.6 The CTLH complex regulates ubiquitination of PKM2 and LDHA ..... 172
4.2.7 The CTLH complex regulates glycolysis ........................................... 172
4.3 Discussion............................................................................................................ 178
4.4 Methods.............................................................................................................. 186
  4.4.1 Cell culture, plasmid construction, and antibodies ......................... 186
  4.4.2 MS sample preparation for global proteomics ................................... 187
  4.4.3 diGLY enrichment ............................................................................... 187
  4.4.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for RanBPM diGLY enrichment .......................................................... 188
  4.4.5 LC-MS/MS for global proteomic and RMND5A diGLY enrichment.... 189
  4.4.6 RanBPM affinity-purification coupled to MS ..................................... 190
  4.4.7 Proteomic data analysis ..................................................................... 192
  4.4.8 In vivo ubiquitination for Western blot ............................................ 194
  4.4.9 Central carbon metabolism profiling ............................................... 195
  4.4.10 PK and LDH activity assays ............................................................... 195
  4.4.11 Measurement of glycolysis in real time .......................................... 196
  4.4.12 Statistics ......................................................................................... 196
4.5 References............................................................................................................ 197

Chapter 5.............................................................................................................. 201

5 Discussion ............................................................................................................ 201
  5.1 Summary of findings .............................................................................. 201
  5.2 Questions remaining about the CTLH complex E3 ligase activity ......... 204
  5.3 Improved model of the human CTLH complex topology defines the utility of multiple substrate receptors ...................................................... 207
  5.4 Do complex subunits share common functions? ................................. 210
  5.5 Possible modes of regulating CTLH complex function ....................... 212
5.6 Implications of the CTLH complex in cancer.................................................... 215
5.7 Significance of the work .................................................................................... 217
5.8 References ........................................................................................................ 219
Appendix A: Permissions from Scientific Reports.................................................. 224
Appendix B: Cross-linked peptides identified......................................................... 225
Appendix C: Mass spectrometry scan settings ....................................................... 227
Curriculum Vitae ...................................................................................................... 228
List of Tables

Table 3.1. List of primers used for mutagenesis of RMND5A RING domain .................... 127

Table 4.1. Proteins increased in RanBPM-dependent proteome that do not have a corresponding change in RNA levels. ............................................................................................... 147

Table 4.2. Previously proposed CTLH substrates in the proteome datasets ...................... 179

Table 4.3. List of functional profiling databases used for g:profiler analysis. ..................... 194

Table 5.1. Summary of E2 selectivity observed in different studies on the yeast Gid or human CTLH complexes .................................................................................................................. 206
List of Figures

Figure 1.1. The complexity of ubiquitination and its generation by a RING E3 ligase-dependent mechanism. ................................................................. 5

Figure 1.2. The canonical RING domain .................................................................................................................. 9

Figure 1.3. Composition and structure of yeast Gid and composition of human CTLH complexes. ................................................................. 13

Figure 1.4. Vacuole and proteasomal degradation mechanisms of Fbp1 orchestrated by the Gid complex during catabolite inactivation in *Saccharomyces cerevisiae* ................. 16

Figure 1.5. LisH domains ................................................................................................................................. 20

Figure 1.6. CTLH and CRA domains ................................................................................................................ 22

Figure 1.7. Current knowledge of pathways and functions linked to the CTLH complex. .... 42

Figure 2.1. Characterization of the CTLH complex. ......................................................................................... 63

Figure 2.2. MAEA and Muskelin co-immunoprecipitate with RanBPM in stringent binding conditions. ........................................................................ 64

Figure 2.3. Localization of CTLH complex subunits. ......................................................................................... 66

Figure 2.4. RanBPM and TWA1 are essential for complex stability. ................................................................. 68

Figure 2.5. Analysis of mRNA levels of proteins that change in figure 2.4......................................................... 69

Figure 2.6. Confirmation of changes of CTLH member protein expression in knockout cells or RanBPM shRNA cells by alternative knockdown/knockout strategies or rescue experiments. ................................................................................. 71

Figure 2.7. The CTLH complex has E3 ligase activity ......................................................................................... 73

Figure 2.8. CTLH complex is not compromised in RMND5A KO cells. ......................................................... 74
Figure 2.9. Recombinant RMND5A and MAEA exhibit E3 ligase activity *in vitro*........... 75
Figure 2.10. Properties of the CTLH complex E3 ligase activity. ........................................ 77
Figure 2.11. Muskelin is a target of the CTLH complex........................................................ 80
Figure 3.1. Figure 3.1. RMND5A and MAEA RING domains............................................... 106
Figure 3.2. RMND5A RING residues critical for controlling muskelin levels. .................... 108
Figure 3.3. RanBPM immunoprecipitation in CTLH complex subunit knockout cells. ...... 112
Figure 3.4. Domains of RanBPM required for association with CTLH complex subunits.. 115
Figure 3.5. BS3 cross-linking mass spectrometry for RanBPM immunoprecipitation. ...... 117
Figure 3.6. Cross-linking mass spectrometry of CTLH complex maps proximity of domains and subunits.. ................................................................. 118
Figure 3.7. Updated model of human CTLH complex architecture.. ................................. 120
Figure 4.1. Strategy to identify CTLH complex ubiquitination substrates in HeLa cells. ... 141
Figure 4.2. The RanBPM-dependent proteome................................................................. 144
Figure 4.3. Comparison of shRanBPM proteome versus previous microarray data.. ....... 145
Figure 4.4. The RMND5A-dependent proteome................................................................. 149
Figure 4.5. The MAEA-dependent proteome................................................................. 152
Figure 4.6. Comparison of MAEA KO, RMND5A KO, and shRanBPM HeLa proteomes. 153
Figure 4.7. The RanBPM-dependent ubiquitinome............................................................ 157
Figure 4.8. The RanBPM-dependent ubiquitinome............................................................ 159
Figure 4.9. The RMND5A-dependent ubiquitinome.......................................................... 162
Figure 4.10. The RMND5A-dependent ubiquitinome....................................................... 164
Figure 4.11. Comparison of RanBPM and RMND5A-dependent diGLY data................. 167

Figure 4.12. The endogenous RanBPM interactome.................................................. 169

Figure 4.13. Comparisons of the RanBPM interactome with increased proteins and decreased ubiquitination .......................................................... 171

Figure 4.14. CTLH complex-dependent ubiquitination of glycolysis enzymes............... 174

Figure 4.15. The CTLH complex regulates glycolysis............................................. 176

Figure 5.1. The human CTLH complex is a RING heterodimer multi-subunit E3 ligase that regulates essential cellular processes via ubiquitination........................................ 203
List of Appendices

Appendix A: Permissions from Scientific Reports ................................................................. 224
Appendix B: Cross-linked peptides identified .................................................................. 225
Appendix C: Mass spectrometry scan settings ............................................................... 227
Chapter 1

Introduction

Human cells experience constant stress yet can maintain homeostasis. A healthy cell deftly adapts to changes in their environment while also executing programmed biological processes, such as cell death, the cell cycle, or stages of embryo development. These biological processes and responses are governed by regulatory proteins in signaling pathways that communicate and transduce a signal by acting as either sensors, transducers, or effectors. Reversible regulation of proteins by post-translational modification (PTM) activates or inhibits these pathways by controlling protein fate and function. PTM regulation provides the opportunity to biochemically coordinate a rapid cellular response to changes in environment or facilitating timed biological processes. This is critical for cells to remain healthy and for organisms to develop properly.

Errors in signaling pathway regulation often cause a diseased state. Cancer develops because of mutations in certain genes (oncogenes and tumour suppressors) give a cell a selective growth advantage. Protein products of oncogenes (activated in cancer and promote tumour development) and tumour suppressor genes (inactivated in cancer, normally function to restrict cancer development) regulate signaling pathways and activities of the cell. These mutations allow cells to acquire the following hallmarks of cancer: sustaining proliferative signaling, evading growth suppressors, avoiding immune detection, enabling replicative immortality, tumour-promoting inflammation, activating invasion & metastasis, inducing angiogenesis, genome instability & mutation, resisting cell death, and deregulating cellular energetics\(^1\). Together, these characteristics permit
cancer cells to survive, metastasize, come in and out of dormancy, and divide uncontrollably.

Deregulation of pathways that result in acquisition of these hallmarks can occur because of mutations that alter the PTM landscape. For example, genes encoding protein kinases, which function to catalyze phosphorylation of specific substrates, are frequently mutated or overexpressed in multiple cancer types and drive the development of the tumour (e.g. RAS, EGFR, PI3K)\(^1\). Similarly, genes encoding proteins that specify the addition of the PTM ubiquitin (Ub) to protein substrates (ubiquitination) are oncogenes and/or tumour suppressors in cancer (e.g., MDM2, VHL, SPOP, FBXW7, BRCA1)\(^2,3\). In contrast to phosphorylation, which involves a small phosphate moiety, Ub is a polypeptide. Its addition has substantial effects on a protein; for example, it can target proteins for degradation or change protein interactors\(^4\). The concept of ubiquitination frames the scope of this thesis and is introduced in section 1.1. This thesis characterizes a new player in ubiquitination and a potential regulator of cancer development, the C-terminal to LisH (CTLH) complex. A review of the current literature on the CTLH complex is presented in section 1.2.

### 1.1 Ubiquitination

Ubiquitination is a dynamic and reversible PTM with varied functional outcomes and fundamental redundancy, specificity, and multiplicity\(^4\). This regulatory capacity makes ubiquitination an essential protein modification that governs nearly all cellular processes. In the typical ubiquitination reaction, Ub is covalently added to a lysine residue of a substrate. It can be a single moiety, called monoubiquitination, or form a
polyubiquitination chain of multiple Ubs linked together (Figure 1.1A). The effects of 
ubiquitination on a substrate vary. It can include tagging the protein for proteasomal or 
lysosomal degradation, re-localizing a protein to a specific subcellular organelle, altering 
enzymatic activity or complex assembly, and changing protein interaction partners.

Ubiquitination is achieved and coordinated by a suite of writers, editors, and 
readers5–7. The writers comprise E1 activating enzymes, E2 conjugating enzymes, and 
E3 ligases. The sequential actions of these three proteins result in ubiquitination (Figure 
1.1B). Like other PTMs, ubiquitination can be reversed in a process called 
deubiquitination. This is achieved by enzymes called deubiquitinases, or DUBs (the 
editors), which are proteases that cleave the isopeptide bond between a ubiquitin 
molecule and a protein, or between ubiquitins in a polyubiquitin chain8.

1.1.1 **Ubiquitin and the ubiquitin code**

Ub is a 8.6 kDa protein that is structurally compact and contains a five-stranded β 
sheet, an α helix, a short 3_{10} helix, and an exposed, flexible six-residue C-terminal tail 
(Figure 1.1A)9. Polyubiquitination chains can be linked through one of the seven internal 
lysines (Lysine (K) 6, K11, K27, K29, K33, K48, or K63) or the N-terminal methionine 
on each ubiquitin (Figure 1.1A)³. Typically, only one lysine is linked in a poly-ubiquitin 
chain (homotypic chains, e.g., all ubiquitins linked only through K48 or only through 
K63), however, branched chains can develop when multiple linkages are used in a single
Ubiquitin code
- K48 link: proteasomal degradation
- K63 link/monoUb: change in function

K48 chain
- Linked through one of 7 lysines or N-term methionine

K63 chain

K48/K63 branched chain

Reversible/editable
Deubiquitinases remove Ub

Dynamic
- Enzymatic
- Signal dependent
- Allosteric regulation

Specific
- E3 ligase recruits specific substrates
- Distinct E2-E3 pairings

Redundancy and multiplicity
- One E3 has many substrates
- One substrate targeted by many E3s
**Figure 1.1. The complexity of ubiquitination and its generation by a RING E3 ligase-dependent mechanism.** A) Ubiquitination products on lysines and its reversal by deubiquitinases. Ubiquitin protein (Ub, the blow up shows its structure) can be attached to lysines as monoubiquitination or as homotypic or heterotypic polyubiquitin chains linked through its lysines (e.g., K48 or K63). The type of ubiquitination dictates functional effects. B) Ubiquitination mediated by really interesting new gene (RING) type-E3 ligases consists of: (1) Ubiquitin (Ub) activation by E1 enzymes involving formation of a thioester bond (~) between catalytic E1 cysteine and C-terminal glycine of Ub; (2) transfer of Ub to the catalytic cysteine of E2 conjugating enzyme via a transthiolation reaction, also forming a thioester bond; and (3) conjugation of Ub to substrate lysine via an aminolysis reaction, forming an isopeptide bond between lysine and C-terminal glycine of Ub. RING E3s form a scaffold, binding the E2 via its RING domain (square space in figure) and one of many specific substrates via a non-RING element (triangle space). Figure created with Biorender.com
chain (heterotypic chains). Additional complexity arises when ubiquitin itself is modified with a PTM.

The ubiquitin code describes how the information imparted by a particular ubiquitination type is transmitted into distinct signaling events. For example, while K48-linked polyubiquitin chains lead to proteasomal degradation, K63-linked chains have non-proteolytic effects such as regulating enzyme activity, protein complex assembly, and protein sorting (Figure 1.1A). The diversity of ubiquitin chains that can be generated in combination with other modifications and cellular context underlies the complexity of the ubiquitin code and the functional outcomes that can occur.

1.1.2 E1 and E2 enzymes

Ubiquitin activation is a multi-step reaction (Figure 1.1B). First, E1 enzymes (in humans, UBA1 and UBA6) bind Ub, adenosine triphosphate (ATP), and a magnesium ion through their inactive and active adenylation domains to catalyze acyl-adenylation of the Ub C-terminus. Following this, the catalytic cysteine in the E1 attacks the adenylated ubiquitin to form a high energy ubiquitin~E1 complex (~ indicates thioester), releasing adenosine monophosphate (AMP). In the last step, the Ub linked to the catalytic cysteine is transferred to an E2 enzyme in a transthiolation reaction.

For ubiquitination involving really interesting new gene (RING) E3 ligases, E2 enzymes accept activated ubiquitin from the E1 and then, in an aminolysis reaction, transfer the ubiquitin from the cysteine~Ub thioester to the lysine of the substrate via scaffolding by the RING protein (Figure 1.1B). The latter reaction depends on a closed E2~Ub conformation and involves the substrate lysine amino group attacking the
thioester linkage, leading to hydrolysis and subsequent isopeptide bond formation between Ub C-terminal glycine and the substrate lysine\textsuperscript{5,13}. E2 interactions with E1 and E3 enzymes are typically transient and mutually exclusive.

The ~40 different human E2s each contain a conserved catalytic Ubiquitin Conjugation (UBC) domain adopting an α/β-fold\textsuperscript{5}. Disordered insertions and extensions flanking the UBC vary between E2 enzymes, classifying E2s based on whether these appendages are absent (class I), or occur N-terminal (class II), C-terminal (class III), or at both ends (class IV) of the UBC domain\textsuperscript{11}. Although a RING E3 is responsible for substrate recruitment, the E2 in most cases determines residue selection, mono- versus poly-ubiquitination, and/or chain linkage type and length. E2 enzymes function as either: (1) chain initiating agents that transfer Ub to a non-Ub lysine only (diverse range of substrates); (2) chain builders that transfer Ub to a Ub lysine only (very specific, repetitive reaction); or (3) can do both. In several cases, the varying N or C-terminal extensions of E2s account for the differences in chain initiating versus building tendencies and their specificity for E3 binding, residue selection, and linkage choice. For example, the E2 UBE2R1/CDC34 interacts with the Skp, Cullin, F-box (SCF) E3 ligases through its unique acidic C-terminal tail\textsuperscript{14}, while the N-terminal extension of the E2 UBE2E restricts the enzyme to monoubiquitination\textsuperscript{15}. Importantly, E2s largely dictate chain topology and therefore have an important role in regulating ubiquitination outcome. Certain E2 chain builders have preferences for a specific ubiquitin linkage, likely due to an interaction with ubiquitin and the E2 that positions the particular ubiquitin lysine in the E2 active site\textsuperscript{16}. UBE2S (K11 chains), UBE2K (K48 chains), UBE2R1 (K48 chains),
and UBE2N (K63 chains) are examples of these types of E2 enzymes. Other E2 chain builders are more promiscuous, and can catalyze more than one type of linkage, such as members of the UBE2D enzyme family.

1.1.3 RING E3 ligases

Of the >600 E3 ligases encoded in the human genome, RING domain (including its variant U-box domain) containing E3 ligases comprise the vast majority and will be the focus of this thesis. In comparison to Homologous to E6AP C-terminus (HECT) and RBR (RING-in-between-RING) E3 ligases, which bear a catalytic cysteine and accept ubiquitin, RING and U-box domains do not participate as directly in the ubiquitin transfer to substrate. Instead, they bind the E2 (and thus place the substrate in close proximity) and can promote the E2–Ub closed conformation through hydrogen bond formation (Figure 1.1B). The canonical RING domain is a zinc finger with a Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2- Cys-X(4-48)-Cys-X2-Cys motif (Figure 1.2). The cysteines and the single histidine in the RING motif form a cross-braced arrangement and coordinate two zinc ions critical for the compact α/β fold of the RING domain (Figure 1.2). Two loops that surround either the first or second zinc coordination site contain conserved hydrophobic residues that typically mediate the E2 interaction. Additional E2-E3 interaction points can exist, including non-RING elements, but are different for each RING E3 ligase and likely dictate E2 selectivity. Unique non-RING components of a RING E3 ligase are responsible for imparting the substrate specificity of the ubiquitination reaction. Thus, in general, each RING protein uses different surfaces for function: non-RING elements for recruitment of their specific substrates and the RING
Figure 1.2. The canonical RING domain. *Top,* the cross-brace structure and sequence of a canonical RING domain coordinating two zinc ions. Created with Biorender.com. *Bottom,* the RING domain structure of BIRC7 coordinating two zinc ions (blue spheres). Image was created with PyMOL (PDB:4AUQ). The cysteines and histidine involved in zinc coordination are colour matched in both images.
domain to bind to an E2. The U-box domain has a very similar structure to the RING
domain, including the two loops, but it does not coordinate Zinc; instead, polar and
charged residues are present that replicate the same structural purpose via hydrogen bond
networks\(^{17}\).

While RING-type E3s do not participate directly in Ub transfer, a RING
“linchpin” residue forms hydrogen bonds with both Ub and the E2 in such a way that
restricts their relative orientations, stabilizing the E2~Ub closed conformation (thus
promoting Ub transfer)\(^{5,17}\). Besides the linchpin residue, additional RING or non-RING
components of a RING E3 can contribute to stimulating the reaction. For instance,
phosphorylated Y363 of the CBL-B E3 ligase, which is located outside of its RING
domain, interacts with Ub and enhances Ub transfer\(^{18}\).

1.1.4 RING heterodimers and multi-subunit E3 ligases

Homo- or hetero-dimerization of RING domain proteins is common. The
interaction is mediated by the RING domain itself and/or non-RING \(\alpha\)-helical regions\(^{17}\).
In several of these cases, dimerization is essential for stability and ubiquitin transfer.
Recent structural insights of RING dimers show that Ub interacts with both subunits\(^{19–22}\).
This interaction locks the otherwise flexible Ub C-terminal tail, priming it for
nucleophilic attack. Interesting examples exist of heterodimerization between active and
inactive (i.e., no E2 binding) RING domain partners. For MDM2/MDMX, MDM2 RING
domain interacts with the E2 (UbcH5b/UBE2D2) and provides the linchpin (R479) for
hydrogen bond stabilization of the E2~Ub conjugate, but both MDM2 and MDMX
interact with Ub\(^{23}\). Together, these interactions stabilize a closed E2~Ub confirmation.
The BRCA1/BARD1 RING heterodimer has increased ligase activity compared to BRCA1 by itself; BARD1 has no intrinsic E3 ligase activity\(^{24,25}\). In the case of UbcH5c/UBE2D2 as the E2, the BRCA1 RING domain provides the linchpin (K65) and mediates E2 binding through its two loops and \(\alpha\)-helix\(^{26-28}\).

The Cullin RING ligase (CRL) represents a family of multi subunit E3 ligase complexes. In humans, they are characterized by a scaffolding cullin protein with an elongated shape that binds a RING protein (either RBX1 or RBX2) at its C-terminus\(^{29}\). At the N-terminus, each cullin protein has a specific adaptor protein that in turn binds their own set of multiple substrate receptors. Therefore, different CRLs comprise a common catalytic core (a cullin with RBX1/2) but have diverse substrates. The architecture of the complex is arranged such that the substrate bound to the substrate receptor is near the active site of the RBX1/2-bound E2 enzyme\(^{30,31}\). The cell cycle regulator anaphase promoting complex/cyclosome (APC/C) is another example of a multi-subunit E3 ligase\(^{32}\). It is a large complex (1.2 MDa) of 14 subunits, organized into the following three lobes: a catalytic core with a small RING protein, a scaffolding subcomplex, and two interchangeable substrate receptors that are regulated by different phases of the cell cycle. The different subunits in multi-subunit E3 ligases allow for multiple points of allosteric regulation and diverse substrate interaction surfaces.

While some generalities exist between different RING E3 ligases, the examples and factors discussed thus far demonstrate that there are clear differences and peculiarities in the structure and mechanisms of each RING protein/complex. Therefore, it is important to investigate the intricacies of each E3 ligase on an individual basis.
Multi-subunit E3 ligase complexes are of particular interest because of their substrate diversity and multiple points of regulation. The CTLH complex, which is the focus of this thesis, is a recently identified multi-subunit E3 ligase complex.

1.2 The CTLH complex

Initially an overlooked set of proteins, the human CTLH complex now represents an emerging E3 ligase that has caught the attention of many labs around the world. Although exact subunit composition was initially unclear\(^{33}\), we now know with certainty (including from work stemming from this thesis) that ARMC8\(\alpha\) (armadillo repeat-containing protein 8), ARMC8\(\beta\), GID4 (glucose-induced degradation protein 4 homolog), MKLN1 (muskelin), RanBPM (Ran-binding protein 9, aka RANBP9), TWA1 (glucose-induced degradation protein 8 homolog, aka GID8), WDR26 (WD repeat-containing protein 26), and the RING domain proteins MAEA (E3 ubiquitin-protein transferase macrophage erythroblast attacher) and RMND5A (E3 ubiquitin-protein transferase required for meiotic nuclear division 5 homolog A) are subunits of the human CTLH complex\(^{34,35}\) (Figure 1.3A). In addition, RanBP10 (Ran-binding protein 10), RMND5B (E3 ubiquitin-protein transferase required for meiotic nuclear division 5 homolog B), paralogues of RanBPM and RMND5A, respectively, and YPEL5 (yippee-like 5) are putative complex members, but their exact involvement is less definitive. Strikingly, 6 of the confirmed members, MAEA, muskelin, RanBPM, RMND5A, TWA1 and WDR26 contain conserved LisH (lissencephaly type-1-like homology) and CTLH domains (hence the name of the complex), with four of those (MAEA, RMND5A, RANBP9, TWA1) also
Figure 1.3. Composition and structure of yeast Gid and composition of human CTLH complexes. A) Schematic of domain maps of yeast Gid and human CTLH complex subunits. Homologues are side-by-side and names are colour matched with the rest of the figure. Size of the domains and proteins are roughly scaled. LisH - lissencephaly type-1-like homology; CTLH - C-terminal to LisH; CRA - CT11-RanBPM; SPRY - SPla and the RYanodine Receptor; RING – Really Interesting New Gene. B) Schematic of the topology of the yeast complex based on cryo-EM data from Qiao et al., 2020. C) Images depicting structure and arrangement of Gid1 (red), Gid5 (purple), Gid4 (pink), and Gid8 (salmon) of the yeast Gid complex from the cryo-EM data from Qiao et al 2020 (PDB: 6SWY). The structure deposited did not contain Gid2, Gid9, or Gid7. Created with PyMOL.
containing a CT11-RanBPM (CRA) domain (Figure 1.3A). RANBP10 and RMND5B also have LisH, CTLH, and CRA domains. Additionally, several protein interaction domains (SPRY (named from the named from SPLa and the RYanodine Receptor), β-barrel, kelch repeats, discoidin, armadillo (ARM) repeats, and WD40 repeats) are present within the subunits RanBPM, GID4, muskelin, WDR26, and ARMC8 (Figure 1.3A).

Despite the existence of a 670 kDa RanBPM-TWA1-muskelin complex first being reported in 2003\textsuperscript{36}, and further expanded to include RMND5A, MAEA, and ARMC8α/β in 2007\textsuperscript{33}, much of the understanding of the structure and function of the CTLH complex has only been gained in the last few years. This section will review previous work on CTLH subunits, and the recent investigations of complex structure and function, by starting with the findings of its yeast homologue, the Glucose-induced degradation-deficient (Gid) complex.

1.2.1 The yeast Gid complex function in catabolite inactivation

In glucose-starved \textit{Saccharomyces cerevisiae} cells undergoing gluconeogenesis, replenishment of glucose induces immediate inhibition and degradation of enzymes that control catabolic metabolic processes, such as the gluconeogenic enzyme Fructose 1,6-bisphosphatase (Fbp1, aka FBPase)\textsuperscript{37}. This phenomenon is called catabolite inactivation and allows \textit{S. cerevisiae} to switch from a state of gluconeogenesis back to a state of glycolysis, thereby preventing needless ATP consumption. Interestingly, Fbp1 degradation involves either the ubiquitin-proteasome system or the vacuole (homologous to the mammalian lysosome), with the mechanism used by the cell hinging on the time of starvation on ethanol prior to glucose rescue: proteasome if <1 day, vacuole if >1 day
(Figure 1.4)\textsuperscript{38}. Remarkably, proteins of the Gid complex are involved in both Fbp1 degradation mechanisms.

1.2.1.1 Vacuolar degradation of gluconeogenic enzymes by the Gid complex

UV mutagenesis screening for \textit{S. cerevisiae} genes required for glucose induced Fbp1 vacuole degradation after long glucose starvation revealed a set of vacuolar import and degradation (\textit{VID}) genes\textsuperscript{39}. In cells with \textit{VID} gene mutations, Fbp1 avoided vacuolar degradation and either accumulated diffusely in the cytosol or formed cytoplasmic punctate structures\textsuperscript{39}. This indicated that intracellular structures containing Fbp1 are formed before they are imported to the vacuole. Over the subsequent years, a variety of yeast mutants and biochemical assays defined the following key steps of this process (Figure 1.4, right): (1) In \textit{S. cerevisiae} starved of glucose for 3 days, a majority of Fbp1 is secreted as extracellular vesicles in the periplasm (if starved between 1-3 days, secretion is minimal); (2) meanwhile, a complex of Gid4 (aka Vid24, homologue of human GID4), Gid5 (aka Vid28, similar to human ARMC8), Gid1 (aka Vid30, homologue of human RanBPM), and COPI coatamer proteins, including Sec28, is localized at intracellular plasma membrane actin patches; (2) glucose addition triggers endocytosis of periplasmic Fbp1, which, together with intracellular Fbp1, localizes to the actin patches where 30-50 nm membrane-bound vesicles (named Vid vesicles) are formed with Fbp1 in the lumen and the Gid1-Gid4-Gid5-Sec28 complex on the periphery; (3) the Vid vesicles aggregate and form endosome-like clusters of varying size that disassociate from actin and are delivered to the vacuole; (4) Fbp1 is degraded in the vacuole but other Vid vesicle proteins are returned to the cytosol via retrograde transport\textsuperscript{38,40–45}. 
Figure 1.4. Vacuole and proteasomal degradation mechanisms of Fbp1 orchestrated by the Gid complex during catabolite inactivation in *Saccharomyces cerevisiae*. In a glucose-starved state, cells are utilizing fructose 1,6-bisphosphatase (Fbp1) and other gluconeogenic enzymes. When replenished with glucose, gluconeogenic enzymes are degraded to prevent needless ATP consumption. *Left*, if glucose starved for less than 24 hours, re-introduction of glucose triggers synthesis of Gid4, which then associates with the glucose-induced degradation-deficient (Gid) complex. Gid4 subsequently binds Fbp1 and recruits it to the complex. Fbp1 is polyubiquitinated by Ubc8 E2 enzyme that is bound to the Gid2-Gid9 RING dimer, causing proteasomal degradation. Gid4 is also ubiquitinated and degraded, returning the complex to the original state. *Right*, if glucose starved for 3 days, glucose addition causes recruitment of Fbp1 to actin patches. Subsequently, Vid vesicles are formed containing at least Gid1, Gid4, and Gid5 with Fbp1 in the lumen. Vid vesicles cluster into larger endosome-like intracellular structures that fuse with the vacuole where Fbp1 is degraded but Gid proteins are returned to the cytosol. Created with Biorender.com.
1.2.1.2 Ubiquitin-proteasomal degradation of Fbp1 by the Gid complex

Starving *S. cerevisiae* of glucose for a much shorter time (≤24 hours) followed by re-introduction of glucose causes rapid Fbp1 ubiquitination in the cytosol followed by 26S proteasomal degradation (Figure 1.4, left)\(^{38,46}\). Initial genetic screens identified the first set of genes required for Fbp1 proteasomal degradation: *GID1, GID2* (homologue of human RMND5A), *UBC8* (Ubiquitin-conjugating enzyme E2-24 kDa, homologue of human Ubiquitin Conjugating Enzyme E2 H (UBE2H)), *GID4, GID5, GID6, GID7* (homologue of human WDR26), *GID8* (homologue of human TWA1), and *FYV10* (aka *GID9*, which is how it will be referred to henceforth; homologue of human MAEA)\(^{47–49}\). Subsequent work characterized that protein products of some of those genes (Gid1, Gid2, Gid4, Gid5, Gid7, Gid8, and Gid9) comprise a >600 kDa complex\(^{46,47,50,51}\). Termed the Gid complex, it acts as an E3 ligase that polyubiquitinates Fbp1 through its RING heterodimer Gid2-Gid9\(^{46,52,53}\). This regulation also occurs for the glucose regulating enzymes Pck1, Icl1, and Mdh2\(^{46,53–55}\).

Rapid induction of Gid4 expression and association after glucose replenishment is required for Gid complex ubiquitination of the metabolic enzymes\(^{46}\). This signal-dependent ubiquitin activation depends on Gid4 molecular recognition of N-terminal proline degrons on the substrates\(^{54–58}\). The crystal structure of its human counterpart, GID4, shows it has an antiparallel β-barrel with a unique insertion of three short helices between β-strands 4 and 5\(^{56}\). The N-terminal proline of a PGLW peptide fits snugly at the bottom of the hydrophobic binding cleft in the GID4 β-barrel in a precise position to mediate a network of hydrogen bonds\(^{56}\). New insights reveal that other hydrophobic
residues, not just proline, can be accommodated in the binding cleft\textsuperscript{58}. Interestingly, once cells return to a normal metabolic state, Gid4 is ubiquitinated and rapidly degraded, a process which is dependent on the proteasome and almost all Gid complex proteins\textsuperscript{46,59}.

In summary, the time of glucose starvation \textit{S. cerevisiae} dictates two different degradation mechanisms for Fbp1: a shorter glucose starvation period causes Fbp1 delivery to the 26S proteasome whereas a longer starvation induces Fbp1 secretion, internalization, and then degradation in the vacuole. There are important requirements that are the same for both processes, including: Vid/Gid proteins, synthesis of Gid4, the N-terminal proline of Fbp1, and K48 and/or K63 poly-ubiquitin chain formation\textsuperscript{38,39,46,48,49,54,60,61}.

\textbf{1.2.2 Gid complex structure and topology}

Protein-Protein Interaction Prediction Engine (PIPE) provided the first insight into Gid complex topology, predicting a core complex of Gid1, Gid5, and Gid8 with a secondary component of Gid9, Gid4, and Gid2\textsuperscript{62}. These findings were then confirmed and expanded on by the Wolf group in binding assays using yeast deletion strains and domain mutants, producing an initial model of complex topology\textsuperscript{50}. Cryo-electron microscopy (Cryo-EM) structural determination of endogenous and reconstituted (without Gid7) Gid complex at 9° and 3.7°, respectively, further expanded the model, providing much needed resolution and clarity\textsuperscript{55}.

The overall architecture of the complex as determined by Cryo-EM and supported by the prior studies shows a clamp-like structure resembling CRLs, with Gid4 at one end of the clamp, a T-shaped Gid2-Gid9 RING dimer on the other end, and a scaffolding unit
of Gid1, Gid8, and Gid5 that connect the two ends together (Figure 1.3B,C)\textsuperscript{55}. Gid7 was not included in these experiments, but based on Menssen \textit{et al.}, 2012\textsuperscript{50}, it is expected to be directly bound to the CTLH domain of Gid1, which projects outward. Complex stability is compromised in yeast deficient of Gid1 or Gid8, highlighting the essential role for both proteins as the core of the complex\textsuperscript{50}. The Gid1-Gid8 interaction forms a trefoil structure, with contacts at the vertex mediated by LisH and the CRA (C-terminal segment) domains of both proteins\textsuperscript{50,55}. A heterodimer of Gid8-Gid9, which both contact each other through their CTLH domains and N-terminal segments of their CRA domain, provides the link between the scaffold unit and the RING proteins\textsuperscript{50,55}. Gid2 and Gid9 proteins stabilize each other \textit{in vivo} and dimerize through their RING domains, akin to other RING heterodimers\textsuperscript{53,55}.

\subsection*{1.2.2.1 Structure of LisH, CTLH and CRA domains}

Nearly 100 \textit{Homo sapiens} proteins contain a LisH domain in the SMART database. This domain was named after its first characterization in the gene product of \textit{LIS1} that is mutated in the neuro-developmental disease type-1 lissencephaly\textsuperscript{63}. The 33-residue LisH domain comprises two \(\alpha\)-helices connected by a loop that often homodimerize as an antiparallel four-helix bundle primarily maintained by a tightly packed hydrophobic core and dimer interface (Figure 1.5A)\textsuperscript{64–68}. For the most part, residues engaged in the hydrophobic interactions are conserved (or replaced by a non-polar amino acid) across \textit{H. sapiens} LisH domain containing proteins, including human CTLH proteins (figure 1.5B). Besides the hydrophobic core, the domain is also stabilized by hydrogen bond formation across the interface (red in Figure 1.5A) and support by a
**Figure 1.5. LisH domains.** A) The Lis1 lissencephaly type-1-like homology (LisH) domain dimer and close-up view of the hydrophobic interface with the hydrogen bond forming glutamate in red (PDB: 1UUJ). B) Alignment of LisH domains. Residues coloured by Clustalx. C) The mouse Muskelin N-terminus with a close-up view of LisH domain interface (PDB: 4OYU). Monomers are coloured separately in each image (magenta and teal). Structure images created with PyMOL. Alignment performed with MAFFT online service and visualized in Jalview.
non-LisH element as observed in structural characterizations of LisH dimers in Lis1, FOP, SSDP, SMU1, and TBL1.

The conservation and secondary structure prediction of LisH domains from CTLH complex proteins has been analyzed by Francis et al., 2013. Briefly, the predicted overall structure of LisH domains of all CTLH subunits (two α-helices separated by a loop) is consistent with those studied in other LisH proteins, though there are minor deviations from the consensus sequences. The crystal structure of muskelin revealed dimerization in part through the LisH N-terminal α-helix (the second helix could not be crystallized), with an interface comprising Cys180 at the center surrounded by hydrophobic residues, which are all in similar positions in each monomer (Figure 1.5C).

Like the LisH domain, CTLH and CRA domains are α-helical. In all the reported structures, the CTLH domain contains 3 helices with several conserved hydrophobic residues and some conserved polar and/or charged residues, including CTLH complex subunits (Figure 1.6A,B). Across different CRA domains, hydrophobic residues are conserved along with prolines in the loop regions (Figure 1.6C). Additionally, a positively charged residue followed by a negatively charged residue in the second helix is strongly conserved.

Structural insight has been gained from the crystal structures of Arabidopsis thaliana TOPLESS (atTPL), rice TOPLESS-related (osTPR), and human SMU1 proteins, which like RanBPM, TWA1, MAEA, and RMND5A, contain all three LisH, CTLH, and CRA domains in that order. Overall dimer shape and domain structures are similar between atTPL, osTPR2, and SMU1: a dimer interface with the typical LisH antiparallel bundle connected to a CTLH domain containing three helices, followed by α-helical CRA
**Figure 1.6. CTLH and CRA domains.** A) Alignment of CTLH domains. Residues are coloured by Clustalx. B) Human SMU1 CTLH domain (PDB: 5EN8). C) Alignment of CRA domains. Residues coloured by Clustalx. D) Crystal structure of human SMU1 dimer with its domains colour coded in each monomer. (PDB: 5EN8). E-F) Crystal structure of Arabidopsis thaliana TOPLESS protein (TPL) as a monomer (E) and tetramer (F) with a close-up view of the tetramer interface. In (E) domains are coloured separately. In (F) monomers are coloured separately (PDB: 5NQV). Structure images created with PyMOL. Alignment performed with MAFFT online service and visualized in Jalview. LisH - lissencephaly type-1-like homology; CTLH - C-terminal to LisH; CRA - CT11-RanBPM.
that supports dimerization by looping back to rest its last helix on top of the LisH first helix; the dimer is symmetrical such that the last CRA helices from each monomer cross over, resembling an X-shape structure (Figure 1.6D,E). The CRA domain in atTPL contains 4 helices, with its second and third helix mediating an interface between dimers in the atTPL tetramer (Figure 1.6F). Hydrophobic grooves in these structures are responsible for interactions with complex partners, but differences in the relative positions of LisH, CTLH, and CRA domains create a distinct conformation of the binding surface. High resolution structural determination of the human CTLH subunits awaits to determine how they interact and what grooves (within and between subunits) and peripheral protein interaction domains are available for potential substrates or interacting proteins, and how this is coordinated with respect to E2–Ub transfer.

1.2.2.2 Structure of kelch, discoidin, SPRY, WD40, and ARM domains

Several protein-protein interaction domains are present in the CTLH complex, each with unique binding preferences that could recruit distinct substrates (Figure 1.3A). The GID4 β-barrel that binds N-terminal hydrophobic peptides was described in section 1.2.1.2. Muskelin has six kelch repeats C-terminal to the CTLH domain that are predicted to form a β-propellor. The muskelin kelch domain has been determined to mediate interaction with p39 and TBX20b. Muskelin also has a discoidin domain, which is present at the N-terminus before the LisH domain. Crystal structures show the muskelin discoidin domain forms a jellyroll fold (similar to a β-barrel), comprising two antiparallel β sheets (a five then three stranded β-sheet) facing each other with a hydrophobic core. In other proteins, discoidin domains have a variety of protein interactors, but
also a wide range of other types of interacting molecules, such as lipids, phospholipids, galactose, and collagen\textsuperscript{77}.

RanBPM and RanBP10 SPRY domain and upstream region are nearly identical, containing two antiparallel β-sheets (composed of 7 β-strands each) held together by hydrophobic and polar interactions, a helix present at each terminus, and a shallow binding pocket\textsuperscript{78}. For RanBPM, the SPRY domain mediates interaction with most of RanBPM’s associated proteins, including Axl, Met, p73, TrkA, p42IP4, Mgl1, TRAF6, Rta from EBV virus, TSSC3, and c-Kit\textsuperscript{79–88}.

WD40 repeats are present on WDR26 C-terminal to its CTLH domain. Like kelch domains, WD40 repeats form a β-propeller\textsuperscript{89}. They can facilitate protein-protein interactions or protein-DNA interactions and are often found in multi-subunit complexes, including other E3 ligases. ARMC8 α and β isoforms contain several Arm repeats that are found in a wide range of proteins, including β-catenin, APC, importin-α, and plakophilin 1. These repeats fold together to form a superhelix of helices that serves as a versatile protein interaction surface\textsuperscript{90}.

1.2.3 MAEA and RMND5A RING domains and E3 ligase activity

As mentioned earlier, the canonical RING motif (Cys-X\textsubscript{2}-Cys-X\textsubscript{(9-39)}-Cys-X\textsubscript{(1-3)}-His-X\textsubscript{(2-3)}-Cys-X\textsubscript{2}- Cys-X\textsubscript{(4-48)}-Cys-X\textsubscript{2}-Cys) is a cross brace structure coordinating two zinc ions that facilitates E2 binding and can stimulate E2–Ub transfer (Figure 1.2). Francis et al., 2013 determined that both MAEA and RMND5A RING domains are conserved and identified key features such as the conserved cysteines and histidine that
are likely to be involved in zinc coordination (or in the case of MAEA, not suspected to coordinate zinc)\textsuperscript{52}.

The \textit{S. cerevisiae}, human (this thesis), \textit{Xenopus laevis}, and \textit{Lotus japonicus} homologues of recombinant RMND5A have all now been demonstrated to have intrinsic ligase activity, even on its own (i.e. without MAEA)\textsuperscript{35,46,91,92}. Mutation of the cysteine in position 3 of the RING domain in the \textit{S. cerevisiae} and \textit{X. laevis} homologues of RMND5A (C379 in yeast Gid2, equivalent to C354 in human RMND5A; zinc site #2), abolished the \textit{in vitro} activity. Gid9, which does not display \textit{in vitro} activity, contributes to Fbp1 polyubiquitination as mutation of the cysteine in position 1 (zinc site #1; C434 in yeast Gid9, C314 in human MAEA) prevents Fbp1, Pck1, and Mdh2 polyubiquitination and degradation \textit{in vivo} but does not change the interaction between Gid2 and Gid9\textsuperscript{53}.

In most of the \textit{in vitro} ubiquitination assays with recombinant RMND5A homologues, the promiscuous UBE2D2 (ubiquitin-conjugating enzyme E2 D2, aka UbcH5b) has been used as the E2\textsuperscript{35,46,91,92}. However, the yeast complex \textit{in vivo} appears to use the E2 Ubc8 for gluconeogenic enzyme ubiquitination\textsuperscript{49}. Only recently was Ubc8 shown to function with the recombinant yeast complex for substrate K48 polyubiquitination of Mdh2 \textit{in vitro}\textsuperscript{55}. No evidence of Ubc8 functioning with other E3 ligases has been reported and ΔUbc8 \textit{S. cerevisiae} have no obvious phenotype besides being deficient in catabolite inactivation of Fbp1\textsuperscript{49,93}. Ubc8 is homologous to the human E2 UBE2H (aka UbcH2) and both contain an acidic C-terminal extension similar to yeast CDC34, and thus are class III E2 enzymes\textsuperscript{94}.

\subsection{Subcellular localization of mammalian CTLH complex subunits}
Overall, complex subunits have been reported (including this thesis) to generally exist diffusely in both cytoplasmic and nuclear compartments, and in specific locations such as the plasma membrane, microtubules, aggresomes, chromatin, mitochondrion, and early and late endosomes. RanBPM subcellular localization has been demonstrated for the endogenous and ectopically expressed protein, showing either a mostly cytoplasmic or a mostly nuclear localization varying by cell type. Leptomycin B treatment leads to an accumulation of endogenous RanBPM in the nucleus, suggesting the protein undergoes nuclear export. Indeed, RanBPM contains a nuclear export sequence (residues 140-155) necessary for the cytoplasmic distribution of the protein. Its LisH/CTLH domains have cytoplasmic targeting function and are required for association with α-tubulin, which is likely bridged by interaction with HDAC6 and/or a complex member since MAEA and TWA1 also associate with microtubules. The C-terminal 35 residues of muskelin are important for its cytoplasmic distribution, since its deletion in transfected muskelin causes the mutant protein as reported by two different groups to be either nearly completely exclusively nuclear or nucleocytoplasmic, as opposed to the non-nuclear wild-type version. Interestingly, these residues are also required for interaction with RanBPM and for the oligomeric state of recombinant muskelin.

Localization of some subunits have been reported to be dynamic and altered by stimuli or overexpression of an interacting protein. In Murine erythroleukemia (MEL) cells, exogenous Wdr26, Twa1, Rmnd5a, and Ranbp10 accumulate in the nucleus in cells treated with leptomycin B, suggesting they shuttle between the nucleus and cytoplasm.
When co-expressed with its interacting partner p39, muskelin changes from a diffuse cytoplasmic staining to enrichment in the perinuclear region, lamellipodia, and along the cell periphery\textsuperscript{74,109}. In Jurkat T cells, endogenous WDR26 is cytosolic but, upon stimulation of the cells by the chemokine SDF1α, translocates to the plasma membrane where it colocalizes with F-actin\textsuperscript{110}. Transfected YPEL5 and MAEA have been independently observed to undergo dynamic changes in the nucleus during cell division, the only localization change so far shared by more than one complex member\textsuperscript{111,112}. Interestingly, RanBPM becomes nuclear enriched during the early time points in cells treated with ionizing radiation (IR) but then relocates to the cytoplasm as treatment persists, eventually colocalizing with aggresomes\textsuperscript{102,107,113}. Overall, these findings demonstrate that the individual subunit localization can be dynamically altered in response to a variety of signals, although whether they do this in unison with the whole complex has been largely unexplored.

### 1.2.5 Functions and ubiquitination targets of the CTLH complex

Since the establishment of the complex as an E3 ligase, discoveries of putative or \textit{in vitro} confirmed ubiquitination targets and functions of the human complex have come to light, such as transcription factor HMG-Box Transcription Factor 1 (HBP1), nuclear matrix protein lamin B, energy regulator AMP-activated protein kinase (AMPK), and (in this thesis chapter 2) its own subunit muskelin\textsuperscript{34,35,108,114}. One surprising certainty is that, despite the overall conservation between the yeast and mammalian complexes, the mammalian complex does not regulate gluconeogenesis, and does not ubiquitinate human
Fbp1 (in fact, recombinant human complex cannot in vitro ubiquitinate yeast Fbp1)\textsuperscript{34,115,116}.

Previous work focused on individual subunits should be re-assessed in light of their - unrealized at the time - involvement in a multi-subunit E3 ligase complex. Here, the past work on functions of the individual subunits is summarized into unifying themes, with emphasis on the recent findings of targets of the complex or common interactions and functions between complex members.

1.2.5.1 Differentiation and development

Various complex subunits have been ascribed functions in developmental and cell differentiation pathways in multiple animal models. Notably, two groups showed a fascinating function and regulation of the entire complex as part of the precise temporal control of the maternal proteome in the maternal-to-zygotic transition (MZT) in *Drosophila melanogaster*. In the early stages of the MZT, the *D. melanogaster* CTLH complex is responsible for degradation of RNA binding proteins Cup, TRAL, and ME31B that are components of a translation inhibiting complex required for oogenesis\textsuperscript{117,118}. Interestingly, the CTLH complex is activated here by egg-induded PNG kinase-dependent translational upregulation of *D. melanogaster* UBE2H homolgue\textsuperscript{117}.

In mice, RanBPM knockout (KO) resulted in both sexes being sterile due to defects in oogenesis and spermatogenesis\textsuperscript{119}. For RanBPM KO female mice, premature ovarian failure was observed due to an arrest of oocyte differentiation during early meiosis. In RanBPM KO males, spermatogonia proliferation after the first week of birth was markedly reduced and at around one month of age there was a loss of all germ cell
types, suggesting an effect on spermatogonia stem cells. The phenotype observed was subsequently attributed to RanBPM interaction and post-transcriptional regulation of c-Kit tyrosine kinase, a protein critical for germ cell development, among other functions. A different group, investigating both a global knockout and germ cell specific RanBPM knockout mice, also observed male infertility and depletion of germ cells (more pronounced in the global knockout). Exploration of RanBPM function in this model determined a direct regulation of alternative splicing by RanBPM via association with RNA binding and alternative splicing factors such as PABPC1 and 2, SF3B3, and HNRNPM.

Though investigated separately, WDR26, RanBPM and RMND5A have both been shown to be important in brain development in a variety of model organisms. *X. laevis* wdr26 and Rmnd5 knockdown animals have been characterized, a species in which Rmnd5 E3 ligase activity has been demonstrated and shown to partially cofractionate with Armc8 at 200-300 kDa in a glycerol density gradient. In *X. laevis*, both Rmnd5 and wdr26 are expressed early and throughout embryonic development, and both show highest expression in the neural regions. Intriguingly, deficiency of both Rmnd5 and wdr26 caused a forebrain formation impairment and reduction in the same neural marker, *pax6*. In zebrafish embryos, RanBPM expression is also highest in the neural regions and its deficiency caused defects in brain development and retinogenesis. Strikingly, there are several reports that showed global *RanBPM*−/− mice have neonatal lethality and postnatal growth retardation. These mice died neonatally because of a
compromised somatosensory system, which caused a sucking defect leading to malnutrition\textsuperscript{123}.

An important role for complex members in red blood cell homeostasis has been well documented. An initial study showed that \textit{Maea} null mouse embryos died perinatally with anemia and differentiation defects in erythroid and macrophage lineages\textsuperscript{124}. The data indicated that the primary cause was defective erythroblast enucleation. Subsequently, Maea was determined to be an adhesion molecule on both macrophages and erythroblasts that through homophillic interaction mediates attachment of the two cell types in erythroblastic islands, where macrophages promote enucleation\textsuperscript{124–126}. However, a recent study with young and conditional adult \textit{Maea} knockout has challenged this view\textsuperscript{127}. No perinatal lethality, anemia, or enucleation defect were observed in young adult \textit{Maea}\textsuperscript{Csf1r-Cre} mice. Instead, macrophage development, erythroblastic islands formation, and erythroblast maturation was impaired if \textit{Maea} was deleted specifically in the monocyte-macrophage lineage, but this phenotype was not observed if \textit{Maea} was deleted in the erythroid lineage. This suggested that Maea instead is critical in macrophages only.

WDR26 has also been linked to regulating red blood cell development. \textit{Wdr26} expression is upregulated in terminally differentiating erythroblasts (MEL cells induced with DMSO), possibly by Gata1, Tal1, CTCF transcription factors that have binding sites in the \textit{Wdr26} promoter\textsuperscript{108}. Knockdown of Wdr26 in mouse primary erythroblasts resulted in severe defects in enucleation, a reduction in hemoglobin production, and blocked differentiation at the basophilic erythroblast stage\textsuperscript{108}. Furthermore, Zebrafish Wdr26
knockouts exhibited profound anemia likely due to defective erythropoiesis, a phenotype shared by the initial study on *Maea* null mouse embryos\textsuperscript{108,124}; however, instead of involvement in erythroblastic island adhesion or macrophage differentiation, a different mechanism failure was determined: Wdr26, and the CTLH complex, polyubiquitinate lamin B (and possibly other nuclear proteins) leading to its degradation, which facilitates transient nuclear opening and nuclear condensation, a prerequisite to enucleation. *In vitro* ubiquitin assays with Wdr26 pulldown (and therefore the CTLH complex) demonstrated it can ubiquitinate Lamin B2 in the presence of UBE2H. Knockout of Ranbp10, Rmnd5a and Twa1 in MEL cells, like Wdr26, also resulted in increased nuclear size\textsuperscript{108}.

RanBP10 has also been linked to blood cell homeostasis. *RanBP10*\textsuperscript{-/-} mice are viable and have no obvious phenotype, but did have defective hemostasis, platelet activation and aggregation, and impaired thrombus formation\textsuperscript{128,129}. Slight decreases in erythrocyte numbers and size were observed\textsuperscript{128} – an anemic-like phenotype shared by *Wdr26*\textsuperscript{-/-} zebrafish and the first report on *Maea*\textsuperscript{-/-} mice\textsuperscript{108,124}. The tubulin-microtubule equilibrium is altered in *RanBP10*\textsuperscript{-/-} mice, which also show an increased level of β1-tubulin protein levels\textsuperscript{129}. Additionally, *GID4* was identified as a novel gene required for hematopoietic stem/progenitor cell specification\textsuperscript{130}. Thus, overall, there is a clear importance of CTLH complex subunits in different aspects of the hematopoietic system. So far, however, the ubiquitination of lamin B is the only direct link with CTLH complex E3 ligase activity.

**1.2.5.2 Cell migration and adhesion**
RanBPM in particular has been linked with several cell migration and adhesion pathways. Reports have shown RanBPM association with various integrin, junctional, receptor, and signaling proteins such as β1- and β2-integrins; adhesion molecule L1CAM and PlexinA1 receptor, and co-localization with ZO-1 and β-catenin in the developing rat neocortex (reviewed in Salemi et al., 2017). Depletion of RanBPM increased HT22 and NIH3T3 cell attachment by disrupting focal adhesion signaling, and breast cancer cell invasiveness by regulating BLT2-mediated reactive oxygen species generation and IL-8 production.

Thus far, the only direct implication involving the entire complex in cell migration is a regulation of histone deacetylase 6 (HDAC6) activity, which mediates the increased cell migration observed in RanBPM-deficient HEK293 cells. Cells depleted of RanBPM, muskelin, and RMND5A showed increased HDAC6 activity and/or increased deacetylation of HDAC6 target α-tubulin, but no change in HDAC6 protein levels, while RanBPM, MAEA, and TWA1 were shown to be colocalized at microtubules with HDAC6; however, in this context ubiquitination was not investigated so the regulatory mechanism of HDAC6 by the CTLH complex remains unclear. Ubiquitination of HDAC6 that alters its activity or ubiquitination of an HDAC6 coregulator are two possible mechanisms underlying HDAC6 regulation by the CTLH complex.

Muskelin was initially identified in a screen for proteins that promoted adherence to thrombospondin-1 substratum in a C2C12 mouse myoblast cell line. In rat lens epithelial cells and brain tissue, muskelin was found in a complex with myosin and p39 and colocalized with actin and stress fibers. In the lens cells, muskelin depletion
reduced Rho-GTP activation, myosin phosphorylation, dissociation of stress fibers, and cell migration\textsuperscript{109}. Muskelin and RanBPM depletion in lung A549 cells adherent on fibronectin caused enlarged cell perimeters and altered morphology and F-actin distribution\textsuperscript{95}. WDR26 has been linked with cell migration in multiple cell types, but with opposing effects observed. In leukocytes treated with chemokines, WDR26 translocated to the plasma membrane where it interacted and colocalized with Gβγ and PLCβ2 at F-actin, and enhanced Gβγ activation of PLCβ2\textsuperscript{138}. In this context, WDR26 was required for SDF1α-induced cell migration and Akt T473 phosphorylation. WDR26 complex formation with Gβγ, AKT2 and PI3Kβ promoted PI3K/Akt signaling-mediated migration and invasiveness of aggressive MDA-MB-231 breast cancer cells\textsuperscript{139}. However, an opposite effect of WDR26 on cell migration was shown in intestinal epithelial cells, where it was found to associate with Formyl peptide receptor 1 (FPR1) and inhibit FPR1-Mediated Cell Migration and Wound Healing\textsuperscript{140}.

### 1.2.5.3 Nuclear functions

Nuclear-specific functions of complex members have been limited in comparison to their effects and interactions in non-nuclear regions, although the CTLH complex is implicated in the nuclear condensation of developing erythroblasts via direct polyubiquitination of lamin B, as mentioned above\textsuperscript{108}. Other nuclear functions such as transcriptional regulation or DNA repair are likely since at least two complex members have been found together in the interactomes of transcription factor SOX2\textsuperscript{141}, cohesin\textsuperscript{142}, nuclear NOTCH1\textsuperscript{143}, transcriptional repressor NFX1\textsuperscript{144}, bromodomain-containing protein BRD1\textsuperscript{145}, and the BRCA1/BARD1 DNA repair dimer\textsuperscript{146}, among others.
A link with the complex and epigenetic regulation was made with early studies on UBE2H that showed it is capable of binding and ubiquitinating histone H2A \textit{in vitro} in an E3-independent manner\textsuperscript{94,147}. Similar to CDC34, UBE2H has an acidic C-terminal extension that is responsible for histone binding\textsuperscript{147}. UBE2H has also been used as an E2 for RAG1 \textit{in vitro} ubiquitation of histone H3\textsuperscript{148}. Interestingly, H2A ubiquitination is decreased in MEL cells lacking WDR26\textsuperscript{108}. Taken together, these findings suggest a role for the complex in histone ubiquitination.

RanBPM has several links to transcriptional regulation, and microarray analysis of RanBPM Hela and HCT116 knockdown cells indicate numerous effects on gene expression\textsuperscript{149}. However, a clear mechanism is lacking. RanBPM has been reported to directly interact with histone acetyltransferase KAT5 (aka Tip60)\textsuperscript{150} and transcriptional coregulator HIPK2\textsuperscript{151}, and shown to be associated and co-localized in the nucleus of neural precursors with TAF4, a subunit of the TFIID transcription initiation complex\textsuperscript{152}. Importantly, RanBPM and/or RanBP10 interaction with steroid and hormone nuclear receptors, such as androgen receptor and glucocorticoid receptor, has been observed, and both have been shown to act as transcriptional co-activators for these proteins\textsuperscript{153–155}. RanBPM also interacted and enhanced transcriptional activities of Epstein–Barr virus (EBV) proteins Rta and Zta and was present on Zta-responsive elements on EBV gene promoters\textsuperscript{86,156}. Sumoylation of the viral transcription factors by Ube9 was regulated by RanBPM, which affected their transcriptional activity\textsuperscript{86,156}. Thus far, that is the only established direct mechanism for any complex member on transcriptional regulation.

1.2.5.4 \textit{Cell proliferation, death, and survival pathways}
Pro and anti-proliferative functions have been documented for individual complex members with regulation of the major signaling pathways, particularly MAPK and WNT, via a variety of interacting proteins. Studies implicating the entire complex have also been conducted. Lampert et al., 2018 described decreased cell proliferation in newly generated WDR26 and MAEA KO retinal pigment epithelium cells manifested by downregulation of cell cycle markers, which then adapted to be indistinguishable from control cells after several days of culturing. This effect on cell proliferation in the newly generated knockouts was attributed to a CTLH complex-dependent regulation of protein stability of HBP1, a transcription factor that regulates expression of cell cycle regulators. In an in vitro ubiquitination assay with UBE2H as the E2, HBP1 was ubiquitinated by the recombinant CTLH complex. This confirmed that HBP1 is a direct ubiquitination target of the complex, the first one that was definitively identified. Increased cell growth opposite to that of Lampert et al., 2018, was observed in RMND5A KO HEK293 cells and RanBPM-depleted HEK293 cells and mouse embryonic fibroblasts. Furthermore, downregulation of RanBPM promoted tumour formation in a mouse xenograft model. In these contexts, the regulation of c-Raf kinase protein levels and downstream activation of MEK1/2 and ERK1/2 phosphorylation is believed to have a contributory role to the phenotype. c-Raf was shown to undergo RMND5A-dependent ubiquitination, but whether this involves direct ubiquitination by the CTLH complex or another E3 ligase is not known.

In a recent study, RMND5A KO NIH-3T3 cells showed increased autophagic flux linked to reduced mTOR activity. This regulation was reported to occur through
RMND5A-dependent K48 polyubiquitination of AMPK, which affected proteasomal degradation of phosphorylated AMPK after cellular adaptation to at least 6 hours of starving conditions (i.e., no serum or glucose in media). Muskelin and RanBP10 were present in AMPK immunoprecipitations in RMND5A KO cells, although whether this effect on AMPK is due to direct ubiquitination by the complex was not clearly established. This is so far the only connection of a mammalian complex member with the AMPK/mTOR pathway. WDR26 has been linked to autophagy but with the opposite effect. In H9c2 cells (rat cardiomyoblasts), WDR26 was shown to promote hypoxia-induced autophagy by increasing Parkin translocation at mitochondria and increasing general ubiquitination of mitochondrial proteins.\(^{159}\)

Several connections of the complex with the WNT pathway have been established, although with inconsistent findings. A recent report claimed RMND5A-MAEA can directly ubiquitinate β-catenin, yet no in vitro ubiquitin assay or binding assay was conducted.\(^{160}\) The same group previously published that WDR26 associated with Axin, but not with β-catenin.\(^{121}\) Depletion of WDR26 increased β-catenin stability in X. laevis and in WNT-stimulated HEK293 cells independently of GSK3β, and regulated β-catenin ubiquitination if co-expressed with Axin. Interestingly, the entire complex was found in the Axin interactome.\(^{161}\) One report, though, demonstrated that TWA1 expression caused sustained activation of the pathway by acting as a β-catenin nuclear retention factor.\(^{162}\)

Some complex members have been linked to the activation of apoptosis in response to cellular stress. In response to IR, RanBPM has been reported to be
phosphorylated in an ATM-dependent manner and initially predominantly nuclear immediately after IR treatment, but then increasingly cytoplasmic as treatment is prolonged\textsuperscript{107,113}. At 72 hours of IR treatment, RanBPM is recruited to perinuclear aggresomes, colocalizing with Ub, dynein, and HDAC6\textsuperscript{102}. Studies in lung cancer cells showed that RanBPM is essential for DNA damage response activation, homologous recombination DNA repair, and sensitivity to genotoxic stressors such as IR and cisplatin treatment\textsuperscript{107,163}. In Ranbp9 germ cell KO testes, enhanced apoptosis of spermatocytes and defective DNA repair is also observed\textsuperscript{120}. On the other hand, RanBPM has been shown to be pro-apoptotic in a variety of cell lines via activation of the intrinsic pathway but as well as through other means, specifically involving regulation of MAPK pathway, aggresome formation, activation of cofilin, and interactions with p73 and TSSC3\textsuperscript{87,102,113,157,164,165}. In keratinocytes, ARMC8 expression had a subtle positive effect on apoptosis induction in response to ultraviolet B radiation\textsuperscript{166}. Meanwhile, WDR26 inhibited oxidative stress-induced cell death in SH-SY5Y cells and cardiomyocytes\textsuperscript{103,167}.

1.2.5.5 \textbf{Functions and disease implications in the central nervous system}

Besides the roles in development of the nervous system mentioned in section 1.2.5.1, most complex members have important functions in the brain and/or have genetic or expression changes in a variety of human neurodevelopmental or mood disorders and/or neurodegenerative diseases. Duplication of a genomic region containing the RMND5A gene was found in a unique case of giant occipitoparietal meningoencephalocele\textsuperscript{168}. WDR26 mutations in LisH, CTLH, and WD40 domains that affect protein stability are observed in a unique neurodevelopmental disorder, termed
Skraban-Deardorff syndrome, which is associated with intellectual impairment, seizures, and other characteristics\textsuperscript{169}. TWA1 is candidate gene contributing to Ring chromosome 20, a rare syndrome characterized by intellectual disability and epilepsy\textsuperscript{170}. Finally, UBE2H mutations are associated with autistic disorder\textsuperscript{171}.

In the rodent brain, muskelin is expressed throughout the central nervous system (highest in hippocampus and cerebellum), is present in the nucleus, and enriched in fractions containing plasma membrane, vesicles, and large protein complexes\textsuperscript{172}. While there were no gross histological abnormalities in the brain of muskelin KO mice, there was membrane accumulation of GABA\textsubscript{A} receptor α1 and altered hippocampal network oscillation\textsuperscript{105}. Muskelin was shown to directly interact with GABA\textsubscript{A} receptor, and muskelin expression mediated GABA\textsubscript{A} receptor trafficking, endocytosis, and lysosomal degradation\textsuperscript{105}. Interestingly, a muskelin polymorphism was associated with early onset bipolar disorder, and it was hypothesized that deregulation of GABAergic signaling as a result of muskelin mutation could be an underlying factor\textsuperscript{173}. A fascinating link with muskelin and prion disease has also been made. Muskelin was found in a complex with cellular prion protein (PrP\textsuperscript{C}), APP, KIF5C, and dynein, which was found to undergo cotransport as vesicles in neurons\textsuperscript{174}. In this context, muskelin expression dictated PrP\textsuperscript{C} targeting to the lysosome for degradation versus being released in exosomes. In line with this function, muskelin KO challenged with pathogenic prions experienced accelerated prion disease progression compared to wild-type (WT)\textsuperscript{174}.

Several interacting partners and functions of RanBPM in the brain have been described, particularly in the context of Alzheimer’s Disease (AD). Like muskelin,
RanBPM was shown associated with APP\textsuperscript{174,175}. RanBPM also was shown to interact with BACE1 and RanBPM expression elevated processing of APP; endocytosis of APP, LRP, and β1-intergrin; and Aβ generation, resulting in increased deposition of amyloid plaques (a hallmark of AD) in RanBPM overexpressing mice\textsuperscript{135,175,176}. RanBPM overexpression also contributed to AD pathogenesis independently of Aβ generation through activation of apoptosis via increased SSH1-mediated dephosphorylation of coflin\textsuperscript{164,177–179}. Interestingly, a fragment of RanBPM (residues 1-392), which encompasses the proline and glutamine rich N-terminal region, and the SPRY and LisH domains, is increased in AD brains and when overexpressed enhanced APP processing and Aβ generation even greater than full length RanBPM\textsuperscript{180}. RanBPM overexpression may also contribute to AD progression by stabilizing Tau protein through interaction with Hsp90/Hsc70\textsuperscript{181}.

1.2.5.6 Immune system

There are some reports of roles of CTLH complex members in immunology, although nothing linking the entire complex. A compelling study discovered that RanBPM is part of a complex with AXL and LRP-1 that facilitates dendritic cell efferocytosis and antigen cross-presentation to T cells\textsuperscript{182}. RanBPM was also shown to interact with TRAF6 and suppress TRAF6 activation of NF-κB signaling\textsuperscript{85}. Finally, the cell surface CD39 protein, which plays a major role in limiting immune responses by hydrolyzing extracellular ATP, interacts with RanBPM and its hydrolyzing activity is inhibited by co-expression with RanBPM\textsuperscript{183}. 
A connection with viruses is suggested by the presence of the entire CTLH complex in the interactomes of viral proteins from Severe acute respiratory syndrome coronavirus 1\textsuperscript{184,185} and Kaposi’s sarcoma-associated herpesvirus\textsuperscript{186}. Additionally, WDR26 was found in the interactome of pUL26 kinase from β-herpesvirus human cytomegalovirus\textsuperscript{187}. As mentioned previously, RanBPM expression regulated transcriptional activities of Rta and Zta transcription factors from EBV and enhanced EBV lytic development\textsuperscript{86,156}. In a similar vein, RanBP10 was found interacting with ICP0 form herpes simplex virus 1 (HSV-1) and was required for HSV-1 replication, cell-cell spread, and viral gene expression by regulation of chromatin remodeling (specifically, regulation of the amount of Histone H3 in HSV-1 promoters)\textsuperscript{188}. Overall, these studies provide evidence that the CTLH complex is implicated in immune and viral regulations.

1.2.5.7 Regulation of protein stability not (yet) linked to CTLH complex E3 ligase activity

Several reports focused on individual subunits have found roles for CTLH complex members in either reducing or more frequently promoting protein stability of interacting partners. These reports identify possible CTLH complex ubiquitination substrates not considered at the time as well as links with protein chaperoning and deubiquitination. The latter is suggested by the identification of USP11 deubiquitinase association with RanBPM and RMND5A\textsuperscript{34,189}. Expression of RanBPM, which itself is a substrate of ubiquitin specific peptidase 11 (USP11), stabilized Mgl1 by promoting USP11 deubiquitination of K48 Ub chains on Mgl1, thus preventing its proteasomal degradation\textsuperscript{189,190}. RanBPM expression also stabilized p73 (by inhibition of its
ubiquitination)\textsuperscript{81}, TSSC3\textsuperscript{87}, and c-Kit\textsuperscript{88}, although whether these effects involve USP11 or any other complex member was not investigated. Similarly, Exportin-5 stability was reported to be enhanced by RMND5A and RanBPM interaction via inhibition of its ubiquitination\textsuperscript{191}. Interestingly, it was shown that RanBPM directly interacts with Hsp90 and Hsc70 and RanBPM expression enhanced their ATPase activities, which stabilized Tau protein levels\textsuperscript{181}. Finally, ARMC8 was found associated with α-catenin and ARMC8 expression promoted α-catenin proteasomal degradation though this is apparently independent of ubiquitination\textsuperscript{192}.

In summary, the CTLH complex E3 ligase activity or individual subunits have been implicated in several biological pathways, processes, or disease mechanisms (Figure 1.7). In a few recent reports, the ubiquitination of a substrate has been linked with function (e.g., lamin B, HBP1, c-Raf, AMPK). Yet, much remains to be elucidated about the functions and mechanisms of the CTLH complex.
Figure 1.7. Current knowledge of pathways and functions linked to the CTLH complex. See text (section 2.5) for details. Ub’n: ubiquitination. In bold are pathways and regulations that linked RanBPM to cancer development. These findings demonstrated RanBPM’s tumour suppressive effects which served as a motivation for this thesis.

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1.3 **Scope of thesis**

At the start of this thesis project, besides reports on individual subunits, research specifically focused on the mammalian CTLH complex was limited. Its formal characterization was in 2007\textsuperscript{32}, identifying the components RanBPM, TWA1, muskelin, ARMC8 α and β, RMND5A, and MAEA (later expanded to include WDR26, GID4, RMND5B and RanBP10). In 2013, phylogenetic analysis demonstrating the conservation of CTLH subunits with yeast Gid proteins, including the RING domains of RMND5A and MAEA, suggested that the human CTLH complex, like the yeast Gid complex, could function as an E3 ligase\textsuperscript{51}; however, proof that the human complex has intrinsic E3 ligase activity was waiting. Multi-subunit E3 ligases, such as the CRL and APC/C complexes, have been implicated in the regulation of numerous signaling pathways owing to its modularity and substrate binding capacity. In contrast to those well-studied E3 ligase complexes, our understanding of the CTLH complex structure and function is in the infancy.

RanBPM has been the most studied CTLH complex member. It has been implicated in a vast array of cellular functions and shown to regulate numerous proteins and pathways (see section 2.5 and review by Salemi *et al.*, 2017\textsuperscript{133}). In the context of cancer development, RanBPM has tumour suppressive properties (bolded in figure 1.7)\textsuperscript{113,134,157,158,193}. In all the reports on RanBPM function at the start of this thesis, the exact mechanism of action was unknown besides being thought to act as an adaptor or scaffold protein.
1.4 Hypothesis & objectives

With increasing knowledge of the yeast Gid complex as a multi-subunit E3 ligase, the conservation of the yeast and mammalian subunits being described, and RanBPM functions in cancer development, this thesis project was designed to address fundamental characterizations of the CTLH complex composition, structure, activity, and function. Based on its conversation with the yeast Gid complex\textsuperscript{52} and the role of RanBPM in cancer development through an unknown mechanism\textsuperscript{134}, I hypothesized that the human CTLH complex has E3 ligase activity that regulates cancer-related pathways via ubiquitination. After first developing and optimizing reagents and tools to study this little characterized complex (e.g., antibodies and knockout cell lines), I then addressed this hypothesis with the following objectives:

1. Demonstrate E3 ligase activity of the human CTLH complex (Chapter 2) – published in Scientific Reports. In this chapter, the first part of the hypothesis was addressed. Human CTLH complex E3 ligase activity was demonstrated and its E2 selectivity and lysine polyUb linkage generation was characterized. Additionally, the composition, stability requirements, and subcellular localization of the complex was assessed. Finally, it was revealed that muskelin protein levels and ubiquitination are regulated by the complex. Overall, this chapter provided an essential first step by confirming and characterizing the human CTLH complex’s E3 ligase activity, which served as a foundation to probe the structure and function of the complex in more detail for chapters 3 and 4.
2. **Characterize/confirm CTLH complex structure and topology (Chapter 3)** – *manuscript in preparation*. We next explored the complex ubiquitination activity and architecture in more detail to better understand its mechanism of ubiquitin transfer. First, the effect of mutations in RMND5A’s RING domain on muskelin protein levels were assessed as a read out of its ubiquitin activity. This identified residues either critical for zinc coordination in the RING domain or ones that mediate E2 binding. Additionally, the initial model of complex topology was clarified using immunoprecipitations in knockout cell lines. Finally, cross-linking mass spectrometry of the RanBPM immunocomplex revealed which parts of the complex are in proximity. In combination with new insights on the Gid complex structure, these experiments revealed an updated model of the CTLH complex architecture that positioned the multiple protein-protein interaction surfaces near the RING-E2 catalytic module. This chapter provided important insights into the complex’s E3 ligase activity which further substantiates it as an E3 ligase, again confirming the hypothesis that complex is an E3 ligase. Furthermore, it helps define the architecture and utility of the protein interaction surfaces on the complex. This provides important opportunities to explore substrate engagement that specify function of the complex, enabling mechanistic investigation of the hypothesis that the complex regulates pathways via ubiquitination.

3. **Identify novel functions and ubiquitination substrates of the CTLH complex in human cancer cells (Chapter 4)** – *manuscript submitted*. With the E3 ligase activity confirmed, we turned to mass spectrometry-based proteomics to identify
ubiquitination substrates of the complex as way to reveal novel functions that may link to RanBPM’s regulation of cancer development. High-throughput analysis was done in multiple complex subunit depleted cells that assessed protein and ubiquitination levels. This revealed that the complex regulates ubiquitination of multiple glycolysis enzymes and validation experiments further demonstrated that the CTLH complex inhibits the activity of these enzymes and glycolysis. Since glycolysis is a pathway frequently upregulated in cancer cells which is believed to be key for their survival\textsuperscript{194}, the ubiquitination and inhibition of glycolysis by the CTLH complex may be one of the mechanisms explaining how loss of RanBPM promotes tumorigenesis. In addition to work that came out during this thesis (CTLH complex regulation of c-Raf, HDAC6, and β-catenin\textsuperscript{100,121,158}), this chapter provides another example of the role of ubiquitination of specific substrates by the CTLH complex in the regulation of cancer-related pathways. With these new functions and targets discovered, exact mechanisms of substrate engagement can be envisioned and tested with the updated architecture model of the complex presented in chapter 3.

1.5 References

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Chapter 2

2 The mammalian CTLH complex is an E3 ubiquitin ligase that targets its subunit muskelin for degradation

A version of this chapter has been published:


2.1 Introduction

Ubiquitination, the addition of the 76 amino acid protein ubiquitin to lysine residues on target proteins, modifies protein function or turnover and regulates a wide spectrum of biological processes in development and disease\textsuperscript{1,2}. The three-step mechanism is initiated by ATP-dependent activation of ubiquitin by E1 enzymes, conjugation to E2 enzymes and covalent attachment to a substrate recruited by an E3 ligase\textsuperscript{3}. Subsequent elongation of ubiquitin molecules forms poly-ubiquitin chains, linked through one of its seven lysine residues or N-terminal methionine. The type of linkage dictates the fate and function of the ubiquitinated protein\textsuperscript{2}. For example, poly-ubiquitinated chains linked through lysine 11 (K11) or 48 (K48) are known to target proteins for proteasomal degradation, while lysine 63 (K63) linked chains can trigger lysosomal degradation, endocytosis, regulate protein trafficking, or alter protein interactions\textsuperscript{2}.

E3 ligases with Really Interesting New Gene (RING) finger domains mediate direct transfer of ubiquitin to the substrate from the E2\textsuperscript{2,3}. The RING domains coordinate
two Zn$^{2+}$ ions in a cross braced arrangement critical for its structure, or without Zn$^{2+}$ coordination in the case of the U-box family of E3 ligases in which case polar and charged residues substitute for the Zn$^{2+}$ ions$^4$. Approximately 600 RING finger E3 ligases exist in the human genome, underlying their importance in cell signaling regulation and the diversity of their substrates. RING E3 ligases exist as monomers, homodimers, heterodimers, or in multi-subunit complexes, such as the Cullin RING ligases, which contain subunits that function either as a scaffold, adaptor, or substrate recognition element, in addition to the RING subunit$^5$.

The glucose-induced degradation deficient (Gid) complex is a multi-subunit E3 ligase in Saccharomyces cerevisiae. Upon replenishment of glucose to starved yeast cells, the Gid complex ubiquitinates gluconeogenic enzymes, such as fructose-1,6-bisphosphatase (Fbp1), phosphoenolpyruvate carboxykinase (Pck1) and malate dehydrogenase (Mdh2), leading to their proteasomal degradation$^7$.$^8$. These substrates are targeted by the complex via recognition of their N-terminal proline residues by Gid4 in the complex by a process termed the Pro/N-end rule pathway$^8$.$^9$. Gid4 is rapidly synthesized after glucose addition and associates with the Gid complex, thus triggering the ubiquitination of the gluconeogenic enzymes and allowing the cells to switch from gluconeogenesis to glycolysis$^7$.

In the Gid complex, Gid2 and Gid9 both contain conserved RING finger domains and form a heterodimer$^7$.$^{10}$.$^{11}$. Ubiquitin ligase activity has been demonstrated in vitro for yeast Gid2$^7$ and its Xenopus laevis homologue$^{12}$. For Gid9, mutation of a cysteine residue in its RING domain abrogates ubiquitination and degradation of the gluconeogenic
targets of the Gid complex, although activity of Gid9 in \textit{in vitro} assays could not be demonstrated\textsuperscript{11}.

The C-terminal to LisH (CTLH) complex is the mammalian homologue of the Gid complex\textsuperscript{10}. It was originally identified by analysis of Ran binding protein M (RanBPM, also known as RanBP9) associated proteins in a high molecular weight fraction of HEK293 extracts, consisting of muskelin, Two Hybrid-Associated Protein 1 (RanBPM, TWA1, also known as GID8), Armadillo Repeat Containing 8 (ARMC8) isoforms α and β and two subunits containing RING domains, Macrophage Erythroblast Attacher (MAEA, also known as EMP; homologue of Gid9) and Required For Meiotic Nuclear Division 5 Homolog A (RMND5A; homologue of Gid2)\textsuperscript{10,13,14}. The CTLH complex was named following the observation that five of the complex members contain LIS1-homology motif (LisH) and CTLH domains\textsuperscript{10,13}.

The most well-studied complex member is RanBPM, a 90 kDa ubiquitously expressed, nucleocytoplasmic and evolutionary conserved protein implicated in a variety of cellular functions\textsuperscript{15}. RanBPM is a regulator of multiple signaling pathways, including the ERK pathway, Transforming growth factor-β (TGF-β), histone deacetylase 6 (HDAC6) activity and the Ataxia Telangiectasia Mutated (ATM)-dependent DNA damage response\textsuperscript{15–17}. Other CTLH subunits, such as muskelin, have been implicated in intracellular protein trafficking, microtubule dynamics and cell adhesion, whereas MAEA has been found to regulate erythroblast and macrophage maturation\textsuperscript{18–21}. Few studies so far have evaluated the biological function of the complex in mammalian cells\textsuperscript{15,22,23}. While most subunits of the complex are conserved within eukaryotic lineages and the
RING domains in MAEA and RMND5A share high levels of conservation with their yeast counterparts\textsuperscript{10}, the composition, activity and function of the complex remain to be characterized.

In this study, we further the characterization of the CTLH complex and investigate its E3 ubiquitin ligase activity in human cells. We find that WD repeat-containing protein 26 (WDR26) and GID4 are components of the CTLH complex and that complex members are differentially distributed within nuclear and cytoplasmic compartments. Through analysis of knockout cell lines of CTLH subunits, we determine that the stability of several complex members is interdependent and that, in particular, RanBPM and TWA1 are critical for complex stability. We show that the complex has E3 ligase activity and that this is dependent on both RMND5A and MAEA. Furthermore, we characterize the E2 pairings of the complex and ubiquitin linkage. Finally, we determine that the stability and ubiquitination of CTLH complex member muskelin is regulated by the complex, suggesting that this may be part of an autoregulatory mechanism.

\section*{2.2 Results}

\subsection*{2.2.1 WDR26 and GID4 are CTLH complex members}

The initial characterization of the CTLH complex determined that it was composed of 6 subunits, RanBPM, TWA1, muskelin, ARMC8 and the RING domain proteins RMND5A and MAEA (Figure 2.1a)\textsuperscript{13,14}. We confirmed the composition of the complex by immunoprecipitation of RanBPM in HEK293 cells and found that CTLH complex members remain associated with RanBPM even under stringent conditions (tested up to 0.5% NP40 and 0.2% triton; Figure 2.1b, Figure 2.2). WD Repeat Domain
**Figure 2.1. Characterization of the CTLH complex.** (a) Schematic representation of the CTLH complex. The model is adapted from the yeast Gid complex (Menssen et al., 2012). Note that the position of muskelin in the complex has not been formally defined. (b) Subunits of the CTLH complex are present in RanBPM immunocomplexes. HEK293 whole cell extracts were incubated with a RanBPM antibody and immunoprecipitated. Immunoprecipitates were analyzed by Western blot with the indicated antibodies. IgG was used as a negative control. (c) WDR26 associates with the CTLH complex. Whole cell extracts were prepared from HeLa cells untransfected (-) or transfected with FLAG-tagged WDR26 (+). FLAG-WDR26 was immunoprecipitated with a FLAG antibody and immunoprecipitates were analyzed by Western blot with the indicated antibodies. (d) GID4 associates with CTLH complex. Whole cell extracts were prepared from HEK293 cells untransfected (-) or transfected with HA tagged GID4 (+). HA-GID4 was immunoprecipitated with an HA antibody and immunoprecipitates were analyzed by Western blot with the indicated antibodies.
Figure 2.2. MAEA and Muskelin co-immunoprecipitate with RanBPM in stringent binding conditions. HEK293 whole cell extracts were adjusted to the indicated final detergent concentrations and then incubated with a RanBPM antibody and immunoprecipitated. Immunoprecipitates were analyzed by Western blot with the indicated antibodies. IgG was used as a negative control.
26 (WDR26) and human GID4 (also known as c17orf39), the homologues of the yeast Gid complex members Gid7 and Gid4, respectively, were not detected in the initial identification of the complex. In contrast, we found that endogenous WDR26 associates with RanBPM (Figure 2.1b) and that CTLH complex members co-immunoprecipitate with exogenously expressed WDR26 and GID4 (Figure 2.1c, d), consistent with recent interactome studies that revealed the human proteins do associate with the complex. Taken together, this work shows that WDR26 and GID4 are CTLH complex subunits.

To compare the subcellular localizations of all complex members, we transfected HA or FLAG tagged constructs of RanBPM, TWA1, ARMC8, RMND5A, MAEA, muskelin, WDR26 and GID4 in HeLa cells (Figure 2.3a). Consistent with a previous report, RanBPM, TWA1, ARMC8 and RMND5A showed nucleocytoplasmic distribution, with a nuclear predominance, while muskelin appeared mostly cytoplasmic and MAEA nearly exclusively nuclear (Figure 2.3b). Interestingly, GID4 displayed a near exclusive nuclear staining and WDR26 was primarily cytoplasmic (Figure 2.3a, b). The differing subcellular localization of CTLH complex members suggests the possibility that several complexes of varying composition may co-exist in the nucleus and cytoplasm.

2.2.2 Interdependence of CTLH complex subunit stability

The RanBPM and TWA1 yeast homologues Gid1 and Gid8, respectively, form the core of the Gid complex with MAEA and RMND5A homologues (Gid9 and Gid2), which form a heterodimer (Figure 2.1a). The remaining 3 subunits, Gid7, Gid5 and Gid4, the yeast homologues of WDR26, ARMC8 and GID4/C17orf39, respectively, were
Figure 2.3. Localization of CTLH complex subunits. (a) Representative images of transfected HA- or FLAG-tagged CTLH complex members in HeLa cells. Scale bar: 10 μm. Hela cells fixed 24h after transfection were incubated with an HA or FLAG antibody and then with an Alexa Fluor 488 secondary antibody. Nuclei were stained with DAPI. (b) Analysis of the subcellular localization of transfected CTLH complex members. Subcellular localization was scored as either, N>>C (completely nuclear), N>C (nuclear greater than cytoplasmic), N = C (nuclear equal to cytoplasmic), C>N (cytoplasmic greater than nuclear) or C>>N (completely cytoplasmic). Data represent averages from three separate experiments, each assessing approximately 50 cells. Error bars represent standard deviation (SD).
predicted to be located on the periphery of the complex\textsuperscript{15,26}. If this topology is similar in the CTLH complex, RanBPM and TWA1 would be expected to be critical for complex stability. To determine how CTLH complex subunit expression influences each other, we assessed the levels of CTLH individual subunits in stable shRNA knockdown or CRISPR knockout (KO) cell lines for six of the complex members. We found that depletion of RanBPM or TWA1 strongly affected each other’s protein levels as well as that of MAEA and RMND5A (Figure 2.4a, b). TWA1 knockout also had a surprising inhibitory effect on the stability of ARMC8β, while ARMC8α was not significantly changed (Figure 2.4b). Interestingly, the RING dimer partners MAEA and RMND5A appeared to require each other for stability as knockout of each one individually significantly reduced the protein levels of the other (Figure 2.4c, d). No other prominent change was seen in RMND5A and MAEA KO cell lines, except that both showed a significantly higher amount of muskelin (Figure 2.4c, d). This was also observed in RanBPM shRNA cells, albeit to a lesser extent potentially owing to the partial downregulation of RanBPM in these cells (Figure 2.4a). Knockout of ARMC8 resulted in downregulation of MAEA, RMND5A and TWA1 (Figure 2.4e), suggesting that TWA1, MAEA/RMND5A and ARMC8 influence each other’s stability. Finally, the knockout of muskelin only had subtle effects, if any, on protein levels of other complex members (Figure 2.4f). To determine whether these changes occurred at the mRNA level, we performed RT-qPCR analyses to evaluate whether the KO of individual subunits had an effect on the transcriptional regulation of other CTLH complex members. We did not detect any change in mRNA expression for most of the subunits tested, except for a small reduction
Figure 2.4. RanBPM and TWA1 are essential for complex stability. Whole cell extracts prepared from control shRNA and RanBPM shRNA HEK293 cells (a), or from control (labelled as C), TWA1, RMND5A, MAEA, ARMC8 and muskelin HEK293 CRISPR knockout cells (b-f) were analyzed by Western blot with antibodies to CTLH complex members, as indicated. Vinculin was used as a loading control. Quantifications are shown below each blot and protein levels are shown relative to control cells set to 1 and normalized to Vinculin levels. Data represent averages from three separate experiments, with error bars indicating SD. P<0.05 (*), P<0.01 (**), P<0.001 (***) , P<0.0001 (****).
Figure 2.5. Analysis of mRNA levels of proteins that change in figure 2.4. Fold changes shown are relative to control cells, and normalized to GAPDH levels. Details described under “Methods”. n=3, *p<0.05, **p<0.01.
of muskelin and MAEA mRNA in RMND5A KO cells, and a slight decrease for RMND5A mRNA in ARMC8 KO cells (Figure 2.5). In all cases, these changes were much smaller than the effects observed at the protein level (or even opposite in the case of muskelin) and therefore unlikely to account for the full extent of the effect observed at the protein level. Altogether, this substantiates that the knockout of individual complex members affects the stability of other complex members mostly at the protein level.

Finally, we used a combination of transient siRNA knockdown and subunit re-expression in knockout cell lines to confirm that these changes were not due to off-targets effects. We confirmed that siRNA downregulation of TWA1, muskelin and RMND5A recapitulated the changes observed in TWA1, muskelin and RMND5A CRISPR KO cells (Figure 2.6). Similarly, RanBPM KO cells showed similar changes in CTLH subunits as the RanBPM shRNA cells and transient re-introduction of Flag-MAEA in MAEA KO cells restored the expression of RanBPM, ARMC8 and muskelin close to the levels observed in WT cells (Figure 2.6).

2.2.3 Characterization of the CTLH complex E3 ligase activity

To determine whether the mammalian CTLH complex has E3 ligase activity, we conducted in vitro ubiquitination assays with the CTLH complex immunoprecipitated from HEK293 cells via a RanBPM antibody (using the stringent conditions identified in Figure 2.1b). For these assays, we supplemented the reactions with the E2 enzyme UBE2D2 (UbcH5b) because it paired with the yeast RMND5A counterpart (Gid2) in in vitro assays and was also identified as an interacting partner for human RMND5B, a paralog of RMND5A, in a large protein interaction screen. Ubiquitination products
Figure 2.6. Confirmation of changes of CTLH member protein expression in knockout cells or RanBPM shRNA cells by alternative knockdown/knockout strategies or rescue experiments. Related to figure 2.4. Western blot analyses of whole cells extracts of: a) HEK293 RanBPM knockout cells. b) HEK293 cells transiently transfected with control or TWA1 siRNA. c) HEK293 cells transiently transfected with control or RMND5A siRNA. d) HEK293 cells transiently transfected with control or muskelin siRNA. e) HEK293 MAEA knockout cells untransfected or transiently transfected with FLAG-MAEA (as indicated at the top).
were observed when the CTLH complex was immunoprecipitated from wild-type HEK293 cells, but not in RMND5A knockout HEK293 cells (Figure 2.7a). As the complex is intact in the RMND5A KO cells (save for RMND5A, Figure 2.8), this result demonstrates that RanBPM immunocomplexes have E3 ligase activity and that it is dependent on RMND5A, a RING domain CTLH complex subunit. To understand the contributions of MAEA to the E3 ligase activity of the CTLH complex, we conducted \textit{in vitro} ubiquitination assays with the RanBPM immunocomplexes in control and MAEA knockout HEK293 cells. As anticipated, limited E3 ligase activity was observed in the MAEA KO cells (Figure 2.7b); however, consistent with the yeast Gid complex topology\textsuperscript{26}, co-IP of RMND5A was not detected in MAEA KO cells. Therefore, the loss of activity could be attributed to the absence of RMND5A.

To further characterize the E3 ligase activity of the RING domain subunits RMND5A and MAEA, we conducted \textit{in vitro} ubiquitination assays with bacterially expressed proteins. Initial experiments using purified GST-RMND5A and SUMO-MAEA failed to show any detectable activity (data not shown), possibly due to poor folding or insolubility of these enzymes. Therefore, we omitted the purification step and conducted E3 ligase assays using crude bacterial extracts as previously done to test the activity of Gid2 and Gid9\textsuperscript{7,11}. In these conditions, human GST-RMND5A exhibited weak, but observable substrate and self-ubiquitination activity (Figure 2.9a). Previous studies reported that the bacterially expressed yeast Gid9, the homologue of MAEA, had no detectable E3 ligase activity \textit{in vitro}\textsuperscript{11}. However, \textit{in vivo}, a cysteine
Figure 2.7. The CTLH complex has E3 ligase activity. (a) RanBPM immunocomplexes have E3 ligase activity that is dependent on RMND5A. Whole cell extracts prepared from wild type (WT; lanes 3-4) or RMND5A KO (lane 5) HEK293 cells were subjected to immunoprecipitation with a RanBPM antibody or an IgG control, as indicated. Immunoprecipitates were resuspended in an ubiquitination assay master mix and E2 enzyme (UBE2D2) was added (+) or omitted (-), prior to incubation at 37°C for 30 minutes. Reactions were run on SDS-PAGE gel and fluorescein-ubiquitin was imaged directly on the gel at 800 nm. Shown below is a Western blot analyzing RanBPM and RMND5A presence in the immunoprecipitates. Asterisk indicates a non-specific band. (b) MAEA is required for CTLH complex activity. RanBPM was immunoprecipitated from control or MAEA KO HEK293 cells and ubiquitination reactions were carried out as described above. The presence of RanBPM and MAEA in the immunoprecipitates was analyzed by Western blot. Asterisk indicates a non-specific band.
Figure 2.8. CTLH complex is not compromised in RMND5A KO cells. WT or RMND5A KO HEK293 whole cell extracts were incubated with a RanBPM antibody and immunoprecipitated. Immunoprecipitates were analyzed by Western blot with the indicated antibodies. IgG was used as a negative control.
Figure 2.9. Recombinant RMND5A and MAEA exhibit E3 ligase activity *in vitro*. (a) Bacterially expressed RMND5A exhibits E3 ligase activity. Bacterial extracts with induced expression of GST (lane 2) or GST-RMND5A (lane 6) were incubated with ATP and His-ubiquitin and with or without, as indicated, His-E1 and UBE2D2 (E2) for 2 hours at 37°C. Reactions were analyzed by Western blot using anti-His antibody (top), or an RMND5A antibody (bottom). The identity of the products is indicated on the right. (b) SUMO-MAEA has *in vitro* ligase activity. His-tagged SUMO MAEA was expressed and incubated in reaction as in (a). His-tagged SUMO-SLP2 was used as a negative control. Analysis was done by Western blot analyzed using an anti-His antibody (top), or a MAEA antibody (bottom).
mutation in the MAEA RING domain abolished Gid complex ubiquitination of FBPase, suggesting that the RING domain of Gid9 is required for the Gid complex activity. Surprisingly, a SUMO tagged recombinant version of human MAEA expressed in *Escherichia coli* exhibited some E3 ubiquitin ligase activity (Figure 2.9b), albeit weaker than that of RMND5A. Thus, both recombinant RMND5A and MAEA display E3 ligase activity *in vitro*.

### 2.2.4 Characterization of E2 pairings and lysine linkage

As E3 ligase ubiquitination activity is dependent on a specific E2 enzyme, we sought to determine which E2 enzymes function optimally with the CTLH complex. In a panel of 11 E2 enzymes, the GST-RMND5A fusion protein exhibited E3 ligase activity only when UBE2D1 (UbcH5a) or UBE2D2 (UbcH5b) are present in the reaction (Figure 2.10a), while RanBPM immunocomplexes were able to function with UBE2D1, UBE2D2 and UBE2D3 (Figure 2.10b). Interestingly, the complex or GST-RMND5A did not exhibit activity when paired with UBE2H (UbcH2) (Figure 2.10a and b), the human homologue of yeast Gid3 (also known as ubc8), which is the E2 required for the glucose-induced ubiquitination of FBPase. Similarly, no activity was detected with CDC34 (Fig. 2.10b), which has a C-terminal extension of acidic residues similar to that of Gid3 that is critical for its activity.

### 2.2.5 Muskelin is a target of the CTLH complex

Muskelin is the only member of the CTLH complex that does not have a yeast homologue in the Gid complex and therefore stands out as a unique difference between the two complexes. Interestingly, muskelin protein levels were significantly increased
Figure 2.10. Properties of the CTLH complex E3 ligase activity. (a) E2 enzymes that function with GST-RMND5A. GST-RMND5A ubiquitination assays were conducted as described in figure 5A with the indicated E2 enzymes. Uncropped image and GST empty vector reactions are shown in Supplementary Figure 5. (b) RanBPM immunocomplexes functionally couple with UBE2D1, UBE2D2 and UBE2D3 but not with UBE2H or CDC34. RanBPM was immunoprecipitated from HEK293 cell extracts, resuspended in ubiquitin assay master mix containing fluorescein-ubiquitin and the indicated E2 enzymes were added and incubated at 37°C for 30 minutes. Reactions were run on SDS-PAGE gel and fluorescein-ubiquitin was imaged directly on the gel using a LICOR imager at 800 nm. Asterisk indicates a non-specific band. (c) GST-RMND5A mediates K48 and K63 polyubiquitination. GST-RMND5A ubiquitination assay reactions (as in Fig. 2.9a) were LysC/trypsin digested and then subjected to analysis by liquid chromatography tandem Mass Spectrometry (LC-MS/MS). Shown is a representative replicate of the MS1 peak extraction for ubiquitin peptides containing GlyGly sites.
in both RMND5A and MAEA KO cells compared to control HeLa cells and this was reversed by the reintroduction of RMND5A and MAEA into their respective knockout cells by transient transfection (Figure 2.4c,d, Figure 2.5, and Figure 2.11a). Treatment with the proteasome inhibitor MG132 resulted in increased muskelin levels in control cells relative to DMSO control treatment, but not in RMND5A KO HeLa cells (Figure 2.11b), suggesting that the accumulation of muskelin in RMND5A KO cells is through the regulation of muskelin proteasome degradation. To confirm that muskelin protein stability was dependent on the CTLH complex, we performed cycloheximide (CHX) treatments to compare muskelin degradation of WT and RMND5A KO cells. Muskelin had a half-life of about 24 hours in WT cells, whereas no significant degradation was detected in RMND5A KO cells, even 36 hours following addition of CHX, suggesting that muskelin degradation is dependent on the CTLH complex activity (Figure 2.11c). This led us to suspect that muskelin could be a ubiquitination target of the complex leading to proteasomal degradation.

To evaluate if ubiquitination of muskelin is regulated by the CTLH complex, we co-transfected HA-ubiquitin and a construct encoding FLAG-muskelin in control or RMND5A KO HEK293 cells, followed by treatment with MG132. FLAG pulldown of transfected muskelin and hybridization with an HA antibody showed a poly-ubiquitination pattern in control cells which was reduced to background levels in RMND5A KO cells (Figure 2.11d). Furthermore, endogenous ubiquitinated muskelin co-immunoprecipitated with transfected HA-ubiquitin in control cells but not in RMND5A KO cells (Figure 2.11e). Together, this suggests that the CTLH complex is required for
Figure 2.11. Muskelin is a target of the CTLH complex. (a) Deregulation of muskelin in RMND5A KO cells can be rescued by restoration of RMND5A expression. HeLa whole cell extracts prepared from control, RMND5A KO, or RMND5A KO cells transfected with HA-RMND5A for 24 hours were analyzed by Western blot with the antibodies indicated. Below, quantification of muskelin levels is shown to the right for untransfected (-) or HA-RMND5A transfected RMND5A KO HeLa cell, normalized to vinculin levels and relative to control cells set to 1. N=3, error bar indicates SD. **P<0.01. (b) Muskelin upregulation is proteasome-dependent. Whole cell extracts were prepared from Control or RMND5A KO HeLa cells treated with DMSO or 10 μM MG132 for 16 hours and analyzed by Western blot. Below, quantifications of muskelin of in MG132-treated control or RMND5A KO HeLa cells, normalized to actin levels and relative to their DMSO control set to 1. N=4, error bar indicates SD. **P<0.01. (c) Degradation kinetics of muskelin. Control or RMND5A KO HeLa cells were treated with 100 μM cycloheximide (CHX) and whole cell extracts were prepared at the times indicated. Samples were run on a 10% SDS-PAGE gel and analyzed by Western blot with the indicated antibodies. Muskelin quantifications are shown below relative to vinculin levels and normalized to untreated samples which were set to 1. N=3, error bars indicate SD. *P<0.05. The error bar for the KO samples at 24h is not visible because it is smaller than symbol size. (d) Ubiquitination of transfected muskelin is regulated by RMND5A. Control or RMND5A KO HEK293 were co-transfected with FLAG-muskelin or HA-ubiquitin (HA-Ub) for 24 hours, followed by 10 μM MG132 treatment for 8 hours. FLAG-muskelin was pulled down in denaturing conditions (lysis in 1% SDS). Note that transfected muskelin migrates higher than endogenous muskelin due to the added tags that amount to about 7kDa. (e) Ubiquitination of endogenous muskelin is regulated by RMND5A. Control or RMND5A KO HEK293 were transfected with HA-Ub for 24 hours, followed by 10 μM MG132 treatment for 8 hours. HA-Ub was pulled down in denaturing conditions.
muskelin ubiquitination in vivo. Overall, the data suggests that the CTLH complex regulates ubiquitination and protein levels of one of its own subunits, muskelin.

2.3 Discussion

In this study, we have characterized the composition and stability of the CTLH complex and demonstrated, using in vitro assays, that the complex has E3 ligase activity. We confirmed that human GID4 and WDR26 associate with the complex and that RanBPM, TWA1, MAEA, RMND5A and ARMC8 each have roles in maintaining complex stability. Importantly, RMND5A and MAEA are completely co-dependent on each other. We found that ubiquitination activity of the complex is dependent on the RING subunits RMND5A and MAEA. Furthermore, both recombinant RMND5A and MAEA also exhibit activity. Additionally, we determined that the complex can pair with ubcH5 family of E2 enzymes and GST-RMND5A can catalyze K48 and K63 ubiquitin chains. Finally, we revealed that CTLH complex regulates ubiquitination and proteasomal degradation of its peripheral subunit muskelin.

Our data suggests that protein expression of several CTLH complex subunits is interdependent, which has previously been observed to some extent for the yeast Gid complex. In the Gid complex, Gid1 (RanBPM) was deemed essential for the stability of the complex as its deletion resulted in decreased levels of Gid8 and Gid2\textsuperscript{26}. Also, Gid2 and Gid9 were found to stabilize each other\textsuperscript{26}. Our analyses of CTLH complex subunits knockdown/knockout cells suggest that this is also true for the CTLH complex. RanBPM knockdown resulted in the decrease of TWA1, RMND5A and MAEA protein levels and RMND5A and MAEA required each other, suggesting that, like in the Gid complex, the
two RING domain subunits are stabilized through heterodimerization. TWA1, like RanBPM, is central to CTLH complex formation, as its knockout affected RanBPM, MAEA, RMND5A and, curiously, ARMC8β. Finally, muskelin knockout did not significantly affect other CTLH complex members’ protein levels. Previous microarray analyses revealed no changes in CTLH complex members’ RNA expression in RanBPM shRNA cells\textsuperscript{32}, and this was confirmed through qPCR analyses. We did not detect any significant changes in mRNA levels in most other CTLH complex knockout cells, except in 2 cases (RMND5A and ARMC8) where slight changes in mRNA expression were detected but were much more subtle than the effect observed at the protein level. Therefore the changes in protein expression induced by the knockout of individual CTLH subunits reflect mostly changes in protein stability rather than at the transcriptional level.

We found that both ARMC8α and β were present in the CTLH complex. These 2 isoforms originate from alternative splicing of the same gene product and have previously been identified as being part of the CTLH complex\textsuperscript{13}. However, it raises the question as to whether both isoforms are present together in the complex or that sub-complexes contain one or the other isoform. Interestingly, the loss of TWA1 had different effects on the two ARMC8 isoforms, with ARMC8β being strongly decreased and ARMC8α slightly increased, albeit not significantly, inferring that the alternate splicing event may be regulated by the CTLH complex. Reversely, ARMC8 knockout significantly reduced TWA1, MAEA and RMND5A levels, suggesting that ARMC8 may stabilize the association of the core complex members.
Immunofluorescence analyses showed that the CTLH complex subunits are localized to different extents in the cytoplasm and nucleus. The subcellular localization observed with ectopically expressed proteins appear to match that of several endogenously expressed CTLH complex members reported in a previous study\textsuperscript{13}. Adding to that, we found that GID4 is nearly exclusively nuclear, whereas WDR26 is mostly cytoplasmic. This suggests the possibility that several distinct CTLH complex variants exist in the cell. However, the fact that the knockout of MAEA, which is mostly nuclear, affects the levels of muskelin, which is mostly cytoplasmic, implies that these two seemingly differentially localized members are connected. Therefore, the dynamics of the nucleocytoplasmic localization of CTLH complex(es) will need further investigation. Also, our analysis involved ectopically expressed proteins, and while our results show that transfected CTLH complex members can interact with the endogenous CTLH complex (see Figure 2.1), it is possible that a significant fraction of the transfected proteins did not associate with the complex and that their observed localization was dictated by their own localization properties rather than by those of the complex as a whole.

Our analyses of the CTLH complex E3 activity through \textit{in vitro} ubiquitination assays using RanBPM immunocomplexes showed that, as expected, RMND5A is essential for activity. MAEA is also likely essential since its KO reduces RMND5A protein levels to background, therefore preventing CTLH complex activity. Also, based on the yeast topology, MAEA links TWA1 to RMND5A and potentially ARMC8 and therefore is essential for complex formation\textsuperscript{26}. 
We found that both bacterially-expressed recombinant RMND5A and MAEA have E3 ligase activity. This was previously reported for the *Xenopus laevis* and yeast homologues of RMND5A\(^7,12\), however, this is the first time that MAEA is shown to have intrinsic activity. The activity of both RMND5A and MAEA was low, and the reason for this may be that both contain RING domains that diverge considerably from the RING consensus\(^10\). Analysis of the consensus sequence for RMND5A homologues suggests its RING domain resembles that of TRIM32, but contains serines instead of cysteines in its second active site\(^10\). TRIM32 exists as a tetramer formed by two separate dimerization events that occur through the RING domains and the coiled-coil region\(^33\). TRIM32 RING dimerization is essential for catalytic activity\(^33,34\). This suggests that this may also be true for the RMND5A-MAEA dimer. Previous analyses with the yeast complex found that the interaction of Gid2 and Gid9 still occurs when the Gid2 CTLH and LisH domains are deleted, suggesting that the interaction may be mediated by the RING domains\(^11\). In our *in vitro* assays, we expressed RMND5A as a fusion protein with GST. Since GST dimerizes, that could be fulfilling the pre-requisite for dimerization. Alternatively, the LisH and CTLH domains could mediate dimerization and/or the RING domain may have similar dimerization capabilities as TRIM32. The MAEA RING domain is longer than conventional RING domains, is not predicted to coordinate zinc and has no obvious similarities to other eukaryotic RING motifs\(^10\). As it was expressed as a SUMO-fusion protein, dimerization, if required for activity, could only have been mediated by the RING and/or the LisH/CTLH domains.
Another study recently described the activity of the CTLH complex through biochemical reconstitution with purified components\textsuperscript{35}. While some of our conclusions are in agreement with their findings, notably with respect to the requirements for MAEA and the other core complex subunits RanBPM, TWA1 and RMND5A for E3 ligase activity, some of our results differ, in particular regarding the identification of the E2 requirement for complex activity. While we detected robust activity with UBE2D1, UBE2D2 and UBE2D3 variants, we did not observe any activity with UBE2H, or CDC34. In agreement with our data, Lampert et al. did not report any activity with CDC34, however, they found that UBE2H was the most efficient E2 and they observed little activity with UBE2D3. While UBE2H was identified as the human homologue of Gid3\textsuperscript{28}, it lacks the C-terminal extension of acidic residues found in Gid3. This acidic tail is reminiscent of CDC34 in which the tail both binds the E3 ligase and promotes ubiquitin transfer\textsuperscript{31}. Most of their assays involved the \textit{in vitro} reconstituted CTLH complex with subunits purified from insect cells, whereas our assays involved bacterially-expressed RMND5A and the endogenous CTLH complex immunoprecipitated from HEK293 cells. Another difference is that we assayed complex activity from endogenous RanBPM immunoprecipitates, whereas they assayed the E3 activity from the complex immunoprecipitated through transfected tagged-RMND5A. The use of different experimental models (recombinant versus native complex) or the difference in complex isolation procedure (potentially yielding different complex conformations) as well as the relative activities of E2 enzymes could potentially account for these differences. However, our \textit{in vitro} assays using recombinant RMND5A are
consistent with previous in vitro ubiquitination assays which showed that yeast Gid2 (homolog of RMND5A) was able to function with UBE2D2\(^7\).

Our finding that muskelin protein levels and ubiquitination are regulated by the CTLH complex provides insight into a possible function and/or regulatory module of the complex. It also raises the question as to whether muskelin has a role in CTLH complex function or if it is just one of its targets. If the former, its appearance later in evolution suggests that it may provide a function to the complex distinct from the yeast Gid complex. As shown in this study and others, muskelin localizes mostly to the cytosol, which is dependent on its LisH domain-mediated dimerization\(^{13,20,36–38}\). Interestingly, its overexpression has previously been shown to promote relocalization of other CTLH nuclear complex members to the cytoplasm\(^{13}\). Therefore, we speculate that the function of muskelin in the CTLH complex may be to direct the complex to specific cytoplasmic targets. Ubiquitination and subsequent degradation of muskelin by the complex could therefore serve as a feedback mechanism under certain conditions. Self-ubiquitination is a common feature of most E3 ligases and in many cases is used as a mechanism of autoregulation\(^{39,40}\).

Muskelin promotes cellular prion protein (PrP\(^C\)) turnover via the lysosome in neurons and muskelin knockout accelerates Prion disease induced by prion infection in mice\(^{19}\). Interestingly, a fragment of RanBPM is found overexpressed in Alzheimer’s Disease (AD) patients and its overexpression promotes Amyloid beta (A\(\beta\)) generation and hallmarks of AD\(^{41–45}\). As PrP\(^C\) propagates the neurotoxic signaling effects of A\(\beta\)\(^{46–48}\), this could implicate a mechanism whereby the effects observed by RanBPM
overexpression in AD are, at least partially, a result of increased ubiquitination and proteasomal degradation of muskelin, leading to a defect in PrP<sup>C</sup> lysosomal degradation. It will be of interest to assess adult mouse models of other CTLH complex members, especially in the context of neurodegenerative diseases, to determine if there is an interplay between the CTLH complex and muskelin ubiquitination that contributes to disease pathogenesis.

In summary, we have extended the characterization of the mammalian CTLH complex, a unique E3 ligase complex containing a RING heterodimer. We confirmed its function as an E3 ubiquitin ligase and determined the requirements for its stability and activity. We have also revealed it regulates ubiquitination of its subunit muskelin, adding to its list of possible targets and identifying a potential autoregulatory mechanism that awaits further investigation. A comprehensive study of the substrates of the CTLH complex will be informative to understand the biological function of this multi-subunit E3 ubiquitin ligase.

2.4 Methods

2.4.1 Cell culture, transfections and treatments

Wild-type HeLa and HEK293 cells, and control shRNA and RanBPM shRNA stable HEK293 cells have been described previously<sup>49,50</sup>. All cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (Wisent Bioproducts, St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (Wisent Bioproducts) at 37°C in 5% CO<sub>2</sub>. Cells were treated with 10 μM MG132 (EMD-CalBiochem, San Diego, CA) for the indicated time points. For cycloheximide (CHX) treatment, cells were treated with 100
µg/ml cycloheximide (BioShop, Burlington, ON, Canada) and collected at the indicated time points. Plasmid transfections were carried out with jetPRIME (Polypus Transfection, Illkirch, France) according to the manufacturer’s protocol. siRNA transfections were carried out as described previously for siMuskelin and siRMND5A and siTWA1 (Silencer, AM16708A, 25822, Ambion, Life Technologies, Burlington, ON, Canada) transfections were performed using the same conditions.

2.4.2 Generation of CRISPR Knockout cells

Single guide RNA (sgRNA) sequences were designed using the Benchling CRISPR tool (https://benchling.com). Top and bottom oligos with overhanging ends containing sgRNA directed against RMND5A

(5’CACCGTGGAGCACCTCTTTCGACA, 5’AAACTGTCGAAAGAAGTGCTCCAC)
MAEA (5’CACCGCGTTTGTTCAGCGTCTCGTA), TWA1

(5’CACCGAGCAGCGGAAGTTTCGAA,
5’AAACTTTCGAAAACCTCTCCGCTGTC), ARMC8

(5’CACCGTTTGGTTCGAATGTGCAGTA,
5’AAACTACTGCACATTCGAACCAAAC), RanBPM

(5’CACCGCGGAAGGGCTGATCGCAGTA,
5’AAACCCCGGCCCCCTAACGCTCGC), or muskelin

(5’CACCGCTATTTTAATGAATCGCACA,
5’AAACTTGGTGCATTACATGAAATAGC) were cloned into pSpCas9(BB)-2A-Puro V2.0 (PX459) digested with BpiI and then transfected into early passage HEK293 or HeLa cells. Forty-eight hours after transfection, cells were put under puromycin selection
(1.2μg/ml for HEK293, 0.3μg/mL for HeLa) for seven days, followed by colony picking and expansion. Control cells were generated by the above approach but using Cas9 alone (i.e. without an sgRNA guide) and pooling colonies together.

2.4.3 Plasmid constructs

The following plasmids were gifts: pCDNA-SBP-FLAG-WDR26\(^{51}\) (Dr. Songhai Chen, University of Iowa, Iowa City, IA, USA), pMT123 plasmid expressing HA-ubiquitin\(^{52}\) (Dr. Lina Dagnino, Western University, London, ON, Canada), pET-His-SUMO-SLP2 (Dr. Stan Dunn, Western University, London, ON, Canada), pSpCas9(BB)-2A-Puro V2.0 (PX459; Dr. Joe Torchia, Western University, London, ON, Canada) and pCMV-HA-muskelin\(^{53}\) (mouse cDNA; Dr. Kai Jiao, University of Alabama at Birmingham, Birmingham, AL, USA). pCMV-HA-RanBPM has been described previously\(^{49}\). Primers for RMND5A (5’

GTACATTCTAGAATGGATCAGTGCGTGACGGTGGAG,
5’GTACATGGATCCTCAGAAAAATATCTGTTTGGCATC), MAEA (5’

GTACATTCTAGAATGGCGGTGCAGGAGTCGGCGGCTCAGTTGTCC,
5’

GTACATGGATCCTACTTGGGCTCCTCAATCACCC), were used to generate inserts amplified from a Jurkat T-cell cDNA library (Clontech, see reference\(^{54}\)). The inserts were digested with XbaI and BamHI enzymes and cloned into pCGN-HA plasmid (created by removal of Oct-1 from pCGN-Oct-1, see reference\(^{55}\)). ARMC8 insert was generated by PCR amplification
(5’ATTGAAGTCGACGATGGAAGTAACAGCTAGCAGTCG,
5’AATGTTCTCGAGATGGCCAGGTACTGCTGCAGTGC) from the same cDNA library, digested with SalI and XhoI and ligated with pCMV-HA (created by removal of RanBPM from pCMV-HA-RanBPM). Muskelin cDNA (derived from pCMV-HA-muskelin (mouse)) was PCR amplified
(5’ATTATAACCGGTATGGCGGCTGGCGGGGCTGTTG,
5’AGTCCGCTCGAGCTACAGTGTGATCAGGTCTACCAG) with Age1 and XhoI sites and subcloned into pCDNA-SBP-FLAG (created by removal of WDR26 from pCDNA-FLAG-SBP-WDR26). GID4 cDNA (Accession: NM_024052) in pEZ-M06 was purchased from GeneCopoeia (EX-E1982-M06; Rockville, MD, USA). RMND5A cDNA was PCR amplified from pCGN-HA-RMND5A
(5’GTACATGAATTCACTGGATCAGTGCAGTGACTGGGAG,
5’GTACATGGATCTCAGAAAAATATCTGTGTTTGCCATC), digested with EcoRI and BamHI and subcloned into pGEX-4T1. MAEA cDNA was PCR amplified from pCGN-HA-MAEA (5’ TATTTAGGTACCATGGCCTGGTGACTGGGAG, 5’ GCCGTAAGCTTCATCATGTGACATGCTGCAGTGCAGTGC), digested with KpnI and HINDIII and subcloned into pET-His-SUMO (created by removal of SLP2 from pET-His-SUMO-SLP2).

2.4.4 RNA extraction, reverse transcription and quantitative PCR

RNA extraction was performed using RNeasy Mini Kit (QIAGEN, Toronto, Canada) in accordance with the manufacturer’s directions. 2 μg of total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied
Biosystems, Foster City, California, United States). Quantitative PCR was completed on Bio-Rad CFX Connect Real-Time System (Bio-Rad) using pre-designed TaqMan probes (4448892, Applied Biosystems) for the following genes: RANBP9 (Hs00170646_m1), GID8 (Hs00215479_m1), RMND5A (Hs00405598_m1), MKLN1 (Hs00992683_m1), MAEA (Hs01028524_m1), and ARMC8 (Hs01046446_m1). Experiments were performed with technical triplicates using GAPDH (4453320, Hs02786624_g1, Applied Biosystems) as a normalization control. Changes in gene expression were determined by the \( \Delta(\Delta Ct) \) method and reported as fold increase or decrease in mRNA levels in knockout cell line samples relative to control cells.

2.4.5 Western blot

Whole cell extracts were prepared as described\(^{49}\) and resolved using 8% or 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to polyvinylidene fluoride membranes, blocked in 5% milk and hybridized with the following antibodies: ARMC8 (E-1, sc-365307; Santa Cruz Biotechnology, Santa Cruz, CA, USA); FLAG (M2, F1804, Sigma-Aldrich, St. Louis, MO, USA); HA (HA-7, H3663 Sigma-Aldrich); MAEA (AF7288, R&D Systems, Minneapolis, MN, USA); Muskelin (C-12, sc-398956, Santa Cruz Biotechnology); RanBPM (5M, 71-001, Bioacademia, Japan); RMND5A (NBP1-92337, Novus Biologicals, Littleton, CO, USA); TWA1 (NBP1-32596, Novus Biologicals); Vinculin (E1E9V, Cell Signaling Technology, Danvers, MA, USA); WDR26 (ab85962, Abcam, Cambridge, UK); \( \beta \)-actin (A5441, Sigma-Aldrich) and His (MAB050, R&D Systems). Blots were developed using Clarity
Western ECL Substrate (BioRad, Hercules, CA, USA) and imaged using a ChemiDoc MP (BioRad). Analysis and quantifications were done using Image Lab (BioRad).

2.4.6 Immunofluorescence microscopy

Cells were plated on coverslips and transfected following overnight incubation. Cells were fixed with 3% paraformaldehyde, permeabilized in 0.5% Triton-X100 for 10 min and blocked in 5% FBS diluted in PBS. Coverslips were incubated overnight with primary antibody HA (HA-7, H9658, Sigma-Aldrich) or FLAG (M2, F1804, Sigma-Aldrich), washed in PBS and incubated with anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen, Life Technologies, Burlington, ON, Canada). Cells were mounted with ProLong Gold antifade with DAPI (Invitrogen). Visualization was done using an Olympus BX51 microscope with a 40x objective and images were captured with the Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). For quantification analysis, images were blinded by a third party.

2.4.7 Immunoprecipitations

For co-immunoprecipitation experiments, extracts were adjusted to 0.25% NP-40, pre-cleared and rotated overnight at 4°C with antibodies to RanBPM (F-1, sc-271727, Santa Cruz Biotechnology), HA (HA-7, H9658, Sigma-Aldrich), or FLAG (M2, F1804, Sigma-Aldrich). Samples were then incubated for 1 hour at 4°C with Dynabeads protein G (10004D, Invitrogen), washed three times in lysis buffer and resuspended in SDS loading buffer. Mouse IgG (sc-2025, Santa Cruz Biotechnology) was used as negative control for RanBPM immunoprecipitations. For ubiquitination assays, RanBPM immunoprecipitations were carried out as above, except extracts were supplemented with
extra NP-40 (final 1.0%) and Triton (final 0.2%) and washed three times in lysis buffer without EDTA.

To assess ubiquitination of FLAG-muskelin, control or RMND5A KO HEK293 cells were co-transfected with pCDNA-FLAG-muskelin and pMT123 plasmid expressing HA-ubiquitin\(^{52}\). Twenty-four hours after transfection, cells were MG132 treated for 8 hours. Cells were lysed in denaturing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1% SDS, 1 mM Na3VO4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of aprotinin, 10 μg/ml of peptatin, 1 μg/ml of leupeptin and 25 mM NEM (N-Ethylmalalide, Bioshop Canada, Burlington, ON, Canada)), passed through a 23G needle ten times and incubated on ice for 30 minutes. For immunoprecipitation, lysates were diluted 1:10 in buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM Na3VO4, 10 mM NaF and 25 mM NEM) and incubated with anti-FLAG (M2; F1804; Sigma-Aldrich) for 2 hours at 4°C, followed by incubation with Dynabeads Protein G for 1 hour. Beads were then washed five times in buffer A and resuspended and boiled in SDS loading dye. Eluates were split in half when loading on SDS-PAGE so that HA and FLAG immunoblotting could be analyzed separately (to avoid overlapping signal). The same procedure was employed for the HA-ubiquitin immunoprecipitation, except that cells were transfected with only HA-ubiquitin plasmid and anti-HA (HA-7, H9658, Sigma-Aldrich) was used for the pulldown.

### 2.4.8 In vitro ubiquitination assays

BL21 *E. coli* expressing GST, GST-RMND5A, His-SUMO-SLP2 and His-SUMO-MAEA were grown at 37°C with 250 μM ZnCl₂ to 0.7-0.8 OD600 and induced
with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside, then grown at 16°C overnight. Cells were lysed in 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM dithiothreitol (DTT), 2 mM PMSF, 20 μg/mL Leupeptin, 1.0 μg/mL aprotinin and 1% Triton-X 100 and passed through a French press twice. 4.5 μL of bacterial extract was used in a 20μL ubiquitination reaction with 5μM UBE2D2 (E2-622, BostonBiochem, Cambridge, MA), 0.1 μM His-tagged E1\textsuperscript{56}, 25 μM His-ubiquitin, 10 mM Mg-ATP (B-20, R&D Systems), 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl\textsubscript{2}, 0.1 mM DTT and 25 μM ZnCl\textsubscript{2}. The UbcH (E2) Enzyme Kit (K-980B, BostonBiochem) and CDC34 (see reference\textsuperscript{57}) was used to screen E2 enzymes. Reactions were incubated in a thermomixer with periodic shaking at 37°C for 2 hours. The entire reactions were run on an 8% SDS-PAGE and analyzed by Western blot. For ubiquitination assays with RanBPM immunocomplexes, beads were resuspended in 0.1 μM His-tagged E1, 5 μM UbcH5b, 22.5 μM His-ubiquitin, 2.5 μM Ub\textsuperscript{800} (see reference\textsuperscript{58}), 10 mM Mg-ATP and 50 mM HEPES. Reactions were incubated in a thermomixer with periodic shaking at 37°C for 30 minutes. Gels were scanned by the Odyssey Imaging system (LiCor, Lincoln, NE, USA) and fluorescence intensity was measured at 800 nm.

**2.4.9 Chain linkage analysis by Mass Spectrometry (MS)**

Sample preparation of GST-RMND5A ubiquitination reactions were performed as described previously\textsuperscript{59,60}. Briefly, reactions were reduced with 10 mM DTT, alkylated with 100 mM Iodoacetamide, followed by methanol precipitation. The protein pellet was resuspended in 0.5% PPS Silent Surfactant (Expedeon Ltd, Cambridge, UK) and digested for 16 hours with 0.5 mg of Lysyl Endopeptidase (LysC; 125-05061, Wako Pure
Chemical Ind., Ltd., Japan) and 0.75 mg of LysC/Trypsin (V5071, Promega, Madison, WI, USA), followed by 4 hours with 0.5 mg Trypsin (V5111, Promega). Quenched digested reactions were then desalted with C18 Stage Tips and eluted peptides were lyophilized, followed by resuspension in 0.1 % Formic acid. Samples (1 μg as measured by BCA) were analyzed by using an M-class nanoAptiy UHPLC system (Waters, Milford, MA, USA) connected to a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). Raw MS files were searched in MaxQuant (version 1.5.8.3) using the Human Uniprot database. MS1 peak areas corresponding to each ubiquitin linkage were extracted using Xcalibur software (Thermo Scientific).

2.4.10 Statistical analysis

Statistical analyses were performed using GraphPad PRISM (GraphPad Software Inc., La Jolla, CA, USA). Differences between two groups were compared using unpaired two-tailed t test. Results were considered significant when $P<0.05$.

2.5 References


56. Condos, T. E. *et al.* Synergistic recruitment of UbcH7–Ub and phosphorylated Ubl
doi:10.15252/embj.2018100014


Chapter 3

3 Characterizing the structure of the human CTLH E3 ligase complex

This chapter constitutes a draft of a manuscript that is in preparation.

3.1 Introduction

Ubiquitination is the covalent attachment of the protein ubiquitin (Ub) to lysine residues of protein substrates either as a singly moiety or as a polyUb chain. Depending on the type of ubiquitination or topology of the polyUb chain, ubiquitination can elicit numerous effects on a protein such as proteasomal degradation, changes in protein interactions, or regulating subcellular localization. Ubiquitination is achieved through the coordination of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases. Reaction specificity is conferred by the E3 ligases via interaction with the substrate. E3 ligases come in a variety of types that differ in their domain structure and direct or indirect participation in the ubiquitin transfer.

E3 ligases with RING (really interesting new gene) domains bridge the interaction between the E2–Ub conjugate and a substrate. The RING domain binds the E2 enzyme, while a non-RING element in RING E3 ligase binds the substrate and orients it such that the accepting lysine is in proximity of the E2 catalytic site. The RING domain can also promote the reaction by hydrogen bond formation via its linchpin residue with the E2 and Ub that restricts the E2–Ub conformation, priming it for Ub transfer to substrate. RING E3 ligases can exist in the form of monomeric, homo or heterodimeric, or within a multi-subunit complex. For the latter, Cullin RING ligases (CRLs) have been well studied as...
a family of multi-subunit E3 ligase complexes, comprising interchangeable substrate receptors, an adapter, scaffold, and RING catalytic modules. An emerging example of a mammalian multi-subunit E3 ligase is the C-terminal to LisH (CTLH) complex. Its yeast homologue, the glucose-induced degradation-deficient (Gid) complex, and the E2 Ubc8, are responsible for ubiquitination of gluconeogenic enzymes during catabolite inactivation in *Saccharomyces cerevisiae*. In this phenomenon, glucose addition induces rapid synthesis of Gid4 (aka Vid24; in humans, GID4). Gid4 then associates with the complex and binds N-terminal proline degrons present on the gluconeogenic enzymes, a requirement for their ubiquitination and subsequent proteasomal degradation. A recent Cryo-electron microscopy (cryo-EM) structure of the endogenous and recombinant Gid complex as well as previous work that used immunoprecipitation in yeast knockout strains, reveals it contains a RING module (in this case, a RING heterodimer of Gid2-Gid9), a scaffold module (Gid1-Gid8-Gid5) and a substrate receptor (Gid4) that is positioned to coordinate binding of the substrate with RING domain-bound E2. In the RING heterodimer, only Gid2 has *in vitro* E3 ligase activity.

Recently, the human CTLH complex was confirmed to also have E3 ligase activity mediated by its RING heterodimers, RMND5A and MAEA, the homologues of Gid2 and Gid9, respectively. Ubiquitination targets confirmed *in vitro* with UBE2H (homologue of yeast Ubc8) as the E2 have been revealed, such as the transcription factor HBP1 and nuclear matrix protein lamin B. In addition to RMND5A and MAEA, the core complex is composed of RanBPM (aka RanBP9, homologue of Gid1), TWA1 (aka
GID8, homologue of Gid8), muskelin (aka MKLN1), WDR26 (homologue of Gid7), ARMC8 (similar to Gid5), and GID4. Strikingly, RanBPM, TWA1, RMND5A, and MAEA each contain, in the same order, LisH (lissencephaly type-1-like homology), CTLH, and RanBPM-CT11 (CRA) domains. Interestingly, the Arabidopsis thaliana TOPOLESS (atTPL) protein also contains these domains in the same sequence. The crystal structure of its tetramer reveals a conformation of the domains that is likely similar in the CTLH complex subunits: LisH domain with two α-helices that homodimerizes as a four-helix antiparallel bundle at the top of the structure, followed by the CTLH domain with three α-helices that connects to the CRA domain of 4 α-helices. The CRA domain forms a tetramer interface at the base with its second helix but loops back to place its last helix on top of the LisH domain, dimerizing with the same helix from the other monomer. Muskelin and WDR26 also contain LisH and CTLH domains, but not the CRA domain. In addition, several protein-protein interaction domains of currently unknown significance are present on different subunits in the CTLH complex, such as the SPRY domain of RanBPM, discoidin domain and kelch repeats of muskelin, β-barrel of GID4, WD40 repeats of WDR26, and armadillo (ARM) repeats on ARMC8.

The reconstitution of the human complex and aforementioned cryo-EM structure of the yeast complex provides important insights into the overall structure of what the human complex may look like. The core of the human complex is composed of the CRA domain subunits RanBPM, TWA1, RMND5A and MAEA, with RanBPM and TWA1 each being critical for core complex stability. Additionally, ARMC8 is required for GID4 association. There are, however, several important differences
between the yeast Gid and human CTLH complexes. Notably, muskelin is not present in the yeast complex\textsuperscript{20,21}. Additional differences include TWA1 as a dimer within the human complex (revealed during the preparation of this chapter)\textsuperscript{16,22}, the coexistence of two human isoforms of ARMC8 (which is also considerably different from Gid5 and is not actually a homologue (revealed after chapter 2 was published)\textsuperscript{17,21}, the likely incorporation of RanBPM and RMND5A paralogues (RanBP10 and RMND5B) in the human complex\textsuperscript{16}, and, in general, the increased size of the yeast counterparts\textsuperscript{20}. Therefore, a clearer depiction of CTLH complex architecture and of its E3 ligase activity is needed to understand the functional relationships between subunits and advance the field on this multi-subunit E3 ligase. To that end, we focused on producing an updated and more accurate topology model of the CTLH complex and requirements of its E3 ligase activity. For the latter, predicted structures of RMND5A and MAEA were analyzed and residues were identified in RMND5A that are necessary for the \textit{in vivo} control of protein levels of muskelin, a presumed ubiquitination target of the complex\textsuperscript{17}. To probe the overall structure of the human complex, we immunoprecipitated endogenous RanBPM and assessed proximal interactions and relative orientations of the human CTLH complex subunits using cross-linking mass spectrometry (CLMS). Immunoprecipitations of RanBPM in various knockout cell lines and RanBPM domain deletion immunoprecipitations also provided clarity to subunit arrangement. Overall, this work provides an updated model of complex topology and determined requirements in the RMND5A RING domain for E3 ligase activity. Together, these findings highlight key
similarities and differences of the structure and activity between the human and yeast CTLH/Gid complexes.

3.2 Results

3.2.1 Analysis of MAEA and RMND5A RING domains

The canonical RING domain is a cross brace structure with cysteine and histidines coordinating two zinc ions. In the consensus RING sequence, the first zinc coordination site is composed of cysteines C1, C2, C4, and C5 and the second site is composed of cysteines and a histidine C3, H1, C6, and C7 (Figure 3.1A). Both human and yeast RMND5A contain the cysteines and histidine that are part of zinc coordination site #2 and this is apparent in the predicted structure (Figure 3.1A, B). For zinc coordination site #1, only C1 is present in human or yeast RMND5A. Leucine, serine, and alanine are present in place of C2, C4, and C5, respectively (Figure 3.1A). Yeast Gid9 does have the first two cysteines, but polar or charged residues are found instead of the other cysteines or histidine, reminiscent of the U-box domain (Figure 3.1A). Human MAEA, on the other hand, has cysteines C1, C2, and C6 but polar residues at positions C3, H1, C5, and C7, and a glycine at position C4 (Figure 3.1A). The predicted structure of human MAEA clearly shows some of these polar residues (N358, Y360, and T381) are positioned for hydrogen bonding for zinc coordination site #2 (Figure 3.1C). Zinc coordination site #1 in the predicted structure of human MAEA is uncertain, but a few cysteines and a histidine are in proximity and it is conceivable that they could coordinate zinc (Figure 3.1C). Human MAEA RING domain and its yeast homologue have an insertion between the first α-helix and first β-sheet (Figure 3.1A,C). Two cysteines, a histidine, and a mix of
**Figure 3.1. RMND5A and MAEA RING domains.** A) Multiple sequence alignment of various known human RING and U-box domains and the RING domains of human and yeast MAEA and RMND5A. Residues are coloured according to their physiochemical properties (Zappo). See methods for details. B) Predicted structure of human MAEA, with a close-up view of the RING domain. C) Predicted structure of human RMND5A, with a close-up view of the RING domain.
hydrophobic, polar and charged residues are present in this insertion for human MAEA, while Gid9 has two histidines along with polar, charged, and hydrophobic residues but no cysteines (Figure 3.1A). This region in human MAEA is predicted to be a disordered structure that loops around to the β-sheet, but currently its significance is unknown (Figure 3.1C). In Qiao et al., 2020, Y514 of Gid9 (equivalent to Y394 of human MAEA, labelled in Figure 3.1C) was determined to be a non-RING priming element, but the other mutations tested suggested Gid9 does not bind the E2\textsuperscript{13}.

To test the roles of potential zinc-coordinating cysteine residues on the function of RMND5A within the CTLH complex, we restored wild-type or mutant RMND5A in RMND5A KO HeLa cells and assessed muskelin protein levels. We previously determined that, in RMND5A KO cells, muskelin proteins levels are increased (and ubiquitination decreased) which can be reversed upon re-introduction of RMND5A in RMND5A KO cells\textsuperscript{17} (Chapter 2). A C354S (C3 in zinc site #2) mutation abolished human RMND5A ability to rescue muskelin protein levels in RMND5A KO cells (Figure 3.2A). This is consistent with studies on \textit{S. cerevisiae} and \textit{Xenopus laevis} RMND5A homologues in which mutation of the equivalent C354 residue eliminated \textit{in vitro} RMND5A activity\textsuperscript{8,35}. A C374S (C6 in zinc site #2) mutation also could not reduce muskelin levels (Figure 3.2A). These cysteine mutations presumably prevent zinc coordination at site #2 and disrupt the folding of the RMND5A RING domain. Intriguingly, a C336S (C1 in zinc site #1) mutation also prevented the rescue of muskelin levels (Figure 3.2A). This was unexpected since the first zinc coordination site is not apparent in the predicted structure or primary sequence.
Figure 3.2. RMND5A RING residues critical for controlling muskelin levels. Wild-type RMND5A and the indicated mutations were transfected into RMND5A KO HeLa cells for 24 hours. A) Cysteine mutations. The levels of muskelin normalized to vinculin loading control relative to mock, as determined by chemiluminescence, is shown in the right. n=3, *p<0.05, **p<0.01. B) Analysis of non-cysteine mutations in the RMND5A RING domain. The levels of muskelin normalized to tubulin loading control relative to mock, as determined by fluorescent signal, is shown in the right. n=4, *p<0.05, ****p<0.0001.
In Qiao et al., 2020, Gid2 RING domain residues were identified that most likely form the hydrophobic E2 binding site for Ubc8 or function to stabilize the closed E2–Ub conformation (indicated in bottom of figure 3.1A). The hydrophobic E2 binding residues of Gid2, V363 and L364, correspond to I338 and L339 of human RMND5A. While the E2 responsible for ubiquitination of muskelin by the CTLH complex is unknown, the I338A/L339A double mutation eliminated the ability of RMND5A to reduce muskelin levels in RMND5A KO cells, suggesting that the E2 platform is consistent between yeast and human RMND5A (Figure 3.2B).

Two linchpin residues were identified in Gid2, K365 and to a lesser extent Y403. Linchpin residues interact with both E2 and Ub to stabilize their closed conformation, thus promoting Ub transfer. In human RMND5A, R340 is in the equivalent position as K365 in yeast Gid2 (Figure 3.1A). Unlike in yeast Gid2, however, mutation of this residue to alanine was indistinguishable from WT RMND5A (Figure 3.2B). Mutation of Y376 in human RMND5A, homologous to Y403 of yeast Gid2, did have a significantly reduced ability to control muskelin protein levels suggesting that it acts as a linchpin for the human complex in this context (Figure 3.2B). Finally, mutation of F418 in the Gid2 C-terminus, considered to be a non-RING priming element, showed a modest effect in Qiao et al., 2020. At the end of human RMND5A, two phenylalanines are present (F390 and F391) and labelled in Figure 3.1B. An F390A/F391A mutation had a slight, though non-significant, effect on muskelin protein levels (Figure 3.2B).
3.2.2 Mapping topology of the human CTLH complex

The architecture of the yeast complex provided by Menssen et al., 2012\textsuperscript{14} and Qiao et al., 2020\textsuperscript{13} provides a foundation to understand the same for the human CTLH complex (Figure 3.3A). The structure of the cryo-EM yeast complex in Qiao et al., 2020\textsuperscript{13} shows Gid1 (RanBPM homologue), Gid8 (TWA1 homologue), and Gid5 (similar to ARMC8) making multiple contacts with each other, providing a scaffold module that links the Gid2-Gid9 RING heterodimer to the substrate receptor Gid4, which is directly bound to Gid5. We present an initial model of the human complex topology (Figure 3.3B) based on the findings of the yeast complex topology from Qiao et al., 2020\textsuperscript{13} and Menssen et al., 2012\textsuperscript{14} and of the recombinant human complex in Lampert et al., 2018\textsuperscript{16}. However, in addition to the differences between the yeast Gid and human CTLH complexes stated in the introduction, several questions remain, including the position of ARMC8 (which in Qiao et al., 2020\textsuperscript{13} model is bound to TWA1 but in Menssen et al., 2012\textsuperscript{14} is bound to MAEA, see figure 3.3A) and details of muskelin and WDR26 association with the complex, which was not covered by the yeast cryo-EM structure in Qiao et al., 2020\textsuperscript{13}. Based on data from Menssen et al., 2012\textsuperscript{14}, Gid7 (WDR26) is positioned bound to Gid1 (RanBPM) (Figure 3.3A,B). Muskelin, initially thought to replace Gid7 in the human complex, but it is now reported to coexist with WDR26 in the recombinant complex\textsuperscript{16}, is also shown bound to RanBPM since their interaction has been documented\textsuperscript{36,37} (Figure 3.3B).

As an initial test of the current model of subunit arrangement, we immunoprecipitated endogenous RanBPM and looked for which CTLH members co-IP
Figure 3.3. RanBPM immunoprecipitation in CTLH complex subunit knockout cells.

A) Models of subunit arrangement for the yeast Gid complex based on data from Menssen et al., 2012 (left) and Qiao et al., 2020 (right). B) Preliminary model of human CTLH complex based on the yeast complex and data from Lampert et al., 2018 (e.g., TWA1 as a dimer). The position of ARMC8 isoforms is unclear. CTLH complex subunits are colour matched to their yeast homologues in (A), although ARMC8 may not be a true homologue of Gid5. Muskelin does not have a homologue or a similar protein in yeast. C) Immunoprecipitation of RanBPM in ARMC8, MAEA, or TWA1 KO cells. WT or KO HEK293 cells were lysed in whole-cell lysis buffer and subjected to immunoprecipitation with a RanBPM antibody DMP crosslinked to beads (to avoid release of light/heavy chains). Elutions were run on a Western blot and analyzed for the presence of the indicated CTLH complex subunits. D) Immunoprecipitation of RanBPM in muskelin, WDR26, or RMND5A KO cells. Same as (C) but using HeLa WT and KO cells. E) RanBPM is not required for muskelin association with MAEA and RMND5A. Flag tagged muskelin was transfected into shControl or shRanBPM HCT116 cells and immunoprecipitated with a flag antibody. F) Updated model of complex architecture based on findings in C-E, clarifying the position of ARMC8 and muskelin. Asterisk and pound sign indicate where in the figure the new positions were derived; the same symbols are in the bottom of the lane in panels C and E showing MAEA is not required for ARMC8 association and muskelin does not require RanBPM for association with RMND5A and MAEA (exact muskelin binding is unknown as denoted by the question mark), respectively.
in CTLH subunit knockout cell lines (Figure 3.3C). We note that because of the stability interdependence of some members as we previously reported\textsuperscript{17}, interpretations in certain cases are limited. Conclusions that can be drawn are summarized below. RanBPM immunoprecipitates in WT or ARMC8 KO cells are indistinguishable (Figure 3.3C), consistent with prior results that ARMC8 is not required for complex formation\textsuperscript{38}. Yet, based on the data from the yeast complex and the requirement of ARMC8 for GID4 binding to the human recombinant complex\textsuperscript{13,14,16}, GID4 is expected to be bound to ARMC8 (we could not probe for GID4 because of a lack of a usable antibody), although whether it is the $\alpha$ or the $\beta$ isoform of ARMC8, or both, that mediates the GID4 interaction is not clear. Interestingly, MAEA was not required for ARMC8 to associate with RanBPM, suggesting that ARMC8 is bound to TWA1 instead (Figure 3.3C), which is in agreement with the yeast complex structure in Qiao et al., 2020\textsuperscript{13}. As expected, TWA1 was necessary for association of MAEA and RMND5A to RanBPM (Figure 3.3C). Presumably, TWA1 is also necessary for ARMC8 association, although we cannot conclude this since it is also not present in the input. Finally, as expected, muskelin, WDR26, and RMND5A are not required for RanBPM association with other members that we tested (Figure 3.3D), suggesting that they are not solely responsible for bridging subunit interactions.

Since muskelin does not have a yeast homologue, we tested further its association with the complex. As mentioned, the current topology model assumes muskelin is bound to only RanBPM (Figure 3.3B). To test the accuracy of that assumption, we transfected full length muskelin in cells with and without RanBPM. As can be seen in figure 3.3E,
MAEA and RMND5A is, surprisingly, still observed associated with Muskelin in shRanBPM cells to the same extent as control cells (relative to their levels in the input). This finding suggests that muskelin interactions within the complex involve more than just RanBPM. Taken all of this data together, an updated model of complex topology is generated taking into account the clarification of ARMC8 position and potential multiple interaction points of muskelin (Figure 3.3F).

3.2.3 Domains of RanBPM required to mediate association in the complex

RanBPM has multiple conserved domains (Figure 3.4A). The SPRY domain, named from the SPla and the RYanodine Receptor, is located near the N-terminus of RanBPM. It is identical to RanBP10, comprising two antiparallel β-sheets, a helix present at each terminus, and a shallow binding pocket\textsuperscript{39}. It is followed by α-helical LisH, CTLH, and CRA domains (details mentioned in introduction)\textsuperscript{40}. To determine which of these domains are required for RanBPM to associate with complex members, we transfected and immunoprecipitated ΔSPRY, ΔLisH, ΔCTLH, and ΔCRA mutants of HA-RanBPM in RanBPM deficient cells (Figure 3.4A). This experiment revealed that the CTLH and CRA domains are both required for Muskelin, ARMC8 (α and β), and WDR26 association, while the LisH domain is required for MAEA association (Figure 3.4B). These findings are consistent with Menssen et al\textsuperscript{14} that did a similar experiment with Gid1 (yeast RanBPM homologue), showing that it required the CTLH and C-terminus for Gid7 (yeast WDR26 homologue) association and LisH domain for association with Gid9 (MAEA) via Gid8 (TWA1) interaction. A notable inconsistency in our data compared to the yeast complex is that Gid5 (similar to ARMC8) bound strongly to the Gid1 ΔCTLH
Figure 3.4.Domains of RanBPM required for association with CTLH complex subunits. A) Schematics of RanBPM deletion mutants. B) The indicated HA-RanBPM mutant constructs were transfected in shRanBPM HCT116 cells for 24 hours and immunoprecipitated with an anti-HA antibody crosslinked to beads (to avoid release of heavy/light chains). Elutions were run on a Western blot and analyzed for the presence of the indicated CTLH complex subunits. C) Refinement of the model of complex architecture based on presumed interactions with RanBPM domains. Asterisk and pound sign indicate where in the figure the new positions were derived; the same symbols are in the bottom of the lane in panels B and C showing CTLH and CRA is required for ARMC8, muskelin, and WDR26 association (question mark indicates exact binding is unclear) and LisH is required for RanBPM association with RMND5A and MAEA (presumably through TWA1 based on yeast complex), respectively.
mutant in Menssen et al\textsuperscript{14}. Taking these findings into consideration, an updated version of the CTLH complex topology shows the RanBPM LisH domain mediating interaction with TWA1 (since LisH was required for MAEA association and in yeast Gid1 is linked to Gid9 through Gid8), while muskelin, WDR26, and ARMC8 co-occupy RanBPM CTLH and/or CRA domains, and no interactions occur with the RanBPM SPRY domain (Figure 3.4C).

### 3.2.4 Cross-linking mass spectrometry of the endogenous human CTLH complex

To map complex interactions in a different way and to determine relative orientations, we used cross-linking mass spectrometry (CLMS) on the endogenous complex by immunopurifying RanBPM under stringent binding conditions in HEK293 cells (Figure 3.5A). The primary amine reactive cross-linker bis(sulfosuccinimidyl)suberate (BS3) that has a spacer length of 11.4 angstroms was used to cross-link intra- and inter-molecular nearby residues (Figure 3.5A). While this is done more commonly on recombinant proteins, we used it for the immunoprecipitated RanBPM complex following a published protocol\textsuperscript{41}. First, we optimized the concentration of the BS3 for RanBPM immunoprecipitates and best observed the production of higher molecular weight species at 1 mM (Figure 3.5B). We then identified, using MS, residues of CTLH complex members cross-linked by BS3 (Figure 3.6, Appendix B). Strikingly, the most links were made in the region between the CRA and RING domains of MAEA (residues 306-314). Here, 4 links are made with muskelin kelch repeats, 2 links with TWA1 (extreme N and C-terminus), 2 links with RMND5A (N-terminus and RING domain), 1 with the C-terminus of ARMC8β, and a link with the SPRY domain of RanBPM. In addition, two
Figure 3.5. BS3 cross-linking mass spectrometry for RanBPM immunoprecipitation. A) RanBPM was immunoprecipitated from HEK293 cells (5 mg protein) in 0.5% NP40/0.2% triton, washed in buffer containing 1M NaCl, 0.5% tween, 0.5% triton, and 1% NP40, then resuspension in 1 mM BS3. After elution in acid/acetonitrile, proteins were digested with trypsin/LysC. The crosslinked peptides were enriched with strong cation exchange and injected into a Q exactive plus mass spectrometer. Crosslinks were identified using xiSearch software and then further assessed for FDR of 5% at residue level using xiFDR. B) Optimization of BS3 crosslinker concentration for RanBPM immunoprecipitation. RanBPM was immunopurified as described above then subjected to on-bead cross-linking with the indicated concentrations of BS3.
Figure 3.6. Cross-linking mass spectrometry of CTLH complex maps proximity of domains and subunits. Network map of filtered mass spectrometry identifications of BS3 cross-linked residues in RanBPM immunoprecipitations drawn in xiNet. Domain positions are determined by the Uniprot database or homology. Ambiguous links arise because the cross-link identified is part of peptide sequence identical between ARMC8α/ARMC8β.
MAEA self-links were observed. Muskelin kelch repeats had links with MAEA and RMND5A, and two self-links. The RMND5A RING domain was linked with RanBPM SPRY domain, WDR26 (between its CTLH and WD40 repeats), and, as already mentioned, MAEA. Interestingly, only a few cross-links (RanBPM CRA with ARMC8β and RMND5A) were observed in any LisH, CTLH, or CRA domains, suggesting that, for the most part, these domains are tightly packed and thus not accessible to the crosslinker. No cross-links with GID4 passed the statistical criteria. Overall, the data indicates previously unrecognized proximity of certain domains of complex members, notably the muskelin kelch repeats, RanBPM SPRY domain, or a region N-terminal to the WD40 repeats of WDR26 with the RING domains of MAEA and RMND5A. Thus, a final (but still incomplete that does not explain all results) model of complex topology incorporates the CLMS derived relative proximities of individual domains of the CTLH complex subunits with the data in this chapter, the findings of TWA1 as a dimer (which was revealed while experiments were ongoing) but, the requirements of complex formation in Lampert et al, 2018, and the recent cryo-EM structure of the yeast complex (Figure 3.7).

3.3 Discussion

In this data chapter, the CTLH complex structure and E3 ligase activity were clarified and expanded, revealing similarities and differences with its yeast homologue, the Gid complex. Using analysis on the yeast Gid2 as a guide, we determined that cysteines C336, C354, and C374 are likely critical for zinc coordination and RING folding, hydrophobic residues I338 and L339 likely form the E2 binding platform, and
**Figure 3.7. Updated model of human CTLH complex architecture.** The new model considers data from this report, the yeast cryo-EM structure from Qiao *et al.*, 2020, and the reconstituted human complex from Lampert *et al.*, 2018 as follows: (1) presumption that the E2 is bound only to RMND5A (Qiao *et al.*, 2020 and figure 3.1 and 2); (2) ARMC8 is bound to TWA1 and RanBPM, and the general arrangement of subunits (Qiao *et al.*, 2020, Lampert *et al.*, 2018, and figure 3.3); (3) CLMS data in figure 3.5 showing proximity of SPRY, WD40, RanBPM CRA, and kelch domains to RING domains; (4) CTLH/CRA domains of RanBPM being required for association with WDR26, muskelin, and ARMC8 isoforms, with LisH required for MAEA association (figure 3.4); (5) TWA1 as a dimer (Lampert *et al.*, 2018); (6) GID4 bound to ARMC8, with its β-barrel in proximity to the RING domains (Qiao *et al.*, 2020 and Lampert *et al.*, 2018); and (7) MAEA-RMND5A have multiple heterodimerization interfaces including the RING domains (Qiao *et al.*, 2020 and CLMS data in figure 3.4). ARMC8 isoforms coexist in the human complex but their exact interactions are unclear.
Y376 serves as a linchpin. Analysis of complex interactions using RanBPM mutants, CTLH knockout cell lines, and CLMS revealed an updated picture of human CTLH complex topology. It uncovered the positioning of multiple candidate substrate receptors near the RING domains, making the overall architecture of the human complex more complicated than the current model of the yeast complex.

Analysis of RMND5A and MAEA RING domain primary sequence and predicted structures showed similarities but also clear deviations from the canonical RING. In RMND5A, we identified C336, C354, C374 as residues critical for control of muskelin protein levels. In the predicted structure, C354 and C374 are clearly part of the same zinc coordination site, while the requirement of C336 is surprising since it is not expected to coordinate zinc. The results imply, however, that C336 is needed for RMND5A activity and likely does have a structure stabilizing role. The C354 residue was required for RMND5A regulation of AMPK, and its equivalent residue in other species (X. laevis and S. cerevisiae) is also necessary for E3 ligase activity. Mutation of the zinc coordinating cysteines likely disrupt fold of the domain and would have wide-ranging effects. Therefore, experiments using these mutants to define ubiquitination targets of the CTLH complex should be interpreted with caution. The mutation of the E2 binding site (I338, L339) or the linchpin (Y376) we defined here is more indicative of a ubiquitin transfer mechanism. In yeast RING domains, the predicted linchpin is most frequently an arginine, but tyrosine is also observed (any amino acids with hydrogen bond donor potential can act as linchpins). We did not observe an effect of the R340 mutation which corresponds to the other linchpin identified in Gid2, K365. Besides the different
amino acid, this could be explained by structural differences between human and yeast MAEA, RMND5A, and/or UBE2H, or it could be that a different E2 is used for muskelin ubiquitination. In the 3D predicted structure of human RMND5A (Figure 3.1B), both R340 and Y376 are positioned near I338 and L339 residues and the side chains project into a space presumably occupied by the E2–Ub conjugate. Since muskelin levels were slightly affected in cells transfected with the Y376A mutant, it remains possible that both R340 and Y376 contribute or otherwise compensate for each other. Thus, a R340A/Y376A double mutation should be tested and compared before ruling out R340 as a linchpin for human RMND5A.

In comparison to several other RING domains, MAEA has an insertion between the first helix and the beta strand which contains several cysteines and a histidine. One possibility is that in the presence of Zinc, this structure may shift such that residues in the insertion (H332 and C333) could form a coordination site with the first two cysteines (C314 and C317). In support of this, mutation of a residue in yeast Gid9 that is equivalent to C314 abolishes Fbp1, Pck1, and Mdh2 polyubiquitination and degradation in vivo\textsuperscript{15}. Alternatively, the insertion may hinder E2 binding, explaining why MAEA was observed to have minimal E3 ligase activity on its own\textsuperscript{17}.

Implicit in the deviation of MAEA and RMND5A from the canonical motif with regards to zinc coordination site #1 cysteines, is the interdependence of the MAEA-RMND5A heterodimer for stability in vivo\textsuperscript{17}. MAEA and RMND5A RING domains may require each other for proper folding in place of a more typical zinc site. Co-crystallization of MAEA and RMND5A proteins at higher resolution than obtained by
Qiao et al., 2020\textsuperscript{13} would be valuable to characterize their RING domains and ascertain exactly how dimerization is mediated – if it is through the RING domains, the LisH domain, and/or other regions. Gid9 in vivo association with Gid2 was not impaired when its LisH or CTLH domain was deleted or if the first cysteine in the RING domain was mutated (presumably unfolding it)\textsuperscript{15}, suggesting that Gid2 and Gid9 may heterodimerize through multiple domains, which is supported by the cryo-EM data\textsuperscript{13}.

In the yeast complex, an inconsistency exists among the literature with regards to the position of Gid5: Menssen et al., 2012\textsuperscript{14} data indicate that Gid9 is required for Gid5 association with Gid1, whereas the cryo-EM structure in Qiao et al., 2020\textsuperscript{13} shows Gid5 interacting with both Gid1 and Gid8 (but not Gid9)\textsuperscript{13,14}. In recent reports of the human CTLH complex\textsuperscript{16,17,44}, diagrams of complex subunits show ARMC8 is linked to the complex through MAEA since this was being based on Menssen et al., 2012\textsuperscript{14}. Our data aligns with Qiao et al., 2020\textsuperscript{13} in that ARMC8 is linked through TWA1, not MAEA. Both Menssen et al., 2012\textsuperscript{14} and Qiao et al., 2020\textsuperscript{13} do demonstrate that Gid4 binds to the complex via interaction with Gid5, which was confirmed for the human complex\textsuperscript{16}.

Specifically, the Qiao et al., 2020\textsuperscript{13} structure shows the conserved C-terminus of Gid4 extends into a channel of Gid5 and an extended conserved region N-terminal to the first beta strand of Gid4 wraps around the outside surface of Gid5. In this arrangement, the Gid4 peptide binding β-barrel is accessible as is its unique inserted helices of currently unknown function\textsuperscript{12,13}. It is likely that the specifics of the interaction with Gid4 and Gid5 is similar for the human complex, yet differences between Gid5 and ARMC\textsuperscript{8,23} and the co-existence of α and β isoforms in the complex prevent definitive interpretations.
Muskelin and WDR26 co-exist in the human complex, which contrasts with the initial presumption that Muskelin replaces Gid7/WDR26\textsuperscript{16,17,20}. Yeast-two hybrid screens have demonstrated direct binding between muskelin and RanBPM, with multiple domains of both RanBPM and muskelin being required for their \textit{in vivo} interaction\textsuperscript{36,37}. For muskelin, the last 35 C-terminal residues and the CTLH domain are required for association with RanBPM\textsuperscript{36}. The interaction between WDR26 and RanBPM has not been investigated in detail. Given their co-existence, the exact positions of Muskelin and WDR26 within the complex requires clarification at high resolution as to the contacts made between those proteins and RanBPM. Given the likelihood that muskelin, WDR26, and RanBPM serve as important substrate receptors for the complex, knowledge of their arrangements and structures is imperative towards envisioning substrate engagement for ubiquitin transfer. A hint establishing muskelin as a substrate receptor is its requirement for CTLH complex binding to at least one of its target substrates (ME31B) in \textit{Drosophila melanogaster}\textsuperscript{45}.

While the structures provided for the yeast complex have produced a good foundation to study the human complex, key differences as observed in this report and others are clear. Firstly, the existence of TWA1 as a dimer in the human complex\textsuperscript{16,22}, which we did not focus on here, presents one important variance. The inclusion of muskelin (no yeast homologue) as a likely substrate receptor is an interesting element and so is its autoregulation by the CTLH complex that we previously reported\textsuperscript{17}. A current complication is the role of the paralogues of RanBPM (RanBP10) and RMND5A (RMND5B), which at present has not been addressed in detail. In comparison to the yeast
structure, we found that ARMC8 isoforms required RanBPM CTLH and CRA domains, not the LisH domain, for complex formation. This suggests that ARMC8 association with TWA1-RanBPM in the human complex deviates from the yeast proteins. Differences also exist between the yeast complex and other species. The drosophila complex does not contain Gid4 or Gid5 and corresponding loops and contacts to these proteins by Gid1 and Gid8 as determined by the cryo-EM structure are also not present in the predicted structures of the drosophila proteins\textsuperscript{45}. Furthermore, Rmnd5 from \textit{X. laevis} expressed in \textit{S. cerevisiae} cannot complement the \textit{gid2Δ} phenotype, and also cannot bind to the Gid complex\textsuperscript{35}.

Multi-subunit E3 ligases present fascinating examples of E3 ligase mechanisms and regulation. CRLs have a diverse range of substrates owing to interchangeable substrate receptors and multiple points of regulation. The overall theme of the CTLH topology resembles that of a CRL, with a scaffold linking a RING domain to a substrate receptor. In the case of the topology and orientation of the complex that we determined in this report, multiple subunits have domains in proximity to the RING module that could conceivably act as substrate receptors. The LisH, CTLH, and CRA domains of each subunit are mostly likely packed in the core of the complex, although it would be of interest to determine if, like in TBL1, TPL and TPR2, grooves between the domains can interact with specific proteins\textsuperscript{19,46,47}. Another unique aspect of the CTLH complex as a multi-subunit complex is the RING heterodimer. Our analysis provided clues of how the two domains cooperate but capturing of a high-resolution structure of RMND5A-MAEA
bound to an E2–Ub complex would provide important insight into CTLH complex mechanisms.

In this report, different techniques were used to depict a more accurate and complete model of the human CTLH complex. The final model (Figure 3.7) shows an overall similarity to the yeast clamp-like structure\textsuperscript{13}, but instead of just the Gid4 β-barrel facing the RING domains, the RanBPM SPRY domain, muskelin kelch repeats, and WDR26 WD40 repeats also do. This helps rationalize how the different subunits of the CTLH complex could each contribute towards substrate recruitment.

3.4 Methods
3.4.1 Cell culture

Generation of KO HeLa and HEK293 cell lines have been described previously\textsuperscript{17,24}. All cells were cultured in high glucose (4.5g/L) Dulbecco’s modified Eagle’s medium (Wisent Bioproducts, St. Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% L-glutamine (Wisent Bioproducts) at 37°C and 5% CO2. Plasmid transfections were carried out with jetPRIME (Polypus Transfection) according to the manufacturer’s protocol.

3.4.2 Cloning

RanBPM domain deletions have been described previously\textsuperscript{25,26}. Mutations of RMND5A were generated via site directed mutagenesis using primers (Integrated DNA Technologies) listed below with KOD polymerase (Novagen) and pCGN-HA-RMND5A (described in Maitland et al\textsuperscript{17}) as the template. The entire cDNA sequence was validated by Sanger sequencing.
Table 3.1. List of primers used for mutagenesis of RMND5A RING domain

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Fwd.</th>
<th>Rev.</th>
</tr>
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<tbody>
<tr>
<td>C336S</td>
<td>CACTCTATATTTGCCCTCCCATTTCTTCGTCAG</td>
<td>CTGACGAAGAATGGGGAGGCAATATAGAGTG</td>
</tr>
<tr>
<td>I338A/L 339A</td>
<td>GTATCACTCTATATTTGCCCTCCCGCTGTCCATC</td>
<td>ATTTGTTATCTGTGTTTTGCTGACGAGCGGGCA</td>
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<tr>
<td></td>
<td>AGCAAAACACAGATAACCAT</td>
<td>GGCAATATAGATGTGATAC</td>
</tr>
<tr>
<td>R340A</td>
<td>GCCCTGCCCATTCTTTGCTCAGAAAACAACAG</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>GGTAGCAAAATTTATGTCCCCGTCCAATG</td>
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<td>AATTGTGCTACC</td>
</tr>
<tr>
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<td>GAAGTTCACGCAGCTATCTGTGTTGGCATCTC</td>
</tr>
<tr>
<td></td>
<td>CTGAGAACCTTC</td>
<td>CTG</td>
</tr>
</tbody>
</table>

3.4.3 Multiple sequence alignment and prediction of protein structure

The indicated protein sequences were obtained from Uniprot\(^{27}\) and aligned using MAFFT online service (version 7)\(^{28}\) then imported into Jalview (version 2.11.1.3)\(^{29}\), coloured using Zappo and sorted by pairwise identity. Protein structure prediction was performed using Phryre2 web portal\(^{30}\) with default settings and analyzed using Pymol.

3.4.4 Extract preparation and immunoprecipitations for Western blot

Cells were lysed in whole cell extract buffer with fresh inhibitors (50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 0.5% NP40, 10 mM DTT, 1 mM
Na3VO4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of aprotinin, 10 μg/ml of pepstatin, and 1 μg/ml of leupeptin) on ice for 20 minutes, then centrifuged for 20 minutes 13,000 rpm at 4 °C. Protein concentration was estimated by Bio-Rad protein assay dye (#5000006) or Pierce 660 nm protein assay (Thermo, #22660). For immunoprecipitations western blots and RMND5A cysteine mutations, protein was loaded onto a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to Polyvinylidene difluoride (PVDF) membrane, hybridized and assessed using chemiluminescence as described previously. For RMND5A non-cysteine mutations, Immobilon-FL PVDF membrane (Millipore, #IPFL00005) was used and the blot was blocked for one hour and hybridized with primary antibody (either muskelin or HA) overnight in 0.5% fish gelatin (Sigma, G7041) in TBST (0.05% tween), and the secondary incubation (Goat anti mouse IgG:Dylight 800, BIO-RAD, STAR74D800GA) contained 0.5% tween and 0.1% SDS with Tubulin-rhodamine (BIO-RAD, #12004166) for loading control. Primary antibodies used in any experiments of this study are: ARMC8 (E-1, sc-365307; Santa Cruz Biotechnology); FLAG (M2, F1804, Sigma-Aldrich); HA (HA-7, H3663 Sigma-Aldrich); MAEA (AF7288, R&D Systems); Muskelin (C-12, sc-398956, Santa Cruz Biotechnology); RanBPM (5M, 71-001, Bio academia); RMND5A (NBP1-92337, Novus Biologicals); TWA1 (NBP1-32596, Novus Biologicals); Vinculin (E1E9V, Cell Signaling Technology); and WDR26 (ab85962, Abcam).

For RanBPM co-immunoprecipitation (co-IP) experiments in figure 3.3, 4 micrograms of mouse IgG (sc-2025, Santa Cruz Biotechnology) or RanBPM (F-1, sc-
271727, Santa Cruz Biotechnology) antibody was conjugated to 15 μL Dynabeads Protein G (10004D, Invitrogen) for 1 hour at 4°C with end-over-end rotation. Afterwards, antibody-bead conjugate was washed three times in 100 mM sodium Borate, pH 9 (M5162, Sigma-Aldrich), then resuspended in 20 mM DMP (Dimethyl pimelimidate dihydrochloride, D8388, Sigma-Aldrich) in 100 mM sodium Borate, pH 9 and rotated at room temperature for 30 minutes. Crosslinking was quenched by washing and incubation in 200 mM ethanolamine, pH 8 (S9640, Sigma-Aldrich) for 2 hours at 4°C with end-over-end rotation. After blocking, beads were washed three times in 1 mL whole cell extract buffer and kept on ice. A volume corresponding to 1 mg of protein was adjusted to 0.25% NP40 and precleared with 3 μL Dynabeads Protein G for 30 minutes at 4°C with end-over-end rotation. Precleared extract was then added to the crosslinked antibody-beads and incubated overnight at 4°C with end-over-end rotation. For HA (RanBPM mutants) IPs, extracts were adjusted to 0.25% NP-40, pre-cleared and rotated overnight at 4°C with HA (HA-7, H9658, Sigma-Aldrich) cross-linked to Dynabeads protein G as described above. For FLAG-muskelin IP, extracts were adjusted to 0.25% NP-40, pre-cleared and rotated overnight at 4°C with FLAG (M2, F1804, Sigma-Aldrich) then incubated for 1 hour at 4°C with Dynabeads protein G. In all IP types, beads were washed three times in wash IP buffer (50 mM HEPES, pH7.4, 150 mM NaCl, 10 mM EDTA, 12% glycerol, 0.25% NP40) and resuspended in SDS loading buffer and boiled at 95°C for 10 minutes to elute proteins.

3.4.5 Immunoprecipitation and sample prep for Cross-linking Mass spectrometry
For cross-linking MS RanBPM immunoprecipitations, exponentially growing HEK293 cells were lysed in fresh BS3 lysis buffer with inhibitors (50 mM HEPES, pH 7.8, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% triton, 0.5% NP40, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, 1 μg/ml of aprotinin, 10 μg/ml of pepstatin, and 1 μg/ml of leupeptin) on ice for 20 minutes, then centrifuged for 20 minutes 13,000 rpm at 4 °C. Protein concentration was estimated by Pierce 660 nm protein assay (Thermo, #22660). Seven micrograms of RanBPM (F-1, sc-271727, Santa Cruz Biotechnology) antibody was conjugated to 30 µL Dynabeads Protein G (10004D, Invitrogen) for 1 hour at 4°C and then crosslinked with DMP exactly as described above. A volume corresponding to 5 mg of HEK293 protein was precleared with 3 µL Dynabeads Protein G for 30 minutes at 4°C with end-over-end rotation. Precleared extract was then added to the crosslinked antibody-beads and incubated overnight at 4°C with end-over-end rotation. Sixteen hours later, beads washed in stringent wash buffer five times (50 mM HEPES, pH 7.8, 1 M NaCl, 1 mM EDTA, 10% glycerol, 0.5% tween, 0.5% triton, and 1% NP40, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, 1 μg/ml of aprotinin, 10 μg/ml of pepstatin, and 1 μg/ml of leupeptin), two times with 20 mM HEPES, pH 7.8 with 1% NP40, then two times in 20 mM, HEPES, pH 7.8. Beads were then resuspended in 100 uL of 1 mM BS3 (Thermo, A3926; diluted in 20 mM, HEPES, pH 7.8) and incubated for 1hr at 23°C shaking at 1250 rpm in a thermomixer (Eppendorf, cat# 2231000667). Supernatant was removed, washed in 100 mM ammonium bicarbonate, then incubated in 100 mM ammonium bicarbonate for 10 minutes at 23°C shaking at 1250 rpm in a thermomixer to quench the cross-linker. Beads were then washed five times in HPLC
grade water and cross-linked proteins were eluted with four rounds of 5-minute incubations in 0.5% formic acid (FA), 30% acetonitrile with 1250 rpm shaking at 23°C on a thermomixer. Pooled elutions were spun down briefly to collect any residual beads, transferred to a new tube and dried in a SpeedVac (Thermo Scientific). Dried protein was resuspended in 6 M Urea and incubated in 10 mM DTT for 30 minutes, followed by 100 mM iodoacetamide for 25 minutes in the dark, and then methanol precipitated as described in Kuljanin et al., 2017\textsuperscript{31}. Protein pellet was resuspended in 200 µL of 50 mM ammonium bicarbonate (ABC) and subjected to a sequential digest first with 250 ng of LysC (125-05061, Wako Chemicals, USA) for 4 hours, then 500 ng of Trypsin/LysC (V5071, Promega) for 16 hours, followed by 500 ng of Trypsin (V5111, Promega) for an additional 4 hours. Digestions were incubated at 37 °C at 600 rpm with interval mixing (30 seconds mix, 2 minutes pause) on a Thermomixer C (Eppendorf, cat# 2231000667). After the last digestion, samples were acidified with 10% FA to pH 3-4 and centrifuged at 14,000 xg to pellet insoluble material. Supernatant was filtered by passing through a > 10 kDa cellulose membrane (UFC501096, Sigma-Aldrich) and then dried in a SpeedVac. Dried peptides were resuspended in 0.1% Trifluoroacetic acid (TFA), desalted with C18 Stage tips and then fractionated with strong cation exchange (SXC)-stage tip as described in Chen and Rappsilber, 2019 using increasing concentrations of ammonium acetate\textsuperscript{32}. Next, the eluted peptides were dried in a SpeedVac, an equivalent volume of water was added, and then peptides dried again. Peptides were resuspended in 20 µL 0.1% FA and 500 ng of each fraction as quantified by BCA assay was injected onto a Waters M-Class nanoAcquity HPLC system (Waters) coupled to an ESI Orbitrap mass spectrometer (Q
Exactive plus) (ThermoFisher Scientific). Buffer A consisted of mass spectrometry grade water with 0.1% FA and buffer B consisted of acetonitrile with 0.1% FA (ThermoFisher Scientific). All samples were trapped for 5 min at a flow rate of 5 uL/min using 99% buffer A and 1% buffer B on a Symmetry BEH C18 Trapping Column (5 mm, 180 mm x 20 mm, Waters). Peptides were separated using a Peptide BEH C18 Column (130 Å, 1.7 mm, 75 mm x 250 mm) operating at a flow rate of 300 nL/min at 35°C (Waters). Samples were separated using a non-linear gradient consisting of 1%–7% buffer B over 1 min, 7%–23% buffer B over 59 min and 23%–35% buffer b over 20 min, before increasing to 98% buffer B and washing. MS acquisition settings are provided in Appendix C.

3.4.6 Identification and analysis of cross-linked peptides

Thermo RAW files with were converted to mgf format using MSConvert and analyzed using xiSEARCH (version 1.7.6.1). Peptides were searched against a database containing human CTLH complex subunits (RANBP9 (Q96S59), RANBP10 (Q6VN20), RMND5A (Q9H871), MAEA (Q7L5Y9), ARMC8α (Q8IUR7), ARMC8β (Q8IUR7-6) GID8 (Q9NWU2), GID4 (Q8IVV7), MKLN1 (Q9UL63), WDR26 (Q9H7D7). BS3 (small scale) was selected as the cross linker MS1 and MS2 mass tolerance was set to 10 ppm and 20 ppm, respectively. Enzyme was set to trypsin with a maximum of 4 missed cleavages. Cysteine carbamidomethylation was included as a fixed modification, and methionine oxidation and BS3 with the other end amidated or hydrolyzed methionine oxidation was included as variable modifications. FDR was assessed and automatically filtered using xiFDR (version 2.1.5.2) on the 5% link level and other settings were left
at default. Links passing the FDR threshold (5%) in two out of three replicates were manually inspected and retained for downstream analysis in xiNET\textsuperscript{34}.

### 3.4.7 Statistics

Statistical analyses were performed using GraphPad PRISM (version 9.0.1). Differences between two groups were compared using unpaired two-tailed t test. Results were considered significant when \( P < 0.05 \).

### 3.5 References

13. Qiao, S. *et al.* Interconversion between Anticipatory and Active GID E3 Ubiquitin
Ligase Conformations via Metabolically Driven Substrate Receptor Assembly. 


Chapter 4

4 Proteomic-based identification of ubiquitination substrates reveals the CTLH E3 ligase complex regulates glycolysis

This chapter constitutes a submitted manuscript.

4.1 Introduction

Metabolism is regulated at multiple levels to maintain homeostasis\(^1\). In cancer cells, however, increased cell proliferation necessitates a high demand for cellular energetics and biosynthetic building blocks\(^2\). To this end, a hallmark of cancer cells is the reprogramming of their metabolism for high glycolytic flux even under anaerobic conditions, a characteristic termed the Warburg effect\(^3\).

Glycolysis proceeds in the cytosol as a series of enzymatic reactions that convert glucose into pyruvate, ATP, and NADH. In a key step of the pathway, pyruvate kinase (PK) converts phosphoenolpyruvate (PEP) to pyruvate. Pyruvate is then converted into either lactate by lactate dehydrogenase (LDH) or transported into the mitochondria and fed into the tricarboxylic acid (TCA) cycle. Additionally, intermediate metabolites of glycolysis can be used as precursors for anabolic pathways, such as the pentose phosphate pathway, glycogen synthesis, fatty acid synthesis, and amino acid synthesis\(^2\).

In *Saccharomyces cerevisiae*, gluconeogenesis, which, in overall effect, is the reversal of glycolysis, is regulated by the glucose-induced degradation deficient (Gid) complex, a multi-subunit RING E3 ligase\(^4,5\). RING E3 ligases confer substrate specificity in the direct transfer of ubiquitin from an E2 conjugating enzyme to a substrate.
Ubiquitin-protein homolog), armadillo (glucose-induced cells demonstrated termed glucose-dependent degradation, enzymes fructose-1,6-bisphosphatase (Fbp1), isocitrate lyase (Icl1), malate dehydrogenase (Mdh2), and phosphoenolpyruvate carboxykinase (Pck1), leading to their proteasomal degradation. Ubiquitination of these enzymes by the Gid complex is triggered by the glucose-dependent induction and association of subunit Gid4, which recognizes the N-terminal prolines of the gluconeogenic enzymes. Recently, cryo-EM structural determination of the Gid complex revealed that it exists in an anticipatory confirmation that can switch to its active conformation, enabling rapid regulation of glucose metabolism in response to stress conditions.

The Gid complex is evolutionarily conserved and its mammalian homologue is termed the C-terminal to LisH (CTLH) complex. We and others have recently demonstrated that the mammalian CTLH complex exhibits E3 ligase activity in human cells and has at least 8 subunits: RanBPM (Ran binding protein M, aka RanBP9), TWA1 (glucose-induced degradation protein 8 homolog, aka GID8), ARMC8 (α and β isoforms; armadillo repeat-containing protein 8), GID4 (glucose-induced degradation protein 4 homolog), MKLN1 (muskelin), WDR26 (WD repeat-containing protein 26), and the RING domain proteins, which are required for its E3 ligase activity, RMND5A (E3 ubiquitin-protein transferase RMND5A) and MAEA (E3 ubiquitin-protein transferase
MAEA)\textsuperscript{11–13}. A fascinating and conserved feature of the CTLH complex is the shared presence and order of appearance of a lissencephaly type-1-like homology (LisH), a C-terminal to LisH motif (CTLH), and CT11-RanBPM (CRA) domains present in RanBPM, TWA1, RMND5A, and MAEA, with muskelin and WDR26 also containing LisH and CTLH domains\textsuperscript{10,11}. Additionally, discoidin, SPRY (named from SP\textit{l}a and the RY\textit{anodine Receptor}), kelch repeats, WD40 repeats and armadillo (ARM) repeats comprise the variety of protein-protein interaction domains on CTLH complex subunits.

A functional understanding of the mammalian CTLH complex is still limited but recent work suggests its ubiquitination targets, or proteins it regulates, include HBP1 (HMG box-containing protein 1)\textsuperscript{13}, LMNB2 (lamin B2)\textsuperscript{14}, PRKAA (5'-AMP-activated protein kinase catalytic subunit alpha-1, aka catalytic subunit of AMP-activated protein kinase (AMPK))\textsuperscript{15}, c-Raf (RAF proto-oncogene serine/threonine-protein kinase, aka RAF1)\textsuperscript{16}, HDAC6 (histone deacetylase 6)\textsuperscript{17}, and its own subunit muskelin\textsuperscript{12}. In the cancer context, individual subunits of the complex have been found to be either growth promoting or suppressive\textsuperscript{13,16,18,19}, which is likely dependent on cell type and contextual determinants. Positive or negative regulation of several oncogenic pathways (e.g. MAPK/ERK, WNT, TGFβ, NFκB, cell cycle) have been linked to individual subunit regulations, although, in almost all cases, whether the regulation involves the ubiquitin activity of the complex was not investigated\textsuperscript{9,19,20}.

Here, we integrated three mass spectrometry-based proteomic techniques to uncover candidate ubiquitination targets and novel functions of the mammalian CTLH complex in HeLa cells. Using global and diglycine (diGLY)-enriched (ubiquitinome)
label-free proteomics and affinity-purification mass spectrometry using a RanBPM antibody, we identified several candidate ubiquitination targets, including four glycolytic enzymes. We show that the complex regulates ubiquitination and activity levels of PKM2 (pyruvate kinase PKM) and LDHA (L-lactate dehydrogenase A chain) glycolysis enzymes and functions to inhibit glycolytic flux and prevent altered metabolism. These results indicate that, like in yeast, the mammalian CTLH complex regulates glucose metabolism, albeit in a different manner, and reveal a novel multi-level post-translational negative regulation of glycolysis.

4.2 Results

To comprehensively identify candidate ubiquitination substrates of the CTLH complex, we used both mass spectrometry-based quantitative global and diglycine (diGLY) -enriched proteomics in various CTLH knockdown/knockout cells in conjunction with the identification of associated proteins of the endogenous complex by affinity purification mass spectrometry (Figure 4.1). The approach was to compare these different screens to confidently identify ubiquitination targets of the complex. For example, a protein that shows an association with the CTLH complex with an increase in protein abundance and/or a decrease in ubiquitination in the knockout/knockdown cell lines would represent a candidate ubiquitination target of the complex.

4.2.1 RanBPM-dependent global proteome

First, we assessed RanBPM-dependent changes in protein abundance by comparing the global proteomes of shControl and shRanBPM HeLa cells using label-free
Figure 4.1. Strategy to identify CTLH complex ubiquitination substrates in HeLa cells. Ubiquitination substrates of the CTLH complex could have: 1. Left, increased abundance in shRanBPM, RMND5A KO, and/or MAEA KO cells as determined by global proteomics; 2. Middle, decreased ubiquitination (as assessed by enrichment of the diGLY remnant on tryptic peptides originating from ubiquitinated lysine) in shRanBPM and/or RMND5A KO cells treated with protease inhibitor MG132, or 3. Right, Associate with the complex as determined by affinity purification mass spectrometry of endogenous RanBPM. In all cases, HeLa cells are used, and peptide analysis is done using liquid chromatography tandem mass spectrometry (LC-MS/MS) and searched and label-free quantified with MaxQuant software.
quantification mass spectrometry. After filtering, a total of 4835 proteins were used for downstream analysis. Sixty-one proteins were significantly increased (p<0.05, log2 shRanBPM/shControl fold change >1.0) and 38 were significantly decreased (p<0.05, log2 shRanBPM/shControl fold change <-1.0) in shRanBPM cells compared to the shControl cells (Figure 4.2A-D). This included RanBPM itself and previously reported protein level changes, such as a decrease in TWA1/GID8\(^{12}\) (Chapter 2). We analyzed the list of RanBPM-dependent increased and decreased proteins for statistical enrichment of gene ontology (GO) terms and pathways. Proteins related to Lipoprotein particle binding, axon and dendrite, amino acid binding, and dopamine receptor binding, among others, were enriched amongst the increased proteins (Figure 4.2E, top). For decreased proteins, several proteins associated with molecular transducer/signaling receptors, inflammatory response pathway, and cardiac progenitor differentiation were included (Figure 4.2E, bottom).

We compared the changes at the proteome level to a previously conducted microarray analysis which was also done using the same shRanBPM HeLa cells\(^{32}\). Proteome versus RNA changes showed a relatively weak correlation (R\(^2\) = 0.301) (Figure 4.3). The candidates that showed the greatest changes (either increasing or decreasing) at the protein level, however, also showed large differences at the RNA level (e.g., THY1 (Thy-1 membrane glycoprotein), ALPL (alkaline phosphatase, tissue-nonspecific isozyme), BCAT1 (branched-chain-amino-acid aminotransferase, cytosolic), OAS3 (2'-5'-oligoadenylate synthase 3), and CRYAB (alpha-crystallin B chain)), indicating that the
**Figure 4.2. The RanBPM-dependent proteome.** Protein extracts of shControl and shRanBPM HeLa cells were subjected to trypsin/LysC digestion and analyzed and quantified using LC-MS/MS. A) The number of proteins quantified in each replicate of shControl or shRanBPM HeLa cells and the number of quantified proteins in total across all samples (dashed line). Error bars represent standard deviation (SD). B) Pearson correlation coefficients between the indicated biological replicates for shControl samples (blue) or shRanBPM samples (orange). C) Heatmap clustering representation of quantified significantly changed proteins (p<0.05) across replicates. D) Volcano plot showing distribution of log2 fold change (x-axis) and –log10 p value (y-axis) of proteins quantified by label-free methods in shControl and shRanBPM HeLa cells, n=5. Blue dots represent proteins significantly decreased (p<0.05, log2 fold change <-1.0), while orange dots represent proteins significantly increased (p<0.05, log2 fold change >1.0). Other proteins are coloured in grey. Significantly changed proteins with separation in the plot or noted in the text are labelled with their gene name. E) Gene ontology terms and pathways significantly enriched (adjusted p <0.05) as determined by g:profiler (see methods) in either RanBPM-dependent significantly increased proteins (top) or decreased proteins (bottom). Terms (nodes) were clustered and grouped (yellow circles) based on their similarity coefficient. Size of the node reflects size of the term, and colour reflects Q-value (FDR) ranging from orange (0.05) to white (0).
Figure 4.3. Comparison of shRanBPM proteome versus previous microarray data. Proteins quantified in the RanBPM-dependent proteome (y-axis) were matched with their RNA change in Atabakhsh et al., 2012 (x-axis). Dots are coloured according to the corresponding p-value in the proteome (legend at bottom).
levels of these proteins were changed due to transcriptional effects rather than post-translational regulations. Therefore, we made note of several proteins that were found significantly increased in the proteome but not at the RNA level in shRanBPM cells since some of these are presumably regulated by ubiquitination resulting in proteasomal degradation (top 15 listed in Table 4.1). LRP8 (low-density lipoprotein receptor-related protein 8) had the greatest protein fold change with a minimal change in RNA levels.

4.2.2 RMND5A and MAEA-dependent global proteomes

We also scored the proteome in knockout cells of RMND5A and MAEA, the two RING CTLH complex subunits. For RMND5A KO cells, 4726 quantified and filtered proteins were used for downstream analysis, including 108 significantly decreased (p<0.05, log2 RMND5A KO/WT fold change <-1.0) and 78 increased (p<0.05, log2 RMND5A KO/WT fold change >1.0) (Figure 4.4A-D). As we previously reported\textsuperscript{12} (chapter 2), protein levels of CTLH subunit muskelin (MKLN1) were significantly increased. EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1), CNN1 (calponin-1), BCAT1, CD63 (CD63 antigen), and NUDT4 (diphosphoinositol polyphosphate phosphohydrolase 2) represented some of the most significantly increased proteins while CPS1 (carbamoyl-phosphate synthase [ammonia], mitochondrial), ALPL, KCTD12 (BTB/POZ domain-containing protein KCTD12), and aldo-ketose reductases (AKR1C3, AKR1C2) were some of the most significantly decreased proteins (Figure 4.4D). Several GO terms and pathways were enriched for both increased and decreased proteins lists (Figure 4.4E). For RMND5A-dependent increased proteins, vasculature and eye development, wound healing, cell adhesion and migration, extracellular
Table 4.1. Proteins increased in RanBPM-dependent proteome that do not have a corresponding change in RNA levels. The top 15 significantly increased proteins where the proteome change is at least 5-fold greater than RNA level change (Atabakhsh et al, 2012 microarray dataset) in shControl vs shRanBPM HeLa cells are listed.

<table>
<thead>
<tr>
<th>GENE SYMBOL</th>
<th>PROTEIN NAME</th>
<th>RNA FOLD CHANGE (LOG2)</th>
<th>PROTEIN FOLD CHANGE (LOG2)</th>
</tr>
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<tbody>
<tr>
<td>LRP8</td>
<td>Low-density lipoprotein receptor-related protein 8</td>
<td>0.12</td>
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<td>Mitochondrial basic amino acids transporter</td>
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<td>Actin-binding LIM protein 1</td>
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<td>Cysteine-rich protein 1</td>
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<tr>
<td>SLC9A3R1</td>
<td>Na(+)/H(+) exchange regulatory cofactor NHE-RF1</td>
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<tr>
<td>VLDLR</td>
<td>Very low-density lipoprotein receptor</td>
<td>0.16</td>
<td>1.10</td>
</tr>
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</table>
Figure 4.4. The RMND5A-dependent proteome. Protein extracts of Control and RMND5A KO HeLa cells were subjected to trypsin/LysC digestion and analyzed and quantified using LC-MS/MS A) The number of proteins quantified in each replicate of Control or RMND5A KO HeLa cells and the number of quantified proteins in total across all samples (dashed line). Error bars represent SD. B) Pearson correlation coefficients between the indicated biological replicates for Control samples (blue) or RMND5A KO samples (orange). C) Heatmap clustering representation of quantified significantly changed proteins (p<0.05) across replicates. D) Volcano plot showing distribution of log2 fold change (x-axis) and –log10 p value (y-axis) of proteins quantified by label-free methods in Control and RMND5A KO HeLa cells, n=4. Blue dots represent proteins significantly decreased (p<0.05, log2 fold change <-1.0), while orange dots represent proteins significantly increased (p<0.05, log2 fold change >1.0). Other proteins are coloured in grey. Significantly changed proteins with separation in the plot or noted in the text are labelled with their gene name. E) Gene ontology terms and pathways significantly enriched (adjusted p <0.05) as determined by g:profiler (see methods) in either RMND5A-dependent significantly increased proteins (top) or decreased proteins (bottom). Terms (nodes) were clustered and grouped (yellow circles) based on their similarity coefficient. Size of the node reflects size of the term, and colour reflects Q-value (FDR) ranging from orange (0.05) to white (0).
matrix/collagen, and MET signaling were pathways enriched (Figure 4.4E, top). Circulatory system, autophagosome membrane, and NRF2 signaling are examples of biological themes enriched in the RMND5A-dependent decreased proteins (Figure 4.4E, bottom).

In the global proteomic screen with MAEA knockouts, 4738 quantified proteins were used in analysis after filtering. Of these, 94 were significantly decreased (p<0.05, log2 MAEA KO/WT fold change <-1.0) and 71 were increased (p<0.05, log2 MAEA KO/WT fold change >1.0) (Figure 4.5A-D). Muskelin (MKLN1) was the most increased protein (Figure 4.5D). GDA (guanine deaminase), ZNFX1 (NFX1-type zinc finger-containing protein 1), NUDT16 (U8 snoRNA-decapping enzyme), FAM129A (protein Niban 1), PAGE1 (P antigen family member 1), and EPHB2 (rphrin type-B receptor 2) are other examples of MAEA-dependent increased proteins (Figure 4.5D). Interestingly, the same Aldo-keto reductases (AKR1C2, AKR1C3) that were found decreased in RMND5A KO cells were also decreased in the MAEA KO cells (Figure 4.4D and 4.5D). Other decreased proteins included KCTD1, SNCG (gamma-synuclein), VAMP8 (vesicle-associated membrane protein 8), CDKN2A (cyclin-dependent kinase inhibitor 2A), L1CAM (neural cell adhesion molecule L1), and TNS4 (tensin-4) (Figure 4.5D). Hydrolase and deaminase activity, collagen binding, Kinase activity, and ERK1/2 cascade were among the GO terms enriched within increased proteins (Figure 4.5E, top). Numerous GO terms and pathways were enriched in the decreased proteins, including plasma membrane, aldo-ketose reductases, GPCR signaling, and ion homeostasis (Figure 4.5E, bottom).
**Figure 4.5. The MAEA-dependent proteome.** Protein extracts of Control and MAEA KO HeLa cells were subjected to trypsin/LysC digestion and analyzed and quantified using LC-MS/MS. A) The number of proteins quantified in each replicate of Control or MAEA KO HeLa cells and the number of quantified proteins in total across all samples (dashed line). Error bars represent SD. B) Pearson correlation coefficients between the indicated biological replicates for Control samples (blue) or MAEA KO samples (orange). C) Heatmap clustering representation of quantified significantly changed proteins (p<0.05) across replicates. D) Volcano plot showing distribution of log2 fold change (x-axis) and –log10 p value (y-axis) of proteins quantified by label-free methods in Control and MAEA KO HeLa cells, n=4. Blue dots represent proteins significantly decreased (p<0.05, log2 fold change < -1.0), while orange dots represent proteins significantly increased (p<0.05, log2 fold change > 1.0). Other proteins are coloured in grey. Significantly changed proteins with separation in the plot or noted in the text are labelled with their gene name. E) Gene ontology terms and pathways significantly enriched (adjusted p < 0.05) as determined by g:profiler (see methods) in either MAEA-dependent significantly increased proteins (top) or decreased proteins (bottom). Terms (nodes) were clustered and grouped (yellow circles) based on their similarity coefficient. Size of the node reflects size of the term, and colour reflects Q-value (FDR) ranging from orange (0.05) to white (0).
Figure 4.6. Comparison of MAEA KO, RMND5A KO, and shRanBPM HeLa proteomes. Overlap of proteins in the different proteomes if p<0.05 and log2 fold change <-0.5849 (top) or >0.5849 (bottom).
4.2.3 Commonalities in the global proteomic screens

We compared the lists of increased and decreased proteins from each cell line that had a log2 KO/WT fold change > 0.5840 or < -0.5840 (Figure 4.6). MAEA KO and RMND5A KO showed an overlap of 79 decreased proteins and 36 increased proteins. Much less overlap (≤10) was found between shRanBPM and either MAEA or RMND5A KO for increased or decreased proteins. The most decreased proteins in shRanBPM cells were also decreased in either MAEA KO or RMND5A KO cells. ALPL, a very decreased protein in shRanBPM cells (log2 shRanBPM/shControl =−3.50) also showed a marked decrease in RMND5A KO cells (log2 RMND5A KO/WT=−3.26). Likewise, THY1 was the most decreased protein in shRanBPM cells (log2 shRanBPM/shControl =−4.62) and was also very reduced in MAEA KO cells (log2 MAEA KO/WT=−1.89). Interestingly, both THY1 and ALPL are significantly decreased in their RNA levels in shRanBPM cells (Figure 4.3) suggesting that their change of expression is due to a transcriptional regulation. Overall, 2 decreased proteins were common in all three screens (NAPRT (nicotinate phosphoribosyltransferase), SORBS2 (sorbin and SH3 domain containing 2)) and 2 increased proteins were common in all three screens (JAK1 (janus kinase 1) and DMD (dystrophin)). Notably, besides muskelin, two other previously identified RanBPM associated proteins, SRC (proto-oncogene tyrosine-protein kinase Src) and the deubiquitinase USP11 (ubiquitin carboxyl-terminal hydrolase 11) were increased in both RMND5A and MAEA KO cells33,34.

4.2.4 RanBPM and RMND5A-dependent ubiquitinomes
Next, we assessed the RanBPM-dependent ubiquitinome by enriching for the diGLY remnant, which is present on lysines in tryptic peptides originating from ubiquitinated proteins. Extracts prepared from shControl and shRanBPM HeLa cells treated with MG132 were subjected to LysC/trypsin and diGLY peptides were enriched using a specific antibody. Using label-free quantification, we quantified a total of 13429 ubiquitin sites, with 5232 used for downstream analysis after filtering (Figure 4.7A-C). We found 205 ubiquitin sites significantly decreased in shRanBPM cells (p<0.05, log2 shRanBPM/shControl fold change <-1.0), and 129 significantly increased (p<0.05, log2 shRanBPM/shControl fold change >1.0) (Figure 4.7D).

We compared the significant (p<0.05) ubiquitin changes to the corresponding total protein change from the proteome analysis (Figure 4.8A). Here, any ubiquitin sites that changed in the same direction as its corresponding protein in the shRanBPM-dependent proteome more than a log2 shRanBPM/shControl fold change of 0.5849 (black dots in Figure 4.8A), or were not detected in the proteome, were omitted from further analysis. This was done so that changing total protein levels would not confound differences in ubiquitination. Using a diGLY enrichment log2 fold change cut-off at ±0.5849, 88 increased and 234 decreased ubiquitin sites were remaining. Surprisingly, no proteins that increased more than a log2 shRanBPM/shControl fold change of 0.5849 at the protein level had at least a 0.5849 log2 shRanBPM/shControl fold decreased ubiquitin site. GJA1 (gap junction protein alpha 1) and SOAT1 (sterol o-acyltransferase 1) were decreased at least 0.5849 log2 shRanBPM/shControl fold at the protein level and had an increased ubiquitination site. All others were not changed at the protein level. EHD4 (EH
**Figure 4.7. The RanBPM-dependent ubiquitinome.** Protein extracts of exponentially growing MG132-treated (10 uM for 4 hours) shControl and shRanBPM HeLa cells were subjected to trypsin/LysC digestion and peptides were enriched with a diGLY antibody and then analyzed and quantified label-free using LC-MS/MS A) The number of ubiquitin sites quantified in each replicate of shControl or shRanBPM HeLa cells and the number of quantified sites in total across all samples (dashed line). Error bars represent SD. B) Pearson correlation coefficients between the indicated biological replicates for shControl samples (blue) or shRanBPM samples (orange). C) Heatmap clustering representation of significantly changed (p<0.05) diGLY sites across replicates. D) Volcano plot showing distribution of log2 fold change (x-axis) and \(-\log10 \ p \) value (y-axis) of ubiquitin sites quantified in shControl and shRanBPM HeLa cells as determined by t-test (see methods), n=3. Blue dots represent sites significantly decreased (p<0.05, log2 fold change <-1.0), orange dots represent sites significantly increased (p<0.05, log2 fold change >1.0). Other sites are coloured in grey.
Figure 4.8. The RanBPM-dependent ubiquitinome. Related to figure 4.7. A) Proteins with a significant diGLY site (p<0.05) in shRanBPM cells plotted with their log2 fold change (y-axis) against the log2 fold change of their matched total protein levels from the proteome (x-axis). Blue dots represent sites decreased (log2 fold change <-0.5849), orange dots represent sites significantly increased (log2 fold change >0.5849). B) Blue dots in A were compared to increased proteins in either RMND5A KO proteome or MAEA KO proteome. Overlap of MAEA and RMND5A KO proteomes was already shown in Figure 4.6. C) Gene ontology terms and pathways significantly enriched (adjusted p <0.05) as determined by g:profiler (see methods) in proteins with either RanBPM-dependent significantly increased diGLY enrichment (top, orange dots in A) or decreased diGLY enrichment (bottom, blue dots in A). Terms (nodes) were clustered and grouped (yellow circles) based on their similarity coefficient. Size of the node reflects size of the term, and colour reflects Q-value (FDR) ranging from orange (0.05) to white (0).
domain containing 4), increased at protein level in MAEA KO and RMND5A KO, had a significantly decreased diGLY site in shRanBPM cells, although it did not change at the protein level in shRanBPM cells (Figure 4.8B). Six proteins were increased in the RMND5A KO proteome or the MAEA KO proteome and had a significantly decreased diGLY site in shRanBPM cells (Figure 4.8B). In RanBPM-dependent increased ubiquitinated proteins, various membrane-related terms, cell adhesion, and hemostasis were terms enriched (Figure 4.8C). Metabolic processes, RNA localization regulators, mitochondrial matrix, and translation factor regulation were enriched within proteins with RanBPM-dependent decreased ubiquitination.

To compare with a second CTLH member, we similarly scored the ubiquitinome in control and RMND5A KO HeLa cells. Here, a combined 5673 ubiquitin sites were quantified, with 4303 sites remaining after filtering (Figure 4.9 A-C). Of those, 175 were significantly decreased (p<0.05, log2 RMND5A KO/WT fold change <-1.0) and 141 significantly increased (p<0.05, log2 RMND5A KO/WT fold change >1.0) (Figure 4.9D). Lysine 260 of muskelin, a CTLH complex member we previously identified as a target of the complex\(^\text{12}\) (chapter 2), exhibited significant decreased ubiquitination (Figure 4.9D). We compared the RMND5A-dependent significant ubiquitin changes (p<0.05) to their corresponding total protein change as was done for the RanBPM dataset, revealing 115 increased ubiquitinated sites and 128 decreased (Figure 4.10A). These increased or decreased ubiquitin sites were used for downstream analysis. Muskelin (MKLN1), ANXA1 (annexin A1), and LAMB1 (laminin subunit beta 1) were increased at their protein level in RMND5A KO cells, but decreased in ubiquitination, while TPM1
Figure 4.9. The RMND5A-dependent ubiquitinome. Protein extracts of exponentially growing MG132-treated (10 μM for 4 hours) Control and RMND5A KO HeLa cells were subjected to trypsin/LysC digestion and peptides were enriched with a diGLY antibody and then analyzed and quantified label-free using LC-MS/MS. A) The number of ubiquitin sites quantified in each replicate of Control or RMND5A KO HeLa cells and the number of quantified sites in total across all samples (dashed line). Error bars represent SD. B) Pearson correlation coefficients between the indicated biological replicates for Control samples (blue) or RMND5A KO samples (orange). C) Heatmap clustering representation of significantly changed (p<0.05) diGLY sites across replicates. D) Volcano plot showing distribution of log2 fold change (x-axis) and –log10 p value (y-axis) of ubiquitin sites quantified in Control and RMND5A KO HeLa cells as determined by t-test (see methods), n=4. Blue dots represent sites significantly decreased (p<0.05, log2 fold change <-1.0), orange dots represent sites significantly increased (p<0.05, log2 fold change >1.0). Other sites are coloured in grey.
**Figure 4.10. The RMND5A-dependent ubiquitinome.** Related to figure 4.9. A) Proteins with a significant diGLY site (p<0.05) in RMND5A KO cells plotted with their log2 fold change (y-axis) against the log2 fold change of their matched total protein levels from the proteome (x-axis). Blue dots represent sites decreased (log2 fold change <-0.5849), orange dots represent sites significantly increased (log2 fold change >0.5849). B) Blue dots in A were compared to increased proteins in either shRanBPM proteome or MAEA KO proteome. Overlap of MAEA and shRanBPM proteomes was already shown in Figure 4.6. C) Gene ontology terms and pathways significantly enriched (adjusted p <0.05) as determined by g:profiler (see methods) in proteins with either RMND5A-dependent significantly increased diGLY enrichment (top, orange dots in A) or decreased diGLY enrichment (bottom, blue dots in A). Terms (nodes) were clustered and grouped (yellow circles) based on their similarity coefficient. Size of the node reflects size of the term, and colour reflects Q-value (FDR) ranging from orange (0.05) to white (0).
tropomyosin 1), AURKB (aurora kinase B), RPN2 (ribophorin II), and MISP (mitotic spindle positioning) showed the opposite effect (Figure 4.10A). Of the 101 proteins with a decreased diGLY site in RMND5A KO cells, four were increased in the MAEA KO proteome (CDK4 (cyclin dependent kinase 4), DHX33 (DEAH-box helicase 33), RICTOR (rapamycin-insensitive companion of mTOR), which also had a decreased diGLY site in shRanBPM cells, and muskelin) and three were increased in the shRanBPM proteome (ABCC1 (multidrug resistance-associated protein 1), ABCD1 (ATP binding cassette subfamily D member 1), and RNF213 (E3 ubiquitin-protein ligase RNF213)) (Figure 4.10B). Among GO terms and pathways, cell migration and actin filament-based process were enriched in proteins with an increased diGLY site (orange dots in figure 4.10A), meanwhile, RNA localization, cell cycle, metabolic processes, and telomere maintenance were terms enriched within proteins with a decreased site (blue dots in figure 4.10A) (Figure 4.10C).

Comparison of the shRanBPM and RMND5A diGLY datasets revealed 15 proteins in common with a decreased ubiquitin site (Figure 4.11). Besides, CBR1 (carbonyl reductase [NADPH] 1) at K157, the other 14 proteins in common have a different ubiquitin site that changed in the datasets. However, we note that 50 of the proteins with a decreased diGLY site in shRanBPM cells do not appear in the RMND5A KO diGLY dataset. Likewise, 12 of the proteins with a decreased diGLY site in RMND5A KO cells do not appear in the shRanBPM diGLY dataset. Despite this, notably, two glycolysis proteins (ENO1 (alpha-enolase) and LDHA), two proteasome subunits (PSMD3 (26S proteasome non-ATPase regulatory subunit 3) and PSMD14 (26S
proteasome non-ATPase regulatory subunit 14)), the ssDNA binding protein RPA1 (replication protein A 70 kDa DNA-binding subunit), LAMB1, and mTOR subunit RICTOR had decreased diGLY sites in both datasets (Figure 4.11). As mentioned, LAMB1 was also significantly increased at the protein level in RMND5A KO cells (Figure 4.10A) and it was also significantly increased in MAEA KO cells although below the cut-off (log2 MAEA KO/WT fold change = 0.52). RICTOR was also increased in the MAEA KO proteome (log2 MAEA KO/WT fold change = 0.67), but not in the other proteomes.

4.2.5 The endogenous RanBPM interactome

To narrow down our list of candidate ubiquitin substrates to those that associate with the complex, we identified CTLH complex associated proteins using a specific RanBPM antibody and affinity purification mass spectrometry. Besides the 9 CTLH members, 85 other associated proteins were identified, including 9 previously reported\(^{13,17,35–38}\) and 76 novel interactors (Figure 4.12A, B). Amongst those CTLH-associated proteins was the coREST transcriptional repressor complex, ribosomal proteins, heat shock chaperones, lysosomal proteins, RNA binding proteins, proteins associated with desmosomes, and glycolysis enzymes (Figure 4.12A). GO cellular compartment analysis revealed that the associated proteins were near equally associated with the cytoplasm and nucleus, consistent with the complex being present in both compartments\(^{11,12}\), with enrichment as well in extracellular organelle, focal adhesion, and vesicles (Figure 4.12C).
Figure 4.11. Comparison of RanBPM and RMND5A-dependent diGLY data.

Yellow circle contains the 1005 proteins common between datasets that had a quantified diGLY site. Grey box lists the 15 proteins with a decreased diGLY site in both screens (p<0.05, log2 fold change < -0.5849). Blue box lists the 12 proteins with a decreased diGLY in the RMND5A KO diGLY dataset (p<0.05, log2 fold change < -0.5849) that were not quantified in the RanBPM diGLY screen. Red box lists the 50 proteins with a decreased diGLY in the RanBPM KO dataset (p<0.05, log2 fold change < -0.5849) that were not quantified in the RMND5A KO diGLY screen.
**Figure 4.12. The endogenous RanBPM interactome.** RanBPM was immunoprecipitated from wild-type HeLa cells and the elution was digested and analyzed by mass spectrometry. Contaminants were removed and protein was identified as a hit if \( p<0.05 \) and more than 2-fold enriched over negative control IgG \( (n=4) \). A) Map of identified associated proteins clustered based on STRING interaction and biological theme. B) Distribution of identified associated proteins as novel or as previously reported. C) Distribution of top 12 significantly enriched cell compartments as determined by g:profiler. Redundant terms were removed. Dots are coloured based on the number of proteins in the term as a percentage of the total proteins identified in the interactome.
Muskelin was the only protein increased in both MAEA and RMND5A KO proteins that was identified in the RanBPM interactome (Figure 4.13A). CKAP4 (cytoskeleton-associated protein 4), an increased protein in MAEA KOs, as well as DSC3 (desmocollin-3), MAP4 (microtubule-associated protein 4) and PKM1, which were increased in RMND5A KOs, and GLUL (glutamine synthetase), an increased protein in shRanBPM cells, were found in the RanBPM interactome (Figure 4.13A). These proteins could represent ubiquitinated targets of the complex that lead to degradation. Our diGLY screens were not deep enough to detect any ubiquitin sites for CKAP4, DSC3, and MAP4 so it remains possible that these proteins could have reduced ubiquitination in either RanBPM or RMND5A depleted cells.

We compared the RanBPM interactome to the list of decreased diGLY sites for either RMND5A KOs or shRanBPM. In this case, any proteins in common would likely be non-degradative ubiquitin targets of the complex. HSPD1 (60 kDa heat shock protein, mitochondrial), PKM2, and PAICS (multifunctional protein ADE2) had RanBPM-dependent decreased ubiquitin sites and were also in the RanBPM interactome (Figure 4.13B). ALDOA (fructose-bisphosphat aldolase A), ANXA2 (annexin A2), JUP (junction plakoglobin), and muskelin had ubiquitin sites decreased in RMND5A KO and were present in the RanBPM interactome (Figure 4.13B). LDHA and ENO1 had ubiquitin sites decreased in both shRanBPM and RMND5A KO cells and were also identified as a RanBPM-associated protein (Figure 4.13B).
Figure 4.13. Comparisons of the RanBPM interactome with increased proteins and decreased ubiquitination. A) Comparison of the RanBPM associated proteins to the proteins increased in the proteome datasets at log2 fold change >0.5849 (Proteome overlaps were already presented in figure 4.6). B) Comparison of the RanBPM associated proteins to the proteins with a decreased ubiquitin site (log2 fold change <-0.5849) in shRanBPM or RMND5A KO cells. The four glycolysis enzymes that overlapped in the datasets are bolded (see next figure).
4.2.6 The CTLH complex regulates ubiquitination of PKM2 and LDHA

Interestingly, four proteins (PKM2, LDHA, ENO1, and ALDOA) that associate with the complex and have ubiquitin sites decreased in one or both diGLY screens, but did not change at the protein level, are key enzymes involved in the glycolysis pathway (Figure 4.14A). We focused on these glycolytic enzymes, intrigued by the connection of the yeast complex with glucose regulation. We validated PKM2 and LDHA as interactors of the complex by immunoprecipitating flag tagged construct of each enzyme and looking for the co-IP of CTLH subunits (Figure 4.14B, C). Additionally, we confirmed the results of the diGLY screens by observing reduced polyubiquitination of transfected PKM2 and LDHA in an IP under denaturing conditions in both shRanBPM HeLa cell and RMND5A KO HeLa cells (Figure 4.14D-G).

4.2.7 The CTLH complex regulates glycolysis

The proteomic data and confirmatory data by IP/Western blot suggested the CTLH complex associates with and regulates ubiquitination of several glycolytic enzymes. However, the global proteome data indicated that proteins levels of PKM2 and LDHA were not changed in shRanBPM, RMND5A KO, or MAEA KO HeLa cells. We confirmed this in shRanBPM cells by Western blot using antibodies directed against LDH and PKM (Figure 4.15A). Thus, the CTLH complex-dependent ubiquitination of PKM and LDHA likely affects these enzymes in ways other than degradation. Since PTM regulation (including ubiquitination) has previously been reported to induce changes in LDH and PKM catalytic activity, we assessed pyruvate kinase (PK) and LDH activities in extracts of shRanBPM HeLa cells. LDH and PK activity assays showed
**Figure 4.14. CTLH complex-dependent ubiquitination of glycolysis enzymes.**

A) Glycolysis pathway with proteins identified as hits in the proteomic screens circled in red and indicated with Ub. The sites that changed either in shRanBPM cells (blue) or RMND5A KO cells (red) are indicated. B and C) LDHA and PKM associate with CTLH complex subunits. Transfected (24 hours) FLAG-LDHA (B) or FLAG-PKM2 (C) in WT HeLa cells were immunoprecipitated (IP) with FLAG antibody and analyzed by western for the co-IP of the indicated antibodies against CTLH complex members. D-G) Reduced polyubiquitination of LDHA and PKM2 in shRanBPM and RMND5A KO HeLa cells. Transfected (24 hours, with 10 uM MG32 in last 4 hours) FLAG-LDHA or FLAG-PKM2 cotransfected with HA-Ubiquitin in the indicated cell lines treated were lysed in denaturing conditions and subjected to IP with FLAG antibody. Western blot was performed by first hybridizing with FLAG antibody then HA antibody to check for ubiquitination. * indicates previous FLAG hybridization. HC, heavy chain of IP antibody.
Figure 4.15. The CTLH complex regulates glycolysis. A) Western blot showing levels of LDH and PKM in untreated shControl and shRanBPM HeLa cells using the indicated antibodies on the right. B) Activities of lactate dehydrogenase (LDH, left; n=6, p=0.005) and pyruvate kinase (PK, right; n=4, p=0.036) in extracts of exponentially growing untreated shControl and shRanBPM HeLa cells. Values are expressed as nmole/min/mL/ug protein (see methods). Error bars represent standard deviation of the mean. C) Glycolysis is upregulated in shRanBPM cells. Top, Extracellular acidification rate (ECAR) tracing of shControl or shRanBPM HeLa cells as measured by Seahorse extracellular flux analyzer. n=3. Error bars represent standard deviation of the mean. Bottom, ECAR measurements for glycolysis (after glucose injection, p=0.037), glycolytic capacity (maximum ECAR after oligomycin injection, p=0.025), and glycolytic reserve (after 2-Deoxy-d-glucose (2-DG) injection, p=0.01); all n=3. Error bars represent standard deviation of the mean. D) Heatmap representation of targeted quantification of central carbon metabolites measured by mass spectrometry (see methods). For each metabolite, values are relative to the highest value of the metabolite across the different samples set to 1 (dark orange). P=phosphate. DHAP=dihydroxyacetone phosphate. TCA= tricarboxylic acid. PPP=pentose phosphate pathway. HBP= hexosamine biosynthetic pathway. FA=fatty acid. See also Fig 4.10A. E) From the same metabolite data as D, relative amounts of relevant nucleotides in shControl (blue) and shRanBPM (orange). n=4. Multiple testing correction using Benjamini-Hochberg FDR method at 5%. Significance for ADP (q=0.03), AMP (q=0.04), ATP (q=0.04), ATP/ADP (q=0.04), GMP (q=0.04), GTP (q=0.04), NADPH (q=0.04), and UMP (q=0.04). Error bars represent standard deviation of the mean.
significantly increased activity of both enzymes in shRanBPM cells (Figure 4.15B). These findings indicate that ubiquitination of PKM and LDHA mediated by the CTLH complex functions to inhibit activity of both proteins.

To determine if the regulation of PKM2 and LDHA by the CTLH complex impacts glycolysis, we measured glycolysis in real time using a Seahorse extra cellular acidification rate (ECAR) assay. Glycolysis, glycolytic capacity, and glycolytic reserve were each significantly higher in shRanBPM HeLa cells compared to control cells, suggesting that the CTLH complex functions to inhibit glycolysis in this cell type (Figure 4.15C). This was further supported by targeted mass spectrometry-based quantification of intracellular central carbon metabolites, which revealed an increase in most of the glycolytic intermediates, such as glucose-6-phosphate, Fructose-6-phosphate, mannose-6-phosphate, glycerate-1,3-bisphosphate, PEP, and pyruvate in shRanBPM HeLa cells compared to control cells (Figure 4.15D). Additionally, metabolites of other pathways were affected, as RanBPM shRNA cells showed increases in glucosamine-6P, erythrose-4p, and phosphocreatine, and decreases in glycerol-3 phosphate and ribose. Surprisingly, little changes were observed in TCA metabolites, although there was an increase in α-ketoglutaric acid accompanied by a decrease in its precursor, α-hydroxyglutaric acid. Amongst nucleotides, ATP, GTP, and ATP/ADP ratio were significantly increased in shRanBPM cells, while ADP, AMP, GMP, and NADPH were significantly decreased (Figure 4.15E). Taken together, we conclude that the CTLH complex functions to negatively regulate glycolysis in HeLa cells.
4.3 Discussion

The human CTLH complex, homologue of the yeast Gid E3 ligase complex\textsuperscript{4,10}, was recently determined to have ubiquitin ligase activity\textsuperscript{12,13}. Since then, a few of his substrates have been identified, including the transcription factor HBP1, nuclear matrix protein lamin B, and its own subunit muskelin\textsuperscript{12–14}. To provide a resource for further interrogation of CTLH complex function, we conducted here a variety of high-throughput proteomic strategies identifying candidate ubiquitination targets.

Within the datasets, previously determined or proposed ubiquitination substrates of the complex can be assessed. Some of these substrates do have changes consistent with their proposed regulation by the complex, such as an increase of Lamin B2 and c-Raf in RMND5A and MAEA KO cells, association of nucleolin with RanBPM, a ubiquitin site (K320) in PRKAA1, the catalytic subunit of AMPK, decreased in shRanBPM cells, and, as previously mentioned, a ubiquitin site (K260) in muskelin decreased in RMND5A KO cells (Table 4.2). Some others, like HBP1, were not detected at the proteome level. In several of the substrates, ubiquitination differences were not observed; however, in these cases it is likely the targeted site is missed in the analysis since only a small portion of the ubiquitinome is detected in our screen. Overall, these comparisons show the value in these datasets as resources to inspect for potential substrates or provide additional information to ones already known. For example, table 4.2 reveals the exact ubiquitination site in AMPK that is regulated by the complex.
<table>
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<th>Substrate</th>
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<th>MAEA KO proteome</th>
<th>shRanBPM diGLY</th>
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Several glycolysis enzymes emerged from the proteomic data as primary candidate targets for ubiquitination by the complex and further analyses revealed that glycolysis is deregulated in cells deficient of RanBPM, an essential CTLH complex member. Our data suggest that the CTLH complex functions to normally restrict glycolytic flux at least in part by inhibiting the activity levels of PKM2 and LDHA, and potentially also ALDOA and ENO1, via ubiquitination. In the *S. cerevisiae* complex, Gid4 binds N-terminal proline residues on gluconeogenic enzymes via its β-barrel domain\(^8,33,41\). Human GID4 also binds N-terminal proline peptides or other non-proline hydrophobic N-terminal peptides\(^{41,42}\). While formally unknown at this time, it is unlikely that the ubiquitination of the glycolysis enzymes by the CTLH complex involves GID4 since PKM2 and LDHA do not fit the GID4 binding criteria. Other confirmed or predicted ubiquitination targets of the human complex identified so far (HBP1, Lamin B2, AMPK, and c-Raf) also do not fit the hydrophobic N-terminal criteria\(^{13–16}\). This implies that the human complex has evolved beyond Gid4 binding of N-terminal proline proteins. The inclusion of muskelin in the vertebrate CTLH complexes and its discoidin and kelch domains is likely to be an important factor differentiating the human complex ubiquitin activity from the yeast complex\(^{10}\).

As part of the catabolite inactivation process in *S. cerevisiae*, the Gid complex ubiquitinates Fbp1 and other gluconeogenic enzymes, resulting in their proteasomal degradation\(^4,8\). Regulation of gluconeogenesis and ubiquitination of gluconeogenic enzymes was previously ruled out for the human complex\(^{13}\). This is further supported by our findings that suggest the mammalian complex evolved to regulate the opposing
glucose regulation pathway. In doing so, it achieves the same overall function: maintenance of glucose metabolism homeostasis. Over evolutionary time, this change in mechanism may have been necessary due to differences in the gluconeogenic enzymes (e.g., human Fbp1 does not have the N-terminal proline) and/or a shift in gluconeogenesis reliance (e.g., in mammals, gluconeogenesis is primarily limited to the liver and kidney).

Glycolysis enzymes are regulated at the transcriptional levels by proteins frequently altered in cancer (e.g. HIF1, c-MYC, p53). Post-translational modifications are also reported to regulate glycolysis enzymes, either by affecting protein stability, subcellular localization, or enzyme activity. For example, high glucose-stimulated acetylation of PKM2 inhibits its kinase activity and promotes its degradation by chaperone-mediated autophagy degradation, while monoubiquitination of PKM2 by Parkin decreases PKM activity. Phosphorylation of LDHA enhances its activity and is required to sustain high glycolytic flux and nuclear translocation of GAPDH is regulated by PCAF-mediated acetylation. Multiple glycolysis enzymes are targeted by p300 for lysine 2-hydroxyisobutyrylation (Khib), which enhances their activities. Our findings uncover a second post-translation modifier that acts at multiple points within the glycolysis pathway, but one that inhibits activities. This ability of multiple glycolytic enzymes to be activated by p300-mediated Khib or inhibited by CTLH complex-mediated ubiquitination likely provides in a healthy cell the opportunity to coordinate and robustly control the amplitude of the glycolysis pathway in response to various stressors. Testing out the glycolysis amplitude of control versus shRanBPM cells in cells with reduced glucose (compared to the high glucose media used in this study) or no sodium
pyruvate would be informative to assess if the CTLH complex inhibition of the pathway can be increased.

Our proteomic data and the findings of the effects of the CTLH complex on glycolysis add to the understanding of this complex and provide a novel function in carbon metabolism. Individual subunits have been linked with various signaling pathways, processes, and diseases, such as the WNT pathway, MAPK signaling, differentiation, development, cancer, and neurodegenerative diseases. The human complex as a unit has been functionally linked with nuclear condensation in developing erythroblasts through ubiquitination of at least lamin B. It has also been determined to ubiquitinate HBP1 to promote cell proliferation. In both cases, CTLH complex-dependent ubiquitination regulates proteasomal degradation and involves in vitro assays that used UBE2H (Ubiquitin-conjugating enzyme E2 H) as the E2 enzyme. The CTLH regulation of three other predicted targets, muskelin, c-Raf, and AMPK, also involve their proteasomal degradation. The drosophila CTLH complex with the UBE2H homologue (named Kdo) are responsible for timed proteasomal degradation of an essential RNA binding complex during the maternal to zygotic transition. This is also consistent with the yeast Gid complex ubiquitination of gluconeogenic enzymes, which results in their proteasomal degradation. Thus, our findings provide the first evidence that CTLH complex ubiquitination affects a protein other than through its degradation, showing the value in not limiting the scope of experiments of E3 ligase substrates to just changes of protein levels. However, the minimal overlap we found between increased proteins and decreased ubiquitination in both RMND5A KO and
shRanBPM cells suggests this might be a more common occurrence than previously anticipated. The diGLY data cannot distinguish between mono versus poly ubiquitination, however our assessment of PKM2 and LDHA ubiquitination by Western blots reveals it to be polyubiquitination. Which E2 enzyme and which ubiquitin linkage type are involved in these ubiquitination events affecting signaling remains an open question. Our observation that recombinant RMND5A can pair with UBE2D2 E2 enzyme to make polyubiquitin chains with K48 and/or K63 linkages \textit{in vitro}\textsuperscript{12} suggests K63 as a possibility. K63-linked polyubiquitination has a wide range of functional outcomes including regulating complex assembly and protein activation\textsuperscript{7}.

CTLH complex subunits contain a variety of protein-protein interaction domains that likely are used to recruit specific substrates: muskelin (discoidin and kelch repeat domains), ARMC8 (ARM repeats), RanBPM (SPRY) and WDR26 (WD40 repeats). The architecture of the yeast complex has been elegantly characterized by Qiao et al\textsuperscript{5} using cryo-EM, however deviations between the yeast and human complex\textsuperscript{10} preclude clear understanding of the positions of the human subunits as substrate receptors in the CTLH complex. Thus, the subunit(s) responsible for recruiting LDHA and PKM2 await future investigation.

While we focused on the regulation of glycolysis enzymes, other interesting findings and patterns emerged from our proteomic data. Subunits of the CoREST repressor complex associated with RanBPM in our interactome. The CoREST complex is a dual epigenetic modifier that is recruited to genomic sites by a transcription factor to regulate gene expression\textsuperscript{53}. The CTLH complex has previously been reported to associate
with HDAC6 and regulate its activity\textsuperscript{17}, but not yet to other HDACs. RanBPM has been established as a transcriptional coactivator for steroid and hormone nuclear receptors, which at least in the case of the androgen receptor also involves RanBP1\textsuperscript{54–56}. RanBPM also enhances transcriptional activity of Zta and Rta transcription factors from Epstein–Barr virus by differentially regulating their Ubc9-mediated sumoylation\textsuperscript{57,58}. Thus, our interactome findings may indicate that the CTLH complex acts to modulate transcription activities via the CoREST complex.

Another interesting finding was the increased protein levels in RMND5A KO and MAEA KO cells of USP11. USP11 interacts and inhibits ubiquitination of RanBPM\textsuperscript{34}. The two also cooperate for the regulation of Mgl-1 (Lethal(2) giant larvae protein homolog 1), with RanBPM being required for Mgl-1 deubiquitination by USP11\textsuperscript{59}. Our data indicates that there may be a reciprocal regulation of USP11 and the CTLH complex: USP11 deubiquitinating RanBPM and the CTLH complex in turn controlling USP11 levels, possibly by regulating its ubiquitination. The increase of USP11 in the knockdown cells can also partially explain increased ubiquitin sites or decreased protein abundance of some proteins. Interestingly, USP11 deubiquitinase activity plays a critical role in the cell cycle by alleviating the suppression of homologous recombination (HR) during the progression from G1 to S/G2 phases\textsuperscript{60}. It is tempting to speculate that the CTLH complex may also be involved in this process since RanBPM accumulates in the nucleus in early time points after ionizing radiation, and its deficiency results in delay of DNA damage signals and inefficient HR\textsuperscript{61}. Also, our finding that RPA1, which is part of the RPA complex involved in DNA replication and DNA repair\textsuperscript{62,63}, has decreased
ubiquitination in shRanBPM and RMND5A KO cells reveals another potential link with the DNA damage response. Our data indicate that the CTLH complex functions to normally restrict glycolytic flux at least in part by inhibiting the activity levels of PKM2 and LDHA, and potentially also ALDOA and ENO1, via ubiquitination. We observed increased glycolysis and glycolytic capacity in HeLa cells depleted of RanBPM accompanied by changes in related metabolites and nucleotides. The Warburg effect is the metabolic reprogramming that cancer cells exhibit of increased glucose uptake and lactate production even under abundant oxygen. Although not completely understood, increased glycolytic flux supports cell proliferation by several proposed mechanisms, such as rapid ATP synthesis and supporting biosynthetic pathways that branch off from glycolysis intermediates (Figure 4.14A)\(^3\). Thus, our results suggest that mutation or loss of expression of the CTLH complex could benefit a cancer cell by providing a means to upregulate glycolysis. We previously have shown that loss of RanBPM and RMND5A increases cell proliferation in HEK293 cells and loss of RanBPM promotes tumour development in a xenograft model\(^{16,18}\).

This report not only provides a resource for identifying substrates of a newly discovered E3 ligase, but also reveals a novel function of the CTLH complex in glycolysis as a post-translational mechanism needed to keep glycolytic flux at an appropriate level in HeLa cells. We determined that two likely substrates of the complex are PKM2 and LDHA, but, instead of proteasomal degradation, their activity is regulated. It is an overall function of regulating glucose metabolism that is shared by the yeast Gid
complex, although on opposing pathways (gluconeogenesis versus glycolysis) and a different mechanism. Cancer cells rely on glycolysis for survival as a quick energy source and supply of intermediates for biosynthetic pathways. Our findings of ubiquitination inhibition at multiple points of the glycolysis pathway may provide an avenue for development of a CTLH complex agonist as a future therapeutic strategy for cancer patients once structural elucidation of the human complex is completed.

4.4 Methods
4.4.1 Cell culture, plasmid construction, and antibodies

Wild-type HeLa, control shRNA stable, RanBPM shRNA stable, CRISPR control, RMND5A KO, and MAEA KO HeLa cells have been described previously. All cells were cultured in high glucose (4.5g/L) Dulbecco’s modified Eagle’s medium (Wisent Bioproducts) supplemented with 10% FBS, 1% sodium pyruvate, 1% L-glutamine at 37°C and 5% CO2. Cells were treated with 10 μM MG132 (EMD-CalBiochem) for the indicated time points. Plasmid transfections were carried out with jetPRIME (Polypus Transfection) according to the manufacturer’s protocol. pCDNA-FLAG-SBP-LDHA was created by PCR amplification of human LDHA cDNA (kind gift from Dr. Rob Cumming) and cloned into pCDNA-FLAG-SBP (generated by digestion of muskelin of pCDNA-FLAG-SBP-MKLN1, described in Maitland et al, 2019) via AgeI and xbaI digestion of both PCR product and vector. pCDNA-FLAG-SBP-PKM2 was created in the same way using PKM2 human cDNA (kind gift from Dr. John Di Guglielmo), but with the PCR product digested with AgeI and NHE1. Antibodies used for Western blot were: FLAG (M2, F1804, Sigma-Aldrich), HA (HA-7,
H3663 Sigma-Aldrich), RanBPM (5M, 71-001, Bioacademia), Vinculin (E1E9V, Cell Signaling Technology), LDH (H-10, sc-133123, Santa Cruz), and PKM (C-11, sc-365684, Santa Cruz).

4.4.2 MS sample preparation for global proteomics

HeLa cells at 75-80% confluency were trypsinized, cell pellet was collected by centrifugation, washed, and then frozen at -80 °C. Cells were lysed by resuspension in 8 M urea, 50 mM ammonium bicarbonate (ABC), 10 mM Dithiothreitol (DTT), 2% Sodium dodecyl sulfate (SDS) and then sonicated with a probe sonicator (20×0.5 s pulses; Level 1). Twenty-five μg of protein lysate, as quantified by Pierce™ 660 nm Protein Assay (ThermoFisher Scientific. #22660, #22663), was reduced in 10 mM DTT for 25 minutes, alkylated in 100 mM iodoacetamide for 25 minutes in the dark, followed by methanol precipitation as described in Kuljanin et al., 201722. The protein pellet was resuspended in 200 μL of 50mM ABC and subjected to a sequential digest first with 250 ng of LysC (125-05061, Wako Chemicals, USA) for 4 hours, then 500 ng of Trypsin/LysC (V5071, Promega) for 16 hours, followed by 500 ng of Trypsin (V5111, Promega) for an additional 4 hours. Digestions were incubated at 37 °C at 600 rpm with interval mixing (30 seconds mix, 2 minutes pause) on a Thermomixer C (Eppendorf, cat# 2231000667). After the last digestion, samples were acidified with 10% formic acid (FA) to pH 3-4 and centrifuged at 14,000 x g to pellet insoluble material.

4.4.3 diGLY enrichment

HeLa cells at 75-80% confluency were treated with 10 μM MG132 for 4 hours and processed exactly as described for global proteomics. After methanol precipitation, 1
mg protein was digested sequentially as described for global proteomics but with 6.66 μg of Lys-C, 20 μg of Trypsin/Lys-C, and 20 μg of Trypsin. Peptides were then dried using a SpeedVac (Thermo Scientific). PTMScan® Ubiquitin Remnant Motif (K-ε-GG, aka diGLY) Antibody Bead Conjugate (Cell Signaling Technology, #5562) (25 uL per sample) was crosslinked and used as described in Udeshi et al., 2013. Briefly, antibody-bead conjugate was washed three times in 100 mM sodium borate, pH 9 (M5162, Sigma-Aldrich), then resuspended in 20 mM DMP (Dimethyl pimelimidate dihydrochloride, D8388, Sigma-Aldrich) in 100 mM sodium borate, pH 9 and rotated at room temperature for 30 minutes. Crosslinking was quenched by washing and incubation in 200 mM ethanolamine, pH 8 (S9640, Sigma-Aldrich) for 2 hours at 4°C with end-over-end rotation. After blocking, beads were washed three times in 1 mL of 1x IAP buffer (provided by the kit) and kept on ice. Dried peptides were resuspended in 1 mL of IAP buffer, centrifuged, and the supernatant was added to the crosslinked antibody-beads and incubated with rotation for 1 hour at 4°C. After enrichment, beads were washed twice with IAP buffer, three times with PBS, and then peptides were eluted with two rounds of 5-minute incubations in 0.15% Trifluoroacetic acid (TFA). The eluted peptides were dried in a SpeedVac and reconstituted in 0.1% FA.

### 4.4.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for RanBPM diGLY enrichment

Approximately 1 μg of peptide sample (as determined by Pierce BCA assay) was injected onto a Waters M-Class nanoAcquity UHPLC system (Waters) coupled to an Orbitrap Elite mass spectrometer (ThermoFisher Scientific). Buffer A consisted of mass
spectrometry grade water with 0.1% FA and buffer B consisted of acetonitrile with 0.1% FA (ThermoFisher Scientific). Samples were trapped for 4 minutes at a flow rate of 5 uL/min using 99% buffer A and 1% buffer B on an ACQUITY UPLC Symmetry BEH C18 Trapping Column (5 mm, 180 mm x 20 mm, Waters). Peptides were separated using an ACQUITY UPLC Peptide BEH C18 Column (130 Å, 1.7 mm, 75 mm x 250 mm) operating at a flow rate of 300 nL/min at 35°C. Samples were separated using a non-linear gradient consisting of 1%–7.5% buffer B over 1 minute, 7.5%–25% buffer B over 179 minutes, 25%–32.5% buffer B over 40 minutes, and 32.5%–40% over 20 minutes, before increasing to 98% buffer B and washing. MS acquisition settings are provided in Appendix C.

4.4.5 LC-MS/MS for global proteomic and RMND5A diGLY enrichment

Approximately 1 µg of peptide sample (as determined by Pierce BCA assay) was injected onto a Waters M-Class nanoAcquity HPLC system (Waters) coupled to an ESI Orbitrap mass spectrometer (Q Exactive plus, ThermoFisher Scientific) operating in positive mode. Buffer A consisted of mass spectrometry grade water with 0.1% FA and buffer B consisted of acetonitrile with 0.1% FA (ThermoFisher Scientific). All samples were trapped for 5 minutes at a flow rate of 5 uL/min using 99% buffer A and 1% buffer B on a Symmetry BEH C18 Trapping Column (5 mm, 180 mm x 20 mm, Waters). Peptides were separated using a Peptide BEH C18 Column (130 Å, 1.7 mm, 75 mm x 250 mm) operating at a flow rate of 300 nL/min at 35°C (Waters). Proteome Samples were separated using a non-linear gradient consisting of 1%–7% buffer B over 1 minute, 7%–23% buffer B over 179 minutes and 23%–35% buffer b over 60 minutes, before
increasing to 98% buffer B and washing. RMND5A diGLY enriched samples were 
trapped for 5 minutes then separated using a non-linear gradient consisting of 1%–7.5% 
buffer B over 1 minutes, 7.5%–25% buffer B over 179 minutes, 25%–32.5% buffer B 
over 40 minutes and 32.5%–40% over 20 minutes before increasing to 98% buffer B and 
washing. MS acquisition settings are provided in Appendix C.

### 4.4.6 RanBPM affinity-purification coupled to MS

Thirty micrograms of mouse IgG (sc-2025, Santa Cruz Biotechnology) or 
RanBPM (F-1, sc-271727, Santa Cruz Biotechnology) antibody was conjugated to 20 µL 
Dynabeads Protein G (10004D, Invitrogen) for 1 hour at 4°C with end-over-end rotation. 
Afterwards, antibody-bead conjugate was cross-linked and quenched exactly as described 
above for the diGLY antibody. After quenching, crosslinked antibody and beads were 
then washed five times in lysis buffer and left on ice until ready. Meanwhile, HeLa WT 
cell pellets were lysed in whole-cell extract buffer (50 mM HEPES, pH7.4, 150 mM 
NaCl, 10 mM EDTA, 10% glycerol, 0.5% NP40, 10 mM DTT, 1 mM Na3VO4, 10 mM 
NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml of aprotinin, 10 µg/ml of 
pepstatin, and 1 µg/ml of leupeptin) for 25 minutes on ice and spun down at 13,000 rpm 
for 20 minutes at 4°C to pellet insoluble material. A volume corresponding to 1 mg of 
protein was adjusted to 0.25% NP40 and precleared with 3 µL Dynabeads Protein G for 
30 minutes at 4°C with end-over-end rotation. Precleared extract was then added to the 
cross-linked antibody-beads and incubated overnight at 4°C with end-over-end rotation. 
The next day, beads were washed and proteins eluted using a protocol adapted from 
Kaboord et al., 2014\textsuperscript{24}. Briefly, beads were first washed three times with IP wash buffer
(50 mM HEPES, pH7.4, 150 mM NaCl, 10 mM EDTA, 12% glycerol, 0.25% NP40),
then three times in 50 mM ABC, followed by five times in HPLC grade water. Proteins
were eluted with two rounds of 5-minute room temperature incubations in 0.5% FA, 30%
acetonitrile with 1250 rpm shaking on a thermomixer. Pooled elutions were spun down
briefly to collect any residual beads, transferred to a new tube and dried in a SpeedVac
(Thermo Scientific). Dried protein was resuspended in 6 M urea and incubated in 10 mM
DTT for 30 minutes, followed by 100 mM iodoacetamide for 25 minutes in the dark, and
then methanol precipitated as described in Kuljanin et al., 2017. Protein pellet was
resuspended in 200 µL of 50 mM ABC and subjected to a sequential digest exactly as
described for global proteomics. After the last digestion, samples were acidified with
10% FA to pH 3-4 and centrifuged at 14,000 x g to pellet insoluble material. Supernatant
was filtered by passing through a > 10 kDa cellulose membrane (UFC501096, Sigma-
Aldrich) and then was dried in a SpeedVac. Dried peptides were then resuspended in
0.1% TFA and desalted with C18 Ziptips (Z720070, Sigma-Aldrich). Eluted peptides
were dried in a SpeedVac, resuspended in 20 µL 0.1% FA and 5 µL was injected onto a
Waters M-Class nanoAcquity HPLC system (Waters) coupled to an ESI Orbitrap mass
spectrometer (Q Exactive plus, ThermoFisher Scientific). Buffer A consisted of mass
spectrometry grade water with 0.1% FA and buffer B consisted of acetonitrile with 0.1%
FA (ThermoFisher Scientific). All samples were trapped for 5 min at a flow rate of 5
µL/min using 99% buffer A and 1% buffer B on a Symmetry BEH C18 Trapping Column
(5 mm, 180 mm x 20 mm, Waters). Peptides were separated using a Peptide BEH C18
Column (130 Å, 1.7 mm, 75 mm x 250 mm) operating at a flow rate of 300 nL/min at
35°C (Waters). Samples were separated using a non-linear gradient consisting of 1%–7% buffer B over 1 min, 7%–23% buffer B over 59 min and 23%–35% buffer B over 20 min, before increasing to 98% buffer B and washing. MS acquisition settings are provided in Appendix C.

### 4.4.7 Proteomic data analysis

All MS raw files were searched in MaxQuant version 1.5.8.3 using the Human Uniprot database (reviewed only; updated May 2017 with 42,183 entries)\textsuperscript{25,26}. Missed cleavages were set to 3, cysteine carbamidomethylation (CAM) was set as a fixed modification and oxidation (M), N-terminal acetylation (protein) and deamidation (NQ) (and for ubiquitin enrichments, diGLY modification of lysine) were set as variable modifications (max. number of modifications per peptide = 5), and peptide length ≥ 6. Protein and peptide FDR was left to 0.01 (1%) and decoy database was set to revert. Match between runs was enabled and all other parameters left at default. Protein groups or GlyGly sites were loaded into Perseus (version 1.6.0.7) and proteins containing peptides only identified by site or matched to reverse and contaminant database were removed. diGLY sites were kept only if there was a localization score ≥0.9. After log2 transformation, protein groups or diGLY sites were only retained if they had valid values in ≥4 samples (out of 5) in either control or knockdown for RanBPM-dependent proteomes, ≥3 (out of 4) samples in either control or knockdown for RMND5A KO proteome, MAEA KO proteome, RMND5A diGLY, or in RanBPM interactome, or ≥3 (out of 3) for RanBPM diGLY. For proteome and diGLY analysis, peptide or protein group LFQ log2 transformed intensities were normalized to the median in each sample.
In all datasets, missing values were imputed from random numbers drawn from a normal distribution using a width of 0.3 and down shift of 1.8 (default parameters), and t-tests were performed in Perseus. Gene ontology and pathway analysis was performed using g:profiler. Protein lists (e.g., the 78 proteins significantly increased in RMND5A KO) were scored against the list of all filtered quantified proteins in the particular dataset as the statistical domain scope (e.g. the 4727 proteins used in the t-test for the RMND5A KO proteome analysis), except for the interactome, which was set to ‘only annotated genes’. Benjamini-Hochberg FDR was set to a significance threshold of 0.05 and “No electronic GO annotations” was enabled. Gene IDs were converted using g:Convert. Data sources selected and their versions are listed in table 4.3. Only significantly enriched terms with a minimum size of 5 and maximum size of 350 were retained. Significant categories were then grouped based on similarity coefficient and visualized using EnrichmentMap (version 3.3.1) and AutoAnnotate (version 1.3.3) applications in Cytoscape (version 3.8.0) following the protocol in Reimand et al., 2019. The RanBPM interactome map was made using Cytoscape (version 3.8.0) and edges were determined by STRING database (confidence score >0.4).
Table 4.3. List of functional profiling databases used for g:profiler analysis.

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4.4.8 In vivo ubiquitination for Western blot

To validate ubiquitination of LDHA and PKM2, cells were transfected with pCDNA-FLAG-LDHA or pCDNA-FLAG-PKM2 and/or pMT123 plasmid expressing HA-ubiquitin. Twenty hours after transfection, cells were treated with 10 μM MG132 for 4 hours then collected, pelleted, and frozen. Cells were lysed in freshly made Buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1% SDS, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, 1 μg/ml of aprotinin, 10 μg/ml of peptatin, 1 μg/ml of leupeptin and 25 mM...
NEM (N-Ethylmaleimide, Bioshop Canada, Burlington, ON, Canada), sonicated with a probe sonicator (30 x 1 second pulses at power level 2) and incubated on ice for 30 minutes, and then insoluble material pelleted by centrifugation. Lysates were quantified using 660 nm protein assay (Thermo, #22660, #22663). For immunoprecipitation, lysates (500 ug) were diluted 1:10 in buffer B (buffer A with no SDS) and incubated with 3 uL of anti-FLAG (M2; F1804; Sigma-Aldrich) overnight at 4°C, followed by incubation with Dynabeads Protein G (10004D, Invitrogen) for 1 hour. Beads were washed five times in buffer B then resuspended and boiled at 95 °C in SDS loading dye. Samples were run on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for LDHA and 8% SDS-PAGE for PKM2 and gels were transferred to polyvinylidene fluoride membrane and blocked in 5% milk.

4.4.9 Central carbon metabolism profiling

Exponentially growing shControl or shRanBPM cells were pelleted by brief centrifugation then snap frozen in dry ice/ethanol bath and stored at -80°C. Samples were processed for quantitation of Central Carbon Metabolism metabolites in cell pellets by UPLC-MRM/MS by University of Victoria Genome BC Proteomics Centre and The Metabolomics Innovation Centre.

4.4.10 PK and LDH activity assays

Colorimetric Pyruvate Kinase Activity Assay Kit (Sigma-Aldrich, MAK072) and Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, MAK066) were used according to the manufacturer’s instructions. Briefly, 1x10^6 cells were collected, washed in PBS, snap frozen and stored at -80°C. Cells were lysed in 250 µL of the assay buffer
and centrifuged. The supernatant was diluted 1:100 and 50 µL of it was used for the assay. Activity was determined as the difference in absorbance (450 nm for LDH, 570 nm for PKM) between the penultimate reading before saturation and $T_{\text{initial}}$, then compared to standard curve and calculated as nmole/min/mL. Activity was normalized to protein concentration in extracts as calculated by BCA assay.

### 4.4.11 Measurement of glycolysis in real time

Glycolysis was measured using Agilent Seahorse XF Glycolysis Stress Test and Extracellular Acidification Rate (ECAR) with a XFe24 Seahorse analyzer according to the manufacturer’s instructions. Briefly, shControl and shRanBPM HeLa cells were seeded onto XFe24 Seahorse plates at $2.5 \times 10^4$ cells per well. Culture media were exchanged for unbuffered media supplemented with 2 mM glutamine one hour before the assay. After basal metabolic readings were recorded, substrates and selective inhibitors were injected to achieve final concentrations of glucose (10 mM), oligomycin (1 µM), and 2-deoxyglucose, (2-DG, 50 mM). Changes in oxygen consumption rate (OCR) and ECAR in response to the addition of substrates/inhibitors were described as the mean change after injection compared with the average OCR or ECAR before the injection. The OCR and ECAR values were normalized to protein concentration from each well.

### 4.4.12 Statistics

Non-proteomic data statistical analyses were performed using GraphPad PRISM (version 9.0.1). Differences between two groups were compared using unpaired two-tailed t test or multiple correction testing as indicated in figure legends. Results were
considered significant when \( p \) or \( q < 0.05 \). Biological replicates are indicated in figure legend.

### 4.5 References


33. Dai, H. *et al.* RanBP9/TSSC3 complex cooperates to suppress anoikis resistance
49. Heisler, F. F. et al. Muskelin Coordinates PrPC Lysosome versus Exosome


Chapter 5

5 Discussion

5.1 Summary of findings

The motivation for this thesis was to identify a molecular mechanism explaining RanBPM function\(^1\). Consequently, this project was designed to characterize the RanBPM-containing CTLH complex, which has a conserved yeast homologue (the Gid complex) that is an E3 ligase targeting proteins for degradation\(^2,3\). Yet, at the time, little was known about the structure, activity, or function of the CTLH complex.

At the start of the project, an initial characterization and confirmation of CTLH complex subunits and E3 ligase activity was required. To that end, in Chapter 2 (Maitland et al., 2019\(^4\)), we determined that WDR26 and GID4 were indeed in a complex with RanBPM, TWA1, muskelin, ARMC8α and β, RMND5A, and MAEA. Next, we identified determinants of complex stability, which turned out to be mainly TWA1 and RanBPM as well as MAEA and RMND5A, in which expression is interdependent. Importantly, we then demonstrated that the endogenous CTLH complex and recombinant human RMND5A have E3 ligase activity. It was also discovered that its own subunit muskelin is a ubiquitination target of the CTLH complex, indicating an intriguing autoregulatory mechanism. At the same time, E3 ligase activity was also demonstrated in Lampert et al., 2018\(^5\), using a recombinant complex and tagged endogenous proteins.

In the second data chapter of this thesis, I probed the E3 ligase activity of the complex and structural arrangement of subunits in more detail, which was aided by publication of the recombinant human complex from Lampert et al., 2018\(^5\) and cryo-EM
structure of the yeast Gid complex in Qiao et al., 2020⁶. Taking together the predicted
structures of human MAEA and RMND5A, data from Qiao et al., 2020⁶, and the results
from the RING mutations’ effects on muskelin protein levels, I was able to draw the
following conclusions: MAEA RING domain is not necessary for E2 binding, while
RMND5A RING domain I338/L339 and Y376 are E2 binding and linchpin residues,
respectively. The results of the immunoprecipitations and CLMS data, as well as the new
yeast Gid complex structure⁶ produced an updated architectural model of the CTLH
complex that positioned the protein interaction surfaces of RanBPM, WDR26, muskelin,
and GID4 each as substrate receptors in proximity of the RING domains (Figure 3.7).

Chapter 4 integrated global proteomes and ubiquitinomes of various CTLH
subunit deficient cells with RanBPM affinity purification MS to identify candidate
ubiquitination targets. This analysis provided a catalogue of proteins that increased in
abundance and/or decreased in site-specific ubiquitination in the knockdowns and
interacted with RanBPM, any of which could represent targets of interest for future
exploration. I focused on the regulation of ubiquitination and activity levels of two key
glycolysis enzymes, PKM2 and LDHA, by the CTLH complex as well as determination
that cells without RanBPM show increased glycolytic flux and deregulated central carbon
metabolism. Taken together it revealed a novel function of the CTLH complex as a
negative regulator of glycolysis via non-degradative ubiquitination.

Collectively, the work in this thesis and reports published during the course of it
produce the following working model (Figure 5.1): (1) the human CTLH complex is an
active multi-subunit E3 ligase through the MAEA and RMND5A RING domains bound
Figure 5.1. The human CTLH complex is a RING heterodimer multi-subunit E3 ligase that regulates essential cellular processes via ubiquitination. This diagram summarizes the work from this thesis and papers published during its completion. The working model is that RMND5A RING domain binds an E2 enzyme, which could be UBE2H, UDE2D2 or others yet to be defined. A substrate is brought to the E2 for ubiquitin (Ub) transfer via specific interaction (dashed line) with any one of the presumed substrate receptor interacting domains of the CTLH complex. Subsequent Ub chain elongation produces poly-ubiquitination, possibly linked through K48 or K63 chains. Implied or in vitro confirmed (asterisk) substrates of the complex are shown with the corresponding effect of the ubiquitination as either proteasomal degradation (orange arrow) or an effect on enzyme activity (blue arrow). The pathway or process regulated by the ubiquitination event is also shown.
to UBE2H or UBE2D2 E2 enzymes, which can generate K48 or K63 polyUb chains, (2) the RING domains are in proximity to several protein interaction domains present in potential substrate receptors (muskelin, WDR26, GID4, RanBPM) via scaffolding by RanBPM-TWA1, (3) the CTLH complex has several ubiquitination targets, which results in either proteasomal degradation or modulation of enzyme activity, and (4) the complex is involved in the regulation of essential biological pathways (e.g., cell proliferation, metabolism) through the ubiquitination of its various targets.

5.2 Questions remaining about the CTLH complex E3 ligase activity

In chapter 3, residues in RMND5A were identified as part of the E2 binding platform or the linchpin. The use of the mutants, particularly the E2 binding double mutant I338A/L339A, can be a useful tool to investigate candidate ubiquitination substrates in future experiments. I planned to do a similar assay with MAEA to identify its important residues, but this was not possible due to complications of the system used to exogenously express MAEA. Without this information, the presumption is the human MAEA RING domain contributes to Ub transfer in a similar manner to the yeast protein. Therefore, it is expected human MAEA does not bind the E2 but does contribute to Ub discharge from the E2 through its non-RING priming element at the extreme C-terminus (in humans, residue Y394). The uniqueness of MAEA RING structure, the mystery of how the fold of the domain is maintained, and uncertainty of how it dimerizes with RMND5A warrant further structural exploration at high resolution. It would be of interest to precisely determine the interactions of the RING subunits with the E2 and Ub molecules, and how the E2–Ub complex is orientated towards substrate lysines brought
to it by the substrate receptors. Capturing of such structures in other dimeric RINGs has provided essential insight of the role of each RING subunit in allosteric activation of Ub transfer\textsuperscript{7–9}.

Conflicting results exist regarding E2 selectivity and the complex (Table 5.1). In Lampert et al., 2018\textsuperscript{5}, the recombinant CTLH complex exhibited activity with UBE2H (the homologue of the yeast E2 for the Gid complex\textsuperscript{10}) and to a lesser extent UBE2D3 (but not with CDC34 or UBE2G) while stably expressed RMND5A immunopurified from HEK293 cells showed activity with UBE2H\textsuperscript{5}. In chapter 2 of this thesis, recombinant RMND5A paired with UBE2D1, UBE2D2, and to a lesser extent UBE2E1 in ubiquitination assays, while endogenous RanBPM immunoprecipitations in HEK293 cells functioned in ubiquitination assays with UBE2D1, UBE2D2, and UBE2D3 (but not CDC34 or UBE2H)\textsuperscript{4}. In a yeast two hybrid screen with 35 E2 and 251 RING human proteins, RMND5B (RMND5A and MAEA were not tested) showed a positive interaction only with UBE2D2, UBE2D3, UBE2D4, UBE2E, UBE2E3, and UBE2W\textsuperscript{11}. Compelling evidence for \textit{in vivo} function of the complex with UBE2H is its presence in ARMC8 and WDR26 co-immunoprecipitates\textsuperscript{5}. These inconsistencies are likely due to the different reaction conditions being used. Most relevant is the E2 used by the complex \textit{in vivo} for a particular substrate, which must be investigated on a case-by-case basis. Thus far, for the two \textit{in vitro} confirmed CTLH complex targets, HBP1 and Lamin B, UBE2H was used as the E2\textsuperscript{5,12}.

An underlying question that is also context-specific is the type of lysine linkage made. In Chapter 2 (Figure 2.10C), MS analysis of an \textit{in vitro} assay with recombinant
Table 5.1. Summary of E2 selectivity observed in different studies on the yeast Gid or human CTLH complexes.

<table>
<thead>
<tr>
<th>Study</th>
<th>UBE2H (or yeast Ubc8)</th>
<th>CDC34</th>
<th>UBE2D1</th>
<th>UBE2D2</th>
<th>UBE2D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Santt et al, 2008 – yeast Gid complex</td>
<td>Not tested in vitro, but presumed in vivo</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Activity with GST-GID2 in vitro Ub assay</td>
<td>Not tested</td>
</tr>
<tr>
<td>Van Wijk et al, 2009 – Y2H screen with human RMND5B</td>
<td>No binding</td>
<td>No binding</td>
<td>No binding</td>
<td>Binding</td>
<td>Binding</td>
</tr>
<tr>
<td>Lampert et al, 2018 – human complex</td>
<td>Full activity with recombinant complex and immunopurified RMND5A in vitro Ub assay</td>
<td>No activity with recombinant complex in vitro Ub assay</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Some activity with recombinant complex in vitro Ub assay</td>
</tr>
<tr>
<td>Maitland et al, 2019 (Ch 2) – human complex</td>
<td>No activity with RanBPM immunocomplex and GST-RMND5A in vitro assays</td>
<td>No activity with RanBPM immunocomplex</td>
<td>Activity with RanBPM immunocomplex and GST-RMND5A in vitro assays</td>
<td>Activity with RanBPM immunocomplex and GST-RMND5A in vitro assays</td>
<td>Activity with RanBPM immunocomplex, no activity with GST-RMND5A in vitro assays</td>
</tr>
<tr>
<td>Qiao et al, 2020 – yeast Gid complex</td>
<td>Activity with recombinant complex</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>
human RMND5A and UBE2D2 as the E2 revealed K48 and K63 polyubiquitination linkages. In E3-independent or RING E3-dependent Ub transfer, polyUb linkage choice is dictated by the E2, and can depend on the orientation of the lysines of the incoming Ub molecule\(^\text{13}\). In E3-independent \textit{in vitro} assays, UBE2D2 can produce K11, K48, and K63 polyUb chains, while UBE2H can produce only K11 and K48 chains\(^\text{14}\). The ubiquitin code is not always straight-forward, but in general K11 and K48 chains mediate proteasomal degradation while K63 chains regulate complex assembly, endocytosis, protein trafficking, and many other effects\(^\text{15}\). Ubiquitination regulation by the CTLH complex of the following substrates affects their proteasomal degradation: c-Raf, muskelin, AMPK, HBP1, and lamin B\(^{4,5,12,16,17}\). Only for AMPK has it been determined that this is through K48 chains, but it is very likely the other candidate substrates listed are also K48 chains. On the other hand, our observation of ubiquitination affecting PKM2 and LDHA activity (Chapter 4) is likely to be through a different polyUb linkage. I speculate that it might be K63 chains orchestrated by UBE2D2 or an unknown E2 (since UBE2H only produces K11 and K48 chains) and that this regulates complex assembly of PKM2 and LDHA, since activities of both enzymes are altered by their oligomeric status\(^{18,19}\).

### 5.3 Improved model of the human CTLH complex topology defines the utility of multiple substrate receptors

RING E3 proteins that are monomeric or dimeric typically contain the RING domain that binds the E2–Ub and a protein interaction surface that binds the substrate. Multi-subunit and multi-domain E3 ligases, on the other hand, have multiple substrate interaction surfaces. CRLs represent the best-studied example of a multi-subunit E3
ligase. This thesis introduces a new human multi-subunit E3 ligase and the investigation of its topology in Chapter 3 helps envision its mechanism of Ub transfer.

Initial diagrams of human complex topology relied on the model generated for the yeast complex from Menssen et al., 2012\textsuperscript{20}. One prevalent issue with this model is that it is too simplistic and does not consider the three-dimensional space occupied by the different subunits. The cryo-EM structural determination of the yeast complex provided a key step towards a more realistic view of the topology of the Gid complex; however, significant deviations of the human and yeast proteins as mentioned in other parts of this thesis meant the human complex architecture required its own investigation. In chapter 3, CLMS was used to get a better visual of human complex topology and complement the Western blot IPs. One of the keys of this technique was that it used the endogenous complex, as opposed to reconstitution of recombinant subunits.

The updated structural model (see figure 3.7), while still incomplete, shows an intriguing arrangement of most of the protein interaction domains of the complex subunits, including RanBPM SPRY, muskelin kelch repeats, and WDR26 in proximity of the RING dimers of RMND5A-MAEA. The finding of a muskelin ubiquitination site at K260 (between its CTLH domain and first kelch repeat) in chapter 4 supports this arrangement in that the E2 bound to RMND5A RING domain is near muskelin kelch repeats. Yet, it is currently still difficult to imagine the complete architecture until higher resolution structures are determined. The cryo-EM structure shows Gid1 (RanBPM homologue) SPRY domain adjacent to the Gid4 β-barrel\textsuperscript{6}, which would be consistent with the CLMS data in that the SPRY domain, like Gid4, is near the catalytic module.
Thus, RanBPM likely has a dual role in the CTLH complex: (1) potentially acting as a substrate receptor via the SPRY domain and (2) as part of the scaffold. The RanBPM SPRY domain as a substrate receptor is intriguing when considering it was not required for association of any CTLH complex subunit (Figure 3.4) but in previous reports determined that it is responsible for mediating most of its interactions of RanBPM with other proteins (e.g., Axl, Met, p73, as highlighted in introduction and Salemi et al., 2017). It is tempting to speculate that a portion of these proteins may in fact be substrates of the complex.

Yet, substrate interacting surfaces may be even more complicated since the CRA domain of RanBPM interacts directly in vitro with c-Raf, which is expected to be a ubiquitination target of the complex. In structures where proteins containing LisH, CTLH, and CRA domains have been crystallized, hydrophobic grooves created by relative positions of the α-helices within or between each domain produce distinct binding surfaces. For example, unique positioning of the LisH domain helices in human TBL1 creates hydrophobic grooves that mediates interaction with SMRT and GPS2. In atTPL and TPR2, a hydrophobic groove between CTLH and CRA domains binds peptides containing the Ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif (LxLxL) that is found in many of the transcriptional regulators interacting with TPL or TPR2. Modeling suggests human RanBPM possesses the same type of groove as TPL/TPR2, as does homologues of TWA1 and WDR26 in A. thaliana.
Work on the complex composition indicates that the presumed substrate receptors (RanBPM, WDR26, muskelin, GID4) co-exist in the same complex\textsuperscript{4,5}. This contrasts with CRLs which have multiple interchangeable substrate receptors\textsuperscript{25}. Thus, in a snapshot image, the CTLH complex architecture is, in general, more complicated than CRLs. Although the total number of substrate receptors is lower, this may give the CTLH complex the advantage of being poised and dedicated for ubiquitination of its substrates immediately upon a signal.

5.4 Do complex subunits share common functions?

In the introduction, reports of functions of individual subunits on various processes and pathways were summarized. Many of these studies used specific model systems and cell lines making it difficult to make comparisons. Yet, there are some interesting overlaps, such as the roles of muskelin and RanBPM in neurodegenerative disease progression and impact on trafficking of specific proteins for endocytosis\textsuperscript{26–29}, and the similar effect of WDR26 and RMND5A knockout in \textit{X. laevis} development\textsuperscript{30,31}. In the paradigm that muskelin and GID4 are separate substrate receptors, they would not be expected to have common functions, but muskelin and MAEA or GID4 and MAEA should. This hypothesis should be tested in future experiments. In the initial characterization of the complex, a sucrose gradient assay determined that all complex members tested (RanBPM, muskelin, TWA1, ARMC8 isoforms, RMND5A, and MAEA) have nearly identical sedimentation behaviours peaking at the 20S fraction and are undetectable at the lower gradients\textsuperscript{32}, suggesting that these subunits are unlikely to exist outside of the complex.
An issue not directly addressed by this thesis is the relationship of RanBP10 and RMND5B with the complex. It is unclear if they replace their paralogues in certain contexts or present as additional entities. Like Gid2-Gid9 in yeast\textsuperscript{20}, MAEA and RMND5A protein stability is interdependent (Chapter 2)\textsuperscript{1}. However, while RMND5A levels are reduced 90% in MAEA KO cells, MAEA is reduced only 50% in RMND5A KO cells (Figure 2.4). It is possible that MAEA stability can be partially maintained due to RMND5A redundancy with RMND5B. A sizeable overlap of differently changed protein abundances in the RMND5A KO and MAEA KO in Chapter 4 indicate, however, that the redundancy/compensation by RMND5B would be minimal. When transfected as GFP fusion proteins, RMND5B is exclusively cytoplasmic while RMND5A is nucleocytoplasmic, suggesting that there are functional differences between them\textsuperscript{30}. Nevertheless, the existence of variations of complex composition or compensation by RMND5B and RanBP10 paralogues is an important consideration going forward. Compensation by RanBP10 upregulation in RanBPM deficient mouse embryonic stem cells has been documented, and vice versa RanBPM expression is upregulated in RanBP10 null mice\textsuperscript{33,34}. RanBP10 compensation may explain a minimal overlap of the RanBPM-dependent proteome compared to the MAEA or RMND5A-dependent proteomes in chapter 4.

Many studies have described interacting proteins and effects of overexpression and/or knockdowns for various complex subunits in different model systems, most prominently for RanBPM. However, careful considerations are needed with ascribing CTLH complex functions with experiments involving overexpressed subunits. There is
clearly a stoichiometric relationship between subunits (e.g., interdependence of RMND5A, MAEA, RanBPM, TWA1)\textsuperscript{4,5}, and an increased subunit, unless it was in a limiting amount prior to induction (e.g., in knockdown cells), would only integrate into the complex and affect its function insomuch as the levels of the other subunits permits. Additionally, subcellular localization of the transfected CTLH subunits in many cases has not been reflective of the subcellular localization of the endogenous protein. This is particularly the case for muskelin and WDR26, both almost completely cytoplasmic when transfected, and MAEA, exclusively nuclear when transfected\textsuperscript{4,32}. Investigations of endogenous muskelin and WDR26 demonstrate it is present in the nucleus (varying amounts depending on cell type), while endogenous MAEA antibody detects the protein in the cytoplasm and plasma membrane\textsuperscript{12,35–38}. This discrepancy provides a cautionary tale of interpreting results using ectopic expression of muskelin, WDR26, and MAEA.

Nevertheless, previous work focused on individual subunits should be re-assessed in light of their - unrealized at the time - involvement in a multi-subunit E3 ligase complex. \textit{UBE2H} and most complex subunits do rank in the top genetic co-dependencies of each other in the Cancer Dependency Map project (DepMap)\textsuperscript{39,40}, confirming common functions of subunits and further substantiates \textit{UBE2H} as the primary cognate E2 enzyme.

5.5 Possible modes of regulating CTLH complex function

A consideration going forward is how the activity of the complex might be regulated by a signal. The control of Gid4 levels in yeast may provide some clues. In \textit{S. cerevisiae}, protein levels of Gid4 are in low amounts under normal conditions, decreased
to undetectable levels when yeast cells are switched to gluconeogenic conditions (i.e. grown under ethanol), but become highly elevated - concomitant with increased RNA levels - immediately after reintroduction of glucose to glucose-starved yeast cells\textsuperscript{3,41,42}. Once \textit{S. cerevisiae} return to a normal metabolic state, Gid4 is ubiquitinated and rapidly degraded (in similar kinetics to Fbp1) that is dependent on the proteasome, Ubc8/Gid3, and all Gid complex proteins except Gid7\textsuperscript{3,41}. The postulated model is that once the gluconeogenic enzymes are degraded, the Gid complex ubiquitinates Gid4 to close the loop and restore the complex to the original state. Gid4 phosphorylation, which is increased in cells grown in ethanol, has been speculated as a mode of regulation in combination with ubiquitination to control Gid4 stability\textsuperscript{41}.

The proteasomal degradation and ubiquitination of muskelin by the complex that we observed in Chapter 2 is reminiscent of the regulation of Gid4 in yeast. It suggests that the human complex may regulate its muskelin-dependent functions by autoubiquitination. This is supported by recent findings on the drosophile CTLH complex regulation of Cup, TRAL, and ME31B during maternal-to-zygotic transition (MZT)\textsuperscript{43,44}. In the late stages of MZT, muskelin is ubiquitinated and its protein levels are rapidly reduced\textsuperscript{43}. Notably, \textit{D. melanogaster} muskelin, but not RMND5A, is required for the association of RanBPM with ME31B\textsuperscript{44}. Thus, just like Gid4 is ubiquitinated by the Gid complex after the gluconeogenic enzymes are degraded, muskelin autoregulation after MZT in drosophila might serve as a switch to channel the complex away from muskelin-dependent substrates.
Interestingly, at the onset of the drosophila MZT, activation of the CTLH complex is triggered by upregulation of a UBE2H homologue\(^{43,44}\). This suggests that modulation of UBE2H levels could be a trigger in other settings as well. UBE2H contains a NF-κB binding site in the promoter and its expression is upregulated by the proinflammatory cytokine tumor necrosis factor α (TNF-α)\(^{45}\). In fact, UBE2H upregulation is part of an overall increased ubiquitin conjugating activity observed upon treatment with TNF-α\(^{45}\). UBE2H upregulation has also been observed during erythroid differentiation by the Tal1 transcription factor\(^{46}\).

Some subunits have been demonstrated to be subjected to post-transcriptional regulation by microRNA (miRNA): RanBPM by mir-101 in mice hippocampus, and mir-26a-5p in rat perihematomal tissues and PC-12 cells\(^{47,48}\); RanBP10 by mir-378 in bovine preadipocytes and mir-196a in N2a cells and mice\(^{49,50}\); ARMC8 by mir-664 in HaCaT cells, which mediates UVB irradiation-induced decreased expression of ARMC8\(^{51}\); and RMND5A, which is targeted by mir-138 in HeLa cells and is a likely target of the oncomir mir-21 in liver cancer\(^{52,53}\). Thus, miRNA modulation is another mechanism by which the complex could be regulated.

Subcellular localization of complex subunits can be dynamic and responsive to changes in the environment as described in the introduction. Additionally, a few cases of PTM regulation of subunits have been described. RanBPM is phosphorylated by ATM which is required for RanBPM nuclear accumulation in the immediate cellular response to ionizing radiation\(^{54}\). Muskelin is phosphorylated by PKC, which may regulate its self-association when exogenously expressed\(^{55}\). Allosteric changes induced by a PTM in the
substrate receptors or RING domain that affect substrate engagement or promotion of Ub transfer are a general feature of E3 ligases\textsuperscript{56}. At present, however, there is no link of subcellular localization change or PTM regulation of the CTLH complex affecting its activity, but it is likely given prior observations of the tight regulations that other E3 ligase complexes such as CRLs are under. For instance, neddylation (conjugation of NEDD8) at a site in any of the cullins at the C-terminus (near Rbx1/2 binding) induces a conformational change that promotes E2 binding\textsuperscript{57}. This site is subject to deneddylation by the COP9 signalosome, an interaction strengthened by the metabolite inositol hexakisphosphate (IP\textsubscript{6})\textsuperscript{58,59}. CRLs can also be regulated be in other ways, such as signal-induced disassociation of the substrate receptor\textsuperscript{60}, CAND1-mediated inhibition of neddylation\textsuperscript{61}, and post-translational modification of the binding motif on the substrate that alter its binding to the substrate receptor\textsuperscript{62,63}. These examples provide some ways in which the CTLH complex could also be regulated.

5.6 Implications of the CTLH complex in cancer

During the preparation of the first data chapter, I contributed to projects that investigated the involvement of the CTLH complex in regulatory events of oncogenic pathways previously linked with RanBPM. The first study involved the cytoplasmic deacetylase HDAC6, which had been shown to interact with RanBPM and which activity was shown to be inhibited by RanBPM\textsuperscript{64}. We were able to show that RMND5A and muskelin were also involved in HDAC6 inhibition, and muskelin also associated with HDAC6, demonstrating the involvement of the CTLH complex in HDAC6 regulation\textsuperscript{65}. RanBPM was also previously identified as an inhibitor of the ERK pathway by
modulating c-Raf protein levels. We subsequently linked RMND5A to regulating c-Raf protein levels and ubiquitination, and inhibition of the ERK pathway. Finally, unpublished results from our lab show that loss of multiple CTLH complex subunits results in increased β-catenin protein levels and hyperactivity of the WNT signaling pathway, which has recently been confirmed by another study. The regulation of glycolysis observed in Chapter 4 adds another tumour suppressive function of the CTLH complex since deregulation of central carbon metabolism is frequently observed in cancer cells to sustain rapid proliferation. All together, these findings point towards the complex being necessary to restrict oncogenesis by acting on multiple pathways and hallmarks of cancer. Yet, the complex also been reported to have cancer-promoting functions, such as its ubiquitination of cell cycle regulator HBP1. Such opposing effects on cancer development are consistent with examples of other proteins (including other E3 ligases) or signaling pathways that act as either tumour promoting or suppressive depending on cellular context. It suggests that the CTLH complex may serve as a hub to maintain homeostasis by regulating a multitude of pathways via ubiquitination of diverse substrates. However, if altered (e.g., by mutation), it could promote either a pro- or anti-proliferative status contingent on the expression levels, PTM status, and subcellular localization of its interacting proteins or substrates, and the dependency of the cell on the affected substrates.

While mutations of individual CTLH complex members are relatively rare, the pool of complex member mutations need to be considered as a whole since CTLH complex members’ stabilities and thus functions are interdependent. For example,
mutations in RanBPM, TWA1, MAEA, or RMND5A would all have the same effect of making the RING dimer unstable. Additionally, mutations of the presumed substrate receptors that would prevent their interactions with the complex or with their recruitment of substrates would also affect complex function. Also, mutations in various cancers types occur in the binding pocket of GID4 that affect its capability of binding N-terminal proline peptides.

Instances of increased expression of a subunit in cancer should be interpreted with caution because of the stoichiometric relationship of subunits as discussed above. Only if the increased subunit is in a limiting amount before mutation relative to the other subunits or the other subunits are also increased in the same diseased individual would it be expected to matter in terms of affecting complex function. Further caution is applied in cases where only RNA expression is considered, since, across 375 cancer cell lines, the RNA to protein correlation of the CTLH complex subunits is low, particularly for MAEA. Mutations disrupting complex integrity or changing activity are more telling, as they would impact the entire complex. Mutations in yet to be identified positive or negative regulators of the complex could also contribute to a diseased state.

5.7 Significance of the work

At the start of this thesis (January 2016), the number of publications mentioning the mammalian CTLH complex could be counted on one hand. At this time, the RING domains in human MAEA and RMND5A were characterized only by their primary sequence and GID4 did not have a proper gene name yet. In fact, the first functional relationship between subunits was made in 2017 by Salemi et al. Prior to this, CTLH
subunits were studied only in isolation and their functional relationship with the complex was not considered, or only speculated. By determining that the CTLH complex is an active E3 ligase, characterizing its architecture and cataloging potential substrates, this thesis sets a foundation for the complex to be investigated on a larger scale. Now, more detailed mechanistic studies of its structure and function can commence.

An important focus going forward should be confirming putative ubiquitination targets in vitro and establishing functional effects beyond one cell type. The impact of chapter 4 is limited because it lacks both of those elements. Future work should investigate the relationship of the CTLH complex with LDHA, PKM2, and glycolysis in an in vivo tumour model to determine if this is a mechanism that cancer cells can hijack to metabolically support tumour growth.

Identification of RanBPM, muskelin, WDR26, and GID4 as substrate receptors warrants interest in identifying their interacting substrates. High throughput proximity interaction assays, such as BioID, in combination with knockout cell lines could capture substrate receptor-dependent transient interactions with substrates at a greater scale than the RanBPM affinity purification MS done in chapter 4. Deciphering which subunit (and domain) recruits the specific substrate of interest should be a priority for all future investigations on CTLH complex ubiquitination targets. High-resolution structural investigation of the subunit engaged with its substrate in the context of the entire complex would be valuable towards understanding details of CTLH complex mediated Ub transfer to specific substrate lysines. For example, characterizing the E2–Ub-bound CTLH complex in association with PKM2 or LDHA could lead to the development of a E3
ligase-substrate interaction trapping molecule. A drug such as this could be used to treat tumours addicted to glycolysis.

5.8 References


Appendix A: Permissions from Scientific Reports

The mammalian CTLH complex is an E3 ubiquitin ligase that targets its subunit muskelin for degradation

Author: Matthew E. R. Maltland et al
Publication: Scientific Reports
Publisher: Springer Nature
Date: Jul 8, 2019
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Appendix B: Cross-linked peptides identified

List of all residue pairs that passed all FDRs and found in at least two samples as
determined by xiSEARCH and xiFDR. Site1: residue in Protein1 crosslinked to Protein2.
Site2: residue in Protein2 crosslinked to Protein1. Score: score for the link based on all
supporting peptide pairs. Link: Between – intermolecular link; Self – intramolecular link.
Fdr - FDR associated with the match score. PEP - Posterior Error Probabilities. Rep# -
indicates which replicate sample. Fraction# - indicates which SCX fractionation.

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<th>Protein1</th>
<th>Protein2</th>
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<td>unassigned, 1, 7, 8, &gt;8</td>
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<tr>
<td>Exclusion mass width low</td>
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<tr>
<td>Exclusion mass width high</td>
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<tr>
<td>Activation time</td>
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<tr>
<td>Minimum signal required</td>
<td></td>
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**RanBPM APMS**

- **Q Exactive Plus**: Setting for Q Exactive Plus spectrometry.
- **Profile**: Scan type for profile acquisition.
- **445.120025**: Mass range for scan acquisition.
- **35K**: Microscan range for acquisition.
- **1.00E+05**: Target AGC for MS2 injection.
- **64 ms**: Injection time for MS2.
- **12**: Loop count for MS2.
- **1.2 m/z**: Isolation width for MS2.
- **0.5 m/z**: Isolation offset for MS2.
- **HCD**: Normalized collision energy.
- **enabled**: Dynamic exclusion setting.
- **Top12**: Exclusion duration setting.
- **unassigned, 1, 2, 8, >8**: Charge exclusion setting.
- **30s**: Minimum signal required.

**Proteomes**

- **Q Exactive Plus**: Setting for Q Exactive Plus spectrometry.
- **Profile**: Scan type for profile acquisition.
- **445.120025**: Mass range for scan acquisition.
- **35K**: Microscan range for acquisition.
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- **HCD**: Normalized collision energy.
- **enabled**: Dynamic exclusion setting.
- **Top12**: Exclusion duration setting.
- **unassigned, 1, 2, 8, >8**: Charge exclusion setting.
- **30s**: Minimum signal required.

**RMND5A diGLY enrichment**

- **Q Exactive Plus**: Setting for Q Exactive Plus spectrometry.
- **Profile**: Scan type for profile acquisition.
- **445.120025**: Mass range for scan acquisition.
- **35K**: Microscan range for acquisition.
- **1.00E+05**: Target AGC for MS2 injection.
- **64 ms**: Injection time for MS2.
- **12**: Loop count for MS2.
- **1.2 m/z**: Isolation width for MS2.
- **0.5 m/z**: Isolation offset for MS2.
- **HCD**: Normalized collision energy.
- **enabled**: Dynamic exclusion setting.
- **Top12**: Exclusion duration setting.
- **unassigned, 1, 2, 8, >8**: Charge exclusion setting.
- **30s**: Minimum signal required.

**RanBPM diGLY enrichment**

- **Orbitrap Elite**: Setting for Orbitrap Elite spectrometry.
- **Profile**: Scan type for profile acquisition.
- **445.120025**: Mass range for scan acquisition.
- **35K**: Microscan range for acquisition.
- **1.00E+05**: Target AGC for MS2 injection.
- **64 ms**: Injection time for MS2.
- **12**: Loop count for MS2.
- **1.2 m/z**: Isolation width for MS2.
- **0.5 m/z**: Isolation offset for MS2.
- **HCD**: Normalized collision energy.
- **enabled**: Dynamic exclusion setting.
- **Top12**: Exclusion duration setting.
- **unassigned, 1, 2, 8, >8**: Charge exclusion setting.
- **30s**: Minimum signal required.
Curriculum Vitae
Matthew Edward Ross Maitland

Education

Doctor of Philosophy (PhD) 2016 – 2021
Department of Biochemistry, The University of Western Ontario, London, Canada
Supervisors: Drs. Caroline Schild-Poulter and Gilles Lajoie

Master of Science (MSc) 2013 – 2015
Department of Biochemistry, The University of Western Ontario, London, Canada
Supervisor: Dr. Joe Torchia

Bachelor of Medical Sciences (BMSc) 2009 – 2013
The University of Western Ontario, London, Canada
Honours Specialization in Biochemistry

Awards and Scholarships

NSERC Post Graduate Scholarship – Doctoral ($21,000/year) 2018-2020
Canadian Cancer Society Travel Award 2018
Ontario Graduate Scholarship ($15,000 – declined) 2018
Cancer Research and Technology Transfer PhD fellowship ($1000) 2017
Ontario Graduate Scholarship ($15,000) 2017
Poster Presentation Award (Cancer) – Robarts Research Retreat 2017
Canadian National Proteomics Network Travel Award ($500) 2017
Teaching Honor Roll – based on student evaluations 2015, 2017

Publications

Notable Conference Presentations (Presenter is underlined)

   *Abstract selected for oral presentation.*
   *National meeting*

   *Invited speaker.*

   *International meeting*

   *Abstract selected for oral presentation.*

   *International meeting*

   *Abstract selected for a travel award.*
   *National meeting*

   *Poster presentation award – cancer category.*

   *Abstract selected for oral presentation.*
   *International meeting*
## Teaching Experience

<table>
<thead>
<tr>
<th>Course</th>
<th>Years</th>
<th>Institution</th>
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</thead>
<tbody>
<tr>
<td><strong>Biochem 3385 (Human Biochemistry) Teaching Assistant</strong></td>
<td>2017 &amp; 2018</td>
<td>Department of Biochemistry, The University of Western Ontario, London, Canada</td>
</tr>
<tr>
<td><strong>Biochem 3380 (Biochemistry Laboratory) Teaching Assistant</strong></td>
<td>2014 – 2017</td>
<td>Department of Biochemistry, The University of Western Ontario, London, Canada</td>
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## Leadership and Professional Experience

<table>
<thead>
<tr>
<th>Role</th>
<th>Years</th>
<th>Institution</th>
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<tbody>
<tr>
<td><strong>Research Information Outreach Team (RIOT)</strong></td>
<td>2018 – 2020</td>
<td>Canadian Cancer Society (CCS), London, Canada</td>
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<tr>
<td>Chair</td>
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<tr>
<td>Team member</td>
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<table>
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<tr>
<th>Role</th>
<th>Years</th>
<th>Institution</th>
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<tbody>
<tr>
<td><strong>Biochemistry Graduate Student Association</strong></td>
<td>2018 – 2019</td>
<td>Department of Biochemistry, The University of Western Ontario, London, Canada</td>
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<tr>
<td>Co-founder, Co-chair, Communications</td>
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<table>
<thead>
<tr>
<th>Role</th>
<th>Years</th>
<th>Institution</th>
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<tbody>
<tr>
<td><strong>Let’s Talk Cancer Organizing Committee</strong></td>
<td>2018 – 2019</td>
<td>Canadian Cancer Society and Let’s Talk Science, London, Canada</td>
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<tr>
<td>Chair</td>
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<tr>
<td>Speaker liaison and logistics</td>
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<tr>
<th>Role</th>
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<tbody>
<tr>
<td><strong>Maud L. Menten &amp; Visiting Speaker Committee</strong></td>
<td>2016 – 2018</td>
<td>Department of Biochemistry, The University of Western Ontario, London, Canada</td>
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<tr>
<td>Student representative &amp; student-speaker meeting host</td>
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<tr>
<th>Role</th>
<th>Years</th>
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<tr>
<td><strong>Robarts Association of Trainees</strong></td>
<td>2016 – 2018</td>
<td>Robarts Research Institute, The University of Western Ontario, London, Canada</td>
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<td>Councilor</td>
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<th>Role</th>
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<tr>
<td><strong>Robarts Research Retreat (RRR) Organizing Committee</strong></td>
<td>2016 – 2017</td>
<td>Robarts Research Institute, London, Canada</td>
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<tr>
<td>Co-chair</td>
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<tr>
<td>Academic content lead</td>
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