Lysosomal interplay between ubiquitination and phosphorylation regulates chaperone-mediated autophagy

Rakshit, Moumita

2017

Rakshit, M. (2017). Lysosomal interplay between ubiquitination and phosphorylation regulates chaperone-mediated autophagy. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72725>

<https://doi.org/10.32657/10356/72725>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**Lysosomal interplay between ubiquitination
and phosphorylation regulates Chaperone-
mediated autophagy**

Moumita Rakshit

School of Biological Sciences

2017

**Lysosomal interplay between ubiquitination
and phosphorylation regulates Chaperone-
mediated autophagy**

MOUMITA RAKSHIT

SCHOOL OF BIOLOGICAL SCIENCES

NANYANG TECHNOLOGICAL UNIVERSITY

SINGAPORE

**A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy**

2017

Acknowledgement

I would like to thank my supervisor Dr. Esther Siew Peng Wong who implanted the germinal seed of this thesis. Her vast horizon of knowledge is infectious and spurred the researcher in me to push the envelope every time I seemed to have hit a dead end. The unmistakable ability to troubleshoot any given problem at any point of time cushioned me at times when I doubted myself. Furthermore, apart from shaping my academic being, Dr. Esther even took interest in my personal well-being.

I am equally thankful to the members of my thesis advisory committee, Dr. Lu Lei and Dr. Valerie Lin, Nanyang Technological University, for their unwavering support and augmenting my thesis with their discerning inputs. Their passion for research is exemplary and commands utmost respect of any biologist, budding or established.

I take this opportunity to thank the two most important people in my life, who have lived my PhD journey as much as I did - my father Mr. Swapan Rakshit and my mother Mrs. Archana Rakshit. They have been mostly patient and sometimes agitated but have been instrumental in the maintenance of my sanity throughout this odyssey of mine. My father has taught me to never give up, and whenever the situation is not in our favor, to keep fighting our way to glory. My mother has taught me to be patient and to never lose hope however adverse the situation is. I would also like to thank my sister Madhurima Rakshit for being by my side at all times. Her cheerfulness always brightened up my spirits and helped me strive through.

I am also blessed to have a gem of a person as my husband Mr. Dwaipayan Das without whose support this journey would have been impossible. His constant care, advices and sacrifices over the years have made life easier during difficult times.

My heartfelt thanks to Nanyang Technological University for the generous scholarship support throughout the four years and for providing excellent learning and research facilities. Many thanks to past and present lab members: Karen, Zhi Wei, Si Jie and Chye Yun. They helped to create a joyful and enjoyable working environment.

I am lucky to have a bunch of genuine friends who form an integral part of this crucial turn-around of my life and have been like family to me away from home. I would like to thank Sandeep Dighe, Manisha Mukherjee, Priya Dharshana Ramani, Tofayel Ahmed, Muneesh Maheshwari, Chandra Mouli Iyer, Karen Lee, Manish Muhuri, Ishani Dasgupta and Bibek Ray Chaudhuri. Special thanks to Nishanth V Menon for helping with the formatting.

Above all, I thank Sai baba and the Almighty for His kind blessings and for instilling in me the motivation to strive harder each day with equal zeal and enthusiasm.

Contents

1. INTRODUCTION	1
1.1 AUTOPHAGY- AT A GLANCE	1
1.1.1 <i>Different forms of autophagy</i>	2
1.2 CHAPERONE-MEDIATED AUTOPHAGY (CMA)	5
1.2.1 <i>The CMA machinery</i>	8
1.2.2 <i>The lysosomal mTORC2/Akt1/PHLPP1 regulatory pathway of CMA</i>	12
1.2.3 <i>Small molecule regulators of CMA</i>	16
1.3 PHYSIOLOGICAL IMPORTANCE OF CMA	16
1.3.1 <i>CMA – a stress response pathway</i>	17
1.3.2 <i>CMA and cellular energetics</i>	19
1.3.3 <i>CMA in DNA damage response</i>	21
1.3.4 <i>Other roles of CMA</i>	21
1.3.5 <i>CMA in Cancer</i>	24
1.3.6 <i>CMA and Leaky Lysosomes</i>	24
1.4 THE LAMP-2A KNOCKOUT MODEL	25
1.5 CONSEQUENCES OF CMA FAILURE	26
1.5.1 <i>Aging</i>	26
1.5.2 <i>Functional failure of CMA in neurodegenerative diseases</i>	28
1.6 CROSSTALK BETWEEN CMA AND OTHER DEGRADATIVE PATHWAYS	32
1.7 UBIQUITINATION	34
1.8 THE E4 LIGASE CHIP	35
1.8.1 <i>Association of CHIP with CMA</i>	37
1.9 THE DE-UBIQUITINATING ENZYME UCH-L1	38
1.10 AIMS	39
1.10.1 <i>Examine association of ubiquitin and ubiquitin modifying enzymes with CMA-active lysosomes</i>	39
1.10.2 <i>Examine the role of E3 ligase CHIP in regulating CMA activity</i>	40
1.10.3 <i>Examine the role of DUB UCH-L1 in regulating CMA activity</i>	40
1.10.4 <i>To elucidate the relationship between CHIP and UCH-L1 in CMA regulation and crosstalk with UPS</i>	41
2. MATERIALS AND METHODS	43
2.1 CELL AND CULTURE	43
2.2 ANTIBODIES AND CHEMICALS	43
2.3 CREATION OF STABLE KNOCKDOWN CELL LINES	44
2.4 CREATION OF STABLE CHIP OVEREXPRESSION CELL LINES	46
2.5 PURIFICATION OF LYSOSOMAL FRACTION	46
2.6 SEPARATION OF LYSOSOMAL MEMBRANE AND MATRIX FRACTIONS	47
2.7 CMA+/ CMA- LYS ISOLATION FROM RAT LIVER AND FROM MICE BRAIN	48
2.8 IMMUNOBLOTTING AND DENSITOMETRIC ANALYSIS	49

2.9 ANALYSIS OF LAMP-2A MULTIMERIC COMPLEXES BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION	51
2.10 IMMUNOPRECIPITATION AND CO-IMMUNOPRECIPITATION	52
2.11 IMMUNOSTAINING AND IMMUNOFLUORESCENCE	54
2.12 REVERSE TRANSCRIPTION (RT-PCR) AND QUANTITATIVE REAL-TIME PCR (qPCR)	55
2.13 CLONING OF UCH-L1 GENE INTO pCDNA VECTOR	56
2.14 SITE-DIRECTED MUTAGENESIS	57
2.15 CALCULATION OF LC3-II FLUX	58
2.16 MEASUREMENT OF PROTEASOME ACTIVITY	59
2.17 ASSESSMENT OF INTRACELLULAR PROTEIN DEGRADATION (PULSE AND CHASE)	59
2.18 STATISTICAL ANALYSIS	61
3. RESULTS	62
3.1 ASSOCIATION OF UBIQUITIN AND UBIQUITIN ENZYME CASCADE WITH CMA-ACTIVE LYSOSOMES	62
3.1.1 <i>Use of LAMP1 as lysosomal loading control</i>	63
3.1.2 <i>Association of ubiquitinated proteins with CMA-active lysosomal membrane changed</i>	65
3.1.3 <i>Greater association of ubiquitin modifying enzymes with CMA active lysosomes</i>	68
3.2 INVESTIGATING THE ROLE OF CHIP AND UCH-L1 IN CMA PATHWAY	69
3.2.1 <i>Creation of knockdown of CHIP and UCH-L1 in SH-SY5Y cells</i>	69
3.2.2 <i>Reduction in CMA activity with CHIP knockdown</i>	73
3.2.3 <i>CHIP KD reduced association of ubiquitinated proteins at the lysosomes</i>	76
3.2.4 <i>Depletion of CHIP affect the levels of CMA associated chaperones</i>	78
3.2.5 <i>Depletion of CHIP affects LAMP-2A multimerization</i>	80
3.2.6 <i>Depletion of CHIP affects the lysosomal levels of mTORC2/Akt1/PHLPP1 pathway</i>	83
3.2.7 <i>Overexpression of CHIP results in increase of PHLPP1 and reduced Akt1 activation</i>	86
3.2.8 <i>CHIP interacts with Akt1 and PHLPP1</i>	89
3.2.9 <i>CHIP regulates lysosomal Akt1 levels via its U-box ubiquitinating domain</i>	91
3.2.10 <i>TPR domain of CHIP interacts with Akt1</i>	94
3.2.11 <i>PHLPP1 interacts with TPR and U-box domains of CHIP</i>	96
3.2.12 <i>CHIP ubiquitinates lysosomal Akt1</i>	98
3.3 LOSS OF UCH-L1 INCREASES CMA ACTIVITY	100
3.3.1 <i>Depletion of UCH-L1 reduces Hsc70 levels</i>	100
3.3.2 <i>Enhanced CMA activity with UCH-L1 KD</i>	102
3.3.3 <i>UCH-L1 KD enhances LAMP-2A multimerization by repression of Akt1 signaling</i>	104
3.3.4 <i>DUB activity of UCH-L1 regulates Akt1 signaling and PHLPP1 gene expression</i>	107
3.4 CHIP REGULATES UCH-L1 LEVELS IN THE LYSOSOMES	110
3.4.1 <i>Presence of CHIP at the lysosomes decreases lysosomal UCH-L1 levels</i>	110
3.4.2 <i>CHIP regulates lysosomal UCH-L1 levels by influencing UCH-L1 degradation</i>	112
3.5 PRESENCE OF CHIP AND UCH-L1 IN THE LYSOSOMES MEDIATES CMA-UPS CROSSTALK DURING STRESS	114
4. DISCUSSION	117
4.1 A ROLE OF UBIQUITIN AND UBIQUITIN MODIFYING ENZYMES IN CMA	117
4.2 CHIP IS A REGULATOR OF CMA PATHWAY	118
4.3 UCH-L1 INHIBITS CMA THROUGH THE DUB ACTIVITY	119
4.4 CHIP AND UCH-L1 REGULATE LAMP-2A DYNAMICS TO AFFECT CMA	120
4.5 RECIPROCAL DYNAMICS BETWEEN CHIP AND UCH-L1 AT THE LYSOSOME UNDERSCORE UPS-CMA CROSSTALK	123
4.6 LIMITATIONS OF THIS STUDY	125
4.6.1 <i>Specificity of Hsp70/Hsc70 antibody</i>	125
4.6.2 <i>Possible presence of contaminating mitochondria and other cytosolic components in the lysosomal fractions</i>	126

5 CONCLUSIONS AND FUTURE DIRECTIONS.....	127
5.1 RAC1 MEDIATED RECRUITMENT OF PHLPP1 TO THE LYSOSOMES.....	128
5.2 CHIP AND UCH-L1 MEDIATED REGULATION OF PHLPP1 EXPRESSION	128
5.3 CMA CROSSTALK WITH MACROAUTOPHAGY PATHWAY	129
5.4 USE OF DENDRA BASED REPORTERS TO VIEW CMA CARGO DEGRADATION OR LAMP-2A MULTIMERIZATION PROCESSES.....	130
6. REFERENCES	131

List of Figures

Fig. 1.1	Different ways of delivering substrates to the lysosome by different autophagy variants.
Fig. 1.2	Basics of CMA.
Fig. 1.3	A model for the dynamic assembly and disassembly of LAMP-2A.
Fig. 1.4	Regulation of CMA by mTORC2/Akt1/PHLPP1 signaling in the lysosome.
Fig. 1.5	Physiological functions of CMA.
Fig. 1.6	The domain structure of CHIP.
Fig. 3.1.1	Levels of LAMP1 remain unaffected upon depletion of CHIP or UCH-L1
Fig. 3.1.2	Association of poly-ubiquitinated (polyUb) proteins and ubiquitin modifying enzymes with CMA active lysosomes.
Fig. 3.2.1	Knockdown of CHIP and UCH-L1 in SH-SY5Y cells.
Fig. 3.2.2	Reduced CMA activity upon CHIP KD.
Fig. 3.2.3	Reduced association of ubiquitinated proteins at lysosomes upon CHIP KD.
Fig. 3.2.4	Depletion of CHIP affects CMA lysosomal levels of chaperones.
Fig. 3.2.5	CHIP KD reduces LAMP-2A multimerization.
Fig. 3.2.6	Depletion of CHIP impacts the mTORC2/Akt/PHLPP1 pathway.
Fig. 3.2.7	Analysis of CHIP overexpression in SH-SY5Y cells stably expressing myc-CHIP.
Fig. 3.2.8	Overexpression of CHIP impacts lysosomal pAkt and PHLPP1 levels.
Fig. 3.2.9	Interaction of CHIP with Akt, Hsc70 and PHLPP1.
Fig. 3.2.10	Overexpression of U-box mutant CHIP causes accumulation of pAkt1.
Fig. 3.2.11	Akt and CHIP interaction is abolished upon mutation of TPR domain.
Fig. 3.2.12	PHLPP1 interacts with WT, TPR and U-box myc-CHIP mutants.
Fig. 3.2.13	Reduced ubiquitination of lysosomal Akt in CHIP KD cells.
Fig. 3.3.1	Depletion of UCH-L1 resulted in lower levels of lysosomal Hsc70.
Fig. 3.3.2	Enhanced CMA activity upon UCH-L1 KD.
Fig. 3.3.3	Increase in LAMP-2A multimers, PHLPP1 and decreased pAkt upon UCH-L1 KD.
Fig. 3.3.4	DUB activity of UCH-L1 regulates lysosomal Akt signaling.
Fig. 3.4.1	CHIP regulates PHLPP1 levels at the lysosomes through UCH-L1.
Fig. 3.4.2	CHIP interacts with UCH-L1 and ubiquitinates UCH-L1.
Fig. 3.5	CHIP drives crosstalk between UPS and CMA pathways.
Fig. 4.1	CHIP and UCH-L1 mediated regulation of CMA and crosstalk.

List of Tables

Table 2.1	Preparation of Nycodenz [®] gradient
Table 2.2	Composition of discontinuous sucrose gradient
Table 2.3	Treatments for measuring rates of protein degradations

Abstract

Chaperone-mediated autophagy (CMA) is a selective autophagic pathway that degrades soluble cytosolic proteins. It utilizes a cascade of chaperones and co-chaperones to recognize and deliver the substrates to the lysosome for degradation. Basal CMA operates in all mammalian cells in order to maintain protein homeostasis and quality control as well as performing specific functions in some cells like kidney cell growth, neuronal survival, antigen presentation and selective degradation of specific transcription factors or metabolic enzymes. CMA is induced upon stress and performs various cellular functions like recycling of raw materials under nutrient starvation, protection against oxidative stress, toxic stress and hypoxic stress. The rate limiting step in CMA is substrate binding to the lysosomal receptor lysosome-associated membrane protein-2A (LAMP-2A) followed by formation of the multimeric LAMP-2A translocation complex. It has been reported that the mTORC2/Akt1/PHLPP1 pathway regulates CMA activity by influencing the formation the LAMP-2A translocation complex. CMA is impaired during aging resulting in metabolic failure and various neurodegenerative disorders. Thus there is tremendous interest in understanding the regulation and deriving ways to regulate CMA function to harness pathological benefits.

Certain evidences suggest the role of ubiquitination in CMA pathway. For instance, rat liver lysosomes depleted of CMA promoted accumulation of ubiquitin-positive aggregates, K63-ubiquitination is necessary for CMA degradation of HIF-1 α and accumulation of K48 ubiquitinated protein in CMA-abolished cells, however, the exact

role of ubiquitination in targeting substrates to CMA pathway remains unclear. The current study has shown the novel role of the ubiquitin system in CMA regulation by uncovering the presence of ubiquitin and a plethora of ubiquitin modifying enzymes associated with the subgroup of lysosomes specifically dedicated to perform CMA (henceforth known as CMA-active lysosomes). Based on these observations, this project aims to understand the potential modulatory role of selected lysosome associated ubiquitin modifying enzymes- E4 ligase carboxyl-terminus of Hsp70 interacting protein (CHIP) and the deubiquitinating enzyme ubiquitin C-terminal hydrolase - L1 (UCH-L1) in different aspects of CMA pathway.

CHIP and UCH-L1 have been previously implicated in regulating the levels of Protein kinase B (Akt) and PH Domain and Leucine Rich Repeat Protein Phosphatase 1 (PHLPP1), but it is not known whether this function of CHIP and UCH-L1 can regulate CMA activity. Addressing this query, the present study has revealed that CHIP is an activator of CMA while UCH-L1 inhibits CMA by regulation of Akt1 and PHLPP1 levels. Knockdown studies revealed that depletion of CHIP leads to changes in lysosomal levels of ubiquitin, CMA associated chaperones as well as accumulation of pAkt1 accompanied by a decrease in levels of PHLPP1. CHIP helps in ubiquitination of pAkt1 leading to its proteasomal degradation. In the absence of CHIP, pAkt1 accumulates which leads to downstream destabilization of LAMP-2A translocation complex resulting in CMA inhibition. In contrast, knockdown studies on UCH-L1 indicate that depletion of UCH-L1 leads to accumulation of PHLPP1 accompanied by a reduction in pAkt1 levels in the lysosomes leading to stabilization of the LAMP-2A complex and promoting CMA activity. Effectively, CHIP and UCH-L1 regulate CMA activity by manipulation of the mTOR/Akt1/PHLPP1 pathway.

Subsequently, it was observed that CHIP regulates the levels of UCH-L1 at the lysosomes by ubiquitination and lysosomal degradation of UCH-L1. Thus, the two enzymes operate in the same pathway; their activities being interconnected. The evidence that the ubiquitin modifying enzymes associate with CMA-active lysosomes and regulate CMA activity could indicate a possible crosstalk between the ubiquitin proteasome system (UPS) and CMA pathway. In the current study, it was observed that upon inhibition of the proteasomal pathway, CHIP localized at the lysosomes, thus reducing lysosomal levels of UCH-L1, pAkt1 and increasing levels of PHLPP1. This could indicate a rise in CMA activity under proteasomal stress. Thus in this study, a novel mechanism of CHIP-mediated crosstalk between the UPS and CMA pathways upon conditions of proteasomal stress has been identified.

This study has been advantageous in understanding the intricate connection between ubiquitination and phosphorylation pathways regulating CMA activity and can be exploited in developing therapeutic benefits by cell type specific studies as UCH-L1 is highly abundant in brain. The current study also provides an insight in understanding the crosstalk between the UPS and CMA degradation pathways both of which are implicated in various neurodegenerative diseases.

List of Abbreviations

3PGK	3- Phosphoglycerate Kinase
6-AN	6-Anisomycin
AD	Alzheimer's Disease
ATG5	Autophagy Related Gene 5
ATG7	Autophagy Related Gene 7
ATP	Adenosine Tri-Phosphate
BAG1	BCL2 Associated Anthogene-1
BAG3	BCL2 Associated Anthogene-3
CASA	Chaperone Assisted Selective Autophagy
CDK	Cyclin Dependant Kinase
CHIP	C-Terminal Of Hsc70 Inteacting Protein
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
CMA	Chaperone-Mediated Autophagy
CNS	Central Nervous System
CTR	Control
Cx	Cycloheximide
DNA	Deoxyribonucleic Acid
EF1 α	Elongation Factor 1 A
EGF	Epidermal Growth Factor
EMT	Epithelial-To-Mesenchymal Transition
e-MI	Endosomal Microautophagy
ER	Endoplasmic Reticulum
ESCRT	Endosomal Sorting Complexes Required For Transport
FOXO3	Forkhead Box O3
gad	Gracile Axonal Dystrophy
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase

GFAP	Glial Fibrillary Acidic Protein
GTP	Guanosine-5'-Triphosphate
HD	Huntington's Disease
HDAC6	Histone Deacetylase 6
HIF1 α	Hypoxia-Inducible Factor 1-Alpha
HIF2 α	Hypoxia-Inducible Factor 2-Alpha
HIP	Hsp70 Interacting Protein
HOP	Hsp70-Hsp90 Organizing Protein
Hsc70	Heat Shock Cognate Protein 70
Hsp40	Heat Shock Protein 40
Hsp90	Heat Shock Protein 90
I κ B	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor
I κ k	I κ b Kinase
IL8	Interleukin 8
K48	Lysine 48 Linkage
K64	Lysine 63 Linkage
KD	Knockdown
KO	Knockout
LAMP-1	Lysosomal-Associated Membrane Protein 1
LAMP-2A	Lysosomal-Associated Membrane Protein 2A
LRRK2	Leucine-Rich Repeat Kinase 2
LSD	Lysosome Storage Disorder
LMP	Lysosomal Membrane Permeabilization
MA	Macroautophagy
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
Mi	Microautophagy
MMP-2	Matrix Metalloproteinase-2
MPA	Mycophenolic Acid

mTOR	Mechanistic Target Of Rapamycin
MVB	Multi Vescicular Bodies
NFkB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
pAkt	Phospho-Akt
PD	Parkinson's Disease
PDTC	Pyrrolidine Dithiocarbamate
PHLPP1	Ph Domain And Leucine Rich Repeat Protein Phosphatase 1
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RAR α	Retinoic Acid Receptor Alpha
RCAN-1	Regulator Of Calcineurin 1
RIPK1	Receptor Interacting Serine/Threonine Kinase 1
RIPK3	Receptor Interacting Serine/Threonine Kinase 3
RND3	Rho Family Gtpase 3
SCA	Spinocerebellar Ataxia
SQSTM1	Sequestosome 1
TCA	Tricarboxylic Acid Cycle
UCH-L1	Ubiquitin Carboxy-Terminal Hydrolase L1
UPS	Ubiquitin Proteasome System
VEGF	Vascular Endothelial Growth Factor
VPS4	Vacuolar Protein Sorting 4
WT	Wildtype

1. INTRODUCTION

1.1 Autophagy- at a glance

Protein turnover is an essential requirement for cellular quality control and supplying raw materials for growth and survival of the cell [1, 2]. This is achieved predominantly by two pathways– the ubiquitin-proteasome system (UPS) and the lysosome-mediated autophagy pathway [3-5]. The UPS mainly degrades soluble short-lived proteins labeled with ubiquitin tags that target them to the proteasome for destruction [6, 7]. On the other hand, autophagy degrades a variety of cellular components such as damaged or unwanted organelles and long-lived proteins inside lysosome - the terminal digestive vesicle that degrades the delivered substrates by virtue of the various proteases localized in the vesicle lumen [8, 9].

Kato et al. have quantitatively estimated the degradation profiles of the long-lived and short-lived proteins in primary hepatocytes. According to the author, the long-labelled protein degradations followed first order kinetics but the short-labelled proteins showed biphasic kinetics where one component degraded very fast and the other component was slowly degrading. It was calculated that 33% of the proteins showed this biphasic degradation profile. Additionally, they have described that about 67% of the total pool of cytosolic proteins have longer half-lives and their degradation is stalled upon addition of weak acids (which neutralize lysosomal proteases) implicating that long-lived proteins usually undergo lysosomal degradation [10].

The optimal activities and cooperation between UPS and autophagy play a critical role in regulating protein degradation to ensure cellular homeostasis and proper functioning

of the cells [11]. Failures in both degradative pathways and the crosstalk between them have been shown to cause excessive accumulation of damaged proteins and organelles in the cytosol seen in aging and many disease conditions [12-15]. Accumulation of such cytosolic toxic materials can lead to formation of protein aggregates and proteotoxicity that contribute to cellular functional decline and cytotoxicity [12, 16]. Hence, understanding ways to regulate the individual degradative systems and the mechanisms of communication between them is important for fine-tuning proteostasis to promote cellular health. To this end, this project has endeavored to elucidate the novel regulation of a highly selective form of autophagy known as chaperone-mediated autophagy (CMA). Besides protein quality control, CMA also protects cells against various stressors and recent studies have revealed CMA as an important anti-aging pathway [17, 18]. This makes the ability to increase or restore the function of CMA to be highly desirable. In this study, a plethora of ubiquitin modulating enzymes has been found highly enriched in the lysosomes that specifically carry out CMA degradation. The exact functions of these ubiquitin enzymes in CMA remain unknown. This study therefore aims to investigate the putative roles of these lysosome-associated ubiquitin enzymes in regulating CMA activity and in mediating crosstalk between CMA and UPS.

1.1.1 Different forms of autophagy

Autophagy (degradation in lysosome) can be broadly classified into three main types – macroautophagy (MA), microautophagy (MI) and chaperone-mediated autophagy (CMA) (Fig. 1.1). In MA, the cargo for degradation is sequestered by a double-membrane vesicle. This compartment is called an autophagosome which subsequently fuses with the lysosome to form the autophagolysosome to bring the cargo in direct

contact with the lysosomal proteases for degradation [19-22]. MI involves internalization of cytosolic cargo by direct invagination of the lysosomal membrane which resembles formation of late endosomal multivesicular bodies (MVB) [23]. The cargo is subsequently digested in the lysosomes. CMA is a non-vesicle based autophagy pathway that degrades substrate proteins bearing a CMA targeting motif that is recognized by cytosolic chaperone heat shock cognate protein of 70kDa (Hsc70) for delivery to the lysosome-associated membrane protein 2A (LAMP-2A) at the lysosomal surface for internalization and degradation. CMA forms the pathway of interest for our study which is further described in Section 1.2.

Recently, autophagy subtypes within the three broad categories of autophagy described above have been reported. These include the chaperone-assisted selective autophagy (CASA) and the endosomal microautophagy (e-MI) [24, 25]. CASA is a type of selective macroautophagy where the substrate is ubiquitinated by C-terminus of Heat shock protein (Hsp) 70-interacting protein (CHIP) and recognized by a chaperone complex consisting of HspB8, Hsc70 and co-chaperone BCL2 associated anthogene 3 (Bag3). Bag3 recruits an autophagy cargo adaptor protein sequestosome p62/SQSTM1 to the substrate-CHIP-HspB8-Hsc70 complex to target the substrate-chaperone complex to the lysosome for degradation [26, 27].

The e-MI takes place in the late endosomes during biogenesis of MVB. It is a selective form of MI. In contrast to the bulk cargo engulfment seen in MI, e-MI selectively entraps cargo delivered by cytosolic Hsc70 to the endosomal membrane via a targeting motif similar to the CMA motif [25].

Fig. 1.1 Different ways of delivering substrates to the lysosome by different autophagy variants. The three different types of autophagy are: (A) **Macroautophagy** engulfs substrates by forming a limiting membrane around the substrate to form an autophagosome. Substrates in the autophagosome are degraded by the lysosomal proteases upon autophagosome fusion with the lysosome. (B) **Microautophagy** involves the direct invagination of the lysosomal membrane to engulf cytosolic substrates for degradation inside the lysosomal compartment. (C) **Chaperone-mediated autophagy** is a selective pathway where the substrate containing the CMA targeting motif is recognized by cytosolic chaperone Hsc70 for transportation to the lysosomal surface for binding to LAMP-2A. Substrate binding initiates LAMP-2A multimerization to form a translocating complex to translocate the substrate into the lysosomal lumen for degradation with the help of a luminal Hsc70 inside the lysosome.

1.2 Chaperone-mediated autophagy (CMA)

CMA is a highly selective form of autophagy that degrades a specific subset of long-lived soluble cytosolic proteins in the lysosomes [28, 29]. The most distinctive features of this pathway are the requirement of a recognition signal in the substrate protein for targeting to the lysosome, and the protein-by-protein translocation of substrate across the lysosomal membrane mediated by LAMP-2A translocating complex for degradation (Fig. 1.2). The discovery of CMA came from the observation that some proteins microinjected into the fibroblast cells underwent different rates of degradation from others, thus suggesting the involvement of specific factors in their degradation [30].

It was further observed that these proteins were degraded faster under conditions of prolonged serum starvation [30]. Among these proteins was RNase A, the first documented CMA substrate [31]. It was found that the first 20 amino terminal residues of RNase A were important for its rapid catabolism, in particular a “KFERQ” pentapeptide motif in this region [32]. Similar “KFERQ”-like pentapeptide motifs were found amongst others in this group of proteins and shown to be necessary and sufficient for their degradation [29, 32]. The “KFERQ”-like pentapeptide motif serves as a recognition signal for binding cytosolic Hsc70 [33] to facilitate delivery of the protein to the lysosomes [34].

Only a subset of lysosomes is competent to perform CMA and these are lysosomes containing an isoform of Hsc70 (lys-Hsc70) in their lumen which is stable at the acidic lysosomal pH of 5.1-5.5 [35]. Under basal conditions, only 20-30% of rat liver lysosomes contain lys-Hsc70 in their lumen and are CMA competent. However, upon prolonged starvation, when CMA is induced, 80% of the total lysosomal pool becomes

CMA competent [36]. This is accompanied by an increase in the levels of LAMP-2A at the lysosomal membrane [37].

Fig. 1.2 Basics of CMA. CMA is a multi-step process that includes: (1) The substrate containing the CMA targeting motif recognized by a chaperone complex comprising of Heat shock cognate protein 70 (Hsc70) and co-chaperones Heat shock protein 90 (Hsp90), (Hsp40), Hsp70 interacting protein (Hip), Hsp70-Hsp90 organizing protein (Hop) and BCL2 associated anthogene 1 (Bag1). (2) The substrate chaperone complex is delivered to the lysosomal receptor LAMP-2A. (3) Binding of the substrate to LAMP-2A elicits multimerization of LAMP-2A to form an active translocon with the help of luminal Hsp90 stabilizing the multimeric LAMP-2A complex. (4) Unfolding of the substrate protein takes place prior to translocation (5) Translocation of the substrate is aided by the lysosomal Hsc70. (5) Once the substrate enters the lumen, it is degraded by the lysosomal proteases.

1.2.1 The CMA machinery

1.2.1.1 The substrate-chaperone complex

Sequence analysis has revealed the presence of CMA targeting motif in about 30% of the total cytosolic proteins and CMA may potentially account for their degradation [28, 31]. The pentapeptide motif is recognized by the cytosolic Hsc70 that further forms a complex with other co-chaperones like Hsp40, Hsp90, Hip, Hop and Bag1 to target the substrate protein to the lysosomes for degradation (Fig. 1.2) [33, 38]. The chaperone-substrate complex then migrates to the lysosomal surface where the substrate binds to LAMP-2A, the receptor responsible for CMA, for substrate internalization. The co-chaperones play a range of diverse roles in the substrate-chaperone complex. Hsp40 stimulates the ATPase activity of Hsc70 which is essential for the binding and release of substrate from Hsc70. Hsp90 recognizes the unfolded regions in the protein substrate and prevents the exposed hydrophobic regions from causing protein aggregation. Bag1 uncouples the binding of substrate proteins from the ATPase cycle of the Hsc70 [39]. Bag1 may regulate the activity of Hsc70 either positively or negatively depending on the type of Bag1 isoform present in the complex [39]. Hop acts as a linker between Hsc70 and Hsp90 and may also contribute as a nucleotide exchanger [33]. Hip interacts with Hsc70 and possibly acts as a scaffold in the chaperone complex [33].

Once the substrate-chaperone complex reaches the lysosomal membrane, substrate unfolding occurs at the lysosomal membrane assisted by the Hsc70 and co-chaperones. The internalization of the cargo through the active LAMP-2A translocating complex into the lysosomal lumen is facilitated by luminal Lys-Hsc70 which acts like a motor pulling the unfolded substrate into the lysosomal lumen (Fig. 1.2). Once inside the lysosomal lumen, the substrates are degraded by lysosomal proteases.

1.2.1.2 CMA receptor LAMP-2A

Another important player in the CMA pathway is the CMA receptor LAMP-2A which acts as a docking and entry point for the substrate into the lysosome [40] (Fig. 1.2). While LAMP-2A is present in all lysosomes, only those lysosomes that have lysosomal-Hsc70 (Lys-Hsc70) in their lumen can execute CMA [35]. LAMP-2A is one of the three splice variants of *lamp-2* gene. The cytosolic portion of LAMP-2A contains four positively charged amino acid residues that helps in binding to the substrate through electrostatic interactions [41]. LAMP-2A is the only splice-variant that can multimerize to form a translocation complex at the lysosomal membrane upon binding of the substrate-chaperone complex to the monomeric LAMP-2A. Substrate binding to LAMP-2A is the rate-limiting step of the CMA pathway. Changes in LAMP-2A levels and configurations regulate the activity of this pathway. When CMA is at basal state, most of the LAMP-2A receptor is concentrated in the cholesterol rich lipid microdomains of the lysosomal membrane (Fig. 1.3). In the lipid microdomains, the LAMP-2A receptors are prone to cleavage by Cathepsin A and a metalloprotease associated with the lysosomal lipid microdomains (Fig. 1.3) [37, 42]. This helps to maintain LAMP-2A at low levels when CMA is not activated. During CMA activation, substrate bound monomeric LAMP-2A starts to migrate out of the lipid microdomains and assemble into a ~700kDa multimeric translocation complex to form a channel across the lysosomal membrane for substrate internalization (Fig. 1.3). The LAMP-2A multimer is highly stable compared to its monomeric form and this stability is mediated by various factors.

1.2.1.3 LAMP-2A dynamics

LAMP-2A forms a dynamic complex at the lysosomal membrane and this dynamicity is conferred by the Hsc70 and Hsp90 chaperones (Fig. 1.3) [43, 44]. The effect of Hsc70

on LAMP-2A is substrate dependent. When Hsc70 is in substrate-bound form, it promotes the multimerization of the LAMP-2A complex (Fig. 1.3). In the absence of substrate, Hsc70 predominantly promotes LAMP-2A disassembly. Hsp90 is another effector involved in the LAMP-2A multimerization. Hsp90 associates with LAMP-2A to stabilize the receptor during transition from monomeric to multimeric form and vice versa (Fig. 1.3). The Hsp90 involved in this function is localized in the lysosomal lumen (Fig. 1.3) [44, 45]. Apart from Hsp90 and Hsc70, LAMP-2A dynamics is also regulated by two other factors, namely glial fibrillary acidic protein (GFAP) and elongation factor 1 α (EF1 α). GFAP stabilizes the 700kDa multimeric LAMP-2A complex by binding to it at the lysosomal membrane. Under starvation conditions that strongly activate CMA, low levels of GTP release EF1 α to bind GFAP. This maintains the GFAP in monomeric state capable of stabilizing the LAMP-2A multimer. (Fig. 1.3). Under basal conditions, when there are high levels of GTP in the cell, EF1 α is GTP bound (Fig. 1.3) and is released from GFAP. This allows the self-assembly of GFAP to form a dimer, resulting in the dissociation of GFAP from LAMP-2A. This induces the mobilization of LAMP-2A into specific cholesterol rich lipid microdomains for degradation (Fig. 1.3) [43-45].

Fig. 1.3 A model for the dynamic assembly and disassembly of LAMP-2A. Under basal conditions, majority of LAMP-2A is in the Lipid-rich microdomains. Upon binding of substrate, LAMP-2A migrates out of the microdomains and luminal Hsp90 helps in this transition. Substrate binding causes LAMP-2A assembly into a translocating complex stabilized by EF1 α -bound GFAP. Under conditions of high GTP, EF1 α dissociates from GFAP and causes GFAP to dissociate from LAMP-2A to form GFAP dimers. Dissociation of GFAP renders the LAMP-2A multimers unstable and promotes LAMP-2A disassembly.

1.2.2 The lysosomal mTORC2/Akt1/PHLPP1 regulatory pathway of CMA

The signaling regulation of CMA has only been recently elucidated. A study has identified retinoic acid receptor alpha (RAR α) signaling as the negative regulator of CMA while the nuclear factor of activated T-cells (NFAT) signaling has been shown to activate CMA in specific subset of the immune cells, particularly the T-cells [46, 47]. Recently, the mammalian target of rapamycin (mTOR) – a serine-threonine kinase that regulates a plethora of signaling cascades has been shown to also regulate CMA [48]. mTOR exists as two distinct complexes – the mTOR complex 1 (mTORC1) when mTOR associates with the raptor protein, and mTOR complex 2 (mTORC2) when mTOR binds the rictor protein [49, 50]. Studies have shown that mTORC1 regulates macroautophagy in response to cellular levels of growth factors, ATP and amino acids by acting as a nutrient sensor at the lysosomes [51]. Activation of mTORC1 in nutrient rich conditions leads to the inhibition of macroautophagy [52].

While mTORC1 is a negative regulator of macroautophagy, recent evidence indicates mTORC2 as an endogenous inhibitor of CMA in CMA-active lysosomes [48]. At basal levels, mTORC2 phosphorylates and activates another lysosomal kinase Protein kinase B (PKB or Akt1), which in turn phosphorylates GFAP, a downstream substrate of Akt1 (Fig. 1.4) [53, 54]. The non-phosphorylated GFAP exists as monomer, is able to bind LAMP-2A translocation complex to stabilize the complex conformation at the lysosomal membrane to facilitate substrate translocation and enhance CMA activity (Fig. 1.4) [44]. However, phosphorylated GFAP has high affinity to form heterodimer with the other GFAP molecules which impedes the interaction of GFAP with LAMP-2A, leading to the disassembly of the multimeric translocation complex (Fig. 1.4) [44].

In this way, the lysosomal mTORC2 negatively regulates CMA activity. When CMA is induced, Ras-related C3 botulinum toxin substrate 1 (Rac1) protein localizes to CMA-active lysosomes through yet unknown mechanisms to recruit the phosphatase PH Domain and Leucine Rich Repeat Protein Phosphatase 1 (PHLPP1) to the lysosomal membrane to dephosphorylate Akt1 and renders it inactive. Inactivation of Akt1 abolishes the downstream phosphorylation of GFAP. Thus, the non-phosphorylated GFAP exists in monomeric forms that can bind LAMP-2A, thereby stabilizing the multimeric translocation complex to upregulate CMA activity (Fig 1.4) [48].

Notwithstanding the identification of mTORC2/Akt1/PHLPP1 signaling in the regulation of CMA, it remains unclear how PHLPP1 level and activity are enriched in the lysosomes during CMA activation. Additionally, the possibility of mTORC2/Akt1/PHLPP1 pathway being regulated by additional signaling mechanisms remains unexplored. Hence, this project aims to examine putative regulatory pathway that could regulate the mTORC2/Akt1/PHLPP1 pathway to control CMA function. Specifically, the mechanism that determines the balance between phosphorylated Akt1 (pAkt) and PHLPP1 levels in the lysosome to fine-tune CMA activity remains unclear. E4 ligase CHIP has been reported to ubiquitinate and degrade pAkt to regulate Akt levels [55]. Additionally, de-ubiquitinating enzyme (DUB) ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) plays a role in regulating PHLPP1 levels by yet unknown mechanisms [56, 57]. Both CHIP and UCH-L1 have been found to be highly enriched in CMA-active lysosomes in our study (Fig 3.1.1 D), however, whether this ubiquitinating and deubiquitinating enzyme pair modulates lysosomal mTORC2/Akt1/PHLPP1 signaling to direct CMA function is unknown. This study thus

aims to elucidate the roles of CHIP and UCH-L1 in influencing lysosomal mTORC2/Akt1/PHLPP1 pathway and CMA activity.

Fig. 1.4 Regulation of CMA by mTORC2/Akt1/PHLPP1 signaling in the lysosome. mTORC2 represses CMA basally by activating Akt1 via phosphorylation. Phosphorylated Akt1 (pAkt1) in turn phosphorylates GFAP (pGFAP) to encourage GFAP dimerization and dissociation from LAMP-2A translocation complex. Without GFAP stabilization effect, LAMP-2A translocation complex undergoes disassembly into LAMP-2A monomers resulting in decreased CMA activity. When CMA is activated, the Rac1 protein migrates to CMA-active lysosomes, at the same time recruits PHLPP1 to dephosphorylate pAkt1. As a result, GFAP is not phosphorylated and exists as monomers able to bind and stabilize the LAMP-2A translocation complex to enhance CMA activity. (Figure adapted from [48].)

1.2.3 Small molecule regulators of CMA

Small molecules that regulate CMA have been reported [58]. These compounds include epidermal growth factor (EGF), cyclohexamide (Cx) and anisomycin (6-AN) that negatively regulate CMA [58]. However, the effects of these compounds on CMA are non-specific and operate through indirect mechanisms [58]. In a recent study, ubiquitously expressed transcription factor RAR α has been shown to inhibit CMA [59, 60]. Development of chemically modified retinoic acid derivatives that displays high stability and efficiency in crossing the membrane lipid bilayer to block RAR α activity has provided a mean to selectively activate CMA by neutralizing RAR α inhibition of CMA [46, 61]. Chemical compounds that modulate lysosomal mTORC2/Akt1/PHLPP1 pathway can also influence CMA activity [48]. However, the currently available Akt inhibitors or PHLPP1 inhibitors exhibit non-specific effects [48]. As a result, this study seeks to gain a better understanding of the regulation of lysosomal mTORC2/Akt1/PHLPP1 pathway so as to identify novel avenues to stimulate CMA in a more targeted fashion.

1.3 Physiological importance of CMA

CMA performs a plethora of functions in cellular homeostasis. Functional decline in CMA activity has been shown to adversely affect cellular wellbeing and contribute to aging and age-related diseases such as neurodegenerative disorders or cancer [17, 18, 62]. A key to understand how alternations in CMA contribute to aging and diseases is an appreciation of the different physiological roles of CMA.

1.3.1 CMA – a stress response pathway

Almost all cell types exhibit basal CMA activity which plays an important role in basal protein quality control where CMA selectively degrades damaged or altered proteins to prevent their toxic accumulation [63, 64]. CMA degrades a diverse range of proteins including glycolytic enzymes [65], transcription factors like c-fos, Pax, MEF2D [66-68], inhibitors of transcription factors [69], trafficking proteins like Tau and α -synuclein [70, 71], annexins [72], lipid associated proteins [73] and also catalytic subunits of the proteasome [74]. CMA also contributes to the quality control and regulation of protein complexes in the cytosol by eliminating the excess subunits. An example is the proteasome. CMA has been shown to selectively degrade the 19S regulatory components of proteasome as a mean to regulate the proteasome activity [74]. Besides basal CMA, CMA is also highly upregulated in response to many cellular stressors. The first observed stress response role of CMA came from early studies where prolonged starvation was shown to maximally induce CMA (Fig. 1.5) [64]. In response to nutrient depletion, CMA is activated to degrade non-essential proteins to recycle amino acids as raw materials for energy supply [64]. Till date, starvation remains one of the best characterized stimuli to induce CMA in animal and cultured cell models [63, 64]. Unlike macroautophagy, which is maximally activated upon 4-6 hours starvation and persists up to 8 hours of nutrient deprivation, CMA requires 16-18 hours prolonged starvation to become maximally induced and can stay activated for up to three days [22, 75]. This temporal difference in CMA activation during nutrient stress is important for orchestrating proteome changes aimed at adapting the cell to changing conditions upon extended period of nutrient scarcity. The bulk removal mechanism of macroautophagy is favored for mass degradation of cellular components to quickly meet metabolic shortfall during initial starvation. However, such bulk self-eating strategy is no longer

sustainable under prolonged starvation. For continuous survival, the cell switches to CMA degradation in order to selectively degrade only non-essential proteins for energy. For example, a well-characterized substrate of CMA is glyceraldehyde 3 phosphate dehydrogenase (GAPDH), which is a principle component of the glycolytic pathway. During starvation, glycolysis is not required and so GAPDH can be degraded to stop glycolysis and also generate raw materials for survival [76, 77]. CMA also degrades inhibitors of transcription factors such as I κ B α during starvation and by so doing may alter the transcriptome to influence survivability against nutrient stress [69]. Starvation enhances CMA activity by upregulating the levels of Lys-hsc70 [36, 64] and LAMP-2A [40, 41]. The increase in LAMP-2A levels is primarily due to increased stability of the LAMP-2A multimers at the lysosomal membrane as well as relocation of the luminal full length LAMP-2A to the lysosomal membrane while the rate of LAMP-2A synthesis remains unchanged [40, 41]. CMA is also activated during oxidative stress where it contributes to the degradation of oxidized proteins (Fig. 1.5) [78]. It was first observed that in the presence of antioxidant pyrrolidine dithiocarbamate (PDTC), the degradation rates of CMA substrates reduced considerably whereas in the presence of oxidative stress inducer H₂O₂, the same substrates were degraded much more efficiently [69]. It was further revealed that oxidized proteins were found in the lysosomal matrix of CMA-active lysosomes [78]. Mild oxidative stress induces protein unfolding that helps to uncover the hidden CMA targeting motifs in the oxidized proteins and preferentially promote their lysosomal targeting for CMA removal [78]. Besides enhancing substrate targeting, mild oxidative stress also increases the levels of Lys-hsc70, Hip and LAMP-2A in the lysosomes to upregulate CMA [78]. Unlike starvation where LAMP-2A levels are stabilized at the lysosomal membrane, under oxidative

stress there is no significant stabilization of LAMP-2A but instead the *lamp-2a* mRNA level increases leading to enhanced *de novo* synthesis of LAMP-2A [78].

CMA activity and LAMP-2A level also increase under other conditions that induce protein damage such as under hypoxic stress (Fig. 1.5). This increase is due to higher levels of *lamp-2a* mRNA synthesis [79, 80]. Hypoxia induced CMA helps to suppress toxic protein aggregation and apoptotic death under hypoxic conditions [79]. *In vivo* studies also showed an increase in neural expression of LAMP-2A in rat brains after ischemic shock with concomitant upregulation of CMA and mitigation of ischemic damage [80]. Thus, CMA plays a cellular protective role under hypoxic conditions.

1.3.2 CMA and cellular energetics

CMA also plays an imperative role in the maintenance of metabolic balance in more than one way (Fig. 1.5). CMA has been implicated in lipid and carbohydrate metabolism pathways [16, 81]. The development of the hepatic LAMP-2A knockout (KO) mouse model has shed light on the role of CMA in maintaining energetic balance [81]. Disruption of CMA in the liver of LAMP-2A KO mice showed distinct metabolic phenotype with reduced glycogen storage and negative energy balance. The animal showed higher overall dependency on carbohydrate oxidation for energy production with an elevated rate of glycolysis. Many glycolytic and tricarboxylic acid (TCA) cycle enzymes are CMA substrates. Thus, these enzymes accumulate in high levels in LAMP-2A KO liver due to loss of CMA thereby leading to enhanced rate of glycolysis [81]. CMA is also crucial for lipid metabolism. LAMP-2A KO liver developed severe hepatosteatosis with overt lipid accumulation and reduced oxidation of fatty acids [16]. The study shows that many enzymes involved in lipid binding and transport, and triglyceride synthesis pathway are also CMA substrates. Therefore, CMA failure results

in increase in lipid uptake and lipogenesis. Furthermore, CMA also degrades lipid droplet associated proteins, perilipin 2 (PLIN2) and 3 (PLIN3) and their degradation is necessary for cytosolic lipases and autophagy-related proteins to assess the lipid droplets to carry out lipolysis [16]. Thus, CMA-incompetent liver also suffers from a reduced rate of lipolysis. Taken together, CMA deficiency perturbs ATP production and promotes metabolic dysfunctions, which are changes that have been observed in physiological aging.

Furthermore, CMA metabolic regulation plays a significant role in promoting tumor growth and metastasis [82]. Many cancer cells possess abnormal high basal CMA that has been shown to fuel the Warburg effect, the aerobic glycolysis in cancer cells [82]. Unlike loss of CMA in normal cells, blockage of CMA in cancer cells by depletion of LAMP-2A surprisingly caused marked reduction of glycolytic enzymes like GAPDH, 3-phospho glycerate kinase (3PGK) and aldolase levels. The reduced levels of these enzymes are not a result of increased degradation, but due to a decrease in the *de novo* synthesis of these enzymes mediated by p53. p53, which accumulates to high levels in the LAMP-2A depleted cells, regulates the transcriptional expression of these glycolytic enzymes [83]. Thus a functional CMA is required to release p53 transcriptional brake on glycolysis to augment Warburg effect to support cancer growth and progression [82].

CMA regulation of glycolytic enzymes can also control cancer cell metabolism. This regulation is mediated by acetylation. Sometimes a substrate containing a partial CMA-targeting motif may be acetylated at the lysine residue to compensate for a missing glutamine and complete the CMA targeting motif [56] leading to CMA degradation of the target. For instance, glycolytic enzyme, pyruvate kinase M2 (PKM2), is important

for cancer cell growth. The end product of PKM2 is pyruvate which can be further reduced to lactate or oxidized to acetyl-coA and fed to the TCA cycle for energy production. PKM2 undergoes acetylation on the lysine K305 residue in the cancer cells turning it into a CMA substrate and resulting in its degradation. This leads to accumulation of glycolytic intermediates and enhances the glycolytic flux in cancer cells to fuel tumor growth [84].

1.3.3 CMA in DNA damage response

In addition to protein quality control, CMA also protects the genome against genotoxicity by regulating DNA damage response. This role of CMA is executed by regulated degradation of phosphorylated checkpoint kinase 1 (Chk1) [85]. During cell division when there is DNA damage, Chk1 delays cell cycle progression to allow DNA repair. Once repair is done, release of cell cycle arrest is necessary. Chk1 is then phosphorylated and tagged for CMA degradation [85]. CMA deficient cells showed nuclear accumulation of Chk1 resulting in deregulated cell cycle progression with persistence of DNA damage upon genotoxic stress. CMA blockage also perturbed the stability of early step DNA repair complex, thereby making CMA deficient cells more vulnerable to etoposide mediated genotoxicity and genome instability due to inefficient DNA repair [85]. Conceivably, a loss of CMA will contribute to the age-related increase in genome instability and malignant transformation observed in aged cells and organisms.

1.3.4 Other roles of CMA

CMA also performs specialized functions related to the client substrates it regulates in certain cell types (Fig. 1.5). In the liver during prolonged starvation, CMA is highly

upregulated in response to heightened production of ketone bodies that incurs massive oxidatively damaged protein to protect the liver against ketogenic stress [86]. In antigen-presenting cells, CMA participates in antigen presentation by the major histocompatibility complex (MHC) class II molecules [87]. CMA activity has also been shown to contribute to organogenesis and development of the kidney [88]. In neurons, CMA maintains the functional pool of myocyte enhancer factor 2D (MEF2D), a neuronal transcription factor important for neuronal survival, by timely removal of dysfunctional MEF2D to prevent the damaged transcription proteins from interfering with MEF2D function. It has been reported that high level of inactive MEF2D accumulates in the cytoplasm of the neuronal cells from CMA compromised models [89]. CMA also controls the regulation of transcription factor NFkB by controlling the degradation of its inhibitor IkB which is a CMA substrate [67].

Fig. 1.5 Physiological functions of CMA. CMA performs multifaceted roles in mediating different stress responses, protein and genome quality control, metabolism and specialized functions in certain cell types.

1.3.5 CMA in Cancer

Many cancer cell types, including those from the lungs, breast, liver and epithelia, display enhanced CMA activity [82]. High CMA activity confers cancer cells with metabolic advantage as CMA is a major regulator of glycolysis [76, 82, 84, 90]. Ablation of CMA in various cancer cells has resulted in reduced cell proliferation and metastatic ability [82]. This is brought about by an increase in the levels of tumor suppressor proteins, p53 and p21, which leads to transcriptional downregulation of various glycolytic enzymes [82]. Studies in gastric cancer cells have revealed that Rho-related GTP-binding protein RhoE (RND3), another tumor suppressor protein, is also a CMA substrate [91]. Thus enhanced degradation of RND3 due to higher CMA activity contributes to the increased proliferation rate of gastric cancer cells [91]. As previously mentioned in Section 1.3.2, CMA also degrades K305 acetylated PKM2 in cancer cells. Reduced level of this enzyme causes accumulation of glycolytic intermediates which can be used by the cancer cells as metabolic raw materials and promotes tumor growth and proliferation [84]. Apart from energetics, high CMA activity also protects the cancer cells against different stressors such as oxidative stress, hypoxia and DNA damaging agents. Hence, CMA is a pro-oncogenic pathway in cancer cells.

1.3.6 CMA and Leaky Lysosomes

In response to endogenous or exogenous stress, lysosomes undergo a phenomenon known as lysosomal membrane permeabilization (LMP) or “leaky lysosomes”. This leads to release of catabolic hydrolases that can cause apoptosis or necrosis [92]. However, cancer cells have developed strategies to counteract LMP. In cancer cells, cytosolic Hsp70 is translocated to the inner leaflet of the lysosomal membranes

preventing LMP [93]. It is thus possible that the greater stabilization of the lysosomes against LMP in cancer cells contributes to the enhanced CMA activity observed in various cancer cells by protecting the pool of CMA-active lysosomes [82]. *Loos et al.* has described a theoretical framework of single-cell fluorescence imaging based approach to accurately assess the pool size of vesicles such as autophagosomes [94]. It will be interesting to exploit this technique in the analysis of changes in the number and size of the CMA-active lysosomes in cancer cells to correlate with the proliferative and metastatic ability of the cancer cells

1.4 The LAMP-2A knockout model

Recently developed liver specific conditional LAMP-2A KO mice was a significant step forward in our understanding of the physiological functions of CMA [81]. These mice displayed higher accumulation of cholesterol esters, triglycerides, free fatty acids and diacylglycerol in the liver. The animals were also more sensitive to chronic high fat diet resulting in larger liver size and accumulation of lipid droplets [81]. Thus the hepatic LAMP-2A KO mice showed altered lipid metabolism and higher susceptibility to lipid stress. Additionally, glycogen storage capacity was also reduced in the hepatic LAMP-2A KO animals where overnight fasting caused a significant drop in blood glucose levels. Liver analysis showed a shift from carbohydrate synthesis and storage towards carbohydrate hydrolysis and utilization [81]. Besides GAPDH identified previously [65], the recent LAMP-2A KO study has further elucidated that 80% of the glycolytic enzymes possess the CMA targeting motif, thus supporting an instrumental role of CMA in regulating glycolysis [81]. Comparative proteomic analysis of the liver confirmed 30% of the proteins were CMA substrates which include proteins involved in carbohydrate and lipid metabolism [81]. Interestingly, LAMP-2A depletion in the liver not only affects the liver but also incurs changes in the peripheral organs.

Therefore, loss of CMA activity in the liver not only perturbs liver protein quality control and functions, it also compromises the overall metabolic homeostasis and adaptation to changing energy demands of the whole animal as what has been observed in aging. In this aspect, the hepatic LAMP-2A KO mice behave more like the aging models at the metabolic level [81]. Thus, modulating CMA activity is a potential anti-aging strategy to delay the onset of aging phenotypes.

1.5 Consequences of CMA failure

1.5.1 Aging

Upon aging, the lysosomes undergo vast changes which include an increase in the lysosomal volume, a decrease in lysosome stability and hydrolase activities, intralysosomal accumulation of indigestible particles (lipofuscin), and impaired regulation of lysosomal pH [11]. A common hallmark of aging cells is the accumulation of damaged or modified proteins in the cytosol resulting in proteotoxicity [95]. This deregulated proteostasis with age is in part due to the functional decline in both macroautophagy and CMA [45, 96]. Decline in CMA activity with age was first observed in primary senescent fibroblasts as well as in lysosomes isolated from various tissues of old rodents [97, 98]. Characterization of CMA-active lysosomes indicates no overt morphological changes with age nor visible accumulation of lipofuscin, the yellowish-brown autofluorescent aged pigment [37]. Recognition of CMA substrates by the hsc70-chaperone complex and their lysosomal targeting, as well as their degradation inside the lysosomal lumen are also well preserved until late in life [37]. Age-dependent CMA failure appears to be mainly caused by a drastic reduction in the LAMP-2A level at the lysosomal membrane to form active CMA translocation complex, thereby decreases CMA substrate binding and uptake into the lysosomes for

degradation [42]. This reduction in the LAMP-2A stability is due to a change in the lipid constituency of the lysosomal membranes [99, 100]. Comparative lipodomics studies of CMA-active lysosomes from young and old mice revealed an age-dependent expansion of the lipid microdomains in CMA-active lysosomal membrane [99]. This undesirable increase in lipid microdomains enhances LAMP-2A degradation by cathepsin A and metalloprotease associated with the microdomains, leading to reduced LAMP-2A level at the lysosomal membrane [45, 99]. As a result, the binding and translocation of CMA substrates by lysosomes are markedly decreased in old organisms. Interestingly, similar lipid alterations can be induced at the lysosomal membrane through high fat diets, hence suggesting the importance of diet in modulating CMA activity and the possible acceleration of its decline with age [100].

In LAMP-2A transgenic mice that maintained an optimal level of LAMP-2A throughout their lifespan, the age-associated CMA decline was prevented [17]. These mice displayed higher CMA activity compared to their control littermates under both basal and oxidative stress conditions. LAMP-2A transgenic mice also demonstrated better proteostasis with marked reduction in the accumulation of poly-ubiquitinated protein aggregates in various tissues compared to their control littermates [17]. Additionally, cellular morphology and function of liver was also better preserved in LAMP-2A transgenic mice with age [17]. Hence, restoring or improving CMA activity through interventions aim at stabilizing LAMP-2A at the lysosomal membrane until advanced ages may prove beneficial in delaying aging and prevention of age-related diseases.

1.5.2 Functional failure of CMA in neurodegenerative diseases

A causative relationship between the gradual loss of CMA functions and the manifestation of aging phenotypes has been observed in physiological aging [17]. On the other hand, pathological aging is associated with severe CMA failure which has been seen in many age-related diseases such as neurodegenerative disorders. CMA plays a critical role in neural homeostasis [18, 62, 101-103]. It has been observed that LAMP-2A is highly expressed in the cortical and midbrain neurons in culture as well as in the central nervous system (CNS) of rats [71]. Failure of CMA has been implicated in the pathogenesis of various neurodegenerative diseases like Parkinson's disease, Alzheimer's disease and various lysosome storage disorders.

Parkinson's disease (PD). PD is the most common neurodegenerative movement disorder characterized by selective loss of dopaminergic neurons in the midbrain *substantia nigra* with accumulation of α -synuclein aggregates in the form of Lewy bodies (LB) [104]. CMA dysfunction plays a major role in both familial and sporadic PD [105-111]. α -synuclein is the first PD-linked protein shown to be degraded by CMA [105, 112]. Both wild type and mutant α -synuclein are degraded by CMA [105]. PD-linked mutations in α -synuclein result in abnormally tight binding of the mutant α -synuclein to the LAMP-2A translocation complex, thereby preventing their translocation into the lysosomal lumen for degradation. Such blockages obstruct not only the translocation and degradation of mutant α -synuclein but also inhibit the degradation of other CMA substrates [105]. Apart from mutations, dopamine-induced post translational modifications in α -synuclein have also been reported to cause similar blockage of CMA [106]. Inhibition of CMA enhances the cytosolic levels of α -synuclein which promotes formation of toxic α -synuclein oligomers and aggregates

[106]. A loss of CMA also sensitizes the neurons toward different stressors leading to enhanced accumulation of oxidized and altered proteins in the cytosol and neuronal death [78].

According to reports, the different isoforms of LAMP-2 protein are differentially altered in early stages of PD preceding loss of neurons and formation of LB [113-116]. The BRAAK staging scheme has been proposed by *Braak et al.* to classify the degree of pathology related to sporadic PD describing abnormal patterns of α -synuclein immunostaining in human brains and proposed a neuropathologic staging scheme [113]. During BRAAK stage IV, which is the earliest stage of disease onset with substantial biochemical α -synuclein changes, there is a selective reduction in LAMP-2A protein levels, along with increased α -synuclein levels [114]. However, there were limited changes in LAMP-2B, LAMP-2C or LAMP1 levels. This selective reduction in neuronal LAMP-2A was accompanied by a reduction in Hsc70 levels and both factors together might result in a deficit in CMA machinery [114].

UCH-L1, a deubiquitinating enzyme, is another causative protein in PD that shows link with CMA [109]. Unlike α -synuclein, UCH-L1 is not known to be a CMA substrate. The I93M mutation in UCH-L1 exhibits dopaminergic cell loss [117] and contributes to the pathogenesis of PD [109]. Instead, UCH-L1 has been shown to interact with Hsc70 and LAMP-2A and proposed to participate in CMA function through yet unknown mechanisms [109]. The PD-linked I93M mutation in UCH-L1 promotes enhanced and aberrant binding of mutant UCH-L1 to LAMP-2A resulting in perturbed organization and function of the translocation complex. This results in CMA inhibition and accumulation of CMA substrates like α -synuclein in I93M UCH-L1 expressing

neurons, leading to accentuated α -synuclein toxicity and neuronal loss thereby contributing to PD pathogenesis [109, 117].

Huntington's disease (HD). HD is another age related neurodegenerative disease which is caused by an abnormal expansion of the highly unstable polyglutamine (polyQ) repeat in the N-terminus of huntingtin (Htt) protein [118]. This causes misfolding of the Htt protein making it more prone to oligomerization and aggregation. The Htt aggregates accumulate in the cytosol triggering neurotoxicity and neurodegeneration [119, 120]. An overt upregulation of CMA has been detected in HD cells and tissues to compensate for the loss of UPS and MA proteolytic flux. This CMA compensatory response may account for the balanced proteostasis observed in young HD animals before the onset of HD phenotype [121]. CMA activity is enhanced by the increased expression and stability of LAMP-2A [121]. Additionally, Htt protein contains a non-canonical CMA targeting motif, which undergoes post translational phosphorylation by Ikk, to convert it into a CMA targeting motif [122, 123]. Thus, the increased CMA in HD cells also helps to reduce the levels of Htt to prevent its toxic accumulation and aggregation. However, the ability of CMA to compensate for the failure of other proteolytic pathways in HD decreases with age due to the age-dependent deterioration occurs in CMA pathway, thereby contributing in part to the onset of HD pathology with age [121].

Alzheimer's disease (AD). AD is another neurodegenerative disease where CMA is implicated. CMA modulates two AD-linked proteins, Tau and regulator of calcineurin 1 (RCAN1). Tau is a CMA substrate and interacts with Hsc70 [70, 124]. Mutant Tau also binds to Hsc70 and is transported to the lysosomal membrane where it binds to the LAMP-2A but does not undergo complete internalization [70]. The partially

translocated mutant Tau undergoes cleavage by cathepsin L inside the lysosomal lumen, leading to generation of pathogenic fragments of Tau on the lysosomal membrane surface. These fragments oligomerize at the lysosomal membrane and affect the membrane integrity. The destabilized lysosomal membrane is prone to leakage and lysis that ultimately results in the release of the toxic lysosomal Tau oligomers into the cytosol to seed further Tau aggregation, thereby potentiating Tau toxicity. The partially internalized Tau also causes CMA translocation blockage to prevent degradation of other CMA substrates, further aggravating the disease condition [70]. RCAN1 is another protein which is overexpressed in the brains of AD patients that results in neuronal cell death [125]. RCAN1 has two CMA targeting motifs and is a CMA substrate [126]. Thus blockade of CMA due to mutant Tau oligomerization can further aggravate the AD condition by promoting the accumulation of RCAN1.

Lysosomal storage disorders (LSDs). LSDs are a group of genetic diseases resulting from the alterations in lysosomal enzyme functions that cause lysosomal dysfunction and massive accumulation of undigested substrates in the lysosomal compartment [77]. CMA failure has been implicated in two forms of LSDs, namely galactosialidosis and mucopolipidosis type IV [42, 77, 127]. Galactosialidosis is caused by the lack of the lysosomal protease cathepsin A. Cathepsin A, besides acting as a chaperone for other lysosomal proteases, it also controls the levels of CMA receptor LAMP-2A by cleaving it at the lipid microdomains [43]. In the absence of this protease, LAMP-2A levels are abnormally high in the lysosomes and result in abnormally high CMA translocation [128]. However, due to the overall reduction in the lysosomal degradative capacity, CMA degradation is overall reduced [28]. In mucopolipidosis type IV, perturbation in lysosomal pH has been proposed to destabilize the luminal Hsc70 chaperone thereby impacting CMA functions [129].

1.6 Crosstalk between CMA and other degradative pathways

The two major pathways for protein degradation inside cells are the autophagy-lysosomal pathway and the ubiquitin proteasomal system (UPS). As described previously, autophagy is further categorized into three major types depending on the mode of substrate selection and uptake. The three major types of autophagy are macroautophagy, CMA and microautophagy. In order to ensure regulated protein turnover, recycling of raw materials and maintenance of cellular homeostasis, a coordinated activity of chaperones and proteolytic pathways is necessary. For example, when a protein is damaged, the chaperones come in to refold them or to direct them to the specific compartments and in case it is beyond repair, it is sent for degradation by the specific proteolytic pathways. There is increasing evidence that UPS and autophagy are functionally coupled processes. Studies show that pharmacological inhibition of autophagy using chloroquine which prevents lysosomal degradation, enhanced proteasomal activity [130]. Similarly, abrogation of macroautophagy by knockdown of autophagy related genes PIK3C3, ATG5 or ATG7 also enhanced UPS activity although induction of autophagy by rapamycin did not reduce UPS activity [130]. In fly model macroautophagy compensates for UPS in HDAC6 dependent manner and controls ER stress accompanied by reduced cell death [131]. UPS also collaborates with CMA to degrade the gene product of regulator of calcineurin 1 (RCAN1) which is overexpressed in conditions like AD and Down's syndrome.

There is extensive communication between macroautophagy and CMA. Both the pathways also share a multitude of common substrates. For example, both LRRK2 and α -synuclein implicated in the pathology of Parkinson's disease are degraded by both CMA and macroautophagy [110, 132]. It has been reported that blockage of macroautophagy either by inhibitor 3-MA or by knockout of autophagy related gene

ATG5 upregulates CMA activity [128]. This is achieved by upregulation of different CMA components like Hsc70, Hsp90 and principally LAMP-2A. There is also an increase in the total number of lysosomes active for CMA [128]. LAMP-2A levels are augmented by increasing the de novo synthesis of LAMP-2A as well as increased stability of LAMP-2A accompanied by an increase in Hsc70. The luminal Hsc70 is most stable in acidic pH. In the absence of ATG5, the fusion of lysosomes and autophagosomes is blocked and as such there is no dissipation of the acidic pH. As a result, the lysosomal pH remains more acidic, and stabilizes the luminal Hsc70. Hence, there are more CMA competent lysosomes and greater CMA activity.

The inverse is also true, selective blockage of LAMP-2A by silencing the *lamp-2a* gene which has been used to impair CMA results in compensatory activation of macroautophagy thus establishing crosstalk between the two pathways [75]. However, these cells show slower growth rates and compromised viability under different stressors due to non-functional CMA pathway.

There are also evidences of crosstalk between UPS and CMA, although not well understood. Acute inhibition of proteasomal pathway by chemical inhibitors activated CMA under starvation, but basal CMA remain unaffected [121]. In addition, some of the subunits of the proteasome are also *bonafide* substrates of CMA [74]. Hence, we aim to investigate the mechanism of crosstalk between UPS and CMA. in our preliminary studies we have observed association of ubiquitin and various ubiquitin modifying enzymes with membranes CMA active lysosomes. Of the ubiquitin modifying enzymes associated to the CMA active lysosomes our studies focus on carboxyl terminus of Hsp70 interacting protein (CHIP) and ubiquitin C-terminal hydrolase L1 (UCH-L1). CHIP- a co-chaperone and an E4 ligase- has been implicated

in Tau degradations and interacts with Hsc70, Hsp40, Hsp90 as well as LAMP-2A. Recently it has been reported that CHIP mediates the degradation of HIF-1 α by CMA pathway. Whereas UCH-L1 is a deubiquitinating enzyme has been implicated in PD pathology. It is also known that UCH-L1 interacts with CMA chaperones Hsp90 and Hsc70, CMA receptor LAMP-2A as well as CMA regulator PHLPP1. Hence we are interested to look at the roles of ubiquitin modifying enzymes CHIP and UCH-L1 in regulating the CMA pathway.

1.7 Ubiquitination

Ubiquitin is a small 76 amino acid protein found in eukaryotes. It contains 7 lysine residues which are important for conjugating to subsequent ubiquitin molecules to form poly-ubiquitin tags. Ubiquitination is the labeling of substrate with poly-ubiquitin tags. These tags act as indicators for the cellular quality control system for their degradation. Poly-ubiquitinated proteins are targeted for destruction by the cellular proteolytic system. A cascade of ubiquitin conjugating enzymes aid in the poly-ubiquitination reaction. E3 ligases play an important role for substrate selection.

Till date, the role of ubiquitination in CMA pathway is unknown. There is evidence that blockage of CMA causes transient impairment of proteasomal pathway [75]. Transgenic mice overexpressing LAMP-2A showed lower accumulation of poly-ubiquitinated aggregates compared to control [17]. These findings highlight the possibility that CMA may also degrade poly-ubiquitinated proteins. Apart from this K63 specific ubiquitination of HIF-1 α , CHIP also prepares it for degradation via CMA pathway [133]. It has recently been reported that downregulation of LAMP-2A in dopaminergic neurons of *substantia nigra* of the rat brain shows accumulation of α -synuclein – a *bona fide* CMA substrate. Interestingly, these α -synuclein puncta were

also ubiquitin positive [134]. All these evidences point towards a possible role of ubiquitination and ubiquitin modifying enzyme in CMA.

1.8 The E4 ligase CHIP

Carboxyl terminus of Hsp70 interacting protein (CHIP) is a co-chaperone and as the name suggests it interacts with the chaperone Hsp70 and is encoded by the *stub1* gene. It has been termed as an E4 ligase as it can bind to oligo-ubiquitinated substrates (proteins that are tagged by only a few ubiquitin molecules) and catalyzes multi-ubiquitin chain assembly in collaboration with E1, E2 and E3 [72]. CHIP is a dual function enzyme - it acts both as a co-chaperone as well as an E4 ubiquitin ligase mediating the degradation of proteins through proteasomal or lysosomal pathways [135, 136]. It has three major domains: an amino terminal tetratricopeptide repeat (TPR) domain for interacting with other proteins, a highly charged central domain necessary for TPR-dependent interactions and a carboxyl-terminal U-box domain for the E3-ligase function (Fig 1.5) [135]. Apart from Hsp70 it also interacts with Hsp90 and LAMP-2A which are CMA associated proteins [136].

Fig 1.5 The domain structure of CHIP CHIP is an E4 ligase with a dual function. It can act as a co-chaperone interacting with chaperones Hsc70 and Hsp90 using its N-terminal TPR domain. It can also function as an E3 ligase using its C terminal U-box domain. The two domains are connected by a highly charged coiled coil structure in between.

1.8.1 Association of CHIP with CMA

CHIP has been reported to interact with various CMA effectors like Hsp90, Hsc70 and LAMP-2A [133]. Initially when we started our investigation, it was known that HIF-1 α is degraded both in the proteasome and the lysosome and the decision was made by CHIP whether the protein should be refolded or should it be degraded [133, 136]. Recent studies have shown that not only HIF-1 α is a CMA substrate but also CHIP is necessary for the degradation of HIF-1 α by CMA [133]. CHIP co-immunoprecipitates with LAMP-2A, Hsc70 and HIF-1 α and this interaction is accentuated in the presence of proteasomal or lysosomal inhibitors [133]. This is because CHIP and Hsc70 form a complex with HIF-1 α and transport it to the lysosomal receptor LAMP2. The role of CHIP was subsequently discovered to ensure that the substrate is K63 ubiquitinated. This specific ubiquitination is necessary for degradation and is a direct proof that ubiquitination might play a role in targeting substrates to CMA degradation and our study aims to focus on the role of ubiquitination and CHIP in CMA pathway.

Wildtype Tau is a CMA substrate but the pathogenic mutant Tau causing Alzheimer's disease binds to the CMA active lysosomes and causes CMA failure [70]. It is known that CHIP is the primary Tau ubiquitin ligase and it helps in ubiquitination of mutant tau and its degradation by the proteasomal machinery [124]. It has been shown recently that Hsp90 also aids in Tau degradation. However, interestingly under Hsp90 inhibitions CHIP specifically targets the mutant tau for ubiquitination and degradation leaving behind the normal Tau species unaffected [137].

Receptor interacting protein kinase 3 (RIPK3) is a key regulator of necroptosis. CHIP is known to ubiquitinate RIPK3 and RIPK1 leading to their degradation and hence under normal conditions prevent their lethality. CHIP interacts with RIPK3

ubiquitinating it and leading to its lysosomal degradation. However, it is not yet clear whether the degradation is via CMA [138]. Thus we see that CHIP has been implicated in various CMA associated neurological disorders. This raises our interest in studying the role of CHIP in the CMA pathway.

1.9 The de-ubiquitinating enzyme UCH-L1

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is a de-ubiquitinating enzyme [116]. It is the most abundant protein in brain and has been implicated in the pathogenesis of neurodegenerative diseases that are caused due to protein aggregations like PD, spinocerebellar ataxia (SCA) and Huntington's disease (HD). It has been reported that UCH-L1 co-immunoprecipitates with LAMP-2 although it is not clear which isoform of LAMP-2 it interacts with. It is reported that mutant UCH-L1 interacts abnormally with LAMP-2A in an enhanced manner blocking CMA, thus causing CMA substrates to accumulate in the cytosol resulting in neurodegenerative diseases like PD (due to accumulation of α -synuclein) [109]. However, the exact function of UCH-L1 in CMA remains unclear.

UCH-L1 has been reported to be an oncogene as well. Overexpression of UCH-L1 enhances rates of lymphoid proliferation and reduces apoptotic cell death in these cancer cells. This is achieved by boosting the signaling through Akt1 pathway. UCH-L1 regulates the activity of Akt1 by targeting the phosphatase PHLPP1 to the UPS for degradation. Depletion of UCH-L1 caused a dramatic increase in PHLPP1 and thus reduced cellular levels p-Akt1 [57].

Hence we see that both CHIP and UCH-L1 are associated with the Akt1 pathway and can interact with CMA associated chaperones and receptor LAMP-2A. This leads us to

form our hypothesis that the ubiquitin modifying enzymes play a regulatory role in CMA activity and we aim to investigate this.

1.10 Aims

1.10.1 Examine association of ubiquitin and ubiquitin modifying enzymes with CMA-active lysosomes

CMA is a selective form of autophagy which degrades cytosolic substrates containing the CMA targeting motif [63] and depletion of this pathway results in various age-related neurodegenerative disorders [18]. Hence, it is of high interest to study the regulation of CMA. There is evidence of post translational modifications like phosphorylation or ubiquitination in CMA pathway [17, 48]. It is reported that Akt1 phosphorylation signaling pathway regulates CMA [48]. Additionally, CMA affects the proteolysis of ubiquitinated proteins like HIF-1 α . It is reported that in addition to presence of the CMA targeting motif, K63-linked ubiquitination of the HIF-1 α is necessary for its CMA degradation under hypoxia conditions.

Nevertheless, the broad role ubiquitination in modulating other substrates to be targeted to CMA-active lysosomes is not yet clear. Hence, in this section we aim to study the role of ubiquitination and ubiquitin modifying enzymes in CMA-active lysosomes. To achieve this goal, we intend to

1. Examine the association of ubiquitinated proteins with CMA-active lysosomes with respect to CMA activity
2. Characterize the types of poly-ubiquitin chains associated with CMA-active lysosomes
3. Investigate the presence of ubiquitin modifying enzymes in CMA-active lysosomes

1.10.2 Examine the role of E3 ligase CHIP in regulating CMA activity

CHIP performs K63-linked ubiquitination of HIF-1 α and targets it for CMA associated degradation [133, 139]. It has been reported that CHIP also ubiquitinates pAkt1 and directs it for proteasomal degradation [55]. It is known that lysosomal pAkt1 is a negative regulator of CMA. Hence it is possible that, in addition to substrate targeting, CHIP also regulates lysosomal pAkt1 and thus regulates CMA activity. This possibility is further supported by the detection and preferential enrichment of CHIP in CMA-active lysosomes which was observed in the current study. Thus it is imperative to study the role of CHIP in regulation of lysosomal mTORC2/Akt1/PHLPP1 signaling pathway regulating CMA activity. Hence, in this section we aim to

1. Examine the effects of CHIP knockdown and overexpression on CMA activity
2. Investigate the role of CHIP in regulating lysosomal mTORC2/Akt1/PHLPP1 signaling
3. Elucidate the putative mechanism underlying CHIP regulation of CMA in the lysosomes

1.10.3 Examine the role of DUB UCH-L1 in regulating CMA activity

The deubiquitinating enzyme UCH-L1 has been reported to play a major role in Parkinson's disease pathogenesis by aberrantly associating with the LAMP-2A receptor and blockage of CMA pathway [109]. UCH-L1 is known to regulate the mTOR complex formations as well as the cellular levels of PHLPP1 [56, 57]. This results in downstream regulation of cellular Akt activity [57]. Thus it is a possibility that like CHIP, UCH-L1 can also regulate the lysosomal mTORC2/Akt1/PHLPP1 pathway and affect CMA activity. Additionally, it has been shown in the current study that UCH-L1

preferentially associates with CMA-active lysosomes. Thus, it is required to study the specific role of UCH-L1 in regulation CMA activity by governing the lysosomal levels of Akt1 and PHLPP1. To achieve this goal, we intend to investigate the following aims

1. Examine the effects of UCH-L1 knockdown on CMA activity
2. Examine the role of UCH-L1 on regulation of lysosomal levels of CMA associated chaperones and LAMP-2A dynamics
3. Study the role of DUB activity of UCH-L1 in regulation of mTORC2/Akt1/PHLPP1 pathway

1.10.4 To elucidate the relationship between CHIP and UCH-L1 in CMA regulation and crosstalk with UPS

Both CHIP and UCH-L1 have also been implicated in regulation of mTORC2/Akt1/PHLPP1 signaling pathway, but in opposing ways by governing Akt and PHLPP1 levels [55-57]. CHIP and UCH-L1 are ubiquitin modifying enzymes with opposing functions in regulation of CMA. CHIP acts as a positive regulator of CMA by regulating the ubiquitination and degradation of pAkt1 and also by stabilizing Akt1 phosphatase PHLPP1. Unlike CHIP, UCH-L1 inhibits CMA by destabilizing PHLPP1 as well as deubiquitination of pAkt1, thus stabilizing it. It has also been shown that both enzymes associate with CMA-active lysosomes and hence. Thus, in this section it was investigated whether these enzymes interact to regulate CMA and how they do so. Additionally, it is important to study that in addition to the known mechanisms of crosstalk between CMA and UPS pathways (as discussed in Section 1.6) [74, 121], does the presence of the ubiquitin modifying enzymes in the CMA-active lysosomes contribute to this crosstalk. Hence the aim of this section is to investigate the following aims

1. Study the effect of CHIP on UCH-L1 levels and vice-versa.
2. Investigate the interaction between CHIP and UCH-L1 in the lysosomes
3. Examine the lysosomal association of CHIP and UCH-L1 during proteasomal stress

2. Materials and Methods

2.1 Cell and culture

SH-SY5Y (human neuroblastoma) and HEK293T cell lines were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) (both from Thermo Scientific HyClone). Growth conditions were 37°C with 5% CO₂ in humidified air. In order to prepare frozen cell stock for long term storage, cells were grown to 80% confluence, washed with Hank's Balanced Salt Solution (HBSS) (Gibco), trypsinized with 0.25% Trypsin (Gibco) and pelleted by centrifugation at 2000rpm using the A-4-62 rotor in Eppendorf centrifuge 5810 R. The cell pellets were resuspended in 1ml of freezing medium comprising of DMEM with 10% FBS and 10% DMSO (Sigma) and transferred to cryovials (Corning). The cryovials were stored in Mr. Frosty (Nalgene) at -80°C for 24 h and subsequently transferred to liquid nitrogen tanks for long term storage.

2.2 Antibodies and Chemicals

The following primary antibodies were used for immunoblotting at the following concentrations: anti-Actin (1:10,000) and anti-LAMP-2A (1:1000) (all from Abcam, UK); anti-LAMP1 (H4A3-Human) (1:3000), anti-c-myc (9E-10) (1:3000), anti-LAMP1(ID4B-mouse) (1:3000) and anti-tubulin (E7) (1:1000) (all from Developmental Studies Hybridoma Bank, USA); anti-LC3 (1:1000, Cell Signaling Technology and Novus Biologicals, USA), anti-Hsp70/Hsc70 (W27) and (13D3) (1:5000, Novus Biologicals, USA); anti-Hsp90 (1:1000; Enzo Life Sciences, USA),

anti-Hsp40 (1:20000, Enzo Life Sciences, USA), anti-UCH-L1 (1:1000, Enzo Life Sciences, USA); anti-CHIP, anti-mTOR, anti-Akt1 and anti-pAkt1 (Ser473), anti-GFAP, anti-pGFAP (all 1:1000, Cell Signaling Technology, USA); anti-ubiquitin (1:1000, Dako, Denmark); anti-K63 ubiquitin (1:250, Millipore, USA); anti-K48 ubiquitin (1:1000, Millipore, USA), anti-PHLPP1 (1:1000, Bethyl Laboratories, USA) and anti-Rictor (1:500; Santa Cruz Biotechnology, USA). Secondary antibodies used for immunoblotting were goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugated IgG (1:10000, Sigma-Aldrich, USA) or Goat anti-Mouse (IRDye® 800CW) and Goat anti-Rabbit (IRDye® 680RD) (both 1:20000, LI-COR®, USA). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, Promega, Merck and Invitrogen (all from USA). The following different chemical inhibitors were used – leupeptin (100uM working, Enzo Life Sciences), Ammonium Chloride (20mM working, Sigma-Aldrich), MG-132 (1uM working, Enzo Life Sciences) and lactacytin (5uM working, Enzo Life Sciences).

2.3 Creation of stable knockdown cell lines

In order to study the role of CHIP and UCH-L1 in CMA pathway, knockdown (KD) studies were performed on SH-SY5Y cell. The CHIP KD and UCH-L1 KD cells were prepared by Ms. Chye Yun Yu and Dr. Esther Wong respectively. The Lentivirus-mediated RNA interference was used to create stable knockdown (KD) of CHIP and UCH-L1 separately in SH-SY5Y. Lentiviral transfer vectors pLKO.1 TRC (Addgene plasmid # 10878) containing shRNAs against human CHIP (NM_005861.1-1069s1c1) or human UCH-L1 (NM_004181.3-522s1c1) from MISSION® shRNA collection (Sigma-Aldrich) or the empty vector were separately co-transfected with the third-generation lentiviral packaging constructs - (pMDLg packaging plasmid encoding viral particles GAG and POL, pRSV-Rev packaging plasmid encoding viral

packaging particle Rev and pMD2.G envelope plasmid encoding envelope protein VSVG) into HEK293T cells. To do this, the HEK293T cells were grown in Iscove's Modified Dulbecco's Medium (IMDM+GlutamaxTM) (Thermo Fisher) containing FBS and PenStrep to 50-60% confluence and transfected using the calcium phosphate method. The cells were incubated with the calcium phosphate-precipitated plasmid DNA at 37°C for 16 h and the media was replaced with 8ml of DMEM (containing FBS and PenStrep). After 24 h the media containing the released lentiviral particles were collected, filtered, aliquoted into 1ml volumes and stored at -80°C.

For each KD or control (CTR), 1ml aliquot of the lentivirus particles (in DMEM) containing the shRNA or empty vector respectively, was used to transduce 2×10^5 SH-SY5Y cells with 5µg/ml Polybrene (Millipore) in 6-well plate format for 72 h. Thereafter, the cells were harvested to examine the efficiency of KD by immunoblotting for either CHIP or UCH-L1 protein levels in the control CTR and respective KD cells. Stable CTR and KD clones were subsequently created via selection with 10µg/ml puromycin (Gibco) as the pLKO.1 TRC vector confers puromycin resistance. Throughout this study CTR, CHIP KD and UCH-L1 KD cells were grown in nutrient-rich conditions using DMEM medium containing FBS and PenStrep for analysis of constitutively active CMA under basal conditions. To study inducible CMA, cells were subjected to prolonged starvation by culturing the cells in DMEM medium without FBS for 16-18 hours after extensive rinsing with Hanks' Balanced Salt Solution (Invitrogen) three times. CMA is highly activated in confluent cells in culture upon long period of serum starvation [75, 113].

2.4 Creation of stable CHIP overexpression cell lines

In order to study the effects of overexpression of CHIP in SH-SY5Y cells, stable cells overexpressing myc-CHIP protein was created. The pcDNA myc-CHIP plasmid was obtained from Dr. K L Lim's Lab as a kind gift. To select the stable clone the cells were co-transfected with pBlast vector conferring blasticidin resistance. 2×10^5 SH-SY5Y cells were grown and co-transfected the empty pBlast vector and pcDNA myc-CHIP using Lipofectamine 2000 (Invitrogen). As a negative control, SH-SY5Y cells were transfected with pBlast vector alone. The cells were incubated in the transfection mix for 72 h and were selected for blasticidin resistance with $10\mu\text{g/ml}$ of blasticidin HCL (Gibco). The blasticidin resistant cells were then seeded at 1×10^4 densities onto 10cm plates to allow growth of cells into single colonies. After 6 days in culture, 6 individual colonies were selected from both the negative control (V) and myc-CHIP overexpressing (OE) plates. These clones were numbered #1- #6 and expanded for selection of positive clones that are blasticidin resistant and overexpressing myc-CHIP. To do this the cells were grown in blasticidin containing media and the different clones were harvested for RIPA lysis followed by immunoblot analysis of myc-CHIP expression. Our immunoblot analysis showed three positive clones overexpressing myc-CHIP – clones OE#3, OE#4 and OE#6. These clones were expanded along with their corresponding vector clones (V#3, V#4 and V#6), frozen down for long term and used for subsequent experiments.

2.5 Purification of lysosomal fraction

Cellular fractionation was performed to analyze the effects of depleting CHIP or UCH-L1 on different markers in the cytosolic (CYT), total cell homogenate (HOM) or lysosomal (LYS) compartments. For this, the CTR and various stable KD cells were

grown in either DMEM containing FBS (S+) or DMEM without FBS (S-). For each condition cells were harvested from 3 confluent 10cm plates. The cell pellets were collected, washed 3 times in ice cold phosphate buffer saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ and 1.8mM K₂HPO₄, pH 7.4) and resuspended in 1ml ice-cold 0.25M sucrose buffer (pH 7.2) with cOmplete, EDTA-free protease inhibitor cocktail (PI, Roche, Switzerland). Cell breakage was done by subjecting the 1ml cell suspension to 18 strokes of homogenization with a tight pestle using a pre-chilled 10ml Dounce homogenizer (Wheaton Industries) on ice. A small aliquot of the resultant homogenate was kept as “total cell homogenate” (HOM) for analysis while the rest of the homogenate was further subjected to a series of sequential centrifugations at 2500, 6800 and 17000 RCF using the FA-45-30-11 rotor and Eppendorf 5810R centrifuge to obtain a crude fraction highly enriched in lysosomes termed as the “lysosome-enriched fraction” (LYS) as the pellet. A “cytosolic fraction” (CYT) was prepared by centrifugation of the supernatant at 100,000 RCF for 1 h at 4°C [140].

2.6 Separation of lysosomal membrane and matrix fractions

Lysosomal membrane (Memb) and matrix (Mtx) were extracted from the lysosomal pellets in order to investigate the association of polyubiquitinated proteins with the different lysosomal components. To do this, the LYS fraction was extracted and divided into two parts. One part was kept aside to examine total lysosomes while the other part was centrifuged at 17000 RCF using the TLA 120.1 rotor in Optima™ MAX Ultraentrifuge (Beckman Coulter, USA) and the pellet was resuspended in water and PI (hypotonic shock) and kept on ice for 30 min. Subsequently, the lysosomal fraction in water was subjected to 10 cycles of rapid freezing (using dry ice) and thawing (using a 37°C waterbath) followed by centrifugation at 67000 RPM using the TLA 120 rotor and Eppendorf centrifuge.[141].

2.7 CMA+/ CMA- lys isolation from rat liver and from mice brain

The different pools of CMA active (CMA+) and CMA inactive (CMA-) lysosomes were separated in order to study the role of ubiquitination and different ubiquitin modifying enzymes in CMA+ lysosomes. This experiment was performed by Dr. Esther Wong. For enrichment in lysosomes active for CMA, Male Wistar Rats or Mice were starved for 48 h but maintained in the presence of water. The animals were suffocated in a CO₂ chamber and the liver or brain was isolated from the rat or mouse respectively. The specific organs were placed in 50 ml falcon tubes with ice cold 0.25M sucrose solution and washed several times to remove any remaining blood. Subsequently the tissues were cut into small pieces using scissors and homogenized in 3 volumes of 0.25M sucrose per gram of tissue using 8-10 strokes of the homogenizer and filtered using double gauze. A small aliquot (200µl) was kept aside as HOM fraction. After two rounds of serial centrifugations at 6800 RCF and 17000 RCF, the pellet and supernatant were collected. The supernatant was kept aside as the CYT fraction while the pellet was resuspended in 0.25M sucrose with a “cold finger” (a glass tube with ice inside) and mixed with 2 volumes of 85.6% Nycodenz[®] (Axis-Shield PoC, Norway). The sample was then loaded at the bottom of a ultra-clear centrifuge tube (Beckman). The following Nycodenz[®] gradient was prepared

Table 2.1 Preparation of Nycodenz[®] gradient

GRADIENT NUMBER	Nycodenz[®] CONCENTRATION	VOLUME (ml)
1 (BOTTOM)	85.6% (mixed with sample)	3.3
2	32.8%	2
3	26.3%	3.3
4 (TOP)	19.8%	3.5

The samples were centrifuged at 28700 RPM for 1:09 h (acceleration 4, deceleration 9) in the SW 41 rotor (Beckman) using the Beckman ultracentrifuge. The CMA+ lysosomes were collected from the topmost interface of the gradients. The next interface contained a mix of CMA+ and CMA- lysosomes which was collected and both fractions were washed with 0.25M Sucrose and centrifuged at 37000 RCF for 15 mins. The pellet from the mixed population of CMA+ and CMA- lysosomes was resuspended in 300µl of 0.25M sucrose and centrifuged for 5min at 10000 RCF. The pellet contained the CMA- lysosomes whereas the supernatant (containing CMA+ lysosomes) was collected to resuspend with the CMA+ lysosomal pellet. This was the total pool of CMA+ lysosomes [36].

2.8 Immunoblotting and densitometric analysis

In order to investigate the various aspects of CMA upon manipulating the levels of CHIP and UCH-L1, we employed immunoblotting technique and examined the levels of different proteins under different conditions and treatments. The HOM and LYS fractions derived from cellular fractionation described above were used to study the

changes at the lysosomal levels. To study the changes in total protein levels, total cellular proteins are extracted by lysing the cells in ice-cold RIPA lysis buffer (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8). Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Inc., USA) using bovine serum albumin (BSA) as a standard. 30-70µg of proteins per sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto nitrocellulose membrane (Whatman, Millipore, USA). Membranes were subjected to reversible Ponceau S staining (0.5% (w/v) Ponceau S in 1.0% (v/v) acetic acid) to visualize total amounts of proteins resolved in each lane prior to blocking with 5% milk in TBST buffer (22.4 g/l Tris base, 80 g/l sodium chloride and 0.05% Tween 20 (BioRad, USA) pH 7.5). Proteins of interest were detected by specific antibodies and visualized using enhanced chemiluminescence (ECL) Western Blot detection reagent (GE Healthcare, USA) and autoradiography with Kodak films (USA) or hyperfilms (GE Healthcare, USA).

Densitometric analysis was performed by quantifying the intensity of the bands using ImageJ software (NIH, USA). Either actin or tubulin was used as the loading control for analyses of total cell homogenates and RIPA lysates while LAMP1 was using for normalizing the total amounts of proteins in the lysosome-enriched fractions.

Alternatively, the proteins of interest on nitrocellulose membranes were detected with Odyssey® CLx Imaging System (LI-COR®, USA). Briefly, membranes were blocked using Odyssey® Blocking Buffer (TBS) (LI-COR®, USA) for 1 h. The primary antibodies were diluted in the same blocking buffer and the membranes were incubated with the respective antibodies overnight. The membranes were then washed three times in TBST and incubated with the respective fluorescent secondary antibodies

(dilution 1:20,000) in TBST for 1 h at room temperature. The membranes were again washed with TBST and the bands were visualized using Odyssey CLx machine (LI-COR®, USA).

2.9 Analysis of LAMP-2A multimeric complexes by discontinuous sucrose density gradient centrifugation

In order to study the effect of CHIP or UCH-L1 KD on LAMP-2A dynamics, the lysosomal membrane proteins were extracted and subjected to discontinuous sucrose density gradient centrifugation to separate the different LAMP-2A monomeric and multimeric complexes followed by western blot analysis. CMA activity is governed by the stability of the multimeric LAMP-2A translocation complex. The monomeric LAMP-2A is 100 kDa while the multimeric LAMP-2A is 700kDa and any intermediate structure ranges in MW between these two. Using this principle, we separated the different LAMP-2A conformers based on their molecular weights to measure CMA activity. Starvation induces CMA and hence majority of the LAMP-2A is in the high MW complex while under basal condition majority of LAMP-2A is in lower MW fraction. We used a discontinuous sucrose density gradient to separate the different complexes based on their molecular weights.

Lysosomal membranes (since LAMP-2A is a membrane protein) were isolated from the CTR or KD cells under starvation (S-) or basal (S+) conditions, and solubilized to release the membrane proteins, by resuspending the lysosomal membranes in 20 mM MOPS (3-(*N*-morpholine)propanesulfonic acid), 150 mM sodium chloride, and 0.5% NP-40 buffer for 15 min on ice. After centrifugation for 15 min at 16,000 RCF, the soluble proteins were recovered in the supernatant. The solubilized lysosomal membranes were loaded on a 5 ml continuous sucrose gradient (10% to 60%) in the following order.

Table 2.2 Composition of discontinuous sucrose gradient

GRADIENT NUMBER	SUCROSE CONCENTRATION	VOLUME (ml)
1 (TOP)	SAMPLE	0.5
2	10%	1
3	20%	1
4	30%	1
5	40%	0.75
6	50%	0.5
7 (BOTTOM)	60%	0.25

The samples were centrifuged at 28,600 RPM for 14 h and 35 min at 4°C in a swinging-bucket rotor (SW55 Ti rotor; Beckman). Aliquots of 500µl were collected from the top to the bottom fraction of the gradient and precipitated with 20% trichloroacetic acid (TCA) and 5µl of 10mg/ml BSA for 30 min, washed with acetone, and resuspended in 20µl 2x sample and boiled at 100°C for 5min. Each aliquot was subjected to SDS-PAGE and immunoblotting. The level of LAMP-2A in each complex was expressed as a percentage of the total amount of LAMP-2A in all the lanes of the gel.

2.10 Immunoprecipitation and co-immunoprecipitation

We performed immunoprecipitation (IP) of either Akt1 or UCH-L1 from the HOM and LYS fractions to study the levels of ubiquitination of these proteins in CTR and CHIP KD cells. To do this, cells were grown on three 10 cm plates per condition and harvested

to get the HOM and LYS fractions. These fractions were then incubated in 210µl of RIPA lysis buffer for 15 min followed by centrifugation at 13,200 RPM at 4°C to solubilize all the proteins. The supernatant containing the soluble proteins was collected and divided into three parts. A small aliquot (10µl) was kept aside as “input” and the rest was divided into 100µl aliquots into two 1.5ml tubes. One aliquot was incubated with 1.2 µg the respective anti-Akt1 or anti-UCH-L1 antibody (experimental) while the other was incubated with anti-mouse HRP (negative control) overnight at 4°C with continuous shaking at 15 RPM to form the antigen-antibody complex. The antigen-antibody complex was then incubated with 30µl of pre-washed protein A beads (GE Healthcare Life Sciences) for 2 h at 4°C. The beads were then pelleted by centrifugation at 16000 RCF for 10mins at 4°C. The flow-through was collected and beads were washed 3 times with RIPA lysis buffer and the proteins were eluted by boiling with 2X SDS sample buffer for 10 min. Samples were resolved using 10% or 12% SDS-PAGE for western blot analysis.

Co-immunoprecipitation (co-IP) was performed to study the interactions of wildtype (WT) and different CHIP mutants with various proteins in the HOM and LYS fractions upon over expression of WT myc-CHIP or mutant myc-CHIP (site-directed mutagenesis to create the mutations described in the next section). For co-immunoprecipitation (co-IP) cells were grown on 10 cm plates and transfected with 4µg of WT myc-CHIP or H260Q myc-CHIP or K30A myc-CHIP expressing plasmids. The cells were transfected for 48 h with Lipofectamine 2000 using DNA: lipofectamine ratio of 1:2. The cells were harvested and the HOM and LYS fractions were collected for each condition. These were used as the starting materials for co-IP. Fractions from the different experimental conditions were resuspended in 1 ml of low stringency PBSCM buffer (100mM sodium phosphate, 150mM sodium chloride, 1mM calcium

chloride, 1mM magnesium chloride, pH 7.2) so that the interactions between different proteins remain intact and immunoprecipitated using 1.2µg of anti-myc antibody similar to the IP protocol.

2.11 Immunostaining and immunofluorescence

We studied the activity of CMA in various CTR, CHIP KD and UCH-L1 KD cells by quantification of the number of colocalizing LAMP-2A/Hsc70 puncta as a measure of the number of CMA+ lysosomes. This was done by immunostaining LAMP-2A and Hsc70 using respective antibodies in the different cells under basal and CMA induced conditions. 1×10^5 cells were seeded on poly-L-lysine (Sigma, USA) coated coverslips and allowed to recover in media containing DMEM and FBS for 24 h. The cells were then treated with either S+ or S- media for 16-18 h. After treatment, the coverslips were washed three times with PBS and treated briefly with 0.1% Digitonin (GE Healthcare, USA) for 10 sec to extract the cytosol preventing non-specific staining of the cytosolic Hsc70, prior to fixation with 4% paraformaldehyde (PFA) (Sigma, USA) for 30 min at room temperature (RT). For staining, cells were first permeabilized with PBS containing 0.1% triton-X (Sigma, USA) for 5 minutes and then washed with PBS 3 times. The permeabilized cells were incubated with respective anti-LAMP-2A or anti-Hsc70 antibodies followed by fluorophore-conjugated anti-mouse Alexa 488 (green) (1:500, Invitrogen, USA) or anti-rabbit Alexa 594 (red) (1:500, Invitrogen, USA) for 1 h at RT. Cell nuclei were visualized by DAPI nuclear stain (blue) contained in the mounting medium (ProLong Gold Antifade Reagent with DAPI, Invitrogen, USA). Z-stack program was used to acquire 15-24 image stacks using Zeiss Live Cell Imaging Microscope under 63X (oil immersion) magnification and the ZEN 2 (Blue Edition) software. The images were deconvoluted using the Deconvolution (default) – Fast Iterative method of the ZEN 2 (Blue Edition) software. Image analyses were carried

out using ImageJ software (NIH, USA). The red and green channels were split, converted to 16 bit images, inverted and a fixed threshold range was set for each channel which was kept constant for all the images in a particular experimental set. The number of colocalizing puncta was analyzed using the “object based method” of analysis in the “JaCOP” plugin of the ImageJ software. Area occupied by each cell was also calculated using ImageJ. For each set of analysis, at least 20 cells from random fields were analyzed per condition.

2.12 Reverse Transcription (RT-PCR) and quantitative real-time PCR (qPCR)

RT-PCR followed by qPCR was performed to examine the levels of expression of PHLPP1 in CTR, CHIP KD and UCH-L1 KD cells. Total RNA was extracted from CTR, CHIP KD and UCH-L1 KD cells using the RNeasy Mini Kit (Life Technology) following the manufacturer's indications, and stored at -80°C until use. The cDNA was synthesized from 2 μg of the total RNA with the RNase H Reverse Transcriptase (Applied Biosystems, USA). Briefly, 2 μg RNA was incubated with dNTP and oligo-(dT) (12-18 bases) primers and heat denatured for 5 min followed by immediate cooling on ice for 3 min. The reaction was then incubated with a mixture containing 10X RT Buffer (Applied Biosystems, USA) and RNase H Reverse Transcriptase at 42°C for 1 h. The resultant cDNA was used for qPCR analysis of gene expression. 90-180bp regions of human actin and PHLPP1 were amplified with specific primers (PHLPP1, 5'-GCCACATAATCCCCTGGAAC-3', 5'-CCATTGCAGTGGGGCTTC-3'; actin, 5'-CCAGAGGCGTACAGGGATAG-3', 5'-CCAACCGCGAGAAGATGA-3') using the SYBR green PCR kit (Life Technologies) on a Quantstudio 6 Flex real time PCR system (Applied Biosystems Inc., Foster City, CA, USA). The final concentrations of each primer used were 50nM (for actin) or 100nM (for PHLPP1).

PCR cycling conditions used were: initial 50°C for 2 min, then 95°C for 10 min, followed by 95°C for 30 sec, 60°C for 1 min and 72°C for 30 sec with real-time data acquisition for 40 cycles. All C_t values were translated into arbitrary units (AU) and relative expression was calculated based on the formula: $AU = 2^{C_t(\text{PHLPP1}) - C_t(\text{actin})}$.

2.13 Cloning of UCH-L1 gene into pcDNA vector

In order to overexpress wildtype UCH-L1 in SH-SY5Y cells, the UCH-L1 gene was cloned into pcDNA. This step was performed by Dr. Nyugen Hong Phuong. The total mRNA was extracted from SH-SY5Y cells using the RNeasy Mini Kit (Life Technology) following the manufacturer's indications. This was followed by generation of total cDNA (as described in section 2.12). Specific primers (fwd 5'-CTCGAGACCATGCAGCTCAAGCCGATGGAGA-3' and rev 5'-AAGCTTGGCTGCCTTGCAGAGAGCCACG-3') and were designed for PCR amplification of UCH-L1 from the cDNA. The primers were constructed in a way that the UCH-L1 gene was flanked by Xho1/HindIII overhangs. Standard PCR conditions were used to amplify the gene. The amplified PCR product was cloned using the pGEM®-T Easy vector (Promega, USA) which was designed based on the T/A cloning method [142]. Successful cloning of the PCR product would disrupt the β -galactosidase gene coding sequence of the the pGEM®-T Easy vector and allowed blue-white screening. The successful recombinant clones would appear white when plated on LB agar supplemented with ampicillin (50 μ g/ml), 0.5mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and 80 μ g/ml X-gal. For subcloning of the gene inserts, 1 μ g of DNA and 0.5 μ g of pcDNA were digested with 1 unit of Xho1 and HindIII restriction enzymes each and the respective restriction buffers. The restricted fragments were subsequently separated by agarose gel electrophoresis. The fragments were then purified using QIAquick® Gel Extraction kit (Qiagen, USA). After recovery of the

restricted fragments, ligation was carried out using T4 DNA ligase (Promega, USA) and transformed into competent *E. coli* cells for amplification and long-term storage.

2.14 Site-directed mutagenesis

Specific functional domains of CHIP (TPR domain or U-box domain) or UCH-L1 (hydrolase/deubiquitinating domain) were mutated, in order to study the functional roles of these enzymes in the CMA pathway. Two CHIP mutations K30A (TPR domain) and H260Q (U-box domain) and one UCH-L1 I93M mutation were generated in the pcDNA myc-CHIP or pcDNA myc-UCH-L1 constructs using site-directed mutagenesis according to previous literatures [109, 133]. The pcDNA myc-UCH-L1 construct and I93M myc-UCH-L1 were developed by Dr. Nyugen Hong Phuong. For each mutation, a pair of oligonucleotide primers containing the intended mutation and annealing to the same sequence on opposite strands of the plasmid were designed. The following mutagenic oligonucleotide primers were designed – for K30A CHIP mutant (forward GCCAGGAGCTCGCGGAGCAG; reverse TGCCCTGCTCCGCGAGCTCCTG), for H260Q CHIP mutant (forward CATCGAGGAGCAGCTGCAG; reverse CTGCAGCTGCTCCTCGATG) and for I93M UCH-L1 mutant (forward CCTGTGGCACAATGGGACTTATTCACGC; reverse GCGTGAATAAGTCCCATTGTGCCACAGG). Mutagenesis reactions were carried out by polymerase chain reaction (PCR) with 100ng of plasmid construct containing either myc-CHIP or myc-UCH-L1 template using KAPA HiFi Fidelity Polymerase enzyme (KAPA bioscience, USA). The final concentrations of each primer used were 300nM and the reactions were carried out in the presence of 5X KAPA HiFi Fidelity buffer (KAPA bioscience, USA), 300μM KAPA dNTP mix (KAPA bioscience, USA) and 5% DMSO. PCR cycling conditions used were: initial 95°C for 2 min, then 98°C for 20 sec, followed by 55°C for 15 sec, 72°C for 4 min and 72°C for

5min for 20 cycles. After thermal cycling, the reaction mixture was incubated with 1 unit of *DpnI* at 37°C for 1 h. This restriction enzyme could only recognize target sequences that are methylated and hence, serves to remove the methylated parental plasmid DNA from the PCR generated non-methylated mutant plasmids. The final mixture was transformed into DH5 α competent *E. coli* strain via heat shock at 37°C for 90s. The bacteria was then plated onto 100 μ g/ml ampicillin containing agar plates and the latter incubated at 37°C for at least 16 h. Single Colony was selected and inoculated in 5ml Lysogeny broth (LB) (Difco LB Broth, BD and company, USA) with 100 μ g/ml ampicillin overnight with shaking at 220 RPM at 37°C. Plasmids were extracted from bacteria using Plasmid Miniprep Kit (Qiagen) following manufacturer's protocol. Purified mutant plasmids were then sequenced to verify the mutations using the CMV-forward primer (5'-CGCAAATGGGCGGTAGGCGTG-3'). The positive mutant plasmids were selected for large-scale purification using HiSpeed Plasmid Maxi Kit (Qiagen) according to manufacturer's protocol and used for transfection.

2.15 Calculation of LC3-II flux

In order to study the activity of macroautophagy (MA), LC3-II flux was calculated to examine whether KD of CHIP or UCH-L1 affects the MA activity. As LC3 II helps in formation of autophagosome as well as degraded by MA, we studied the rate of turnover of LC3-II in different cell lines to determine the MA activity. For this, we treated different CTR and KD cells with or without lysosomal inhibitors (NH₄Cl and leupeptin) for 4 h. Dividing the amount of LC3 II that accumulates upon lysosomal inhibition with the amount of LC3 II in cells without lysosomal inhibition helps us to estimate the rate of MA turnover. The cells were harvested and lysed using RIPA buffer and analyzed with immunoblotting.

The LC3-II flux was calculated using the following formula;

$$LC3II\ FLUX = \frac{\text{Amount of LC3 II in NL treated condition}}{\text{Amount of LC3 II in untreated condition}}$$

2.16 Measurement of proteasome activity

Proteasome activities of the CTR and various stable KD cells were determined using Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assays (Promega, USA) to examine whether the depletion of CHIP and UCH-L1 adversely affects proteasomal function. Various cell lines were seeded in triplicate in 96-well plate at a cell density of 30,000 cells per well. As a negative control cells were treated with 5μM lactacystin to inhibit proteasome activity. After 24 h, the proteasome activities in various cell lines were directly assayed addition of 100μl of the proprietary luminogenic proteasome substrates that emit luminescence when cleaved by chymotrypsin-like, trypsin-like and caspase-like protease activities of the proteasome complex in cultured cells. After incubation for 10 min at RT, the level of luminescence was measured using the Infinite M200 PRO (TECAN, Singapore).

2.17 Assessment of intracellular protein degradation (Pulse and Chase)

In order to evaluate the effect of depletion of CHIP and UCH-L1 on lysosomal activity, the degradation of the pool of long-lived cytosolic proteins (half-lives >6h) was measured. This was attained through pulse and chase experiments by metabolic labeling using a radiolabeled amino acid and inhibitors of autophagic pathways to discriminate those proteins undergoing degradation in lysosomes [140]. CTR, CHIP KD and UCH-L1 KD cells were seeded in duplicates into 12-well plates at 20,000 cells/well density. After 24 h, the cells were washed once using HBSS and “pulsed” with 2μCi/ml of radioactive ³H-Leucine (5mCi stock; Perkin Elmer, USA) supplemented to the media

Materials and Methods

(DMEM). The cells were incubated in the pulse-media for 48 h to maximize the labeling of the long lived proteins. At the end of labeling, the media was discarded, washed twice with HBSS and supplemented with 700µl of the “chasing” medium containing 2.8mM leucine and following treatments;

Table 2.3 Treatments for measuring rates of protein degradations

No.	Treatment conditions	Nomenclature
1	Basal media without lysosomal inhibitors	S+
2	Serum-starved media without lysosomal inhibitors	S-
3	Basal media with lysosomal inhibitors	S+NL
4	Serum-starved media with lysosomal inhibitors	S-NL

The chasing medium contains an excess of leucine which replaces the radioactive leucine in the cells. The radioactive leucine was thus, released into the media after lysosomal degradation of the long-lived proteins and exclusion by the cells. The cells were incubated in the chasing medium at 37°C and 70µl aliquots of the media were collected in duplicates for different time points namely 0h, 4h and 24. The aliquoted media (duplicates), from different time points were collected in pre-wet 96-well filter plates (Millipore, USA) and precipitated with 10% TCA and 0.5 mg/ml BSA for 1 h at 4°C. The media was then vacuum-filtered using the Millipore Manifold. After the 24h time point, the cells were washed and lysed with 0.1N NaOH/0.1%Na deoxycolate overnight. 20µl of the filtered media as well as the solubilized cell lysates (duplicates) were collected in 96-well plastic reading plates and 180µl of scintillation liquid (Perkin

Elmer, USA) was added in each well. The plates were then sealed and the number of radioactivity counts was measured in a Liquid Scintillation counter (Perkin Elmer, USA). Proteolysis is considered the amount of acid precipitable radioactivity (protein) transformed in acid soluble radioactivity (peptides and amino acids) during the incubation time. The rate of lysosomal proteolysis was calculated by dividing the proteolysis in untreated cells by the proteolysis in cells treated with lysosomal inhibitors for each condition (S+ and S-).

2.18 Statistical analysis

All results are presented as mean + S.E.M. and represent a minimum of three independent experiments unless otherwise stated. Statistical significance of difference between groups was determined by the two-tailed unpaired Student's t-test and considered significant if $p < 0.05$.

3. RESULTS

3.1 Association of ubiquitin and ubiquitin enzyme cascade with CMA-active lysosomes

There are various reports of crosstalk between the ubiquitin proteasome system (UPS) and chaperone-mediated autophagy (CMA) pathway [17, 74, 134]. Acute blockage of the ubiquitin proteasome system leads to transient upregulation of macroautophagy as well as CMA [121]. Some of the subunits of the proteasome are *bona fide* CMA substrates [64]. There is also evidence that blockage of CMA causes transient impairment and dysregulation of the proteasomal pathway [74]. However, the first evidence of a link between ubiquitination and CMA was observed in transgenic mice overexpressing LAMP-2A, which showed lower accumulation of poly-ubiquitinated aggregates compared to control littermates [17]. This was further supported by evidence that the E4 ligase CHIP causes K63-linked ubiquitination of HIF-1 α and this ubiquitination is imperative for the degradation of HIF-1 α by CMA pathway [139]. This suggested that specific ubiquitination topology was involved in substrate targeting to CMA+ lysosomes. These findings highlight a close connection between the ubiquitin modifying system and CMA. However, it remains unclear if CMA degradation of ubiquitinated substrates is independent of known factors like presence of CMA targeting motif, phosphorylation or acetylation [65, 143]. It is also not clear if the role of ubiquitination in CMA is specific to conditions of stress or it acts all the time at basal levels. Additionally, not only ubiquitination but also ubiquitin modifying enzymes like CHIP and UCH-L1 are known to interact with CMA components like Hsp90, Hsp40

and LAMP-2A [109, 133] which indicates a possible regulatory role of these enzymes in the CMA pathway. Moreover, CHIP is an E4 ligase and promotes degradation of HIF-1 α by K63-linked ubiquitination. Thus it is possible that K63-linked ubiquitination may be specific for CMA degradation of substrates. Hence, in this section we study the role of ubiquitination and ubiquitin modifying enzymes in CMA.

3.1.1 Use of LAMP1 as lysosomal loading control

In the current study LAMP1 has been shown as a loading control in the western blot analyses of the lysosomal fractions as it does not undergo any change due to changes in LAMP-2A levels or CMA activity [41] and has been used in previous CMA publications [37, 48]. Moreover, in the present study, depletion of CHIP or UCH-L1 did not affect the levels of LAMP1 in total cell lysates (Fig. 3.1.1 A, B). Hence, LAMP1 has been used to normalize the levels of different proteins in the lysosomal fractions. These results can be further affirmed by showing the levels of Cathepsin D as an alternative lysosomal marker.

Fig. 3.1.1 Levels of LAMP1 remain unaffected upon depletion of CHIP or UCH-L1 Representative immunoblots of the HOM fractions show the levels of LAMP1 in (A) CTR and CHIP KD cells or (B) CTR and UCH-L1 KD cells. Graphs show the levels of LAMP1 in (A) CTR and CHIP KD cells (bottom) or (B) CTR and UCH-L1 KD cells (bottom). Values are expressed as mean $\pm$ S.E.M, N=5,3

3.1.2 Association of ubiquitinated proteins with CMA-active lysosomal membrane changed

In this section, the novel function of the ubiquitin system in CMA pathway was examined. In order to study the role of ubiquitination at the lysosomes, the localizations of total ubiquitinated proteins at the CMA⁺ were investigated. It has been well characterized that rat liver provides high yield of CMA⁺ lysosomes upon prolonged starvation for 48 h [36]. Hence, CMA⁺ lysosomes were isolated from fed (basal CMA) and starved (induced CMA) rat liver for this study. The lysosomal membranes (Memb) were separated from the lysosomal lumen/matrix (Mtx) and analyzed by immunoblotting. It has been reported that upon starvation there is an increase in the lysosomal pool of CMA⁺ lysosomes as well as LAMP-2A and Hsc70 [37]. Hence, the presence of high levels of LAMP-2A and Hsc70 upon starvation has been shown as positive control in this experiment. It was observed that majority of the poly-ubiquitinated proteins were associated with the Memb fractions and not with the Mtx (Fig. 3.1.2 A). The association of poly-ubiquitinated proteins with the Memb of CMA⁺ lysosomes was further enhanced upon starvation (to induce CMA) (Fig. 3.1.2 B). This indicates a role of ubiquitination in the CMA pathway since induction of CMA promoted lysosomal association of polyubiquitinated proteins.

Subsequently, since K63-linked ubiquitination has been implicated in HIF-1 α degradation by CMA [139] suggesting a preferential role of K63 ubiquitination in CMA, the specific associations of the K63- and K48-linked ubiquitinated proteins with CMA⁺ lysosomes was examined. Upon starvation and inhibition of lysosomal proteolysis, to capture those proteins that are either binding to or uptaken by CMA⁺ lysosomes, it was observed that both K63- and K48-linked ubiquitinated proteins

associated with CMA+ lysosomal membranes, but there was a marked enrichment of K63-linked ubiquitinated proteins over K48-linked ubiquitinated proteins in the Memb fractions of the CMA+ lysosomes (Fig. 3.1.2 B). Densitometric analysis of the preliminary results showed that there was 6 times greater association of K63-linked than K48-linked ubiquitinated proteins (Fig. 3.1.2 C). Since blockage of lysosomal proteolysis resulted in enhanced enrichment of K63-linked poly-ubiquitinated proteins at the lysosomal membranes rather than K48-linked counterparts, there is a possibility that K63-linked ubiquitination serves special function in CMA and the K63-linked ubiquitinated proteins may be potentially sent to the lysosomes for degradation by CMA. In (Fig. 3.1.2 A,B) the LAMP1 loading control has not been shown, however, the results in the following sections have been confirmed with proper loading controls.

Fig. 3.1.2 Association of poly-ubiquitinated (polyUb) proteins and ubiquitin modifying enzymes with CMA active lysosomes (A) The levels of polyUb proteins, Hsc70 and LAMP-2A in the Memb and Mtx fractions of CMA+ lysosomes under fed and starvation conditions were shown (B) Analysis of CMA+ lysosomes purified from rats injected with leupeptin revealed very little polyUb proteins in lysosomal lumen. There is a marked enrichment of K63 ubiquitinated proteins over K48 ubiquitinated proteins in lysosomal membrane. Lane 5 and 6 were loaded with synthetic K48- and K63-linked ubiquitin₁₋₇ chains to ascertain the specificity of anti-K63 and anti-K48 antibodies. (C) Graph shows the folds of enrichment of differently ubiquitinated proteins in lysosomal membrane over those represented in the homogenates. (D) Immunoblots of different ubiquitin modifying enzymes in lysosomal (CMA+; CMA-) fractions isolated from mouse brains. 1-3 denotes samples from 3 different mice.

3.1.3 Greater association of ubiquitin modifying enzymes with CMA active lysosomes

Apart from ubiquitinated proteins, the other important players in the ubiquitination pathway are the ubiquitin modifying enzymes. These are the different enzymes that help in ubiquitinating or de-ubiquitinating a substrate. Hence, the levels of different ubiquitin modifying enzymes like E4 ligase - CHIP, deubiquinating (DUB) enzyme - UCH-L1, K63-linked E3 ligase - Parkin and corresponding E2 enzyme partner - UBC13 in the different pools of CMA+ and CMA- lysosomes were examined. The different pools of CMA+ and CMA- lysosomes were isolated from mouse brain as UCH-L1 is mainly a brain protein and remains undetected in liver. However, purification of CMA+ lysosomes from rat brain is less pure due to the thick myelination of the rat neurons. Hence, the different pools of lysosomes were extracted from mouse brain. It is reported that LAMP-2A levels are upregulated upon prolonged starvation and upregulation of CMA while LAMP1 levels remain unchanged [37, 144]. Hence, LAMP-2A levels have been indicated to specify the CMA+ lysosomes whereas LAMP1 levels have been indicated as normalizing control. It was revealed that there was greater association of the array of ubiquitin modifying enzymes CHIP, UCH-L1, Parkin and UBC13 with CMA+ lysosomes compared to CMA- lysosomes (Fig. 3.1.2 D). Interestingly, both CHIP and UCH-L1 have been associated with CMA as mentioned earlier. Both enzymes are known to interact with various CMA effectors like Hsc70, LAMP-2A and Hsp90 [109, 133, 136]. As discussed previously, CHIP has been implicated in guiding substrates towards lysosomal degradations. For instance, CHIP helps in HIF-1 α degradation by CMA while mutant UCH-L1 leads to enhanced binding to LAMP-2A and blocks CMA pathway [109]. CHIP and UCH-L1 have been implicated in CMA pathway, and based on the observation of preferential enrichment

of the ubiquitin modifying enzymes at CMA+ lysosomes, in the current study, the possible roles of these enzymes in CMA pathway are investigated.

3.2 Investigating the role of CHIP and UCH-L1 in CMA pathway

It has been observed in the previous section that CHIP and UCH-L1 associate preferentially with the CMA+ lysosomes. Both CHIP and UCH-L1 are not known to be lysosomal substrate and do not contain any CMA-targeting motif. Hence, in this section we aim to investigate whether these ubiquitin modifying enzymes play a role in regulation of CMA. To elucidate the role of CHIP and UCH-L1 in CMA pathway, we aim to manipulate the levels of these enzymes in SH-SY5Y cells and examine their effect in CMA activity, CMA-associated chaperones, receptors and the mTORC2/Akt1/PHLPP1 pathway.

3.2.1 Creation of knockdown of CHIP and UCH-L1 in SH-SY5Y cells

In order to investigate the roles of CHIP and UCH-L1 in CMA, the effects of reducing CHIP and UCH-L1 expressions were examined separately on CMA pathway in SH-SY5Y cells via lentiviral shRNA interference. SH-SY5Y cells stably knocked down for either CHIP or UCH-L1 and selected using puromycin resistance post-lentivirus infection. Immunoblot analysis of CHIP and UCH-L1 levels in the RIPA lysates of the respective knockdown (KD) cells indicated >80% reduction in the levels of both proteins when compared to the control (CTR) cells (Fig. 3.2.1 A).

To study how the depletion of CHIP or UCH-L1 affected CMA, which is a lysosomal pathway, it was pertinent to check that there was also efficient knockdown of both enzymes in the lysosomes. For this purpose, lysosome-enriched (LYS) fractions were purified from the total cell homogenates (HOM) of CTR and various KD cells. Cellular

fractionation and immunoblot analysis verified knockdown of CHIP and UCH-L1 in the lysosomal (LYS) and cytosolic (CYT) fractions (Fig 3.2.1 B top and bottom).

CHIP is an E4 ligase while UCH-L1 is a de-ubiquitinating enzyme, and depletion of these enzymes might have an impact on ubiquitination pathway. Ubiquitinated proteins are often tagged for either UPS-mediated or CASA-mediated degradation pathways. Hence, we examined the effect of KD of CHIP and UCH-L1 on UPS and MA activities. To measure the activity of the MA pathway, the turnover of LC3 II (flux) was investigated under basal condition. CTR, CHIP KD and UCH-L1 KD cells were treated with or without lysosomal inhibitors (NL) for 4hr and then harvested the cells for RIPA lysis and subsequent western blot analysis. The flux/ turnover of LC3 II was calculated by dividing the amount of LC3 II that accumulated (with NL) by (without NL) for each condition. LC3 II helps in formation of the limiting membrane around the MA substrate. Subsequently, when the autophagosome fuses with the lysosomes it degrades along with the substrate. By calculating the turnover (flux) of LC3 II, the MA activity can be estimated. It was observed that there was no significant change in LC3 II flux between the CTR and the various KD cells (Fig. 3.2.1 C left).

In order to determine the proteasome activity, the activity of one of the protease – chymotrypsin was measured. The assay was performed on live cells under basal conditions. The Proteasome-Glo Chymotrypsin-like Cell-Based Assay Kit (Promega, USA) was used to measure the activity. The kit contains a chymotrypsin substrate in a buffer which is optimized for permeabilization of live cells. The substrate, on being acted upon by the chymotrypsin in the cells, produces a luminescent product (Fig. 3.2.1 D left). To determine the proteasome activity, luminescence was measured - greater the cleavage, greater is the luminescence. A lactacystine (LACTA; proteasome inhibitor)

treated condition was added to act as a negative control. It was indicated that there was no change in luminescence and hence proteasome activity due to the KD of CHIP and UCH-L1 compared to control (Fig. 3.2.1 D right).

Taken together, it was shown that knockdown of CHIP or UCH-L1 does not adversely affect the MA and CMA pathways. The reason why no change was observed could be due to the fact that there is a plethora of different ubiquitin modifying enzymes and as such, the depletion of one of them does not have any effect on activities of the UPS or MA pathways globally. CHIP may reduce the degradation of certain substrate proteins through UPS, but not the activity of the UPS pathway. In MA, CHIP only assists in a specialized CASA pathway which degrades a very small pool of substrates and hence, does not affect MA activity. Additionally, it has been reported that deficiency of UCH-L1 caused diminished levels of ubiquitin due to lower stability of the ubiquitin molecules, but did not have any effect on UPS pathway [145]. Moreover, UCH-L1 has not been implicated in MA pathway. These results established that both CHIP and UCH-L1 KD cells serve as good cellular models for studying the consequences of depleting CHIP and UCH-L1 levels on CMA function and lysosomal properties.

Fig. 3.2.1 Knockdown of CHIP and UCH-L1 in SH-SY5Y cells. Levels of CHIP and UCH-L1 were knocked down by lentivirus mediated transduction of scrambled, shRNA CHIP or shRNA UCH-L1 respectively. (A) Representative immunoblots showing the endogenous levels of CHIP and UCH-L1 in the RIPA lysates of scramble control (CTR) cells and the corresponding knockdown (KD) cells. Graphs show the relative fold reduction in the levels of CHIP and UCH-L1 in KD cells compared to control cells. Densitometric analysis shows >80% knockdown of both proteins. (B) Immunoblots show knockdown of CHIP and UCH-L1 in the LYS and CYT fractions. (C) Representative immunoblots (left) show levels of LC3 II in CTR, CHIP KD and UCH-L1 KD cells treated with or without NL for 4hrs. Graph (right) shows the lysosomal flux of LC3 II in CTR, CHIP KD and UCH-L1 KD cells. (D) Schematic (left) depicting

the principle behind luminescence assay for measuring proteasome activity *in situ* in cell culture. Measurement of luminescence (right) shows proteasome activity does not change with the KD of CHIP and UCH-L1 compared to CTR. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.2.2 Reduction in CMA activity with CHIP knockdown

In this section, the effect of CHIP KD on CMA activity was examined. In order to do so, the pool of CMA+ lysosomes was analyzed by quantifying the number of Hsc70/LAMP-2A colocalizing puncta. CMA+ lysosomes are characterized by the presence of Hsc70 in their lumen and by quantification of the number of CMA+ lysosomes CMA activity can be estimated. CTR and CHIP KD cells were grown under S+ and S- conditions. For each condition, a minimum of 20 cells were analyzed using the JACoP plugin of ImageJ software to count the number of colocalizing puncta. Under S+ condition higher number of colocalizing puncta per cell area was observed in the CTR cells compared to CHIP KD cells (Fig 3.2.2 A). Upon starvation (S-) to induce CMA, in the CTR cells as much as twice the number of colocalizing puncta was observed per cell area compared to S+. However, in the CHIP KD cells, there was no increase in the number of colocalizing puncta even in S- condition. This indicated that in the CHIP KD cells there was lower CMA activity under both basal and CMA induced conditions.

Another way to measure CMA activity is to measure the rate of proteolysis of long-lived proteins in live cells as most of the long-lived proteins are degraded by lysosomal proteolysis [114]. This is done through metabolic labeling of the long-lived proteins and measuring the rates of degradation by pulse and chase method [140]. In order to quantify the lysosomal turnover of long-lived proteins, the percentage proteolysis upon lysosomal inhibition (NL treated) was obtained and divided with the percentage

proteolysis without NL. It was observed that under basal condition there was no change in the rate of lysosomal degradation upon CHIP KD, but under starvation when CMA was induced, there was a 3-fold decrease in lysosomal degradation (Fig. 3.2.2 B).

Taken together, depletion of CHIP resulted in reduction of CMA activity, and this effect was more pronounced under starvation condition. Another way to examine CMA activity is to assess the degradation of candidate CMA substrates in the CHIP KD cells.

However, common CMA substrates such as GAPDH or RNase H are highly abundant in the cells and small change in CMA activity might not affect the cellular levels of these proteins significantly. Other CMA substrates such as LRRK-2 or α -synuclein can be degraded via UPS or MA, and since UPS and MA activities are not affected by CHIP KD, it is not certain if we would be able to observe any global change in the levels of these proteins.

Fig. 3.2.2 Reduced CMA activity upon CHIP KD. Representative fluorescent images of (A) CTR and CHIP KD cells (S+ and S-) were stained with LAMP-2A (red) and Hsc70 (green) antibodies. Corresponding graph (right) shows the number of colocalizing puncta per cell area for the different conditions. (B) Graph shows rate of protein degradation (flux) of long-lived proteins in CTR and CHIP KD cells under S+ and S- conditions. A minimum of 20 cells were analyzed for each condition to count colocalizing puncta using JACoP plugin of ImageJ. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ against CTR or ### $p < 0.0005$ against S+

3.2.3 CHIP KD reduced association of ubiquitinated proteins at the lysosomes

CHIP is an E4 ligase and it performs ubiquitination and hence, directs substrates to the proteasomal or lysosomal pathways. It has been previously observed in our preliminary results that poly-ubiquitinated proteins associated with the CMA⁺ lysosomes and that K63-linked ubiquitinated proteins associated more than the K48-linked ubiquitinated proteins. Hence the effects of CHIP KD on the ubiquitination profile at the lysosomes were examined. There were lower levels of total poly-ubiquitinated proteins associated with the HOM and LYS fractions of CHIP KD cells compared to the CTR cells (Fig. 3.2.3 A top). Densitometric analysis showed that there was a 20% reduction in total ubiquitinated proteins associated to lysosomes of CHIP KD cells compared to CTR cells (Fig. 3.2.3 A bottom). However, there was no change in the profiles of the K63- or K48-linked ubiquitinated proteins between CTR and KD cells (Fig. 3.2.3 A top). Densitometric analysis showed that there was no significant difference in the profiles of K48- and K63-linked ubiquitinated proteins (Fig. 3.2.3 A bottom). Depletion of CHIP may reduce the level of ubiquitination of different substrates as CHIP acts as an E4 ligase. Our results have also shown a concomitant reduction in lysosome associated poly-ubiquitinated proteins. This could indicate that CHIP may play a role in the association of poly-ubiquitinated proteins to the lysosomes independent of specific linkages.

Fig. 3.2.3 Reduced association of ubiquitinated proteins at lysosomes upon CHIP KD. (A) Representative immunoblots show levels of total ubiquitin (TOTAL Ub), K48 Ub and K63 Ub in HOM and LYS fractions of CTR and CHIP KD cells (top). Corresponding graph shows lysosomal levels of total ubiquitin (Total Ub), K48 Ub and K63 Ub in CTR and CHIP KD cells (bottom). Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$

3.2.4 Depletion of CHIP affect the levels of CMA associated chaperones

CMA is a multistep pathway which involves two major components – the chaperones/co-chaperones and CMA receptor LAMP-2A. Perturbation in the levels or activity of any of these components can potentially affect CMA activity. It is possible that CHIP directly or indirectly affects one or more components of this pathway. Hence, the effect of CHIP KD on the different CMA associated chaperones was examined. Upon comparing the levels of Hsc70 between CTR and CHIP KD in the HOM and LYS fractions, a reduction of Hsc70 in the CHIP KD cells was observed in both the fractions (Fig 3.2.4 A). Reduced Hsc70 in the lysosomes could implicate that there are lower number of CMA-active lysosomes in the CHIP KD cells which could be the reason why we see lesser number of Hsc70/LAMP-2A colocalizing puncta in CHIP depleted cells in the previous section (Fig. 3.2.2 A).

Additionally, the effect of CHIP KD on Hsp40 and Hsp90 was examined. In the HOM fraction, a steep rise was seen in the levels of Hsp40 in CHIP KD cells with no significant change in Hsp90 levels (Fig 3.2.4 A). However, in the lysosomal fraction, a significant reduction in levels of Hsp40, and a rise in levels of Hsp90 were observed (Fig 3.2.4 A). Reduction in lysosomal Hsp40 could lead to lower ATPase activity of the Hsc70 and hence reduced intake of substrates into the lysosomal lumen. Additionally, increase in lysosomal Hsp90 can lead to greater stability of the LAMP-2A transition stage complexes. Hence, in the next section we aim to investigate the effect of CHIP KD on LAMP-2A levels and conformations.

Fig. 3.2.4 Depletion of CHIP affects CMA lysosomal levels of chaperones. (A) Representative immunoblots show levels of Hsp40, Hsc70, Hsp90, LAMP1 and actin in HOM and LYS fractions of CTR and CHIP KD cells (left). Corresponding graph shows lysosomal levels of Hsp40, Hsc70 and Hsp90 in CTR and CHIP KD cells (right). Values are expressed as mean $\pm$ S.E.M, N=5. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.2.5 Depletion of CHIP affects LAMP-2A multimerization

Substrate binding to LAMP-2A is the rate limiting step in CMA [41]. With age, there is a decline levels of LAMP-2A in lysosomal membrane [42] while during oxidative stress, transcriptional induction leads to enhanced de novo synthesis of LAMP-2A [78]. Thus the levels of LAMP-2A in lysosomes directly correlate with CMA function. Therefore, the effect of depleting CHIP on LAMP-2A levels and dynamics was studied. The levels of LAMP-2A between CTR and KD cells in HOM and LYS fractions were examined and no change was observed between the CTR and KD either in HOM or LYS fractions (Fig 3.2.5 A). Hence, CHIP KD does not influence the levels of LAMP-2A directly. Apart from LAMP-2A levels, the stability of the multimeric LAMP-2A translocation complex also determines CMA activity [17]. Thus, it becomes pertinent to investigate the LAMP-2A conformations upon CHIP KD to decipher the mechanism of CHIP mediated regulation of CMA activity.

The distribution of LAMP-2A in lysosomal membranes isolated from CTR and CHIP KD cells in S+ and S- conditions were compared. The active LAMP-2A translocon is a 700kDa complex whereas the inactive monomeric LAMP-2A is 100kDa. In order to separate the various LAMP-2A conformers we performed a discontinuous sucrose density gradient analysis. Post centrifugation, 12 aliquots were collected from the lightest to heaviest fractions, acid precipitated and subjected to western blot analysis. It is reported that under S+ condition most of the LAMP-2A is in monomeric form and once CMA is induced, the LAMP-2A multimerizes [43]. We observed that in the CTR cells, under S+ condition most of the LAMP-2A were present in the lighter fractions whereas when CMA was induced by starvation (S-), majority of the LAMP-2A shifted towards the heavier fractions (Fig. 3.2.5 B). However, in the CHIP KD cells, we

observed that under both S+ and S- conditions, most of the LAMP-2A were concentrated in the lighter and middle fractions ranging between fractions #1 - #7 (Fig. 3.2.5 B). This indicated that in CHIP KD cells, majority of the LAMP-2A were trapped in the transition stage between monomeric and multimeric conformations. Our results indicated that in upon CHIP KD, the LAMP-2A does not multimerize which is why a lower CMA activity is seen in the CHIP KD cells.

Fig. 3.2.5 CHIP KD reduces LAMP-2A multimerization. (A) Immunoblot show levels of LAMP-2A in HOM and LYS fractions of CTR and CHIP KD cells (left). Corresponding graph shows levels of LAMP-2A in CTR and CHIP KD cells (right) in HOM and LYS. (B) Representative immunoblots show the levels of acid precipitated LAMP-2A in fractions 1-12 after sucrose density centrifugation in increasing order of sucrose density from 10% to 60% (top). Corresponding graph shows the percentages of LAMP-2A in each fraction in CTR and CHIP KD cells (S+ and S- conditions). Values are expressed as mean $\pm$ S.E.M, N=4. Differences are significant for * p <0.05, ** p <0.005, *** p <0.0005 for S+ and # p <0.05, ### p <0.005, #### p <0.0005 for S-

3.2.6 Depletion of CHIP affects the lysosomal levels of mTORC2/Akt1/PHLPP1 pathway

Recently, it has been reported that the mTORC2 pathway can regulate CMA activity by regulating the phosphorylation of Akt1 (Fig. 1.4) [48]. Activation of Akt1 by phosphorylation, results in downstream de-stabilization of the multimeric LAMP-2A translocation complex and hence, it downregulates CMA activity [48]. The phosphatase PHLPP1 acts as a CMA activator by dephosphorylation of pAkt1 [48] (Fig. 1.4). As such, the effect of CHIP KD on the different players of the mTORC2/Akt1/PHLPP1 pathway was examined.

No change was observed in total mTOR levels with KD of CHIP (Fig. 3.2.6 A). Rictor binds to mTOR forming mTORC2 and acts as an upstream kinase for Akt1. No change was observed in Rictor levels in HOM or LYS fractions (Fig. 3.2.6 A). Hence, depletion of CHIP did not affect the total or lysosomal levels of mTORC2. mTORC2 is a kinase that phosphorylates Akt1 (pAkt1) [66]. It has been reported that pAkt1 is ubiquitinated by CHIP in the cytosol [55]. Our results showed an enhanced accumulation of pAkt1 with KD of CHIP in both HOM and LYS while there was no change in total Akt1 levels (Fig. 3.2.6 B). The levels of pAkt1 were normalized against total Akt1 to get the exact amount of pAkt1 in the different fractions represented as pAkt1/Akt1 (Fig. 3.2.6 B). The activation of Akt, besides being regulated by mTORC2 kinase, is also influenced by de-phosphorylation activity of PHLPP1.

PHLPP1 is a positive regulator of CMA. Under basal conditions, PHLPP1 is recruited to the lysosomal membrane by Rac1 protein to activate CMA. Upon depletion of CHIP, there was a marked reduction in levels of PHLPP1 in both HOM and LYS fractions

(Fig. 3.2.6 D). This data suggests that CHIP may influence the activation of Akt1 by determining the levels of PHLPP1. This may underscore the observed increase of pAkt1 in CHIP KD cells.

Thus far, our results indicated that reducing the levels of CHIP in the lysosomes can lead to enhanced activation of Akt1. This led us to further analyze the phosphorylation status of GFAP, the downstream substrate of pAkt1 at the lysosomes. The activation status of GFAP plays a crucial role in determining the dynamics of LAMP-2A receptor [48]. The levels of total GFAP and pGFAP remained unchanged in the HOM fractions of CHIP KD cells compared to CTR cells (Fig. 3.2.6 C). In contrast, a distinct increase in the levels of pGFAP in the LYS fractions of the CHIP KD cells was observed but not the CTR cells (Fig. 3.2.6 C). Taken together, reduction in levels of CHIP in the lysosomes significantly increased GFAP phosphorylation by promoting Akt activation. Increased pGFAP in the lysosomes will facilitate formation of GFAP dimers and prevent it from binding and stabilizing the LAMP-2A translocation complex.

Fig. 3.2.6 Depletion of CHIP impacts the mTORC2/Akt/PHLPP1 pathway. (A) Immunoblots show levels of mTOR, Rictor, (B) pAkt1, total Akt1, (C) p-GFAP, total GFAP and (D) PHLPP1 in CTR or CHIP KD cells in the HOM and LYS fractions. Corresponding graphs show the HOM and LYS levels of (A) mTOR, Rictor, (B) pAkt1, total Akt1, (C) p-GFAP, total GFAP and (D) PHLPP1 normalized against actin for the

HOM and LAMP1 for LYS fractions. Values are expressed as mean $\pm$ S.E.M, N=3-6. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.2.7 Overexpression of CHIP results in increase of PHLPP1 and reduced Akt1 activation

It has been shown that depletion of CHIP affects the lysosomal mTORC2/Akt1/PHLPP1 pathway. Increased pAkt in the lysosomes, upon CHIP depletion can lead to inhibition of CMA activity. Thus overexpression of CHIP might lead to reduction in pAkt1 levels. To verify this, stable myc-CHIP overexpressing SH-SY5Y cells (OE) were created along with corresponding control cells containing empty vector (V). Vector stable clones were denoted as V #1-V#6 and myc-CHIP stable clones were denoted as OE#1-OE#6. Upon screening, it was revealed that OE #3, #4 and #6 were stably overexpressing myc-CHIP and these were selected for further analysis along with corresponding vector controls V#3, #4 and #6 (Fig. 3.2.7 A). Upon selecting the three clones for V and OE, the lysosomal expressions of myc-CHIP was verified for the OE#3, OE#4 and OE#6 clones (Fig. 3.2.7 B).

Overexpression of CHIP resulted in reduced levels of pAkt1, specifically at the LYS fractions (Fig. 3.2.8 A) although the total Akt1 levels remained unchanged (Fig. 3.2.8 A). The pAkt levels were normalized against total Akt1, as mentioned in previous section, and represented as pAkt/Akt (Fig. 3.2.8 B). Reduced activation of Akt1 could be a result of increase in PHLPP1 activity and it was observed that there was a concomitant increase in lysosomal levels of PHLPP1 in CHIP OE cells compared to vector control (V) (Fig. 3.2.8 A). Accordingly, it was established that the changes observed in the levels of pAkt1 and PHLPP1 were due to changes in the cellular levels of CHIP.

Fig. 3.2.7 Analysis of CHIP overexpression in SH-SY5Y cells stably expressing myc-CHIP. SH-SY5Y cells were transfected with pBlast plasmid or co-transfected with empty pBlast plasmid and pcDNA myc-CHIP and selected against blasticidin resistance. 6 clones were selected for each condition. (A) Representative immunoblots show the levels of myc-CHIP in all the clones (V#1-V#6, OE#1-OE#6). (B) Three vector control clones V#3, V#4, V#6 and three OE clones OE#3, OE#4, OE#6 were selected and the HOM and LYS fractions were harvested for western blot analysis. Representative immunoblots show myc-CHIP OE at the HOM and LYS fractions.

Fig. 3.2.8 Overexpression of CHIP impacts lysosomal pAkt and PHLPP1 levels. HOM and the LYS fractions were isolated from three vector control clones #3, #4, #6 and three OE clones #3, #4, #6 (A) Immunoblots show levels of pAkt, Akt and PHLPP1 in vector (V) or CHIP overexpression (OE) clones in the HOM and LYS (B) Graphs showing the LYS levels of pAkt/Akt, total Akt or PHLPP1 in clones #3, #4 and #6. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.2.8 CHIP interacts with Akt1 and PHLPP1

CHIP has been reported to interact with Akt1 in total cell lysate [55]. However, it is not known if they also interact at the lysosomes, thus influencing the pAkt1 and PHLPP1 levels. In order to confirm that CHIP can interact with Akt1 and PHLPP1 at the lysosomes, co-immunoprecipitation was performed in the myc-CHIP OE stable cells. It was first verified that the CHIP OE cells are stably overexpressing the myc-CHIP protein in HOM and LYS fractions (Fig. 3.2.9 A,B). Upon immunoprecipitation of myc-CHIP using anti-myc antibody, it was observed that Akt1 and PHLPP1 interact with CHIP in both HOM (Fig. 3.2.9 C) and LYS (Fig. 3.2.9 D) fractions. Taken together, the KD and OE of CHIP resulted in changes in the lysosomal levels of pAkt1 and PHLPP1, with the observation that CHIP can interact with both Akt1 and PHLPP1 at the lysosomes it can be concluded that CHIP directly influences the activity of the mTORC2/Akt1/PHLPP1 pathway which can lead to dysregulation of CMA.

Fig. 3.2.9 Interaction of CHIP with Akt, Hsc70 and PHLPP1. Expression of myc-CHIP was detected in (A) HOM and (B) LYS fractions of vector control (V) and CHIP OE (OE) cells. Myc-CHIP protein was immunoprecipitated with anti-myc antibody from the (C) HOM and (D) LYS fractions and immunoblotted for Akt, Hsc70 and PHLPP1. For negative controls, the representative fraction were incubated without anti-myc antibody and subjected to the same procedure.

3.2.9 CHIP regulates lysosomal Akt1 levels via its U-box ubiquitinating domain

CHIP has been shown to interact with lysosomal Akt1 and PHLPP1 regulating their lysosomal levels and influencing the mTORC2/Akt1/PHLPP1 pathway. CHIP is a dual purpose enzyme with two distinct functional domains – the U-box domain which mediates its ubiquitination function and the TPR domain which mediates its role as a co-chaperone [135]. Subsequently, specific domains of CHIP responsible for modulating the Akt1 activation were explored. It has been reported previously that H260Q mutation of CHIP abolishes the activity of U-box domain while K30A mutation abolishes activity of the TPR domain (Fig. 3.2.10 A) [133]. Hence, empty pcDNA, WT myc-CHIP, K30A myc-CHIP or H260Q myc-CHIP were transiently overexpressed in SH-SY5Y cells. It was noted that overexpression of the WT CHIP showed no effect on the pAkt1 levels in the HOM (Fig. 3.2.10 B) but there was a marked reduction of pAkt1 levels at the lysosomes (Fig. 3.2.10 C). This aligns with our previous results that overexpression of CHIP leads to reduction of pAkt1 levels at the lysosomes. The total Akt1 levels remained unaffected in both HOM and LYS fractions (Fig. 3.2.10 B,C).

The K30A mutant myc-CHIP did not affect the levels of Akt1 in both the HOM or the LYS fractions (Fig. 3.2.10 B,C) but there was slight accumulation pAkt1 at the lysosomes (Fig. 3.2.10 C). Upon densitometric analysis, it was revealed that the increase in lysosomal pAkt1 levels was not significant in the K30A mutant overexpression (Fig. 3.2.10 D right). However, a marked increase in accumulation of pAkt1 was observed in both the HOM and LYS fractions when H260Q mutant myc-CHIP was overexpressed (Fig. 3.2.10 B,C). Densitometric analysis reflected a 2 fold increase in the lysosomal levels of pAkt1 in the H260Q mutant overexpression condition (Fig. 3.2.10 C right). This data suggests that when there is a deficiency of U-

box domain of CHIP, pAkt1 accumulates. This response is similar to CHIP KD cells, showing increased accumulation of pAkt1 at the HOM and LYS fractions. Taken together, it can be gathered that pAkt1 accumulates in the absence of the E3 ligase activity of CHIP.

Fig. 3.2.10 Overexpression of U-box mutant CHIP causes accumulation of pAkt1. (A) Schematic diagram representing the different domains of CHIP and the effect of mutation of the two domains. SH-SY5Y cells were transfected transiently with pcDNA, WT myc-CHIP, K30A myc-CHIP or H260Q myc-CHIP. (B) Immunoblots show levels of pAkt, total Akt and myc-CHIP expression in HOM and (C) LYS fractions. (D) Graphs show the levels of pAkt/Akt in HOM (left) and LYS (right) fractions. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$

3.2.10 TPR domain of CHIP interacts with Akt1

CHIP regulates the levels of different players of the mTORC2/Akt1/PHLPP1 pathway by interacting with PHLPP1 and Akt1. Additionally, the U-box domain of CHIP regulates the lysosomal levels of Akt1, however it is unclear which domain of CHIP is necessary for its interaction with Akt1. Subsequently, the specific domain of CHIP that regulates its interaction with Akt1 was investigated. Empty pcDNA, WT myc-CHIP, K30A myc-CHIP or H260Q myc-CHIP were transiently overexpressed in SH-SY5Y cells. Upon immunoprecipitation of the WT and different CHIP mutants using anti-myc antibody, it was observed that interaction of Akt1 with K30A myc-CHIP was completely abolished in both HOM (Fig. 3.2.11 A) and LYS fractions (Fig. 3.2.11 B). However, Akt1 was co-immunoprecipitated with H260Q myc-CHIP in the HOM (Fig. 3.2.11 C) and LYS fractions (Fig. 3.2.11 D). This established that CHIP interacts with Akt1 using its TPR domain and not the U-box domain.

Fig. 3.2.11 Akt and CHIP interaction is abolished upon mutation of TPR domain. SH-SY5Y cells were transfected transiently with pcDNA, WT myc-CHIP, K30A myc-CHIP or H260Q myc-CHIP. myc-CHIP or K30A myc-CHIP or H260Q myc-CHIP proteins were immunoprecipitated using anti-myc antibody and immunoblotted for Akt (A) Representative immunoblots show Akt pulled down with myc-CHIP but not the K30A myc-CHIP in the HOM fraction or (B) LYS fraction. (C) Representative immunoblots show Akt pulled down with both myc-CHIP and H260Q myc-CHIP in the HOM fraction or (D) LYS fraction. For negative controls, the representative fractions were incubated without anti-myc antibody and subjected to the same procedure.

3.2.11 PHLPP1 interacts with TPR and U-box domains of CHIP

Fluctuations in the levels of CHIP lead to variations in the total and lysosomal levels of PHLPP1. It has been shown in this study that CHIP can interact with PHLPP1. In this section, the domain of CHIP which is important for interaction with PHLPP1 was investigated. Upon transient overexpression and immunoprecipitation of WT, K30A and H260Q mutant myc-CHIP, it was observed that PHLPP1 was co-immunoprecipitated with WT myc-CHIP, K30A mutant myc-CHIP and H260Q mutant myc-CHIP in the HOM (Fig. 3.2.12 A) and LYS (Fig. 3.2.12 B) fractions. This indicated that in the absence of either TPR domain or U-box domain, PHLPP1 can retain its interaction with CHIP. It remains unclear how PHLPP1 interacts with CHIP and needs to be explored further.

Fig. 3.2.12 PHLPP1 interacts with WT, TPR and U-box myc-CHIP mutants. SH-SY5Y cells were transfected transiently with pcDNA, WT myc-CHIP, K30A myc-CHIP or H260Q myc-CHIP. myc-CHIP or K30A myc-CHIP or H260Q myc-CHIP proteins were immunoprecipitated using anti-myc antibody and immunoblotted for PHLPP1, Akt and Hsc70 (A) Representative immunoblots show PHLPP1 pulled down with WT CHIP, H260Q CHIP and K30A CHIP mutants in the (A) HOM or (B) LYS fraction. Akt and Hsc70 are shown as positive controls. For negative controls, the representative

fraction were incubated without anti-myc antibody and subjected to the same procedure.

3.2.12 CHIP ubiquitinates lysosomal Akt1

It is reported that CHIP ubiquitinates pAkt and tags it for proteasomal degradation [55]. In the previous section, it has been shown that CHIP interacts with Akt1 through the TPR domain (Fig. 3.2.13). Additionally, abolishing the E3-ligase function (by H260Q mutation at U-box domain) of CHIP resulted in accumulation of pAkt1 at the lysosomes (Fig. 3.1.13). It is possible that CHIP ubiquitinates lysosomal pAkt1 leading to its degradation and hence, in the absence of CHIP or the U-box domain of CHIP, pAkt1 accumulates. Thus it was necessary to compare the ubiquitination of Akt1 in CTR and CHIP KD cells.

Endogenous Akt1 was immunoprecipitated from HOM and LYS fractions of CTR and CHIP KD cells. Due to the fact that the levels of pAkt1 are two-thirds more in the CHIP KD than the CTR (Fig. 3.2.6 C), the starting amount of lysates were normalized before immunoprecipitation. It was observed that the levels of ubiquitinated Akt1 remained unchanged in the HOM fraction (Fig. 3.2.13 A) whereas, in the LYS fraction, there was a marked reduction in the levels of ubiquitinated Akt1 (Fig. 3.2.13 B) in CHIP KD cells compared to CTR. Densitometric analysis of the ubiquitin bands normalized by Akt1 showed 30% reduction in ubiquitination of Akt1 in the lysosomes of CHIP KD compared to CTR cells (Fig. 3.2.13 C). Taken together, upon depletion of CHIP there is reduced ubiquitination of lysosomal pAkt1 resulting in lower degradation and increased stability. This increase in the levels of lysosomal pAkt1 leads to increased phosphorylation of GFAP forming GFAP dimers and lower stability of the LAMP-2A translocon. Thus, lower CMA activity is observed in CHIP KD cells.

Fig. 3.2.13 Reduced ubiquitination of lysosomal Akt in CHIP KD cells. total endogenous Akt was immunoprecipitated with anti-Akt antibody and immunoblotted for Ubiquitin, Akt and pAkt (A) Immunoblots show total ubiquitinated Akt and pAkt pulled down with Akt in (A) HOM and (B) LYS fractions. (C) Representative graph shows the total ubiquitinated Akt pulled down normalized against the levels of endogenous Akt in the HOM and LYS fractions. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.3 Loss of UCH-L1 increases CMA activity

The de-ubiquitinating enzyme UCH-L1 plays a major role in CMA associated pathogenesis of PD [116]. It has been reported to interact with LAMP-2A, Hsc70 and Hsp90 [109]. UCH-L1 also regulates the formation of the different mTOR complexes and determines the cellular levels of PHLPP1 [57]. Additionally, in our preliminary experiments, it has been observed that UCH-L1 preferentially associates with CMA+ lysosomes (Fig. 3.1.1 D). However, the role of UCH-L1 in the CMA pathway remains unclear. Hence, in this section the role of UCH-L1 in the CMA pathway was examined.

3.3.1 Depletion of UCH-L1 reduces Hsc70 levels

It has been reported that UCH-L1 interacts with CMA associated chaperones Hsc70 and Hsp90 [109]. In PD, mutant UCH-L1 shows enhanced interaction with chaperones Hsc70 and Hsp90, however, it is unclear whether UCH-L1 influences the lysosomal levels of the different chaperones. Hence, to examine the effect of UCH-L1 in CMA associated chaperones, the cytosolic (CYT) and LYS fractions were isolated from CTR and UCH-L1 KD cells. It was revealed that the levels of Hsp40 and Hsp90 remained unchanged in the CYT and LYS fractions of CTR and UCH-L1 KD cells (Fig. 3.3.1 A left). However, upon UCH-L1 KD, there was reduction in the levels of Hsc70 in both CYT and LYS fractions (Fig. 3.3.1 A left). Densitometric analysis revealed a 15% reduction in Hsc70 levels in the UCH-L1 KD cells (Fig. 3.3.1 A right). Reduced levels of Hsc70 may indicate a decrease in the number of CMA+ lysosomes.

Fig. 3.3.1 Depletion of UCH-L1 resulted in lower levels of lysosomal Hsc70. (A) Immunoblots showing the levels of Hsc70, Hsp40 and Hsp90 in cytosolic (CYT) and LYS fractions of CTR and UCH-L1 KD cells (left). Corresponding graph shows the lysosomal levels of Hsc70, Hsp40 and Hsp90 in CTR and UCH-L1 KD cells. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.3.2 Enhanced CMA activity with UCH-L1 KD

It has been reported that UCH-L1 interacts with various CMA associated players like LAMP-2A, Hsc70 and Hsp90 [109]. In the previous section, it was observed that KD of UCH-L1 influenced the levels of lysosomal Hsc70. The lysosomal Hsc70 helps substrates to enter the CMA⁺ lysosomes for degradation. Hence, in this section the activity of CMA was examined by quantification of CMA⁺ lysosomes in CTR and UCH-L1 KD cells under basal (S⁺) and CMA induced (S⁻) conditions. The cells were co-stained with LAMP-2A and Hsc70 and a minimum of 20 cells were analyzed per condition. Under S⁺ condition, a higher number of colocalizing puncta per cell area was observed in the UCH-L1 KD cells compared to CTR cells (Fig. 3.3.2 A). Upon starvation (S⁻) to induce CMA, an even higher number of colocalizing puncta were detected compared to CTR cells (Fig. 3.3.2 A). This indicated that in the UCH-L1 KD cells there was a greater number of CMA⁺ lysosomes and hence, greater CMA activity compared to CTR cells.

Lysosomal activity was measured by quantification of lysosomal turnover of long-lived proteins. The percentage proteolysis upon lysosomal inhibition (NL treated) was divided with the percentage proteolysis without lysosomal inhibitions to get the rate of lysosomal degradation. It was observed that under basal condition (S⁺) there was only a slight increase in the rate of lysosomal degradation upon UCH-L1 KD, but upon starvation (S⁻) when CMA was induced, there was a 3-fold increase in lysosomal degradation (Fig. 3.3.2 B).

Taken together, depletion of UCH-L1 resulted in increased CMA activity, and this effect is further pronounced upon starvation. This indicates that UCH-L1 may play an inhibitory role in CMA pathway.

Fig. 3.3.2 Enhanced CMA activity upon UCH-L1 KD. Representative fluorescent images of (A) CTR and UCH-L1 KD cells (S+ and S-) stained with LAMP-2A (red) and Hsc70 (green) antibodies. Corresponding graph (right) shows the number of colocalizing puncta per cell area for the different conditions. (B) Graph shows rate of degradation (flux) of long-lived proteins in CTR and UCH-L1 KD cells under S+ and S- conditions. For colocalizations a minimum of 20 cells were analyzed for each condition to count number of puncta using JACoP plugin of ImageJ. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$ against CTR or ## $p < 0.005$ against S+

3.3.3 UCH-L1 KD enhances LAMP-2A multimerization by repression of Akt1 signaling

In contrast to CHIP KD, where CMA is inhibited, depletion of UCH-L1 upregulates CMA activity. It was observed that depletion of CHIP enhanced Akt signaling which caused destabilization of the LAMP-2A translocon (Fig. 3.1.6). It has been reported that UCH-L1 helps to translationally upregulate PHLPP1 levels which represses Akt1 signaling [56]. Repression of the Akt signaling can affect LAMP-2A dynamics.

Lysosomal proteins were extracted from CTR and CHIP KD cells under S+ and S- conditions and subjected to discontinuous sucrose density gradient centrifugation similar to our previous experiment with CHIP KD cells (Section 3.2.5). In the CTR cells, under basal conditions (CTR S+) most of the LAMP-2A were present in the lighter fractions, whereas, upon starvation (CTR S-), majority of the LAMP-2A shifted towards the heavier fractions (Fig. 3.3.3 A). Upon UCH-L1 KD, under basal conditions (UCH-L1 KD S+) a greater amount of LAMP-2A were in the multimeric conformations (Fig. 3.3.3 A) and starvation induced even more monomeric LAMP-2A to migrate to the high molecular weight multimeric conformation (Fig. 3.3.3 A). Hence we observed that UCH-L1 KD cells have higher LAMP-2A multimer stability. This correlates with the observed higher CMA activity due to more substrate translocation through the multimeric LAMP-2A translocon. This increased LAMP-2A stability could be a result to regulation of the mTORC2/Akt1/PHLPP1 pathway by UCH-L1.

Cytosolic PHLPP1 has been reported to be regulated by UCH-L1, however, the effect of UCH-L1 on lysosomal PHLPP1 and Akt1 is unknown. The effect of UCH-L1 KD on PHLPP1 and Akt1 phosphorylation was examined in HOM and LYS fractions. It was observed that upon KD of UCH-L1 there was an increase in PHLPP1 levels and reduction of pAkt1 levels in both HOM and LYS fractions (Fig. 3.3.3 B). This indicated

that UCH-L1 is also a key player in regulating the lysosomal mTORC2/Akt1/PHLPP1 pathway. High levels of lysosomal PHLPP1 can result in inactivation of Akt1, and hence lower levels of pAkt1 were observed. This can lead to lower phosphorylation of GFAP and greater stability of LAMP-2A translocon.

Fig. 3.3.3 Increase in LAMP-2A multimers, PHLPP1 and decreased pAkt upon UCH-L1 KD. (A) Representative immunoblots show the levels of acid precipitated LAMP-2A in fractions 1-12 after sucrose density centrifugation in increasing order of sucrose density from 10% to 60% in CTR and UCH-L1 KD under S+ and S- conditions (B) Immunoblots showing the levels of UCH-L1, PHLPP1, pAkt and total Akt in HOM and LYS fractions of CTR and UCH-L1 KD cells (left). Corresponding graph shows the HOM (top) and LYS (bottom) levels of PHLPP1, pAkt/Akt and total Akt in CTR and UCH-L1 KD cells. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$

3.3.4 DUB activity of UCH-L1 regulates Akt1 signaling and PHLPP1 gene expression

Unlike the E4 ligase CHIP, UCH-L1 is a DUB enzyme and both enzymes perform opposing functions in the ubiquitination pathway. So far, it has been observed that in CMA pathway too CHIP and UCH-L1 have opposing effects. CHIP has been shown to upregulate CMA through ubiquitination, while UCH-L1 inhibits CMA. It is possible that this inhibition is due to the de-ubiquitination activity of UCH-L1.

It is reported that the I93M mutation of UCH-L1 abolishes its DUB activity [109, 116]. SH-SY5Y cells were transiently transfected with pcDNA (-), Wildtype (WT) myc-UCH-L1 and I93M mutant myc-UCH-L1 (I93M). Upon overexpression of UCH-L1 there was a rise in pAkt1 levels accompanied by a drop in PHLPP1 levels (Fig. 3.3.4 A). However, overexpression of I93M mutant UCH-L1 led to reduction of pAkt1 and increase in PHLPP1 compared to WT (Fig. 3.2.4 A). Densitometric analysis showed significant rise in pAkt/Akt levels and reduction of PHLPP1 levels in I93M compared to WT (Fig. 3.2.4 A right). This indicated that the DUB function of UCH-L1 mediates the changes in the levels of these proteins.

Hussain et al. have reported that UCH-L1 deubiquitinates Raptor impairing the formation of mTORC1 and thus, promotes formation of mTORC2 which phosphorylates Akt1 [68]. Apart from this, lower levels of mTORC1 also lead to impaired translation of PHLPP1 [68]. Reports on KMS-28 cells, derived from cancerous plasma cells, show that UCH-L1 has no effect on PHLPP1 transcription levels [57]. However, our experiment in neuroblastoma SH-SY5Y cells revealed a 2-fold increase in PHLPP1 expression in UCH-L1 KD cells compared to CTR (Fig. 3.3.4 B). This UCH-L1-mediated control of PHLPP1 expression is different from published

data, which can be due to the different cell line used or different experimental conditions and needs to be further explored.

Fig. 3.3.4 DUB activity of UCH-L1 regulates lysosomal Akt signaling. (A) SH-SY5Y cells were transiently transfected with pcDNA (-), WT myc-UCH-L1 (WT) and I93M mutant UCH-L1 (I93M). Immunoblots showing the levels of UCH-L1, pAkt, Akt and PHLPP1 in LYS fractions. Corresponding graph shows the lysosomal levels of pAkt/Akt and PHLPP1 in pcDNA, WT and I93M myc-UCH-L1 transfected cells. (B) Graph shows the levels of mRNA expression of PHLPP1 in CTR and UCH-L1 KD. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to CTR or pcDNA and # $p < 0.05$ compared to WT

3.4 CHIP regulates UCH-L1 levels in the lysosomes

The mTORC2/Akt1/PHLPP1 pathway regulates CMA activity [48] and another layer of this regulation has been uncovered via the ubiquitin modifying enzymes CHIP and UCH-L1. While CHIP is a positive regulator of CMA, UCH-L1 inhibits CMA. However, it is unclear if the CMA associated functions of CHIP and UCH-L1 are interconnected. Hence, in this section the aim is to decipher the connection between CHIP and UCH-L1 in regulating CMA activity.

3.4.1 Presence of CHIP at the lysosomes decreases lysosomal UCH-L1 levels

In order to investigate the connection between CHIP and UCH-L1, the levels of each enzyme was evaluated upon KD or OE of the other. Depletion of UCH-L1 did not have any effect on the levels of CHIP in the HOM and LYS fractions (Fig. 3.4.1 A), however, upon depletion of CHIP there was a steep increase in UCH-L1 levels in both HOM and LYS fractions (Fig. 3.4.1 B left). Densitometric analysis of the lysosomal levels of UCH-L1 reflected a 1.5 fold increase in the CHIP KD cells compare to CTR (Fig. 3.4.1 B right). The mRNA expression of PHLPP1 was also enhanced in CHIP KD cells (Fig. 3.4.1 C). UCH-L1 KD promotes the expression of PHLPP1 and upon CHIP KD the expression is diminished. This can either be due to rise in UCH-L1 levels upon CHIP KD or independent of UCH-L1 levels and can be explored further. Subsequently, the levels of UCH-L1 were examined in vector (V#3, V#4 and V#6) and corresponding myc-CHIP OE (OE#3, OE#4 and OE#6) cells and it was revealed that there was a reduction in UCH-L1 levels upon CHIP OE (Fig. 3.4.1 D). Thus it was established that CHIP directly influences the levels of total and lysosomal UCH-L1, although UCH-L1 had no effect of levels of CHIP.

Fig. 3.4.1 CHIP regulates PHLPP1 levels at the lysosomes through UCH-L1. (A) Immunoblots showing the levels ofCHIP in HOM and LYS fractions of CTR and UCH-L1 KD cells (top). Corresponding graph shows the lysosomal levels of CHIP in CTR and UCH-L1 KD cells (bottom). (B) Immunoblots showing the levels of UCH-L1 in HOM and LYS fractions of CTR and CHIP KD cells (left). Corresponding graph shows the lysosomal levels of UCH-L1 in CTR and CHIP KD cells (right). (C) Graph shows the levels of mRNA expression of PHLPP1 in CTR and CHIP KD. (D) Immunoblots

show levels of UCH-L1 in HOM and LYS fractions of different pBlast vector and CHIP OE clones (3,4 and 6) (top). Corresponding graph shows lysosomal levels of UCH-L1 in different pBlast vector (V) and myc-CHIP OE clones. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

3.4.2 CHIP regulates lysosomal UCH-L1 levels by influencing UCH-L1

degradation

It was been shown that CHIP regulates the levels of UCH-L1. CHIP being an E4 ligase, directly interacts with the substrates and ubiquitinates them to tag them for degradation [72]. In order to investigate how CHIP regulates the lysosomal levels of UCH-L1, the interaction and ubiquitination of UCH-L1 were analyzed.

Endogenous UCH-L1 was immunoprecipitated from HOM and LYS fractions of myc-CHIP OE stable cells and it was revealed that CHIP co-immunoprecipitates in both HOM (Fig. 3.4.2 A left) and LYS fractions (Fig. 3.4.2 A right). Thus, CHIP directly interacts with UCH-L1. Subsequently, endogenous UCH-L1 was immunoprecipitated from HOM and LYS fractions of CTR and CHIP KD cells to examine the ubiquitination of UCH-L1 and it was observed that there was a reduction in ubiquitinated UCH-L1 levels in both HOM (Fig. 3.4.2 B left) and LYS fractions (Fig. 3.4.2 B right) of CHIP KD cells compared to CTR. Taken together, it can be said that CHIP directly interacts with UCH-L1 and ubiquitinates it for degradation. Hence, in the absence of CHIP greater accumulation of UCH-L1 in the HOM and LYS fractions was observed.

Fig. 3.4.2 CHIP interacts with UCH-L1 and ubiquitinates UCH-L1. (A) Endogenous UCH-L1 was immunoprecipitated using anti-UCH-L1 antibody from HOM and LYS of stable CHIP OE cells. Representative immunoblots show that CHIP is immunoprecipitated with UCH-L1 in HOM (left) and LYS (right) fractions (N=2). (B) anti-UCH-L1 antibody was used to pull down total endogenous UCH-L1 and immunoblotted for ubiquitin and UCH-L1. Immunoblots show amount of total ubiquitinated UCH-L1 in HOM (left) and LYS (right) fractions (N=2).

3.5 Presence of CHIP and UCH-L1 in the lysosomes mediates CMA-UPS crosstalk during stress

Both CHIP and UCH-L1 have been shown to regulate the mTORC2/Akt1/PHLPP1 pathway, although they have an opposing effect [48]. CHIP promotes CMA and UCH-L1 inhibits it. Additionally, CHIP regulates the lysosomal levels of UCH-L1. However, it is not clear how the level of CHIP is determined at the lysosomes to regulate CMA. CHIP has been implicated in both CMA and UPS pathways and it is possible that CHIP can shuttle between the two pathways. There have been various evidences of crosstalk between CMA and UPS [74, 121, 126]. CMA is a multistep process and it is highly likely that it is interconnected with other protein degradation pathways like UPS.

In order to examine crosstalk between CMA and UPS, CTR and CHIP KD cells were treated with or without proteasomal inhibitor MG-132 (MG). It was observed that in the CTR cells, upon MG treatment, there was higher lysosomal association of CHIP but no change in the total CHIP levels in the HOM (Fig. 3.5 A). This indicated that when proteasome is inhibited, CHIP might be directed towards the lysosomes. Furthermore, upon MG treatment there were reduced lysosomal levels of UCH-L1 and pAkt1 while there was an increase in lysosomal PHLPP1 levels (Fig. 3.5 A). In CHIP KD cells, MG treatment induced no change in lysosomal levels of UCH-L1 and pAkt1. PHLPP1 levels were very low in CHIP KD cells under both MG treated and untreated conditions (Fig. 3.5 A). Taken together it was indicated that upon proteasomal inhibition when CHIP accumulated at the lysosomes, it reduced the lysosomal levels of pAkt1 and UCH-L1, and enhanced levels of PHLPP1. But when CHIP was depleted, this effect was abolished. Hence it can be said that CHIP is a key player in the crosstalk between CMA and proteasomal pathways.

It has been shown that there was a reduction in lysosomal UCH-L1 with MG treatment, hence, it was examined whether UCH-L1 is degraded by the lysosomal or proteasomal pathway. SH-SY5Y cells were treated with MG, NL or a combination of MG and NL (MG+NL). Upon NL treatment or MG+NL treatments UCH-L1 accumulates at the lysosomes (Fig. 3.5 B) indicating that it is a lysosomal substrate and upon MG treatment when CHIP accumulates at the lysosomes, it directs the lysosomal degradation of UCH-L1. Taken together, in this study it has been shown that apart from regulating CMA activity, CHIP and UCH-L1 are also major players in the crosstalk between CMA and UPS.

Fig. 3.5 CHIP drives crosstalk between UPS and CMA pathways. (A) CTR and CHIP KD cells were treated with or without MG-132. Immunoblots showing the levels of CHIP, UCH-L1, pAkt, total Akt, and PHLPP1 in HOM and LYS fractions under different conditions (top). Corresponding graphs show the lysosomal levels of UCH-L1, pAkt/Akt and PHLPP1 in CTR and CHIP KD cells under UT and MG conditions (bottom). (B) Immunoblots showing the lysosomal levels of UCH-L1 and CHIP under untreated (UT), MG-132 (MG), MG-132+NL (MG+NL) and NL treated conditions (left). (B) Graphs show lysosomal levels of UCH-L1 and CHIP under the different conditions. Values are expressed as mean $\pm$ S.E.M, N=3. Differences are significant for * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

4. Discussion

In this study, ubiquitin modifying enzymes CHIP and UCH-L1 have been identified as novel regulators of the CMA pathway. Lysosomal levels of these enzymes regulate the mTORC2/Akt1/PHLPP1 pathway, influencing LAMP-2A dynamics and CMA activity. CHIP associates with pAkt1 using its TPR domain and ubiquitinates it by virtue of its U-box domain. As a result, pAkt1 undergoes degradation and is not able to phosphorylate GFAP downstream. This helps in association of GFAP with the LAMP-2A translocation complex stabilizing it and assisting in substrate translocation leading to greater CMA activity. The current study also showed that CHIP regulates lysosomal levels of UCH-L1 affecting the levels of PHLPP1, thus indirectly regulating the Akt1 signaling pathway. CHIP and UCH-L1 play antagonistic roles at the lysosomes to regulate CMA where CHIP activates CMA and UCH-L1 inhibits the pathway. Our results for the first time demonstrate CHIP as one of the factors which drive crosstalk between the UPS and CMA pathways.

4.1 A role of ubiquitin and ubiquitin modifying enzymes in CMA

The current study has highlighted the association of ubiquitinated proteins to CMA active lysosomes. Specific ubiquitin linkages, namely K63-linked ubiquitinated proteins have shown higher affinity towards CMA-active lysosomes. This indicated an explicit role of ubiquitination in CMA pathway. Recent reports corroborate that certain CMA substrates are K63-linked ubiquitinated for degradation, like HIF-1 α [139]. Apart from ubiquitinated proteins, this study has provided evidence of specific enrichment of ubiquitin modifying enzymes at the CMA active lysosomes. It was found that two of

these enzymes E4 ligase CHIP and deubiquitinating enzyme UCH-L1 have opposing regulatory roles in CMA pathway. CHIP is a stimulator of CMA while UCH-L1 inhibits CMA. Additionally these enzymes also affect integral CMA associated lysosomal components like chaperones Hsp40, Hsp90 and Hsc70. It has been previously reported that both CHIP and UCH-L1 can interact with CMA components Hsc70, Hsp90 and LAMP-2A [109, 133]. CHIP and UCH-L1 may stabilize the lysosomal chaperones by directly interacting with them and hence, upon depletion of these enzymes we see dysregulation in the levels of the chaperones at the lysosomes.

4.2 CHIP is a regulator of CMA pathway

CHIP has multiple roles in the CMA pathway in substrate targeting, regulating chaperone levels and the signaling pathway. Previously it was only known that CHIP helps in targeting specific substrate HIF-1 α for CMA degradation by K63-linked ubiquitination [133, 139, 146]. However, in the current study, CHIP has been identified to also regulate the signaling mechanisms that control LAMP-2A active complex formation at the lysosomes. CHIP directly interacts with the CMA inhibitor pAkt1 using its TPR domain and ubiquitinates it for proteasomal degradation. In the absence of inhibitory control, CMA is upregulated. CHIP also enhances the levels of CMA activator PHLPP1. PHLPP1 is an Akt1 phosphatase and is recruited to the lysosomal membrane by the GTPase, Rac1, under stress [48]. CHIP can directly interact with PHLPP1, although the mode of interaction remains unclear, since abolishing either the TPR domain or U-box domain retains the interaction. Apart from PHLPP1 protein levels, it has also been shown that depletion of CHIP reduces PHLPP1 transcript levels, underlining another level of control. Apart from the direct control of the mTORC2/Akt1/PHLPP1, CHIP also regulates this pathway indirectly. The current study has highlighted that CHIP targets UCH-L1 for degradation by ubiquitinating it.

UCH-L1 inhibits the CMA pathway by promoting mTORC2 formation and Akt phosphorylation while downregulating PHLPP1 translationally [56, 57].

Taken together, this study has revealed a novel mechanism of regulation of CMA pathway through ubiquitin modifying enzymes CHIP and UCH-L1. However, it remains unknown how CHIP is transported and recruited to the lysosomes. CHIP is primarily a cytosolic protein and as such, there needs to be an adaptor to recruit it to the lysosomes.

4.3 UCH-L1 inhibits CMA through the DUB activity

In this part of the study, it has been shown that UCH-L1 is an inhibitor of CMA activity. UCH-L1 destabilizes the LAMP-2A active complex at the lysosomal membrane through dysregulation of the mTORC2/Akt1/PHLPP1 pathway. Previous studies have reported that UCH-L1 plays a two-fold role in regulation of mTOR complexes. UCH-L1 acts as an antagonist to E3 ligase DBB1-CUL4 and prevents ubiquitination of Raptor impeding the formation of mTORC1. Impaired mTORC1 formation results in greater number of mTOR available for binding to Rictor and formation of mTORC2 [57] which is an inhibitor of CMA [48]. The present study also highlighted that the de-ubiquitination activity of UCH-L1 promoted lysosomal accumulation of pAkt1 acting as an antagonist to E4 ligase CHIP. It is reported that Akt1 is phosphorylated by mTORC2 [147], thus corroborating with our results that increase in UCH-L1 promotes mTORC2 formation which enhances Akt1 phosphorylation.

Apart from regulating Akt1 phosphorylation, another role of UCH-L1 is in downregulation of PHLPP1. It has been shown that depletion of UCH-L1 resulted in accumulation of lysosomal PHLPP1, while overexpression of UCH-L1 depleted the lysosomes of PHLPP1. Upon overexpression of the mutant UCH-L1, where the DUB

function is abolished, accumulation of lysosomal PHLPP1 was encountered, highlighting that the DUB function of UCH-L1 was also mediating lysosomal PHLPP1 levels. Furthermore, the present study also showed upregulation of PHLPP1 gene expression upon depletion of UCH-L1. It has been reported previously that UCH-L1 expression leads profound downregulation of PHLPP1 in cancer cells but the mechanism was unclear [57]. The author further states that UCH-L1 does not regulate the gene expression of PHLPP1 [56] which contradicts our observation. It is possible that due to different cell lines used or different experimental conditions, we observe different effects. Thus the effect of UCH-L1 of PHLPP1 gene expression needs to be further explored under different conditions and different cell lines.

4.4 CHIP and UCH-L1 regulate LAMP-2A dynamics to affect CMA

It has been reflected in our results that CHIP and UCH-L1 regulate CMA through modulating the LAMP-2A dynamics. There are two different mechanisms that contribute to those changes in the lysosomal levels of LAMP-2A: the lysosomal degradation of LAMP-2A and its dynamic distribution between the lysosomal membrane and matrix [37]. The current study has indicated that upon depletion of CHIP, the lysosomal levels of LAMP-2A are unaffected, however, the LAMP-2A multimeric translocon is unstable under both basal and CMA induced conditions. It was also reflected that in the CHIP KD cells, majority of the LAMP-2A was trapped in transition state. Under basal conditions, when CMA activity is low, the monomeric LAMP-2A localizes to lipid-rich microdomains of the lysosomal membranes and is prone to internalization into the matrix followed by degradation via lysosomal proteases [99]. In the current study, we did not encounter any reduction in LAMP-2A lysosomal levels and thus it can be inferred that the LAMP-2A was not degraded in CHIP KD cells. Interestingly, it was observed that upon knockdown of CHIP, majority of the

LAMP-2A was found trapped in transition stage (Fig. 3.2.5 B). This can explain why we do not encounter degradation of LAMP-2A, since it is not in the monomeric state and hence, does not localize at the lipid-rich microdomains. The current study also shows an increase in lysosomal levels of Hsp90 chaperone upon CHIP KD. It has been previously reported that lysosomal Hsp90 stabilizes the LAMP-2A at transition stage [43]. Hence, it is possible that due to increased levels of lysosomal Hsp90 in CHIP KD cells, LAMP-2A is stabilized in the transition stage and thus neither it is degraded nor does it form the multimeric translocon.

The CHIP and UCH-L1 mediated regulation of CMA has been described diagrammatically in Fig. 4.1.

Fig. 4.1 CHIP and UCH-L1 mediated regulation of CMA and crosstalk. CHIP localizes to the lysosomal membrane upon proteasome inhibition. At the lysosomes, CHIP ubiquitinates and degrades pAkt1 and UCH-L1. This promotes increase in lysosomal levels of PHLPP1. PHLPP1 dephosphorylates Akt1. Hence, GFAP is not phosphorylated and binds to LAMP-2A translocon stabilizing it leading to increase in CMA activity.

4.5 Reciprocal dynamics between CHIP and UCH-L1 at the lysosome underscore UPS-CMA crosstalk

The current study has identified CHIP as a novel regulator of UCH-L1 levels at the lysosomes. Knockdown of CHIP resulted in accumulation of UCH-L1, while overexpression of CHIP depleted the UCH-L1, thus revealing that the two enzymes are interconnected. It was further deciphered that CHIP directly interacts with UCH-L1 and ubiquitinates it for degradation. However, treatment with proteasomal inhibitor did not have any effect on UCH-L1 levels, while treatment with lysosomal inhibitors led to accumulation of UCH-L1 revealing that UCH-L1 undergoes lysosomal degradation. It was also revealed that CHIP is a mediator of crosstalk between UPS and CMA. Our results, for the first time indicated that upon proteasomal stress, CHIP localizes to the lysosomes resulting in inhibition of Akt1 signaling and upregulation of CMA. It is currently known that certain subunits of the proteasome contain the KFERQ-like CMA targeting motif [29] and are degraded in the lysosomes with the help of Hsc70, upon prolonged starvation [74] through CMA, indicating crosstalk between proteasome and CMA pathways. Thus our studies show yet another mechanism of crosstalk between the two pathways.

Upon proteasomal stress, when CHIP accumulates at the lysosomes there is a concomitant reduction of lysosomal UCH-L1. It has been shown in this study that CHIP ubiquitinates UCH-L1 and UCH-L1 accumulates upon lysosomal inhibition. This indicates that UCH-L1 undergoes lysosomal degradation upon ubiquitination. However it is unclear which mode of lysosomal degradation it undergoes. It is reported that ubiquitinated proteins are recruited by the ESCRT complex and targeted for late endosomal microautophagy [25, 148]. The ubiquitination profile of UCH-L1 can also be further explored to examine if it undergoes specific K48- or K63-linked

ubiquitination. K63-linked ubiquitination can tag substrates for MA or CMA degradations [139, 149].

There are various evidences of crosstalk through which the different degradation pathways communicate. There is increasing evidence that UPS and autophagy pathways are functionally coupled processes. It is reported that upon chemical inhibition of autophagy, there is enhanced proteasomal activity [130]. The author also reported that upon knockdown of various autophagy related genes like PIK3C3, ATG5 or ATG7 there was enhanced UPS activity. The collaborative activities of UPS and CMA regulate the degradation of RCAN1 implicated in Alzheimer's disease [126]. MA and CMA also share a multitude of common substrates including LRRK2 and α -synuclein [110, 132]. Blockage of MA results in CMA upregulation, while, selective blockage of CMA by LAMP-2A depletion, upregulates MA [75, 128]. Acute inhibition of proteasomal pathway by chemical inhibitors activated CMA under starvation [121]. In addition, some of the subunits of the proteasome are also *bonafide* substrates of CMA [74]. Hence, in the absence of one pathway the others are upregulated as a compensatory response. This explains our results, whereby upon inhibition of proteasome, CHIP localizes to the lysosomes and upregulate CMA.

It can be further explored if the CHIP-UCH-L1 mediated crosstalk between UPS and CMA, also affects the macroautophagy pathway. Furthermore, UCH-L1 is an oncogene which is expressed only in neurons of the CNS, distal renal tubules, islets of Langerhans, spermatogonia in testes and upregulated in certain cancer cells like lung cancer or lymphoma [57, 109, 115, 150]. CMA is also highly upregulated in neurons and cancer cells [82]. Thus it needs to be investigated whether this crosstalk is specific to certain cell lines like neurons and cancer cells in order to gain pathological benefits.

4.6 Limitations of this study

4.6.1 Specificity of Hsp70/Hsc70 antibody

In the current study, the effects of CHIP KD or UCH-L1 KD on the levels of Hsc70 were examined in the HOM and LYS fractions by western blot analysis. The number of CMA active lysosomes was also examined by quantification of the number of LAMP-2A/Hsc70 colocalizing puncta. The Hsc70 antibody used in the western blot and immunofluorescence analyses of Hsc70, although same as the one used in CMA publications [85, 121, 140], could cross-react with the inducible Hsp70 form besides the constitutive Hsc70.

For the immunofluorescence studies, the cytosolic proteins were removed by the use of a weak detergent digitonin which permeabilized the plasma membrane leaving the lysosomal membranes intact [151]. Further staining of the cells thus resulted in only the lysosomal Hsc70 being stained as the Hsp70 isoform is not present in the lysosomes. Hence the immunofluorescence results in Section 3.2.2 (Fig. 3.2.2) and Section 3.3.2 (Fig. 3.3.2) indicate Hsc70 staining signifying that depletion of CHIP or UCH-L1 affects the number of CMA active lysosomes. However, for our western blot analyses, in the lysosomal fractions, there might be cytoplasmic contamination and hence, a chance for the Hsp70 isoform to be detected as well. Hence, to further corroborate that the preliminary changes observed with anti-Hsp70/Hsc70 antibody for the western blot experiments, are indeed specific to Hsc70 response, additional confirmatory analyses using an antibody recognizing only Hsc70 need to be performed.

4.6.2 Possible presence of contaminating mitochondria and other cytosolic components in the lysosomal fractions

In this current study, lysosome enriched fractions (LYS) were extracted from the total cell homogenate to investigate the effect of CHIP KD or UCH-L1 KD on different aspects of CMA, like CMA associated chaperones, CMA receptor LAMP-2A and the different players of the CMA regulatory mTORC2/Akt1/PHLPP1 pathway in the lysosomes. As the LYS fractions represent crude lysosomal enriched portions that may still contain light mitochondrial or cytosolic components, the results of this study might reflect an effect on not only the lysosomes, but also on the light mitochondrial and cytosolic proteins. To address this issue, confirmation of preliminary lysosomal changes derived from LYS preparation studies need to be further affirmed by studying the changes in purified CMA+ lysosomes. Purified CMA+ lysosomes can be isolated from LYS fractions using a previously described variable metrizamide gradient based ultracentrifugation method [140]. Using this method, CMA+ lysosomes can be separated from CMA- lysosomes and the contaminating light mitochondria.

5 Conclusions and Future Directions

In our current study, the novel role of ubiquitin modifying enzymes CHIP and UCH-L1 in regulation of CMA pathway has been identified. These enzymes perform opposing functions in mediating the lysosomal levels of PHLPP1 and pAkt, thereby affecting LAMP-2A dynamics. It is reported that CMA is downregulated during aging which brings the onset of various neuronal disorders like Parkinson's disease or Alzheimer's disease [45, 152]. With age, lysosomes undergo a vast range of changes like decrease in lysosome stability, changes in some hydrolases activity, intralysosomal accumulation of indigestible particles (lipofuscin), and impaired regulation of lysosomal pH [11]. Deficiency of CHIP in mice led to reduced life span and accelerated age-related pathophysiological phenotypes like accelerated senescence and increased oxidative stress [153]. In addition, CHIP knockout mice exhibit deregulation of protein quality control and exhibit partial perinatal lethality [153]. Deficiency of UCH-L1 causes gracile axonal dystrophy (gad), in which the mice are unable to swallow food and encounter posterior paralysis ultimately being lethal [154]. Thus we see that depletion of both CHIP and UCH-L1 result in neurodegenerative conditions. Loss of either CMA function or CHIP activity can lead to accelerated age related phenotypes. Hence, CHIP can be targeted to upregulate CMA and harness pathological benefits against age related neurodegenerative diseases. The current study opens up new avenues in regulation of CMA pathway which can have implications in the pathology of various neuronal disorders. Although we have identified two possible enzymes

Conclusions and Future Directions

which can be targeted to regulate CMA, further studies are needed obtain a more holistic picture.

5.1 Rac1 mediated recruitment of PHLPP1 to the lysosomes

It has been reported that upon nutrient stress, the Rho GTPase Rac1 migrates from the cytosol to the lysosomal membrane and recruits PHLPP1 to upregulate CMA [48]. In has been witnessed in this study that depletion of CHIP reduces the lysosomal levels of PHLPP1 while depletion of UCH-L1 enhances lysosomal PHLPP1 levels. Hence it is possible that the lysosomal levels of Rac1 modulate the recruitment of PHLPP1 to the lysosomal membrane and investigating the lysosomal Rac1 levels can provide a more comprehensive picture of CHIP/UCH-L1 mediated CMA regulation.

5.2 CHIP and UCH-L1 mediated regulation of PHLPP1 expression

The current study has highlighted that depletion of CHIP reduces PHLPP1 expression while depletion of UCH-L1 enhances the expression of PHLPP1. However, both CHIP and UCH-L1 are ubiquitin modifying enzymes, hence, it remains unclear how they regulate gene expression of PHLPP1. It is known that CHIP is a dual function enzyme, and apart from being an E4 ligase, CHIP also acts as a co-chaperone [135, 155]. It has been reported that CHIP upregulates Hsp70 expressions by activation of transcription factor Heat Shock Factor-1 (HSF-1) [156]. CHIP binds to HSF-1 and Hsp70 forming trimeric complex which can directly bind to the DNA after induction by heat shock [156]. It can be further explored if CHIP can similarly regulate transcription of PHLPP1. Additionally UCH-L1 has been widely recognized as an oncogene [57], and it is known to mediate metastasis by promoting epithelial-to-mesenchymal transition (EMT) [157]. It has been reported that UCH-L1 upregulates the transcriptional

expression of EMT promoting genes Snail, Twist, MMP9 and MMP7 via its ubiquitin hydrolase activity, however the mechanism is yet unknown [157]. The current study has also shown that UCH-L1 regulates PHLPP1 levels through its ubiquitin hydrolase functions. Hence it is possible that CHIP and UCH-L1 can indirectly influence the transcriptional activation or repression of certain genes and further investigations are required to have a holistic idea of the mechanism of action.

5.3 CMA crosstalk with macroautophagy pathway

The present study has reflected a CHIP mediated crosstalk between CMA and UPS pathways. However, it is not clear whether CHIP or UCH-L1 can also mediate crosstalk between CMA and other pathways like macroautophagy. There are various evidences of crosstalk between CMA and MA [75, 128] as discussed in previous sections. However, the mechanisms that underlie this intercommunication between the two pathways remain unknown. The two regulatory molecules described in this study - Akt and mTOR, have also been implicated in MA pathway [158]. mTORC1 is an inhibitor of MA, while mTORC2 inhibits CMA [48]. mTORC2 has also been implicated in the biogenesis of autophagosomes [159]. Moreover, mTORC2 may stimulate macroautophagy through activation of PKC [159] and inhibit CMA through activation of Akt [48]. Hence mTOR is a prominent switch in maintaining balance between CMA and MA. It has been reported that UCH-L1 dysregulates formation of mTORC1 and thus promotes mTORC2 formation [56]. The current study indicates that CHIP does not have any effect on total or lysosomal levels of mTOR or Rictor, although interaction studies between mTOR, Rictor and Raptor can reveal whether CHIP can influence the different complex formations, and thus determine crosstalk between CMA and MA.

5.4 Use of Dendra based reporters to view CMA cargo degradation or LAMP-2A multimerization processes.

In this study, CMA activity has been assessed by quantifying LAMP-2A/Hsc70 colocalizations or by studying the rate of protein degradation (pulse and chase). The limitation in studying CMA in the above methods is the difficulty in monitoring CMA activity in intact cells. To this end, Dendra based CMA reporters can be used to monitor the lysosomal intake of cargo and degradation. As previously discussed, the KFERQ motif is necessary and sufficient for a protein to be degraded by CMA [29]. This concept was put to use while developing the CMA reporter [121]. The first 20 amino acids of ribonuclease A containing the KFERQ motif were fused to the Dendra reporter to develop CMA reporter (dendra). Dendra is a photoactivatable protein and the photoconversion step allows using the pulse and chase approach. This will allow following the lysosomal association of only the subset of proteins converted to the other fluorescent state which has a different excitation and emission spectra. All the proteins synthesized after photoconversion have a different excitation and emission spectra allowing easy visualization of the lysosomal puncta [121]. An added advantage of these monomeric photoexcitable proteins is that they don't form aggregates and preserve their monomeric features. Similarly, Dendra based LAMP-2A fusion proteins can be developed which can allow us to view the turnover of LAMP-2A multimers and viewed under high resolution microscopes like scanning electron microscopes to monitor CMA activity.

6. References

1. Belle, A., et al., *Quantification of protein half-lives in the budding yeast proteome*. Proc Natl Acad Sci U S A, 2006. **103**(35): p. 13004-9.
2. Toyama, B.H. and M.W. Hetzer, *Protein homeostasis: live long, won't prosper*. Nat Rev Mol Cell Biol, 2013. **14**(1): p. 55-61.
3. Etlinger, J.D. and A.L. Goldberg, *A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes*. Proceedings of the National Academy of Sciences of the United States of America, 1977. **74**(1): p. 54-58.
4. de Duve, C., *The lysosome turns fifty*. Nat Cell Biol, 2005. **7**(9): p. 847-9.
5. Bainton, D.F., *The discovery of lysosomes*. J Cell Biol, 1981. **91**(3 Pt 2): p. 66s-76s.
6. Ciechanover, A., *The ubiquitin-proteasome proteolytic pathway*. Cell, 1994. **79**(1): p. 13-21.
7. Laney, J.D. and M. Hochstrasser, *Substrate Targeting in the Ubiquitin System*. Cell, 1999. **97**(4): p. 427-430.
8. Knecht, E., et al., *Pathways for the Degradation of Intracellular Proteins Within Lysosomes in Higher Eukaryotes*. Advances in Molecular and Cell Biology, 1998. **27**: p. 201-234.
9. Li, F. and R.D. Vierstra, *Autophagy: a multifaceted intracellular system for bulk and selective recycling*. Trends Plant Sci, 2012. **17**(9): p. 526-37.
10. Tasset, I. and A.M. Cuervo, *Role of chaperone-mediated autophagy in metabolism*. FEBS J, 2016. **283**(13): p. 2403-13.
11. Martinez-Vicente, M., G. Sovak, and A.M. Cuervo, *Protein degradation and aging*. Exp Gerontol, 2005. **40**(8-9): p. 622-33.
12. Schneider, J.L., et al., *Loss of hepatic chaperone-mediated autophagy accelerates proteostasis failure in aging*. Aging Cell, 2015. **14**(2): p. 249-64.
13. Koga, H. and A.M. Cuervo, *Chaperone-mediated autophagy dysfunction in the pathogenesis of neurodegeneration*. Neurobiol Dis, 2011. **43**(1): p. 29-37.
14. Zhang, L., et al., *CHIP promotes thyroid cancer proliferation via activation of the MAPK and AKT pathways*. Biochem Biophys Res Commun, 2016. **477**(3): p. 356-62.
15. Xilouri, M., et al., *Impairment of chaperone-mediated autophagy induces dopaminergic neurodegeneration in rats*. Autophagy, 2016: p. 0.
16. Kaushik, S. and A.M. Cuervo, *Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis*. Nat Cell Biol, 2015. **17**(6): p. 759-70.
17. Zhang, C. and A.M. Cuervo, *Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function*. Nat Med, 2008. **14**(9): p. 959-65.
18. Cuervo, A.M. and E. Wong, *Chaperone-mediated autophagy: roles in disease and aging*. Cell Res, 2014. **24**(1): p. 92-104.
19. Mizushima, N. and M. Komatsu, *Autophagy: renovation of cells and tissues*. Cell, 2011. **147**(4): p. 728-41.
20. Mizushima, N., et al., *Autophagy fights disease through cellular self-digestion*. Nature, 2008. **451**(7182): p. 1069-75.

21. Asanuma, K., et al., *MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis*. *FASEB J*, 2003. **17**(9): p. 1165-7.
22. Cuervo, A.M. and F. Macian, *Autophagy, nutrition and immunology*. *Mol Aspects Med*, 2012. **33**(1): p. 2-13.
23. Marzella, L., J. Ahlberg, and H. Glaumann, *Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation*. *Virchows Arch B Cell Pathol Incl Mol Pathol*, 1981. **36**(2-3): p. 219-34.
24. Behl, C., *BAG3 and friends: co-chaperones in selective autophagy during aging and disease*. *Autophagy*, 2011. **7**(7): p. 795-8.
25. Sahu, R., et al., *Microautophagy of cytosolic proteins by late endosomes*. *Dev Cell*, 2011. **20**(1): p. 131-9.
26. Carra, S., S.J. Seguin, and J. Landry, *HspB8 and Bag3: a new chaperone complex targeting misfolded proteins to macroautophagy*. *Autophagy*, 2008. **4**(2): p. 237-9.
27. Gamerding, M., et al., *BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins*. *EMBO Rep*, 2011. **12**(2): p. 149-56.
28. Kaushik, S. and A.M. Cuervo, *Chaperone-mediated autophagy: a unique way to enter the lysosome world*. *Trends Cell Biol*, 2012. **22**(8): p. 407-17.
29. Dice, J.F., *Peptide sequences that target cytosolic proteins for lysosomal proteolysis*. *Trends Biochem Sci*, 1990. **15**(8): p. 305-9.
30. Auteri, J.S., et al., *Regulation of intracellular protein degradation in IMR-90 human diploid fibroblasts*. *J Cell Physiol*, 1983. **115**(2): p. 167-74.
31. Cuervo, A.M., *Chaperone-mediated autophagy: Dice's 'wild' idea about lysosomal selectivity*. *Nat Rev Mol Cell Biol*, 2011. **12**(8): p. 535-41.
32. Backer, J.M., L. Bourret, and J.F. Dice, *Regulation of catabolism of microinjected ribonuclease A requires the amino-terminal 20 amino acids*. *Proc Natl Acad Sci U S A*, 1983. **80**(8): p. 2166-70.
33. Agarraberes, F.A. and J.F. Dice, *A molecular chaperone complex at the lysosomal membrane is required for protein translocation*. *J Cell Sci*, 2001. **114**(Pt 13): p. 2491-9.
34. Chiang, H.L., et al., *A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins*. *Science*, 1989. **246**(4928): p. 382-5.
35. Agarraberes, F.A., S.R. Terlecky, and J.F. Dice, *An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation*. *J Cell Biol*, 1997. **137**(4): p. 825-34.
36. Cuervo, A.M., J.F. Dice, and E. Knecht, *A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins*. *J Biol Chem*, 1997. **272**(9): p. 5606-15.
37. Cuervo, A.M. and J.F. Dice, *Regulation of lamp2a levels in the lysosomal membrane*. *Traffic*, 2000. **1**(7): p. 570-83.
38. Callahan, M.K., et al., *Differential acquisition of antigenic peptides by Hsp70 and Hsc70 under oxidative conditions*. *J Biol Chem*, 2002. **277**(37): p. 33604-9.
39. Alberti, S., C. Esser, and J. Hohfeld, *BAG-1--a nucleotide exchange factor of Hsc70 with multiple cellular functions*. *Cell Stress Chaperones*, 2003. **8**(3): p. 225-31.
40. Cuervo, A.M. and J.F. Dice, *A receptor for the selective uptake and degradation of proteins by lysosomes*. *Science*, 1996. **273**(5274): p. 501-3.
41. Cuervo, A.M. and J.F. Dice, *Unique properties of lamp2a compared to other lamp2 isoforms*. *J Cell Sci*, 2000. **113 Pt 24**: p. 4441-50.
42. Cuervo, A.M., et al., *Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor*. *EMBO J*, 2003. **22**(1): p. 47-59.

43. Bandyopadhyay, U., et al., *The Chaperone-Mediated Autophagy Receptor Organizes in Dynamic Protein Complexes at the Lysosomal Membrane*. Molecular and Cellular Biology, 2008. **28**(18): p. 5747-5763.
44. Bandyopadhyay, U., et al., *Identification of regulators of chaperone-mediated autophagy*. Mol Cell, 2010. **39**(4): p. 535-47.
45. Kiffin, R., et al., *Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age*. J Cell Sci, 2007. **120**(Pt 5): p. 782-91.
46. Anguiano, J., et al., *Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives*. Nat Chem Biol, 2013. **9**(6): p. 374-82.
47. Valdor, R., et al., *Chaperone-mediated autophagy regulates T cell responses through targeted degradation of negative regulators of T cell activation*. Nat Immunol, 2014. **15**(11): p. 1046-54.
48. Arias, E., et al., *Lysosomal mTORC2/PHLPP1/Akt Regulate Chaperone-Mediated Autophagy*. Mol Cell, 2015. **59**(2): p. 270-84.
49. Hara, K., et al., *Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action*. Cell, 2002. **110**(2): p. 177-89.
50. Sarbassov, D.D., et al., *Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton*. Curr Biol, 2004. **14**(14): p. 1296-302.
51. Sancak, Y., et al., *Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids*. Cell, 2010. **141**(2): p. 290-303.
52. Pattingre, S., et al., *Regulation of macroautophagy by mTOR and Beclin 1 complexes*. Biochimie, 2008. **90**(2): p. 313-23.
53. Sarbassov, D.D., et al., *Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex*. Science, 2005. **307**(5712): p. 1098-101.
54. Obenauer, J.C., L.C. Cantley, and M.B. Yaffe, *Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs*. Nucleic Acids Res, 2003. **31**(13): p. 3635-41.
55. Su, C.H., et al., *Akt phosphorylation at Thr308 and Ser473 is required for CHIP-mediated ubiquitination of the kinase*. Cell Signal, 2011. **23**(11): p. 1824-30.
56. Hussain, S., et al., *Ubiquitin hydrolase UCH-L1 destabilizes mTOR complex 1 by antagonizing DDB1-CUL4-mediated ubiquitination of raptor*. Mol Cell Biol, 2013. **33**(6): p. 1188-97.
57. Hussain, S., et al., *The de-ubiquitinase UCH-L1 is an oncogene that drives the development of lymphoma in vivo by deregulating PHLPP1 and Akt signaling*. Leukemia, 2010. **24**(9): p. 1641-55.
58. Finn, P.F., et al., *Effects of small molecules on chaperone-mediated autophagy*. Autophagy, 2005. **1**(3): p. 141-5.
59. Chambon, P., *A decade of molecular biology of retinoic acid receptors*. FASEB J, 1996. **10**(9): p. 940-54.
60. Duong, V. and C. Rochette-Egly, *The molecular physiology of nuclear retinoic acid receptors. From health to disease*. Biochim Biophys Acta, 2011. **1812**(8): p. 1023-31.
61. Rochette-Egly, C. and P. Germain, *Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs)*. Nucl Recept Signal, 2009. **7**: p. e005.
62. Xilouri, M. and L. Stefanis, *Chaperone mediated autophagy in aging: Starve to prosper*. Ageing Res Rev, 2016.
63. Dice, J.F., et al., *Regulation of catabolism of microinjected ribonuclease A. Identification of residues 7-11 as the essential pentapeptide*. J Biol Chem, 1986. **261**(15): p. 6853-9.

64. Cuervo, A.M., et al., *Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation*. Am J Physiol, 1995. **269**(5 Pt 1): p. C1200-8.
65. Cuervo, A.M., et al., *Selective binding and uptake of ribonuclease A and glyceraldehyde-3-phosphate dehydrogenase by isolated rat liver lysosomes*. J Biol Chem, 1994. **269**(42): p. 26374-80.
66. Sooparb, S., et al., *Suppression of chaperone-mediated autophagy in the renal cortex during acute diabetes mellitus*. Kidney Int, 2004. **65**(6): p. 2135-44.
67. Yang, Q., et al., *Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy*. Science, 2009. **323**(5910): p. 124-7.
68. Aniento, F., et al., *Selective uptake and degradation of c-Fos and v-Fos by rat liver lysosomes*. FEBS Lett, 1996. **390**(1): p. 47-52.
69. Cuervo, A.M., et al., *IkappaB is a substrate for a selective pathway of lysosomal proteolysis*. Mol Biol Cell, 1998. **9**(8): p. 1995-2010.
70. Wang, Y., et al., *Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing*. Hum Mol Genet, 2009. **18**(21): p. 4153-70.
71. Vogiatzi, T., et al., *Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells*. J Biol Chem, 2008. **283**(35): p. 23542-56.
72. Cuervo, A.M., et al., *Selective degradation of annexins by chaperone-mediated autophagy*. J Biol Chem, 2000. **275**(43): p. 33329-35.
73. Cuervo, A.M., et al., *Direct lysosomal uptake of alpha 2-microglobulin contributes to chemically induced nephropathy*. Kidney Int, 1999. **55**(2): p. 529-45.
74. Cuervo, A.M., et al., *Degradation of proteasomes by lysosomes in rat liver*. Eur J Biochem, 1995. **227**(3): p. 792-800.
75. Massey, A.C., et al., *Consequences of the selective blockage of chaperone-mediated autophagy*. Proc Natl Acad Sci U S A, 2006. **103**(15): p. 5805-10.
76. Aniento, F., et al., *Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes*. J Biol Chem, 1993. **268**(14): p. 10463-70.
77. Bejarano, E. and A.M. Cuervo, *Chaperone-mediated autophagy*. Proc Am Thorac Soc, 2010. **7**(1): p. 29-39.
78. Kiffin, R., et al., *Activation of chaperone-mediated autophagy during oxidative stress*. Mol Biol Cell, 2004. **15**(11): p. 4829-40.
79. Kobayashi, Y., et al., *Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing truncated androgen receptor protein with expanded polyglutamine tract*. J Biol Chem, 2000. **275**(12): p. 8772-8.
80. Dohi, E., et al., *Hypoxic stress activates chaperone-mediated autophagy and modulates neuronal cell survival*. Neurochem Int, 2012. **60**(4): p. 431-42.
81. Schneider, J.L., Y. Suh, and A.M. Cuervo, *Deficient chaperone-mediated autophagy in liver leads to metabolic dysregulation*. Cell Metab, 2014. **20**(3): p. 417-32.
82. Kon, M., et al., *Chaperone-mediated autophagy is required for tumor growth*. Sci Transl Med, 2011. **3**(109): p. 109ra117.
83. Jiang, P., et al., *p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase*. Nat Cell Biol, 2011. **13**(3): p. 310-6.
84. Lv, L., et al., *Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth*. Mol Cell, 2011. **42**(6): p. 719-30.
85. Park, C., Y. Suh, and A.M. Cuervo, *Regulated degradation of Chk1 by chaperone-mediated autophagy in response to DNA damage*. Nat Commun, 2015. **6**: p. 6823.
86. Finn, P.F. and J.F. Dice, *Ketone bodies stimulate chaperone-mediated autophagy*. J Biol Chem, 2005. **280**(27): p. 25864-70.

87. Zhou, D., et al., *Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens*. Immunity, 2005. **22**(5): p. 571-81.
88. Franch, H.A., et al., *A mechanism regulating proteolysis of specific proteins during renal tubular cell growth*. J Biol Chem, 2001. **276**(22): p. 19126-31.
89. Smith, P.D., et al., *Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2*. J Neurosci, 2006. **26**(2): p. 440-7.
90. Xia, H.G., et al., *Degradation of HK2 by chaperone-mediated autophagy promotes metabolic catastrophe and cell death*. J Cell Biol, 2015. **210**(5): p. 705-16.
91. Zhou, J., et al., *Chaperone-mediated autophagy regulates proliferation by targeting RND3 in gastric cancer*. Autophagy, 2016. **12**(3): p. 515-28.
92. Kroemer, G. and M. Jaattela, *Lysosomes and autophagy in cell death control*. Nat Rev Cancer, 2005. **5**(11): p. 886-97.
93. Nylandsted, J., et al., *Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization*. J Exp Med, 2004. **200**(4): p. 425-35.
94. Loos, B., A. du Toit, and J.-H.S. Hofmeyr, *Defining and measuring autophagosome flux—concept and reality*. Autophagy, 2014. **10**(11): p. 2087-2096.
95. López-Otín, C., et al., *The Hallmarks of Aging*. Cell, 2013. **153**(6): p. 1194-1217.
96. Donati, A., et al., *Age-related changes in the autophagic proteolysis of rat isolated liver cells: effects of antiaging dietary restrictions*. J Gerontol A Biol Sci Med Sci, 2001. **56**(9): p. B375-83.
97. Dice, J.F., *Altered degradation of proteins microinjected into senescent human fibroblasts*. J Biol Chem, 1982. **257**(24): p. 14624-7.
98. Okada, Y. and I. Nakanishi, *Effect of colchicine on mouse synovioblasts (B-cells)*. J Electron Microsc (Tokyo), 1984. **33**(2): p. 160-7.
99. Kaushik, S., A.C. Massey, and A.M. Cuervo, *Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy*. EMBO J, 2006. **25**(17): p. 3921-33.
100. Rodriguez-Navarro, J.A., et al., *Inhibitory effect of dietary lipids on chaperone-mediated autophagy*. Proc Natl Acad Sci U S A, 2012. **109**(12): p. E705-14.
101. Wang, G. and Z. Mao, *Chaperone-mediated autophagy: roles in neurodegeneration*. Transl Neurodegener, 2014. **3**: p. 20.
102. Xilouri, M., et al., *LAMP2A as a therapeutic target in Parkinson disease*. Autophagy, 2013. **9**(12): p. 2166-8.
103. Schneider, J.L. and A.M. Cuervo, *Chaperone-mediated autophagy: dedicated saviour and unfortunate victim in the neurodegeneration arena*. Biochem Soc Trans, 2013. **41**(6): p. 1483-8.
104. Lees, A.J., *The Parkinson chimera*. Neurology, 2009. **72**(7 Suppl): p. S2-11.
105. Cuervo, A.M., et al., *Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy*. Science, 2004. **305**(5688): p. 1292-5.
106. Martinez-Vicente, M., et al., *Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy*. J Clin Invest, 2008. **118**(2): p. 777-88.
107. Xilouri, M., et al., *Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy*. PLoS One, 2009. **4**(5): p. e5515.
108. Baba, M., et al., *Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies*. Am J Pathol, 1998. **152**(4): p. 879-84.
109. Kabuta, T., et al., *Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy*. J Biol Chem, 2008. **283**(35): p. 23731-8.

110. Khan, N.L., et al., *Mutations in the gene LRRK2 encoding dardarin (PARK8) cause familial Parkinson's disease: clinical, pathological, olfactory and functional imaging and genetic data*. Brain, 2005. **128**(Pt 12): p. 2786-96.
111. Berg, D., et al., *Type and frequency of mutations in the LRRK2 gene in familial and sporadic Parkinson's disease**. Brain, 2005. **128**(Pt 12): p. 3000-11.
112. Mak, S.K., et al., *Lysosomal degradation of alpha-synuclein in vivo*. J Biol Chem, 2010. **285**(18): p. 13621-9.
113. Majeski, A.E. and J.F. Dice, *Mechanisms of chaperone-mediated autophagy*. Int J Biochem Cell Biol, 2004. **36**(12): p. 2435-44.
114. Cuervo, A.M., *Autophagy: many paths to the same end*. Mol Cell Biochem, 2004. **263**(1-2): p. 55-72.
115. Kim, H.J., et al., *Ubiquitin C-terminal hydrolase-L1 is a key regulator of tumor cell invasion and metastasis*. Oncogene, 2009. **28**(1): p. 117-27.
116. Liu, Y., et al., *The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility*. Cell, 2002. **111**(2): p. 209-18.
117. Setsuie, R., et al., *Dopaminergic neuronal loss in transgenic mice expressing the Parkinson's disease-associated UCH-L1 I93M mutant*. Neurochem Int, 2007. **50**(1): p. 119-29.
118. Tsubuki, S., et al., *Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine*. J Biochem, 1996. **119**(3): p. 572-6.
119. Hipp, M.S., et al., *Indirect inhibition of 26S proteasome activity in a cellular model of Huntington's disease*. J Cell Biol, 2012. **196**(5): p. 573-87.
120. Martinez-Vicente, M., et al., *Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease*. Nat Neurosci, 2010. **13**(5): p. 567-76.
121. Koga, H., et al., *A photoconvertible fluorescent reporter to track chaperone-mediated autophagy*. Nat Commun, 2011. **2**: p. 386.
122. Dimri, G.P., et al., *A biomarker that identifies senescent human cells in culture and in aging skin in vivo*. Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9363-7.
123. Kraft, C., M. Peter, and K. Hofmann, *Selective autophagy: ubiquitin-mediated recognition and beyond*. Nat Cell Biol, 2010. **12**(9): p. 836-841.
124. Petrucelli, L., et al., *CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation*. Hum Mol Genet, 2004. **13**(7): p. 703-14.
125. Harris, C.D., G. Ermak, and K.J. Davies, *RCAN1-1L is overexpressed in neurons of Alzheimer's disease patients*. FEBS J, 2007. **274**(7): p. 1715-24.
126. Liu, H., et al., *Degradation of regulator of calcineurin 1 (RCAN1) is mediated by both chaperone-mediated autophagy and ubiquitin proteasome pathways*. FASEB J, 2009. **23**(10): p. 3383-92.
127. Venugopal, B., et al., *Chaperone-mediated autophagy is defective in mucopolipidosis type IV*. J Cell Physiol, 2009. **219**(2): p. 344-53.
128. Kaushik, S., et al., *Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy*. Mol Biol Cell, 2008. **19**(5): p. 2179-92.
129. Cuervo, A.M., *Chaperone-mediated autophagy: selectivity pays off*. Trends Endocrinol Metab, 2010. **21**(3): p. 142-50.
130. Wang, X.J., et al., *A novel crosstalk between two major protein degradation systems: regulation of proteasomal activity by autophagy*. Autophagy, 2013. **9**(10): p. 1500-8.
131. Pandey, U.B., et al., *HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS*. Nature, 2007. **447**(7146): p. 859-63.
132. Orenstein, S.J., et al., *Interplay of LRRK2 with chaperone-mediated autophagy*. Nat Neurosci, 2013. **16**(4): p. 394-406.

133. Ferreira, J.V., et al., *STUB1/CHIP is required for HIF1A degradation by chaperone-mediated autophagy*. Autophagy, 2013. **9**(9): p. 1349-66.
134. Xilouri, M., O.R. Brekk, and L. Stefanis, *Autophagy and Alpha-Synuclein: Relevance to Parkinson's Disease and Related Synucleopathies*. Mov Disord, 2016. **31**(2): p. 178-92.
135. Edkins, A.L., *CHIP: a co-chaperone for degradation by the proteasome*. Subcell Biochem, 2015. **78**: p. 219-42.
136. Shin, Y., et al., *The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways*. J Biol Chem, 2005. **280**(25): p. 23727-34.
137. Dickey, C.A., et al., *The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins*. J Clin Invest, 2007. **117**(3): p. 648-58.
138. Seo, J., et al., *CHIP controls necroptosis through ubiquitylation- and lysosome-dependent degradation of RIPK3*. Nat Cell Biol, 2016. **18**(3): p. 291-302.
139. Ferreira, J.V., et al., *K63 linked ubiquitin chain formation is a signal for HIF1A degradation by Chaperone-Mediated Autophagy*. Sci Rep, 2015. **5**: p. 10210.
140. Kaushik, S. and A.M. Cuervo, *Methods to monitor chaperone-mediated autophagy*. Methods Enzymol, 2009. **452**: p. 297-324.
141. Storrie, B. and E.A. Madden, *Isolation of subcellular organelles*. Methods Enzymol, 1990. **182**: p. 203-25.
142. Clark, J.M., *Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases*. Nucleic Acids Res, 1988. **16**(20): p. 9677-86.
143. Kaushik, S. and A.M. Cuervo, *Chaperone-mediated autophagy: a unique way to enter the lysosome world*. Trends in cell biology, 2012. **22**(8): p. 407-417.
144. Murphy, K.E., et al., *Lysosomal-associated membrane protein 2 isoforms are differentially affected in early Parkinson's disease*. Mov Disord, 2015. **30**(12): p. 1639-47.
145. Walters, B.J., et al., *Differential effects of Usp14 and Uch-L1 on the ubiquitin proteasome system and synaptic activity*. Molecular and Cellular Neuroscience, 2008. **39**(4): p. 539-548.
146. Hubbi, M.E., et al., *Chaperone-mediated autophagy targets hypoxia-inducible factor-1alpha (HIF-1alpha) for lysosomal degradation*. J Biol Chem, 2013. **288**(15): p. 10703-14.
147. Moore, S.F., R.W. Hunter, and I. Hers, *mTORC2 Protein-mediated Protein Kinase B (Akt) Serine 473 Phosphorylation Is Not Required for Akt1 Activity in Human Platelets*. The Journal of Biological Chemistry, 2011. **286**(28): p. 24553-24560.
148. Saksena, S. and S.D. Emr, *ESCRTs and human disease*. Biochem Soc Trans, 2009. **37**(Pt 1): p. 167-72.
149. Tan, J.M., et al., *Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases*. Hum Mol Genet, 2008. **17**(3): p. 431-9.
150. Devi, L., et al., *Conserved expression of ubiquitin carboxyl-terminal esterase L1 (UCHL1) in mammalian testes*. Indian J Exp Biol, 2015. **53**(5): p. 305-12.
151. Adam, S.A., R.S. Marr, and L. Gerace, *Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors*. J Cell Biol, 1990. **111**(3): p. 807-16.
152. Yang, D.-S., et al., *Neuronal Apoptosis and Autophagy Cross Talk in Aging PS/APP Mice, a Model of Alzheimer's Disease*. The American Journal of Pathology, 2008. **173**(3): p. 665-681.

153. Min, J.-N., et al., *CHIP Deficiency Decreases Longevity, with Accelerated Aging Phenotypes Accompanied by Altered Protein Quality Control*. Molecular and Cellular Biology, 2008. **28**(12): p. 4018-4025.
154. Kurihara, L.J., et al., *Loss of Uch-L1 and Uch-L3 leads to neurodegeneration, posterior paralysis and dysphagia*. Hum Mol Genet, 2001. **10**(18): p. 1963-70.
155. McDonough, H. and C. Patterson, *CHIP: a link between the chaperone and proteasome systems*. Cell Stress Chaperones, 2003. **8**(4): p. 303-8.
156. Dai, Q., et al., *CHIP activates HSF1 and confers protection against apoptosis and cellular stress*. The EMBO Journal, 2003. **22**(20): p. 5446-5458.
157. Jang, M.J., S.H. Baek, and J.H. Kim, *UCH-L1 promotes cancer metastasis in prostate cancer cells through EMT induction*. Cancer Letters, 2011. **302**(2): p. 128-135.
158. Jung, C.H., et al., *mTOR regulation of autophagy*. FEBS Lett, 2010. **584**(7): p. 1287-95.
159. Renna, M., et al., *IGF-1 receptor antagonism inhibits autophagy*. Hum Mol Genet, 2013. **22**(22): p. 4528-44.